chair and boat forms of ring A are listed in Table V. These nonbonded interactions are considerably decreased in the planar forms. Planar 1 is more favorable than planar 2 since a stronger interaction between the 19-methyl and 4β-methyl groups is present in the latter case.

TABLE V

Conformation		Important nonbonded interactions
Chair	C	2β-Br:19-Me:4β-Me
Boat 1	B-1	CO:19-Me:2α-Br:4α-Me
Boat 2	B-2	19-Me:4β-Me:2α-Br:5α-H

We thus find that the conclusions based on dipole moment data and n.m.r. spectra are in substantial agreement with the results of conformational analysis. Each of these methods has important limitations and the results derived from a consideration of only one of these methods could be unreliable. Bond angle distortions of a few degrees can occur at different points throughout the molecule, and consequently one cannot expect dipole moment or other physical measurements to correspond exactly to the calculated value. In the cases studied here, however, all of these methods essentially pointed to the same stereochemistry. These findings can therefore be accepted with confidence as a first approximation to the real state of affairs.

Acknowledgment.—We are thankful to Professor A. Vystrčil and Dr. J. Klinot for samples of allobetulone derivatives and to Professor G. Ourisson,[19] Dr. J. M. Lehn, and Dr. N. L. Allinger for valuable discussions. The computations were programmed by Dr. P. Funke and carried out at the Computer Center[20] of Stevens Institute of Technology, for which we record our appreciation. This work was supported in part by a grant (G-13290) from the National Science Foundation and a grant (CA-05079) from the Cancer Institute of the U. S. Public Health Service.

(19) In a paper presented before the Second International Symposium on the Chemistry of Natural Products, Prague, 1962, J. M. Lehn, J. Levisalles, G. Ourisson, and P. Witz described their studies on triterpenes which are parallel to ours.

(20) Supported in part by a grant from the National Science Foundation.

[CONTRIBUTION FROM THE DEPARTMENT OF BIOPHYSICS, WEIZMANN INSTITUTE OF SCIENCE, REHOVOTH, ISRAEL]

Cleavage of Histidyl Peptide Bonds by N-Bromosuccinimide[1]

BY SHMUEL SHALTIEL[2] AND ABRAHAM PATCHORNIK

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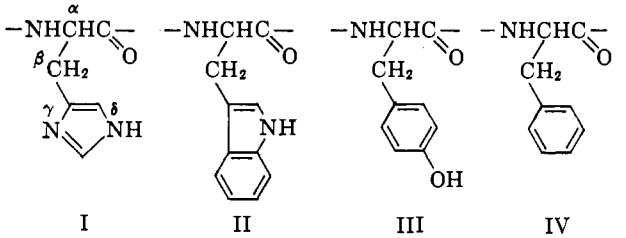
A nonenzymatic method for cleavage of C-histidyl peptide bonds was developed. The imidazole ring of histidine residues is oxidized by N-bromosuccinimide (NBS) in pyridine acetate buffers (pH 3–4) at room temperature. After destruction of excess NBS the reaction mixture is heated for 1 hr. at 100°. Yields of cleavage were found to be 50–65% in histidyl dipeptide models and 30–55% in several synthetic polypeptides consisting of 5–10 amino acids. The histidyl-prolyl bond in sperm-whale myoglobin was cleaved in 53% yield. Tryptophyl and tyrosyl peptide bonds are also cleaved by NBS. By adjustment of reaction conditions and the use of masking groups, differentiation of cleavage of peptide bonds next to tryptophan, tyrosine, and histidine was achieved.

Introduction

In the last few years a new approach to sequence analysis and fragmentation of proteins has been developed. The specific chemical reactivity of certain amino acid residues, permits cleavage of peptide bonds at these sites by nonenzymatic reagents. In many cases, the amino acid residue is selectively modified prior to cleavage. This modification in itself is of potential value in studying the correlation between structure and biological function of proteins. Several nonenzymatic methods for selective cleavage of peptide bonds are reported in the literature. Some of these methods were successfully applied to polypeptides and proteins.[3-14] The work done in this field was recently reviewed by Witkop.[15]

We wish to report here an oxidative method for cleavage of histidyl peptide bonds. No proteolytic enzyme is known to cleave these bonds selectively. Furthermore, histidyl residues are thought to be involved in the biological activity of several enzymes.[16] Therefore, a selective method of cleavage may serve as a tool for the determination of sequence near the active sites of these proteins.

Histidyl residues (I) have a double bond in the γ–δ position relative to the carbonyl group of the peptide bond. A similarly located double bond is found in tryptophyl (II), tyrosyl (III), and phenylalanyl (IV) residues.



I II III IV

When brominating reagents, such as N-bromosuccinimide (NBS), N-bromoacetamide, or bromine, react with tryptophyl or tyrosyl residues, cleavage of their C-peptide bond occurs.[3,17,18] The mechanism sug-

(1) Presented in part in the 1961 and 1963 Meetings of the Israel Chemical Society (S. Shaltiel and A. Patchornik, *Bull. Res. Council Israel*, **10A**, 48 (1961), and "Proceedings of the XXXII Meeting of the Israel Chem. Soc.," 1963, p. 19).

(2) Part of a Ph.D. thesis to be submitted to the Hebrew University, Jerusalem.

(3) A. Patchornik, W. B. Lawson, E. Gross, and B. Witkop, *J. Am. Chem. Soc.*, **82**, 5923 (1960).

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(5) E. Gross and B. Witkop, *J. Biol. Chem.*, **237**, 1856 (1962).

(6) A. Patchornik and M. Sokolovsky, "Proceedings of the Vth European Peptide Symposium," 1962, in press.

(7) S. Sarid and A. Patchornik, "Proceedings of the XXXII Meeting of the Israel Chem. Soc.," 1963, p. 18.

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(15) B. Witkop in "Advances in Protein Chemistry," Vol. 16, C. B. Anfinsen, M. L. Anson, K. Bailey, and J. T. Edsall, Ed., Academic Press, Inc., New York, N. Y., 1961, pp. 221–321.

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(17) G. Schmir, L. Cohen, and B. Witkop, *J. Am. Chem. Soc.*, **81**, 2228 (1959).

(18) E. J. Corey and L. F. Haefele, *ibid.*, **81**, 2225 (1959).

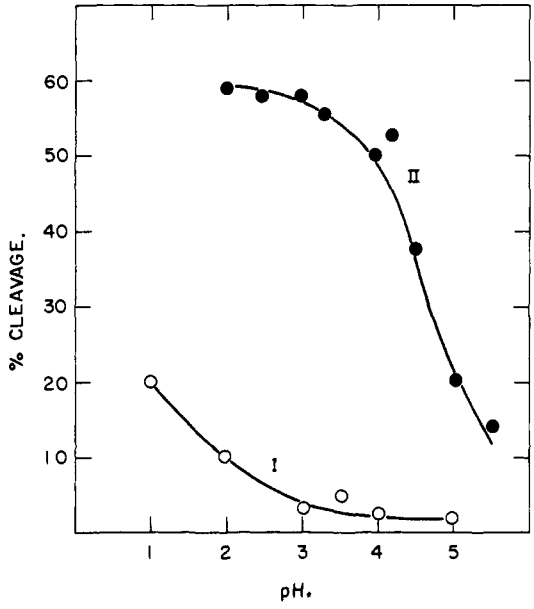
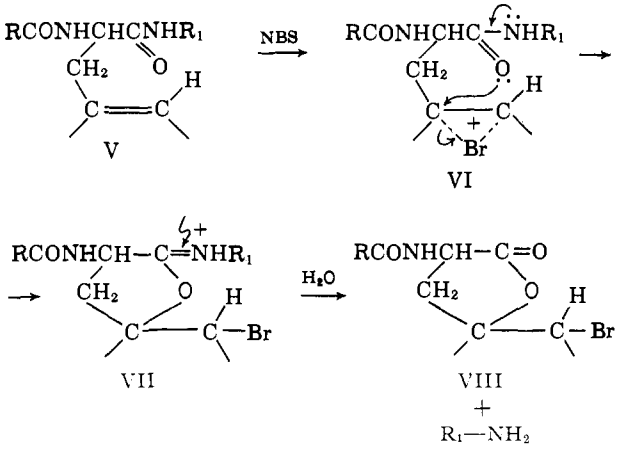


Fig. 1.—Effect of pH on cleavage of N(α)-Z-histidyl-glycine treated with 3 moles of NBS/mole: I, at room temperature; II, after heating for 1 hr. at 100°. The solvents for the reaction were prepared by adding pyridine to 25% aqueous acetic acid. The experiment at pH 1 (curve I) was performed in 0.1 *N* HCl.

gested[3,17,19] for this cleavage involves the formation of a five-membered imino-lactone ring (VII) which is readily hydrolyzed.



