(57%) of N-methylindole-3-propionic acid, m.p. 122–124°. Recrystallization from methanol–water afforded an analytical sample, m.p. 124–126°.

Anal. Calcd. for $C_{12}H_{13}NO_2$: C, 70.91; H, 6.45; N, 6.89. Found: C, 70.67; H, 6.30; N, 7.03.

5-Bromo-N-methyldioxindole-3-propionic Acid Lactone (XIb).—A solution of 1.63 g. (9 mmoles) of N-bromosuccinimide in 20 ml. of acetonitrile was added to a solution of 609 mg. (3 mmoles) of N-methylindole-3-propionic acid (Xb) in a mixture of 20 ml. of acetonitrile and 40 ml. of 0.4 *M* acetate buffer, *p*H 4. After 1 hour at room temperature, the acetonitrile was evaporated *in vacuo*, 50 ml. of 1.0 *N* potassium bicarbonate added and the mixture extracted twice with 25-ml. portions of ethyl acetate. The dried (sodium sulfate) extract was evaporated, and the residue crystallized by addition of ether. The yield of crude, dry lactone was 930 mg. (78%). After recrystallization from methanol, 530 mg., m.p. 161–163°, was obtained.

Anal. Calcd. for $C_{12}H_{10}NO_3Br$: C, 48.67; H, 3.40; N, 4.63; Br, 26.99. Found: C, 48.93; H, 3.51; N, 4.79; Br, 26.62.

[CONTRIBUTION FROM THE NATIONAL INSTITUTE OF ARTHRITIS AND METABOLIC DISEASES, NATIONAL INSTITUTES OF HEALTH, BETHESDA 14, MD.]

The Use of N-Bromosuccinimide and N-Bromoacetamide for the Selective Cleavage of C-Tryptophyl Peptide Bonds in Model Peptides and Glucagon[1]

BY ABRAHAM PATCHORNIK,[2] WILLIAM B. LAWSON, ERHARD GROSS[3] AND B. WITKOP

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Fifteen model peptides III–XVII (Table I) derived from indole-3-propionic acid or N-carbobenzyloxy- and N-benzoyltryptophan have been subjected to cleavage by N-bromosuccinimide and N-bromoacetamide in aqueous acetic–formic acid buffer systems as well as in 10.0 *M* lithium acetate solution of *p*H 4.0. Maximal yields of 60–90% of liberated amino acids have been obtained. Of the 28 peptide bonds present in the pancreas hormone glucagon, only the one following the single tryptophan present was cleaved with liberation of the known tetrapeptide Leu-Met-Asp-Thr.

The reactivity of proteins with brominating agents has been known for some time,[4] and the reaction of amino acids with hypobromite, hypochlorite[5] or with N-bromosuccinimide[6] has been used as a method for decarboxylation of amino acids[7,8] and for the analysis of terminal carboxyl groups on peptides and proteins.[6] On the other hand, the action of sodium hypobromite on simple peptides converts the N-terminal amino acid to a substituted nitrile,[9] an interesting method which so far has found little application. The prolonged action of excess hypohalite in all of these reactions causes many side reactions. For instance, after treatment of ovalbumin with sodium hypobromite, cystine, lysine, tyrosine and tryptophan were destroyed.[9c]

A novel, much milder and more selective use of positive halogen for the cleavage of tryptophyl peptides was suggested by the intramolecular participation reaction which occurs in the transformation of indole-3-propionic acid (I) to the lactone II of 5-bromodioxindole-3-propionic acid during oxidative bromination with N-bromosuccinimide.[1,10] This paper describes the conditions for the selective cleavage of C-tryptophyl peptide bonds in model compounds and in the polypeptide hormone glucagon.[1,11]

When carbobenzyloxytryptophan (Cbz-Try) was treated with successive increments of N-bromosuccinimide (NBS) the initial indole spectrum (Fig. 1A, I), after the addition of 1.53 moles of NBS, changed to an oxindole spectrum (Fig. 1A, II) similar to that of the bromospiroöxindole II ($\lambda\lambda_{max}^{EtOH}$ 261 (ϵ 10,300), 309 mμ (ϵ 1,630)). Figure 1B, I, II, III presents these same changes expressed as difference spectra, whereby the reference cell contains the starting material in the same concentration as the observation cell.