Phenylalanyl peptide bonds undergo a similar cleavage[20] after partial reduction of the benzene ring. Since imidazole and its derivatives are known to react readily with brominating reagents,[21] an analogous reaction could be expected with histidine residues.

Results

When N(α)-carbobenzyloxy-histidyl-glycine was treated with NBS in acidic medium, glycine was liberated in low yield. In order to find the optimal conditions for cleavage, we treated histidyl dipeptide models with various molar ratios of NBS in different solvents and hydrogen ion concentrations. Figure 1 (curve I) describes the pH-dependency of the cleavage when the reaction is performed at room temperature. From this curve it is evident that very low pH values are required in order to attain maximal yields of cleavage (20%). As for the amount of reagent required, we found that 3 moles of NBS/mole of peptide brought about optimal cleavage (Fig. 2, curve I). Similar yields of cleavage (18–28%) were obtained when N(α)-carbobenzyloxy-histidyl-glycine (and other histidine-containing dipeptides) were cleaved in 50% aqueous acetic acid (Table I).

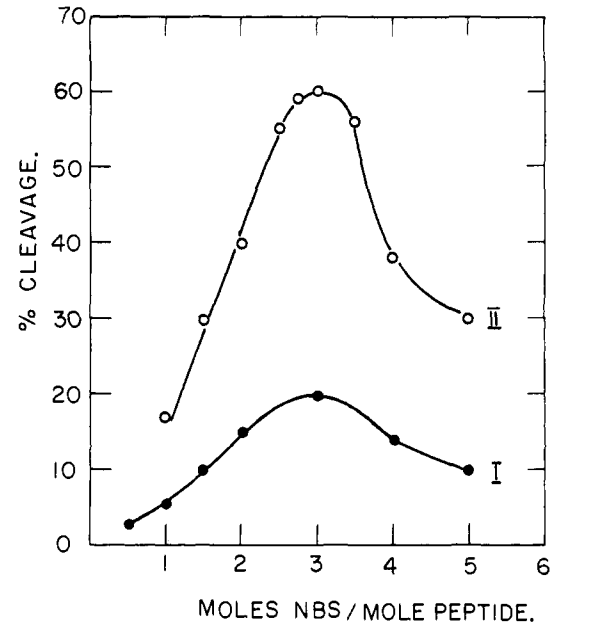


Fig. 2.—Cleavage of N(α)-Z-histidyl-glycine as a function of addition of NBS (I) at room temperature; (II) after heating for 1 hr. at 100° (in pyridine–acetic acid–water, 1:10:19, v./v., pH 3.3).

TABLE I

YIELDS OF CLEAVAGE OF HISTIDYL PEPTIDES

Peptide	Cleavage product	Cleavage, % 50% AcOH Room temp.[b]	50% AcOH 1 hr. 100°	Pyr.-acet.[a] Room temp.[b]	Pyr.-acet.[a] 1 hr. 100°
Imidazole-3-propionyl-glycine	Gly	24	63	3	65
N(α)-Z-L-Histidyl-glycine[22]	Gly	20	59	2	59
N(α)-Z-L-Histidyl-glycine OEt	Gly-OEt + Gly	17	55	0	50
N(α)-Z-L-Histidyl-L-alanine	Ala	21	65	2	63
N(α)-Z-L-Histidyl-L-leucine	Leu	23	60	2	58
N(α)-Z-L-Histidyl-L-phenyl-alanine	Phe	18	53	2	56
N(α)-Z-L-Histidyl-DL-phenyl-alanine	Phe	19	50	1	50
N(α)-Z-L-Histidyl-L-phenyl-alanine amide	Phe-NH_2	18	53	0	50
N(α)-Z-L-Histidyl-L-(ε-Z)-lysine	(ε-Z)-Lys	28	60	3	58

[a] Pyridine–acetic acid–water, 1:10:19 v./v. (pH 3.3). [b] Yield of cleavage was determined after 30 min. (see Experimental).

An attempt was made to replace NBS by other brominating or oxidizing reagents. These included N-bromoacetamide, iodine, hydrogen peroxide, and performic acid. Imidazole-propionyl-glycine was treated with 3 moles/mole of each of these reagents in 50% acetic acid. The yields of cleavage were found to be essentially the same with NBS, N-bromoacetamide, and bromine (24%, 22%, and 19%, respectively), whereas the other reagents cleaved the peptides to a negligible extent only (less than 5%).

When N(α)-carbobenzyloxy-histidyl-glycine (Z-His-GlyOH)[22] was treated with 3 moles/mole of NBS in 50% aqueous acetic acid and the reaction mixture was allowed to stand at room temperature, we observed that after the initial cleavage of 20% (Fig. 3) the yield rose slowly with time and reached 50% after 6 days. This

(19) N. Izumia, J. E. Francis, A. V. Robertson, and B. Witkop, *J. Am. Chem. Soc.*, **84**, 1702 (1962).

(20) M. Wilchek and A. Patchornik, *ibid.*, **84**, 4613 (1962).

(21) E. S. Schipper and A. R. Day in "Heterocyclic Compounds," Vol. 5, R. C. Elderfield, Ed., John Wiley and Sons, Inc., New York, N. Y., 1957, p. 207.

(22) Z = carbobenzyloxy.

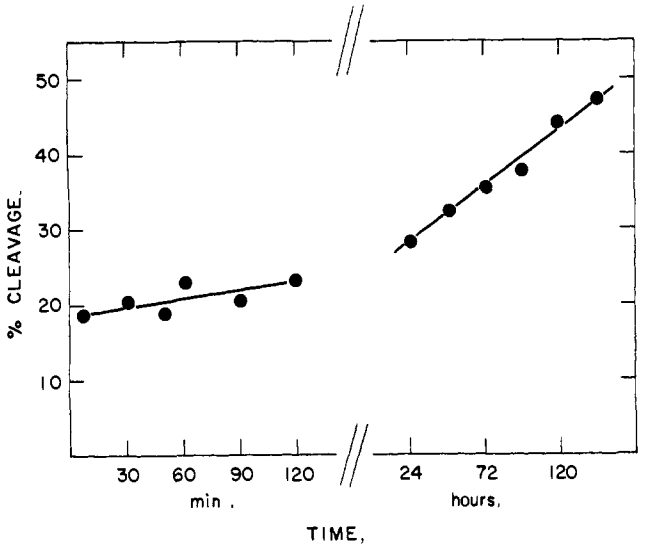


Fig. 3.—Release of glycine with time from N(α)-Z-histidyl-glycine treated with 3 moles of NBS/mole in 50% aqueous acetic acid.

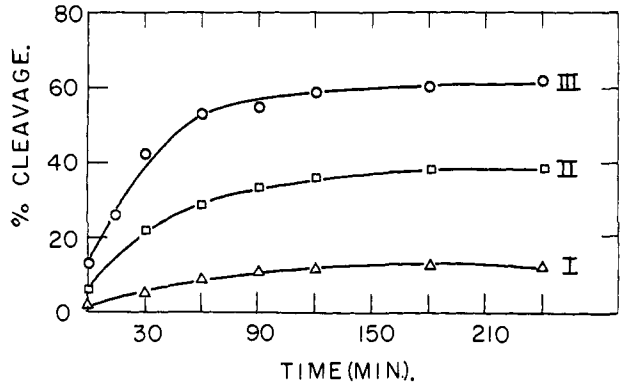


Fig. 4.—Yield of cleavage of N(α)-Z-histidyl-glycine (in 50% aqueous AcOH) on heating the reaction mixture for different times: I, with 2 moles of NBS/mole of peptide; II, with 2 moles of NBS/mole of peptide; III, with 3 moles of NBS/mole of peptide.

slow rise in yield cannot be attributed to hydrolysis of the histidyl peptide since no free glycine was detected in a parallel control experiment (without NBS). Similar high yields of cleavage were obtained when, instead of the long incubation period, the reaction mixture was heated to 100° for 1 hr. (Fig. 4). By this procedure, yields up to 65% were obtained with histidyl dipeptide models (Table I). The fact that the reaction does not proceed to completion (Fig. 4) is not due, in this case, to partial destruction by NBS[23,24] of the released amino acid. On total hydrolysis (with 6 *N* HCl) of aliquots from the same reaction mixture we recovered 90–96% of the glycine. If excess NBS is used (more than 3 moles/mole of peptide), yields of cleavage are lowered (Fig. 2) owing to partial oxidation[23] of the free amino acid released. By destroying excess NBS (before heating) with formic acid,[3] sodium thiosulfate, or imidazole, this difficulty can be overcome without affecting the yields of cleavage.

Heating of peptides or proteins in aqueous acetic acid is liable to cause some hydrolysis of other peptide bonds.[25] However, when the cleavage was performed in a buffer consisting of pyridine, acetic acid, and water (1:10:19 v./v., pH 3.3), yields were not lowered (Table I) and no hydrolysis was detected.

(23) E. W. Chappelle and J. M. Luck, *J. Biol. Chem.*, **229**, 171 (1957).

(24) N. Konigsberg, G. Stevenson, and J. M. Luck, *ibid.*, **235**, 1341 (1960).

(25) A. Yaron, A. Patchornik, and M. Sela; see ref. 15, p. 229.

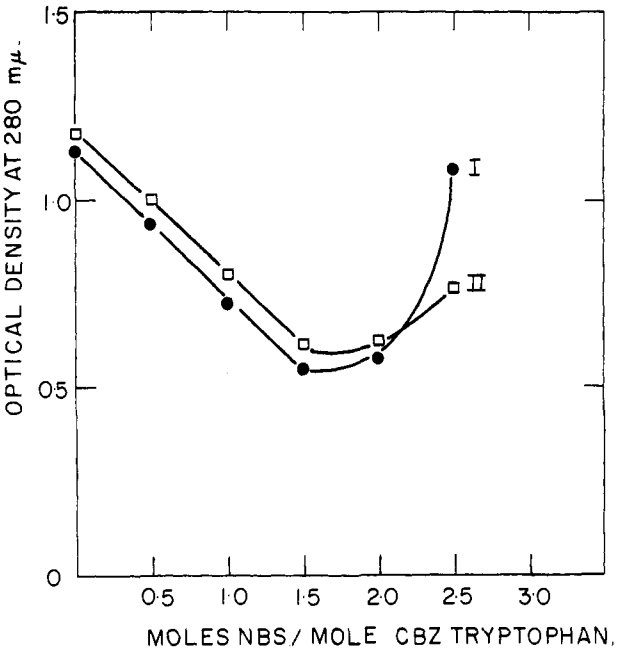


Fig. 5.—Competition between tryptophan and histidine residues for NBS at pH 4.0: I, spectrophotometric "titration" of Z-tryptophan with NBS; II, "titration" of Z-tryptophan in the presence of 5 moles of N(α)-Z-histidine.

Selectivity.—Since tryptophyl and tyrosyl peptide bonds are also cleaved by NBS[3,17] in acidic media, it was necessary to find a way for differentiating between cleavage next to tryptophyl and tyrosyl residues and cleavage next to histidines. Among these residues, tryptophans react fastest with NBS. This reaction is accompanied by characteristic spectral changes.[3] On adding NBS to carbobenzyloxytryptophan (Z-Try), a decrease in absorption at 280 mμ is observed that reaches a minimum when the amount of NBS added is 1.53 moles/mole. On further addition of NBS there is a rise in the absorption (Fig. 5, curve I). In order to determine the competition between tryptophyl and histidyl residues for NBS, we followed the reaction between Z-Try and NBS spectrophotometrically, in the presence of increasing amounts of $N_{(\alpha)}$-carbobenzyloxyhistidine[26] (Z-His). As shown in Fig. 5, the reaction between Z-Try and the first 1.5 moles of NBS is so fast that even 5 moles of Z-His do not interfere with it. However, the reaction with additional amounts of NBS is markedly retarded by the presence of Z-His.

Tyrosyl residues react with NBS more slowly than tryptophyl residues. The reaction between tyrosine and NBS can also be followed spectrophotometically by measuring the increase in optical density at 260 mμ.[17] N-Carbobenzyloxytyrosine (Z-Tyr) was treated with NBS in the presence of increasing amounts of Z-His and it was found (Fig. 6) that histidyl residues interfere with this reaction to a great extent.

An even greater competition of the histidyl residues for NBS has been observed in mixtures of tryptophyl, tyrosyl, and histidyl dipeptides as well as in oligopeptides containing these residues. Thus, the synthetic peptide His-Phe-Arg-Try-GlyOH[27] consumed 1.9 moles of NBS (instead of 1.53 moles) for maximal decrease of indole absorbance at 280 mμ. Similarly, a tyrosine–histidine competition was observed in the pep-

(26) Histidyl residues or their oxidation products do not absorb at this wave length and thus their presence (even in excess) does not interfere with this determination.

(27) This peptide was kindly supplied by Prof. K. Hofmann, University of Pittsburgh.

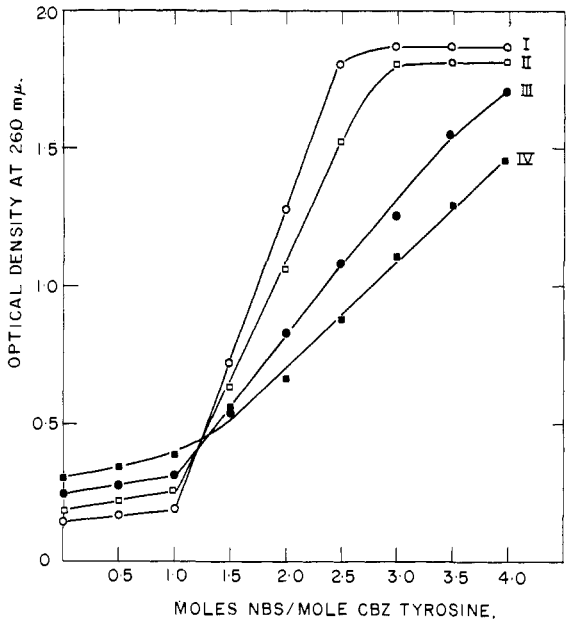


Fig. 6.—Competition between tyrosine and histidine residues for NBS at pH 3.0. I, spectrophotometric "titration" of N Z-tyrosine; II, III, and IV, "titration" of N-Z-tyrosine in the presence of 1, 3, and 5 moles (respectively) of N(α)-Z-histidine.

tide Z-Val-Tyr-Val-His-Pro-PheOMe.[28] This peptide required 4.2 moles of NBS (instead of 3 moles) to reach the theoretical spectral changes at 260 mμ.