The ability of peptide bonds to participate in the lactonization reaction was demonstrated, *e.g.*, with N-carbobenzyloxy-L-tryptophylglycine III. Figure 2 shows that the decrease in optical density at 280 mμ and the liberation of glycine go parallel and reach a maximum after the addition of 1.5–2.5 moles of N-bromosuccinimide.

In an alcoholic aqueous acetate buffer of *p*H 4, glycine was liberated in 39% yield. The maximum yield of glycine was obtained between *p*H 3 and 5. The carbobenzyloxy group in III apparently adversely affects the cleavage,[12] since

(1) *Cf.* A. Patchornik, W. B. Lawson and B. Witkop, THIS JOURNAL, **80**, 4748, 4747 (1958).

(2) The Weizmann Institute of Science, Rehovoth, Israel. Visiting Scientist of the USPHS, 1957–1958.

(3) Visiting Scientist of the USPHS, 1958–1960.

(4) *Cf.* Z. H Skraup and R. Witt, *Monatsh.*, **28**, 605 (1907).

(5) K. Langheld, *Ber.*, **42**, 2360 (1909).

(6) E. W. Chappelle and J. M. Luck, *J. Biol. Chem.*, **279**, 171 (1957).

(7) P. A. Plattner and U. Nager, *Helv. Chim. Acta*, **31**, 2192 (1948).

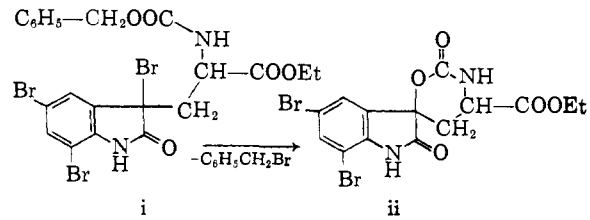
(8) J. C. Sheehan, H. G. Zachu and W. B. Lawson, THIS JOURNAL, **80**, 3349 (1958).

(9) (a) S. Goldschmidt, *et al.*, *Ann.*, **456**, 1 (1927); (b) S. Goldschmidt and K. Strauss, *ibid.*, **471**, 1 (1929); *Ber.*, **63**, 1218 (1930); (c) S. Goldschmidt, *et al.*, *Z. physiol. Chem.*, **189**, 193 (1930).

(10) W. B. Lawson, A. Patchornik and B. Witkop, THIS JOURNAL, **82**, 5918 (1960).

(11) For the application of the tryptophyl peptide cleavage reaction to proteins, *cf.* L. K. Ranachandran and B. Witkop, *ibid.*, **81**, 4028 (1959); T. Peters, Jr., *Compt. rend. trav. lab. Carlsberg*, **31**, 227 (1959).

(12) Apart from the fact that benzyl ethers are oxidized by N-bromosuccinimide [D. G. Markees, *J. Org. Chem.*, **23**, 1490 (1958)], N-carbobenzyloxytryptophan ethyl ester with N-bromosuccinimide in acetic acid gave a labile bromo compound which on recrystallization from petroleum ether lost benzyl bromide (smell). The analysis of the resulting colorless needles, m.p. 235°, agreed with the formula $C_{14}H_{12}N_2O_5Br_4$, possibly the 2-ketotetrahydrooxazine ii formed *via* the labile bromoöxindole i.