In this connection it should be mentioned that histidine residues with a protected carboxyl group differ in reactivity from histidine residues with a free carboxyl group.[29,30] Thus Z-His absorbs 2 moles of NBS in about 2 min. and a third mole within 45 min., while a histidine residue in peptides, such as Z-His-Gly-OH or Z-His-Phe-NH_2, absorbs 3 moles of NBS within 30 sec. For this reason, when we studied the competition between tryptophan, tyrosine, and histidine residues we always compared analogous models, *i.e.* Z-Try (or Z-Tyr) was compared with Z-His, and tryptophyl (or tyrosyl) peptides were compared with histidyl peptides.

In spite of the competition between tryptophyl, tyrosyl, and histidyl residues for NBS, it is possible to differentiate between the cleavage of tryptophyl, tyrosyl, and histidyl peptide bonds. By performing the reaction with NBS at room temperature in pyridine–acetic acid–water (1:10:19 v./v.), rupture of tryptophyl and tyrosyl linkages occurs, whereas histidyl residues are oxidized but not cleaved. After destroying the excess NBS and heating the reaction mixture, cleavage of histidyl linkages occurs without increase in the yields of cleavage of the other peptide bonds. This was proved using equimolar mixtures of tryptophyl, tyrosyl, and histidyl dipeptides (Table II). The extent of cleavage of each of the peptides could be determined by paper chromatography since the dipeptides were chosen so that a different amino acid was liberated from each of the peptides in the mixtures.

Differentiation between cleavage next to tryptophyl and tyrosyl residues was also attempted. Cleavage of peptide bonds next to tyrosine can be prevented by O-carbobenzyloxylation[1] or O-acetylation of these residues. Thus, when O-carbobenzyloxy tyrosine or N,O-dicarbobenzyloxy-tyrosyl-glycine was treated with NBS, no increase in the optical density at 260 mμ (characteristic of lactone formation and of cleavage[17]) could be detected. The above-mentioned peptide as well as N,O-dicarbobenzyloxy-tyrosyl-alanine benzyl ester were not cleaved by NBS (in pyridine–acetic acid–water, 1:10:19, v./v.) even after heating for 1 hr. at 100° in the same solvent. However, after removal of the O-carbobenzyloxy group (by treatment with alkali (pH 13) for 5 min.), cleavage by NBS proceeded in 45–50% yield. The masking and unmasking of tyrosyl residues was followed spectrophotometrically.[31]

TABLE II

CLEAVAGE OF MIXTURES OF TRYPTOPHYL, TYROSYL, AND HISTIDYL PEPTIDES[a]

Peptide mixture[b]	NBS, moles	Bond cleaved	Cleavage,[c] % Room temp.[d]	1 hr. at 100°
Z-L-Tryptophyl-glycine	6	Try–Gly	41	44
N(α)-L-Z-Histidyl-L-phenylanine		His–Phe	2	40
Z-L-Tryptophyl-L-alanine	6	Try–Ala	52	53
N(α)-Z-L-Histidyl-glycine		His–Gly	2	55
N-Z-L-Tyrosyl-glycine	6	Tyr–Gly	42	45
N(α)-Z-L-Histidyl-L-(ε-carbobenzyloxy)-lysine		His–Lys	3	50
N-Ac-L-Tyrosyl-L-alanine	6	Tyr–Ala	41	44
N(α)-Z-L-Histidyl-glycine		His–Gly	1	50
Z-L-Tryptophyl-glycine		Try–Gly	43	46
N-Ac-L-Tyrosyl-L-alanine	10	Tyr–Ala	42	43
N(α)-Z-L-Histidyl-L-(ε-carbobenzyloxy)-lysine		His–Lys	2	47
Z-L-Tryptophyl-L-alanine		Try–Ala	55	53
N-Z-L-Tyrosyl-glycine	9	Tyr–Gly	48	46
N(α)-Z-L-Histidyl-L-leucine		His–Leu	1	55

[a] In pyridine–acetic acid–water (1:10:19, v./v., pH 3.3). [b] Equimolar mixtures of the peptides were used. [c] Yield of cleavage was determined by paper chromatography of the reaction mixture and a quantitative ninhydrin assay (see Experimental). [d] Extent of cleavage was determined after standing for 30 min. at room temperature.

Cleavage in Oligopeptides and in Sperm-Whale Myoglobin.—The applicability of this method to larger molecules was tested by performing the cleavage on four synthetic polypeptides and one protein, sperm-whale myoglobin.[32] Yields of cleavage were found to be 30–53% (see Table III). The extent of cleavage was measured by three different methods. When as a result of the cleavage a free amino acid was released, we used paper chromatography and quantitative ninhydrin assay (*e.g.*, cleavage of the his–leu bond in Val^5-Hypertensin I). To estimate the cleavage of his–pro bonds we used the enzyme proline imino-peptidase[33] (PIP) to cleave the new N-terminal proline which was then determined colorimetrically.[33] The extent of cleavage of the his–phe bond in the peptide His-Phe-Arg-Try-GlyOH was determined by quantitative dinitrophenylation[34] (DNP). In this case, the α-amine of histidine was acetylated before the cleavage to prevent possible side reactions between the α-amine and NBS or products of oxidized imidazole.

Using our method of cleavage we were able to confirm the existance of a his–pro bond in sperm-whale myoglobin. The primary sequence of this protein was

(28) This peptide was kindly supplied by Dr. B. Iselin of CIBA, Ltd., Basle.

(29) *Cf.* ref. 16, p. 58.

(30) As determined by iodometric titrations; *cf.* M. Z. Barakat and M. F. Abd El-Wahab, *Anal. Chem.*, **26**, 1973 (1954).

(31) S. Shaltiel and A. Patchornik, in preparation.

(32) A sample of this protein was kindly supplied by Dr. J. C. Kendrow, Cavendish Laboratory, Cambridge, Eng.

(33) S. Sarid, A. Berger, and E. Katchalski, *J. Biol. Chem.*, **234**, 1740 (1959); *ibid.*, **237**, 2207 (1962).

(34) H. Fraenkel-Conrat, J. I. Harris, and A. L. Levy in "Methods of Biochemical Analysis," Vol. 2, Interscience Publishers, Inc., New York, N. Y., 1955, pp. 359–425.

TABLE III

CLEAVAGE OF SYNTHETIC POLYPEPTIDE MODELS[a]

Peptide	NBS, moles/mole pep.	Bond cleaved	Cleavage, %: Room temp.	Cleavage, %: 1 hr. at 100°	Method of determn.[c]
Ac-His-Phe-Arg-Try-GlyOH[b,27]	6	His–Phe	3	30	DNP
		Try–Gly	45	47	NIN
Z-Val-Tyr-Val-His-Pro-PheOMe[b,28]	6	Tyr–Val	24	28	DNP
		His–Pro	0	42	PIP
Asp(NH_2)-Arg-Val-Tyr-Val-His-Pro-PheOH[35]	6	Tyr–Val	15[d]	..[e]	DNP
(Val[5]-Hypertensin II-asp-β-amide)		His–Pro	2	48	PIP
Asp(NH_2)-Arg-Val-Tyr-Val-His-Pro-Phe-His-LeuOH[35]	10	Tyr–Val	..[e]	..[e]	...
		His–Pro	3	45	PIP
(Val[5]-Hypertensin I)		His–Leu	3	53	NIN

[a] In pyridine–acetic acid–water (1:10:19, v./v.), pH 3.3. [b] Ac = acetyl; Z = carbobenzyloxy. [c] NIN = ninhydrin; DNP = dinitrophenylation; PIP = proline imino-peptidase. [d] Determined by G. L. Schmir and L. Cohen (*cf.* ref. 36). No correction factor for hydrolytic or chromatographic losses has been applied to this result. [e] Extent of cleavage was not determined.

recently elucidated using chemical[37] and X-ray[38] methods. According to these studies there are four proline residues in the protein: one is situated after isoleucine, two residues after alanine, and one after histidine. When the protein was treated with NBS at room temperature (pH 3.3) no terminal proline could be detected, indicating that a try–pro or tyr–pro sequence is unlikely. However, on heating the reaction mixture for 1 hr. at 85–90° (after destruction of excess NBS with imidazole) a new terminal proline was found. After incubation of the cleaved protein with proline iminopeptidase we determined 53% cleavage of the his–pro bond (assuming one his–pro bond in the molecule).

Discussion

When a peptide or a protein is treated with NBS, several amino acid residues are liable to be attacked. These include: cysteine, cystine, methionine, tryptophan, tyrosine, and histidine. Cysteine and cystine residues react rapidly with NBS, and different products may be formed[10] with different amounts of the reagent. However, by prior oxidation of the protein with performic acid,[39–41] cysteines and cystines are converted quantitatively into cysteic acid residues which are resistant to NBS. Methionines are oxidized by excess NBS into methionine sulfone residues[36]; therefore, in the determination of bonds cleaved, a sulfone marker should be included.

Tryptophyl, tyrosyl, and histidyl peptide bonds are all cleaved by NBS. The method we propose does not make it possible to cleave histidyl bonds without cleaving tryptophyl and tyrosyl bonds as well. From the available experimental evidence, it seems that also tryptophyl and tyrosyl bonds cannot be cleaved without, at least, partial oxidation of histidyl residues. In a protein, where certain residues may be activated while others are inaccessible to the reagent, such competition for NBS is liable to cause misleading results. Nevertheless, it is possible to differentiate between cleavage of tryptophyl and tyrosyl peptide bonds, on the one hand, and of histidyl peptide bonds on the other. This is achieved by performing the cleavage in two steps: (a) cleavage of tryptophyl and tyrosyl bonds at room temperature with concomitant oxidation of histidyl residues, (b) cleavage of histidyl peptide bonds by heating. This method proved adequate when tested on peptides (see Tables II and III).

As for the competition between tryptophan and tyrosine, it was reported[3,36] that by using a limited amount of NBS, tryptophyl residues may be cleaved predominantly. Our results indicate that tryptophyl bonds cannot by cleaved in high yields without causing some cleavage of tyrosyl bonds. For instance, when increasing amounts of NBS were added to an equimolar mixture of tryptophyl, tyrosyl, and histidyl peptides and the cleavage of each peptide was followed (Fig. 7), we found that in order to achieve maximal cleavage of the tryptophyl bond (42%) 6 moles of NBS were needed. Under these conditions the tyrosyl bond was cleaved to some extent (15%) and the histidyl residues were partially oxidized (an aliquot of the same reaction mixture was heated and the histidyl bond was cleaved in 24% yield). O-Carbobenzyloxylation or O-acetylation may be a means of preventing cleavage of tyrosyl linkages.[1] A drawback of this masking group is that it usually renders the protein less soluble. Moreover, the literature concerning the reactivity of phenolic hydroxyls on acetylation is contradictory. Some authors[42] state that phenolic hydroxyls are acetylated more readily than aliphatic hydroxyls, while others[43] insist that phenolic

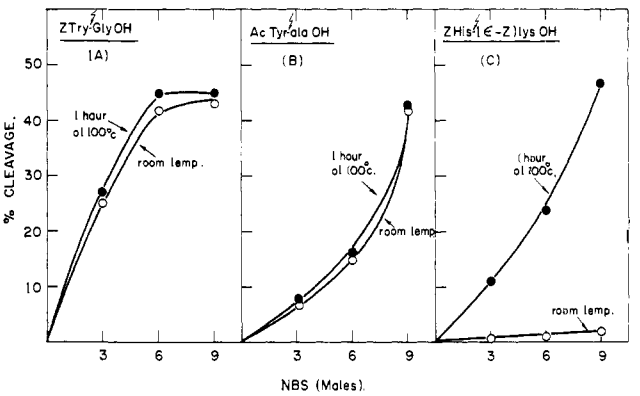


Fig. 7.—Cleavage of an equimolar mixture of the peptides Z-tryptophylglycine, N-Ac-tyrosyl-alanine, and N(α)-histidyl-(ε-Z)-lysine with increasing amounts of NBS. The solvent was pyridine–acetic acid–water, 1:10:19, v./v., pH 3.3: A, extent of cleavage of the try–gly bond at room temperature, —O—, and after heating, —●—; B, extent of cleavage of the tyr–ala bond at room temperature, —O—, and after heating, —●—; C, extent of cleavage of the his–lys bond at room temperature, —O—, and after heating, —●—.

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TABLE IV
HISTIDINE-CONTAINING PEPTIDES

Peptide	Yield, %	Solvent of recrystn.[a]	M.p., °C. [lit.]	Molecular formula	Calcd. C	Calcd. H	Calcd. N	Found C	Found H	Found N	Mol. wt., calcd.	Mol. wt. (found) $HClO_4$ (glac. AcOH)	Mol. wt. (found) $HClO_4$ (dioxane)	Mol. wt. (found) $NaOCH_3$ (MeOH benzene)
$N(\alpha),N_{(Im)}$-Z_2-L-Histidyl-glycine OEt	70	B. or E.A.	109	$C_{26}H_{28}N_4O_7$	61.41	5.55	11.02	61.02	5.73	10.94	509	510	Tr.[b]	Tr.
$N(\alpha),N_{(Im)}$-Z_2-L-Histidyl-L-alanine OBz	74	B	114	$C_{32}H_{32}N_4O_7$	65.74	5.52	9.58	65.92	5.61	9.71	585	592	Tr.	Tr.
$N(\alpha),N_{(Im)}$-Z_2-L-Histidyl-L-phenylalanine OMe	80	B. or E.A.	123 [123–124][50]	$C_{32}H_{32}N_4O_7$	65.74	5.52	9.58	65.70	5.70	9.44	585	578	Tr.	Tr.
$N(\alpha),N_{(Im)}$-Z_2-L-Histidyl-L-phenylalanine OBz	82	B	127 [126–127][51]	$C_{38}H_{36}N_4O_7$	69.08	5.49	8.48	68.90	5.61	8.56	661	670	Tr.	Tr.
$N(\alpha),N_{(Im)}$-Z_2-L-Histidyl-L-(ϵ-Z)-lysine OMe	75	E.A.	112	$C_{37}H_{41}N_5O_9$	63.51	5.91	10.01	63.20	5.71	10.17	700	690	Tr.	Tr.
$N(\alpha),N_{(Im)}$-Z_2-L-Histidyl-L-(ϵ-Z)-lysine OBz	70	B	158–160	$C_{43}H_{45}N_5O_9$	66.56	5.85	9.03	66.49	6.01	9.04	776	760	Tr.	Tr.
$N(\alpha),N_{(Im)}$-Z_2-L-Histidyl-L-leucine OMe	76	B	103 [101–103][49]	$C_{29}H_{41}N_5O_6$	63.26	6.22	10.18	63.30	6.41	10.19	551	558	Tr.	Tr.
$N(\alpha),N_{(Im)}$-Z_2-L-Histidyl-DL-phenylalanine OMe	71	B. or E.A.	106	$C_{32}H_{32}N_4O_7$	65.74	5.52	9.58	65.93	5.67	9.90	585	587	Tr.	Tr.
$N(\alpha)$-Z-L-Histidyl-glycine	75	El.–W.	239 dec. [237–238][49]	$C_{16}H_{18}N_4O_5$	55.48	5.24	16.18	55.58	5.30	16.19	346	342	340	348
$N(\alpha)$-Z-L-Histidyl-L-alanine	80	W or El.–W.	215 dec. [211–214][53]	$C_{17}H_{20}N_4O_5$	56.66	5.59	15.55	56.76	5.41	15.50	360	362	357	360
$N(\alpha)$-Z-L-Histidyl-L-phenylalanine	78	El.–W.	240 dec. [228][54]	$C_{23}H_{24}N_4O_5$	63.29	5.54	12.84	63.40	5.70	12.87	436	438	431	436
$N(\alpha)$-Z-L-Histidyl-L-(ϵ-Z)-lysine	70	El.–Er.	208 dec.	$C_{28}H_{33}N_5O_7$	60.97	6.03	12.70	60.68	5.93	12.60	552	564	558	562
$N(\alpha)$-Z-L-Histidyl-L-leucine	80	W or El.–W.	189 [185–188][53]	$C_{20}H_{26}N_4O_5$	59.69	6.51	13.92	59.77	6.52	13.81	402	396	398	402
$N(\alpha)$-Z-L-Histidyl-DL-phenylalanine	83	El–W.	220 dec.	$C_{23}H_{24}N_4O_5$	63.29	5.54	12.84	63.19	5.52	12.66	436	440	431	432