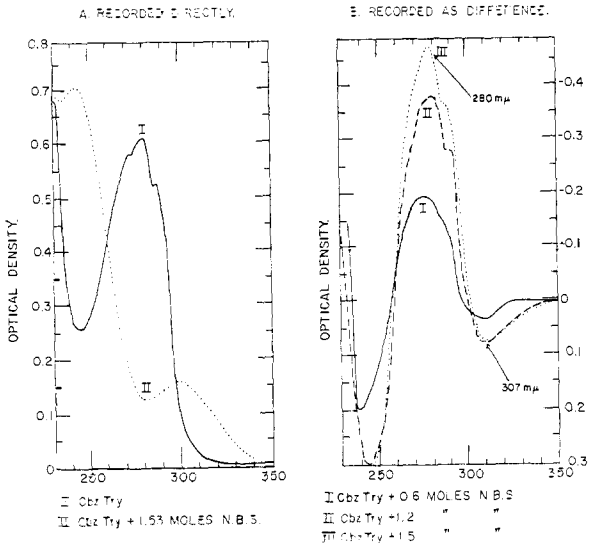


Fig. 1.—Ultraviolet spectra of Cbz-Try with N-bromosuccinimide in aqueous acetate buffer of *p*H 4.0.

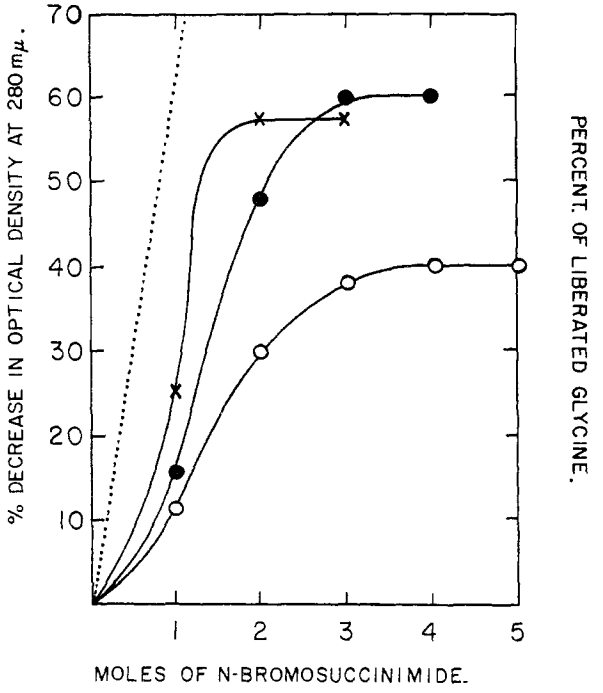
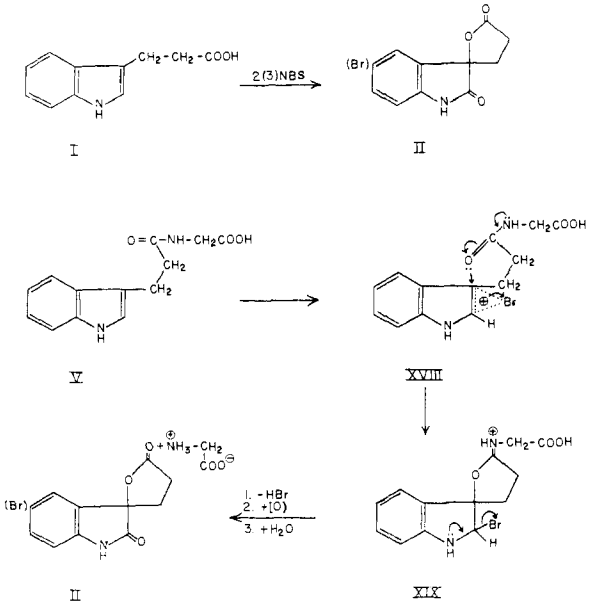


Fig. 2.—The liberation of glycine from N-benzoyltryptophylglycine (IV, -●-●-●-), indole-3-propionylglycine (V, -X-X-X-) and carbobenzyloxytryptophylglycine (III, -O-O-O-) as a function of the addition of N-bromosuccinimide to the solution of the peptides in acetate–formate buffer at *p*H 4. The decrease in optical density at 280 mμ (.....) reaches 100% after the addition of 1.53 moles of NBS.