[a] B, benzene; E.A., ethyl acetate; El, ethanol; W, water; Er, ether. [b] Tr. = traces.

hydroxyls are not affected by acetylation. The applicability of this method for masking tyrosins should be further investigated.

Preliminary experiments indicate that cleavage of histidyl peptides is feasible on paper chromatograms (see Experimental). This method may therefore be of use for quick identification and determination of histidyl peptides in partially hydrolyzed or enzymatically digested proteins.

The products formed on treatment of various histidine models with NBS and the mechanism suggested for this cleavage will be the subject of a forthcoming publication.

Experimental[44]

Synthesis of Model Compounds.—$N_{(\alpha)},N_{(Im)}$-Dicarbobenzyloxy-histidyl amino acid esters were synthesized from dicarbobenzyloxy-L-histidine[45] and the appropriate amino acid ester[46] by the dicyclohexylcarbodiimide method.[47–51] The peptides prepared and the corresponding analytical data are described in Table IV. Nonaqueous titrations were used for the characterization of these peptides and for the determination of their purity[45,52] (see Methods).

$N_{(\alpha)}$-Carbobenzyloxyhistidyl Peptides.—Dicarbobenzyloxyhistidyl peptide esters (3–5 mmoles) were suspended in methanol (25 ml.) and 2 N NaOH (2.5 equiv.) was added, whereupon the suspension cleared. The reaction mixture was allowed to stand at room temperature for 1 hr. and then it was cooled (0°), diluted with water (25 ml.), and acidified with 1 N HCl until crystallization started (pH 5.0–5.5). If crystallization did not start within 30 min., the solution was concentrated *in vacuo* and the peptide was precipitated by addition of acetone. The peptides were recrystallized, dried over P_2O_5 (at 50° for 2–3 hr.), and characterized by nonaqueous titrations (see Methods).

$N_{(\alpha)}$-Carbobenzyloxy-L-histidyl-L-phenylalanine Amide.—Absolute methanol (150 ml.) was saturated with ammonia at 0°. To this solution, 14 g. (24 mmoles) of dicarbobenzyloxy-L-histidyl-L-phenylalanine methyl ester was added. The reaction mixture was allowed to stand at room temperature (in a tightly stoppered flask) for 4 days. The precipitate formed was filtered, washed with cold methanol, and recrystallized from 50% aqueous methanol. After drying over P_2O_5 for 2 hr. (at 60°) the yield was 8.5 g. (82%), m.p. 214–216° (lit.[55] 203–204°).

Anal. Calcd. for $C_{23}H_{25}N_5O_4$: C, 63.43; H, 5.79; N, 16.08; neut. equiv., 435. Found: C, 63.27; H, 5.89; N, 15.80; neut. equiv., 438 (with $HClO_4$ in glac. acetic acid) and 436 (with $HClO_4$ in dioxane).

$N_{(\alpha)}$-Carbobenzyloxy-L-histidyl-glycine Ethyl Ester.—Dicarbobenzyloxy-L-histidyl-glycine ethyl ester (1 g., 2 mmoles) was suspended in 10 ml. of absolute ethanol and 1.9 ml. of 0.55 N $NaOC_2H_5$ was added with vigorous stirring. After 30 min., the reaction mixture was neutralized with 1 N HCl and evaporated to dryness. The peptide was extracted into hot chloroform. The extract was washed with water and dried over Na_2SO_4. On treatment with petroleum ether a solid paste was precipitated which was recrystallized from ethanol and dried over P_2O_5; yield 0.57 g. (75%), m.p. 113° (lit.[55] 113–114°).

Anal. Calcd. for $C_{18}H_{21}N_4O_5$: C, 57.89; H, 5.67; N, 15.01; neut. equiv., 373. Found: C, 58.35; H, 5.82; N, 14.91; neut. equiv., 376 (with $HClO_4$ in glac. acetic acid) and 368 (with $HClO_4$ in dioxane).

4(or 5)-Imidazole-propionic Acid.—A sample of α-chloroimidazolepropionic acid[56] (10 g., 57 mmoles) was dissolved in 150 ml. of 5% aqueous HCl, to which 1 g. of 5% Pd–C catalyst was added. The reaction mixture was transferred into a Parr apparatus for reduction. After 24 hr., the catalyst was filtered off and washed

(44) All melting points are uncorrected.

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with water. The filtrate and the washings were concentrated *in vacuo* until the first crystals appeared. On cooling, a heavy white precipitate was formed which was recrystallized twice from aqueous acetone; m.p. 130° (lit.[57] 105°), yield 9.7 g. (87.5%).

Anal. Calcd. for $C_6H_8N_2O_2 \cdot HCl \cdot H_2O$: C, 37.02; H, 5.70; N, 14.40; Cl, 18.22. Found: C, 36.98; H, 5.70; N, 14.30; Cl, 18.65.

The hydrochloride hydrate (3.9 g., 20 mmoles), was dissolved in a minimum volume of hot methanol. On addition of 2.8 ml. of $(C_2H_5)_3N$ (20 mmoles) precipitation began. After filtering and washing with absolute ethanol, the sample was recrystallized by dissolving in water and dilution with acetone. The sample was dried over P_2O_5 at 60° for 2 hr.; m.p. 212° (lit.[58] 212°), yield 2.2 g. (78%).

$N_{(1m)}$-Carbobenzyloxy-imidazole-propionyl-glycine *p*-Nitrobenzyl Ester.—Imidazole-propionic acid hydrochloride hydrate (9.7 g., 50 mmoles) was dissolved in 100 ml. of 1 *N* NaOH, and 75 ml. of 2 *N* Na_2CO_3 was added. The solution was cooled to 0° and carbobenzyloxy chloride (8.5 ml., 50 mmoles) was added portionwise with vigorous shaking during 20 min. The reaction mixture was allowed to stand for 30 min. and then it was acidified until a gelatinous mass precipitated. This was extracted into 100 ml. of ethyl acetate. An aliquot of this solution was titrated with $HClO_4$ in glacial acetic acid (using thymol blue as indicator) and thus the concentration of $N_{(1m)}$-carbobenzyloxy-imidazole-propionic acid in the ethyl acetate was determined to be 30 mmoles/100 ml. (yield 60%). This compound reacted with 30 mmoles of glycine *p*-nitrobenzyl ester (prepared from 7.4 g. of its hydrobromide by $(C_2H_5)_3N$) in the presence of dicyclohexylcarbodiimide according to the method described for the synthesis of dicarbobenzyloxy-histidyl peptide esters.[49]

$N_{(1m)}$-Carbobenzyloxy-imidazole-propionyl-glycine *p*-nitrobenzyl ester was recrystallized from ethyl acetate–petroleum ether; m.p. 118°, yield 7.1 g.