benzoyltryptophylglycine (IV) and indole-3-propionylglycine (V) liberated glycine to the extent of 55%. The extent of cleavage in acetate buffer (Fig. 2), measured colorimetrically, when plotted *versus* the amounts of N-bromosuccinimide, passed through a maximum. After complete oxidation of tryptophan, excess N-bromosuccinimide degraded glycine. In order to avoid such secondary oxidations a protective agent had to be added. Formic acid is known to be rapidly oxidized by N-bromosuccinimide in aqueous solution to yield CO_2, HBr and succinimide.[13] Accordingly, the addition of formate to the buffer solution used for the cleavage reaction to some extent protected the liberated amino acid from further destruction (Fig. 2), even when large amounts of N-bromosuccinimide were used. Still, a large excess of N-bromosuccinimide should be avoided. In the cleavage of model peptides III, IV and V maximum yields were obtained after the addition of 2–3 moles of oxidant (Table I). Similar yields, 47–60%, have been obtained for various other model peptides (V to IX), such as the N-indole-3-propionyl derivatives of glycine, alanine, leucine, phenylalanine and proline (V–IX). Variation of the length of the indole β-side chain showed that maximal cleavage occurs when 1–5 interaction is possible: The yields for 1,5- 1,6- and 1,4-interaction decreased in the order 53% (ethyl indole-3-propionylglycinate, XI), 17% (indole-3-butyrylglycinate, XII) and 3% (ethyl indole-3-acetylglycinate, XIII).

This peptide cleavage probably proceeds by a mechanism analogous to that proposed[10] for the formation of the bromolactone II from indole-3-propionic acid (I). In the case of a peptide, such as indole-3-propionylglycine (V), interaction of the amide carbonyl group with the 3-position of the hypothetical bromonium intermediate XVIII leads to the salt of an iminolactone XIX, which after further oxidation of an intermediate indolenine[14] and hydrolysis of the unstable iminolactone[15] gives the lactone XX and free glycine. Since ethyl carbobenzyloxy-L-oxytryptophylglycinate (XIV) is



(13) M. Z. Barakat, M. F. A. El-Wahab and M. M. El-Sadr, THIS JOURNAL, **77**, 1670 (1955).

(14) Such indolenine intermediates are known to di- and trimerize easily or to add anionoid species [*cf.* B. Witkop and J. B. Patrick, THIS JOURNAL, **73**, 713 (1951)]. An unstable, non-basic addition product, m.p. 215–220° dec., λ_{max}^{EtOH} 263 mμ, $\lambda\lambda_{max}^{Nujol}$ 5.60, 5.88, 5.99, 6.20 μ, crystallized from the reaction of N-benzoyltryptophan with 3 moles of N-bromosuccinimide in aqueous acetonitrile.

(15) *Cf.* R. Kuhn and D. Weiser, *Angew. Chem.*, **69**, 371 (1957).

TABLE I

YIELDS OF LIBERATED AMINO ACIDS IN THE CLEAVAGE OF VARIOUS TRYPTOPHAN PEPTIDES UNDER FOUR DIFFERENT SETS OF CONDITIONS

Peptide	Cleavage product	Yield of liberated ninhydrin-positive cleavage products % With NBS in formate–acetate buffer *p*H 4.0	With NBA in formate–acetate buffer *p*H 4.0	With NBS in 10.0 *M* LiOAc at *p*H 4.0	With NBA in 10.0 *M* LiOAc at *p*H 4.0
Carbobenzyloxytryptophylglycine (III)	Gly	39	36	67	63
Benzoyltryptophylglycine (IV)	Gly	55	47	65	84
Indole-3-propionylglycine (V)	Gly	55	69	60	91
Indole-3-propionyl-L-alanine (VI)	Ala	53	65	75	90
Indole-3-propionyl-L-leucine (VII)	Leu	53	56	67	90
Indole-3-propionyl-D,L-phenylalanine (VIII)	Phe	47	49	70	71
Indole-3-propionyl-L-proline (IX)	Pro	60	52	72	80
Ethyl indole-3-acetylglycinate (X)	Gly-OEt	3	2	0	0
Ethyl indole-3-propionylglycinate (XI)	Gly-OEt	53	55	62	71
Ethyl indole-3-butyrylglycinate (XII)	Gly-OEt	17	10	7	10
Ethyl carbobenzyloxy-L-tryptophylglycinate (XIII)	Gly-OEt	39	28	53	53
Ethyl carbobenzyloxy-L-oxytryptophylglycinate (XIV)	Gly-OEt	13	22	15	57
Ethyl benzoyltryptophylglycinate (XV)	Gly-OEt	51	44	59	53
N-Methylindole-3-propionylglycine (XVI)	Gly	65	73	87	98
Indole-3-propionyl-*p*-nitroaniline (XVII)	*p*-Nitroaniline	0[a]			

[a] No *p*-nitroaniline detected in the ultraviolet.

cleaved in much lower yield (13%) than ethyl carbobenzyloxy-L-tryptophylglycinate (XIII, 39%), the former cannot be a major intermediate in the cleavage reaction. The possibility of an N-bromoindole intermediate was eliminated by the demonstration that N-bromosuccinimide reacts with N-methylindole-3-propionylglycine (XVI) to give glycine in good yield. The participation of secondary amino acids, *e.g.*, of proline in IX, and the intermediate formation of quaternary iminolactones poses no difficulties to judge from the smooth cleavage of indole-3-propionyl-L-proline (IX).

Substituents at the amide nitrogen with strong electron-attracting groups influence the amide $\rightleftarrows$ imidol tautomerism and prevent intramolecular participation and elimination. Thus, the *p*-nitroanilide of indole-3-propionic acid (XVII) was not cleaved by N-bromosuccinimide.

N-Bromoacetamide, which reacts much more slowly[16] than N-bromosuccinimide with indoles, cleaves peptides of indole-3-propionic acid and of acyltryptophans in yields comparable with those obtained with N-bromosuccinimide (Table I). Slight variations fall within the limit of error ($\pm 5\%$) of the analytical method. In a medium of 8–10 *M* lithium acetate at *p*H 4, however, the extent of cleavage of the model peptides with both N-bromosuccinimide and N-bromoacetamide increased markedly (Table I). This increase in yield is probably due to the greatly decreased activity of water in concentrated salt solutions.[17] Under such conditions opening of the bromonium intermediate XVIII by water competes poorly with intramolecular participation of the imidol group.

Although the cleavage in 8–10 *M* lithium acetate gives maximal yields for small peptides, markedly decreased yields are observed under such conditions in the cleavage of tobacco mosaic virus protein, lysozyme and other proteins.[11] The probable explanation is that lithium acetate, like lithium bromide, brings about the formation of maximum intramolecular hydrogen bonds which in turn makes for a more rigid packing or for a tightening of the helical screw.[18] The ensuing steric restraints make the 1,5-interaction of the tryptophyl side chains difficult or impossible. Steric inhibition of 1,5-interaction, as reflected by low cleavage yields, is especially noticeable in cyclic peptides, such as tyrocidin B.[19]

The polypeptide hormone glucagon,[20] which contains one tryptophan among 29 amino acids, was selected to demonstrate the usefulness of the C-tryptophyl cleavage reaction as a tool in peptide structure work. Treatment of glucagon with N-bromosuccinimide led to the liberation of a major new ninhydrin-positive peptide, which gave a positive platinic chloride reaction for methionine[21] and negative reactions for histidine and arginine. Its hydrolysis gave aspartic acid, threonine, methionine and leucine in equimolar amounts. The tetrapeptide, which arises from the C-terminal sequence -Try-Leu-Met-Asp-Thr has been obtained by the action of chymotrypsin[22] and trypsin[23] on glucagon. The yield of the tetrapeptide

(16) The half-life of a 0.0001 *M* solution of indole-3-propionic acid (I) in the presence of 3 moles of N-bromoacetamide is about 7 min. at room temperature, while N-bromosuccinimide reacts instantaneously. This is at variance with the *general* statement in the literature (ref. 13) that N-bromoacetamide is considerably more reactive than N-bromosuccinimide; *cf.* E. Schmidt, W. v. Knilling and A. Ascherl, *Ber.*, **59**, 1280 (1926); R. E. Buckles, R. C. Johnson and W. J. Probst, *J. Org. Chem.*, **22**, 55 (1957).