Anal. Calcd. for $C_{23}H_{22}N_4O_7$: C, 59.22; H, 4.75; N, 12.01; neut. equiv., 466. Found: C, 59.55; H, 4.80; N, 12.02; neut. equiv., 476 (with $HClO_4$ in glac. acetic acid).

Imidazole-propionyl-glycine.—$N_{(1m)}$-Carbobenzyloxy-imidazole-propionyl-glycine *p*-nitrobenzyl ester (2.3 g., 5 mmoles) was dissolved in 25 ml. of methanol To this solution 6 ml. of 2 *N* KOH was added. The reaction was allowed to proceed for 30 min. at room temperature and the pH was adjusted to 5.0 with 10% $HClO_4$. The precipitate which formed immediately ($KClO_4$) was filtered and washed with methanol. The filtrate and the washings were concentrated *in vacuo* and the resulting oil was dissolved in 40 ml. of methanol. Part of the substance (residual $KClO_4$) which did not go into solution was filtered off. On addition of ether to the filtrate, a white paste was precipitated. This paste was crystallized twice from a minimal volume of hot methanol, and dried over P_2O_5 at 60°; yield 0.42 g. (42%), m.p. 209°.

Anal. Calcd. for $C_8H_{11}N_3O_3$: C, 48.72; H, 5.62; N, 21.31; neut. equiv., 197. Found. C, 48.42; H, 5.42; N, 21.03; neut. equiv., 201 (with $HClO_4$ in glac. acetic acid) and 200 (with $NaOCH_3$ in methanol–benzene).

Tryptophyl and tyrosyl peptides were synthesized by the dicyclohexyl carbodiimide method: Z-L-tryptophylglycine ethyl ester, m.p. 119° (lit.[55] 120°); Z-L-tryptophyl-L-alanine benzyl ester, m.p. 150° (lit.[59] 153°); N,O-Z_2-L-tyrosylglycine ethyl ester, m.p. 164° (lit.[60] 164–165°). By saponification of these peptides we obtained: Z-L-tryptophylglycine, m.p. 157° (lit.[61] 158–159°); Z-L-tryptophyl-L-alanine, m.p. 156° (lit.[62] 155°); N-Z-L-tyrosylglycine, m.p. 98° (lit.[63] 100°); N-Ac-L-tyrosyl-L-alanine, m.p. 110°.[64]

Methods. Characterization and Determination of Peptides Containing Histidine by Nonaqueous Titrations.—The titrimetric properties of imidazoles and acylimidazoles[45] were used for characterization and determination of peptides containing histidine. Each of the peptides was titrated with three reagents: (a) $HClO_4$ in glacial acetic using crystal violet (C.V.) as indicator, (b) $HClO_4$ in dioxane using thymol blue (T.B.) as indicator, (c) $NaOCH_3$ in methanol–benzene using thymol blue as indicator.

The titrations were performed on samples of 0.1–0.15 mmoles, dissolved in the appropriate solvent. The solvent was previously neutralized with the reagent and an aliquot was set aside as a standard for color change. Standard solutions (0.1 *N*) of the reagents were used.

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(64) A sample of this peptide was kindly supplied by Mr. M. Wilchek.

The peptides that we synthesized fall into three groups, the titrimetric properties of which are summarized in Table V. Data concerning molecular weight determinations of these peptides are given in Table IV.

TABLE V

TITRIMETRIC PROPERTIES OF PEPTIDES CONTAINING HISTIDINE

General formula	$HClO_4$ (AcOH), moles/mole	$HClO_4$ (dioxane), moles/mole	$NaOCH_3$ (CH_3OH), moles/mole
Z-NH-CH(-CH_2-Im-N-Z)-CO-NH-CH(R_1)-$COOR_2$	1	0	0[a]
Z-NH-CH(-CH_2-Im-NH)-CO-NH-CH(R_1)-$COOR_2$	1	1	0
Z-NH-CH(-CH_2-Im-NH)-CO-NH-CH(R_1)-COOH	1	1	1
Indicator	C.V.[b]	T.B.[b]	T.B.[b]
Color change	Violet → green	Yellow → red	Yellow → blue

[a] $NaOCH_3$ removes the carbobenzyloxy group from the imidazole catalytically.[45] [b] C.V.—crystal violet; T.B.—thymol blue.

Paper chromatography was carried out on Whatman No. 1 chromatography paper by the descending technique. The solvent used was usually 1-butanol–acetic acid–water (25:6:25, v./v.). Amino acids or amino acid esters were revealed by spraying with a solution of 0.5% ninhydrin in 85% aqueous acetone. Proline was detected on paper by spraying with a solution containing 1% ninhydrin and 17% trichloroacetic acid in ethanol.[33]

High-voltage electrophoresis[55] was performed in pyridine–acetic acid buffer (pH 3.5) at a potential gradient of 40 v./cm. **Ultraviolet spectra** were taken in a Beckman model DK1 recording spectrophotometer. **Total hydrolysis** of peptides was carried out with 6 *N* HCl in sealed tubes at 110° for 22 hr.

Determination of Yields of Cleavage in Dipeptides.—The method used for the determination of yields of cleavage in dipeptide models is exemplified in the following experiment:

Solutions of Z-His-Gly (0.05 *M*) and NBS (0.05 *M*) in pyridine–acetic acid–water (1:10:19, v./v.) were prepared. To 1 ml. of the peptide solution was added 3 ml. of the NBS solution (3 moles/mole) (solution A). Another portion of the peptide solution was diluted with 3 ml. of the solvent (solution B). A 0.125 *M* solution of glycine in the same solvent was prepared (solution C). The concentration of the glycine in solution C was equal to the concentration of the peptide in solutions A and B.

After standing for 30 min. at room temperature, a 1-ml. sample of each of solutions A, B, and C was transferred into test tubes which were sealed and heated to 100° for 1 hr.

Band-like spots (length 2 cm.) of each of the unheated solutions A, B, and C were put on chromatography paper (Whatman No. 1). The spots from solutions A and B were of 50λ whereas from solution C there were spots of 10λ, 20λ, 30λ, and 40λ corresponding to 20, 40, 60, and 80% of the glycine in solutions A and B. Another chromatogram was likewise "spotted" with the heated solutions A, B, and C.

The two chromatograms were run for 12 hr. with butanol–acetic acid–water. After drying for 30 min. in a ventilated oven at 60°, the chromatograms were dipped in a 0.5% ninhydrin solution in 85% aqueous acetone and heated again for 20 min. at the same temperature. The spots formed were cut and eluted with 7 ml. of aqueous ethanol 75%. The color intensity was determined in a Klett–Summerson photoelectric colorimeter with a filter No. 56. The results are summarized in Table VI. A calibration curve was drawn for each chromatogram (Fig. 8) from which the percentage of cleavage was determined.

It should be mentioned that different calibration curves are obtained for different chromatograms owing to several factors, such as the duration and temperature of drying and developing with ninhydrin. In our experiment the freed amino acid (for which the calibration curve was drawn) underwent a similar procedure to that of the peptide in order to minimize the sources of

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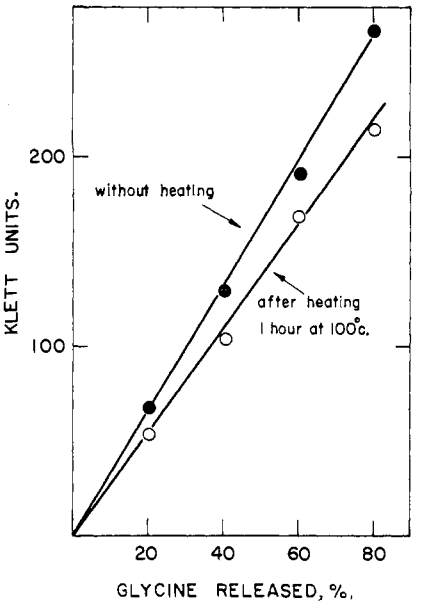

Fig. 8.—Calibration curve for the determination of yield of cleavage in N(α)-Z-histidyl-glycine.

error. Results of cleavage were found to be reproducible within ±5%.

Cleavage of a Peptide on a Chromatogram.—A spot of 100λ 0.0125 *M* Z-His-GlyOH (in 0.1 *N* HCl) was put on a strip of chromatography paper (Whatman No. 1) which was then dipped for 1 min. in a solution of bromine in petroleum ether. After airing and drying, the paper was put in a 100° oven over water vapors for 1 hr. On developing we found glycine.

TABLE VI

DETERMINATION OF CLEAVAGE IN Z-His-Gly

		—At room temp.—		—After heating—	
Solution	Volume "spotted," λ	Color intensity, K.U.[a]	Cleavage, %	Color intensity, K.U.	Cleavage, %
A	50	7	2	160	58
B	50	0	0	0	0
C	10	66	20	54	20
C	20	130	40	104	40
C	30	193	60	168	60
C	40	266	80	215	80

[a] K.U. = Klett units.

Cleavage of the His–Pro Bond in Z-Val-Tyr-Val-His-Pro-Phe OMe.—A sample of the peptide (3.65 mg., 4 μmoles) was dissolved in 4 ml. of pyridine acetate buffer (pH 3.3, solution A.). A 6 μmoles/ml. solution of NBS was prepared by dissolving 10.7 mg. of the reagent in 10 ml. of the same buffer (solution B).

To a test tube containing 2 ml. of solution A we added 2 ml. of solution B (6 moles NBS/mole peptide). The turbidity and yellow color initially formed disappeared on standing at room temperature. After 15 min. no NBS could be detected in the reaction mixture by KI–starch paper; 2 ml. of the reaction mixture was transferred into another test tube, sealed, and heated for 1 hr. at 100°. After cooling, the contents were washed into a round-bottomed flask and lyophilized; 4 ml. of water was added and 2 aliquots of the resulting solution (each of 1 ml. containing 0.25 μmole of the original peptide) were taken out. These were incubated for 20 hr. at 37°, one of them with proline iminopeptidase[33] and the other without the enzyme. In the portion which was digested with the enzyme 0.105 μmole of proline was determined colorimetrically, using acidic ninhydrin. This corresponds to 42% cleavage.

The following control experiments were performed: An aliquot of the reaction mixture (containing 0.25 μmole of peptide) which was oxidized with NBS but not heated, and an aliquot of the peptide which was not oxidized but only heated (1 hr. at 100°, pH 3.3), underwent the same procedure (with PIP). No proline was detected.

Cleavage of the His–Pro Bond in Val[5]-Hypertensin II-asp-β-amide.—The procedure described above was applied to 9 μmoles (9.3 mg.) of this peptide with 6 moles/mole of NBS. Excess of the reagent was destroyed (before heating) with imidazole. The yield of cleavage was found to be 48% after heating and 2% before heating. It should be mentioned that in this case some hydrolysis (13%) of the his–pro bond was detected in the control experiment.

Cleavage of the His–Pro Bond in Val[5]-Hypertensin I.—The procedure described above was applied to 3.3 μmoles (4.27 mg.) of this peptide with 10 moles/mole of NBS. Excess of the reagent was destroyed (before heating) with imidazole. The yield of cleavage of the his–pro bond was 45% after heating and 3% before heating (at room temperature).

Cleavage of the Tyr–Val Bond in Z-Val-Tyr-Val-His-Pro-Phe OMe.—A sample of 9.02 mg. (10 μmoles) of this peptide was treated with 60 μmoles of NBS in pyridine acetate, pH 3.3. The reaction mixture was divided into two parts: one of these was heated for 1 hr. at 100° and then lyophilized, and the other was lyophilized without heating. Both samples were treated with fluorodinitrobenzene in bicarbonate solution.[34] The resulting mixture was hydrolyzed at 110° for 16 hr. Dinitrophenol was removed by sublimation and the residual yellow material was separated by two-dimensional chromatography with valine as a standard. The amount of DNP valine was determined by elution of the spots and spectrophotometric assay. The yield of cleavage was 28% in the heated sample and 24% in the sample which was not heated. These results were corrected for hydrolytic and chromatographic losses. Correction factors were estimated from a parallel control experiment.[34]

Cleavage of the His–Phe Bond in His-Phe-Arg-Try-Gly.—A sample of 7 mg. (10 μmoles) of this peptide was dissolved in bicarbonate solution (400 μmoles/ml.) and 0.01 ml. of acetic anhydride (100 μmoles) was added under cooling (0°). After 1 hr, the reaction mixture was acidified to pH 4 with dilute HCl. Lyophilization was carried out and the resulting acetylated peptide was dissolved in 10 ml. of pyridine acetate (pH 3.2); 5 ml. of this solution was treated with 10 ml. of a 3 μmoles/ml. solution of NBS (6 moles of NBS/mole of peptide). Cleavage and dinitrophenylation were carried out as described above. The his–phe bond was cleaved in 3% yield at room temperature and in 30% yield on heating.

Cleavage of the Try–Gly Bond in His-Phe-Arg-Try-Gly and His–Leu Bond in Val[5]-Hypertensin I.—By cleavage of these bonds, a free amino acid is liberated. The method described for the determination of yields in dipeptides was applied in these cases. Yields of cleavage are given in Table III.

Cleavage of the His–Pro Bond in Sperm-Whale Myoglobin.—The sample on which the cleavage was performed was a paste precipitated by $(NH_4)_2SO_4$ which contained *ca.* 40% protein according to Dr. J. C. Kendrew. This was confirmed by colorimetric determination[66,67] of the iron content in our sample (0.12% Fe as compared with 0.31% Fe in the pure protein).[68]

A sample of 125.8 mg. of myoglobin paste[32] (2.8 μmoles) was suspended in 10 ml. of pyridine–acetate buffer (pH 3.3). A solution of NBS in the same solvent (35 mg. in 5 ml.) was added portionwise with vigorous stirring. After standing for 15 min. at room temperature, excess NBS was destroyed by adding crystals of imidazole until no more NBS could be detected on KI–starch paper. The reaction mixture was heated for 1 hr. at 85–90°. After lyophilization, 20 ml. of water was added, the pH of the mixture was adjusted to 8.5, and most of the substance was dissolved. The color of the solution was red while that of the precipitate was dark red. After centrifugation the precipitate was washed with water, and the washings combined with the solution were lyophilized again to yield a yellowish powder. By incubation with proline iminopeptidase, it was determined that the powder contained 1.48 μmoles of proline or that cleavage in 53% yield occurred.

In a parallel experiment in which the reaction mixture was not heated no proline was detected.

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(66) F. Scheibl and D. Saffer, *Z. physiol. Chem.*, **298**, 272 (1954).
(67) We thank Mrs. S. Rogozinski for performing this determination.
(68) Assuming a molecular weight of 18,000; *cf.* ref. 37.