(17) *Cf.* R. A. Robinson and R. H. Stokes, "Electrolyte Solutions," Academic Press, Inc., New York, N. Y., 1955.

(18) *Cf.* W. F. Harrington and J. A. Schellman, *Compt. rend. trav. lab. Carlsberg, Ser. Chim.*, **30**, 167 (1957).

(19) E. Gross, L. C. Craig and B. Witkop, unpublished observation.

(20) W. W. Bromer, L. G. Sinn and O. K. Behrens, THIS JOURNAL, **79**, 2807 (1957). We are greatly indebted to Dr. Behrens of the Lilly Research Laboratories for his interest and coöperation.

(21) G. Toennies and J. J. Kolb, *Anal. Chem.*, **23**, 823 (1951).

(22) W. W. Bromer, L. G. Sinn and O. K. Behrens, THIS JOURNAL, **79**, 2798 (1957).

(23) W. W. Bromer, A. Staub, L. G. Sinn and O. K. Behrens, *ibid.*, **79**, 2801 (1957).

TABLE II

Compound	M.p., °C.	Solv. of crystn.[b]	Formula	Analyses, % Calcd. C	Calcd. H	Calcd. N	Found C	Found H	Found N
Ethyl indole-3-acetylglycinate (X)	87–89[a]	B	$C_{14}H_{16}N_2O_3$	64.60	6.20	10.76	64.70	6.26	10.49
Ethyl indole-3-butyrylglycinate (XII)	115–116	B	$C_{16}H_{20}N_2O_3$	66.64	6.99	9.72	66.34	6.96	9.69
Indole-3-propionyl-L-alanine (VI)	179–180	W	$C_{14}H_{16}N_2O_3$	64.60	6.20	10.76	66.44	5.01	10.70
Indole-3-propionyl-L-leucine (VII)	164	EB	$C_{17}H_{22}N_2O_3$	67.36	7.33	9.27	67.59	7.30	9.27
Indole-3-propionyl-D,L-phenylalanine (VIII)	155–156	EB	$C_{20}H_{20}N_2O_3$	71.41	5.99	8.33	71.51	5.93	8.23
Ethyl benzoyl-L-tryptophylglycinate (XV	159–160	EW	$C_{22}H_{23}N_3O_4$	67.16	5.89	10.68	67.45	6.10	10.52
Benzoyl-L-tryptophylglycine (IV)	210–212	EW	$C_{20}H_{19}N_3O_4$	65.74	5.24	11.50	65.70	5.28	11.39
Ethyl carbobenzyloxy-L-tryptophylglycinate (XIII)	116–117	EP	$C_{23}H_{25}N_3O_5$	65.23	5.95	9.92	65.03	5.91	9.77
N-Methylindole-3-propionylglycine (XVII)	112–113	EC	$C_{14}H_{16}N_2O_3$	64.60	6.20	10.76	64.85	5.91	10.42
Indole-3-propionyl-*p*-nitroanilide	216–219	ET	$C_{17}H_{15}N_3O_3$	66.01	4.89	13.59	65.85	4.79	13.20

[a] The lower homolog β-indoleacetylglycine which happens to have the same m.p. (86–87°) as the ester has been prepared by the mixed anhydride method [T. Wieland and G. Hörlein, *Ann.*, **591**, 192 (1955)] and by Schotten–Baumann acylation of glycine with 3-indoleacetyl chloride [L. E. Weller and H. M. Sell, *J. Org. Chem.*, **23**, 1776 (1958)]. [b] B = benzene, W = water, EB = ethyl acetate–benzene, EW = ethanol–water, EP = ethyl acetate–petroleum ether, EC = ethyl acetate–cyclohexane, and ET = ethanol.

in our work was low (6–14%) but the cleavage was selective. After cleavage in formate–acetate buffer of *p*H 3.5 with equivalents of N-bromosuccinimide, N-terminal analysis by the method of Sanger[24] gave the expected dinitrophenylleucine in 14% yield, and a trace (0.1%) of dinitrophenylthreonine. After cleavage with 4 equivalents of N-bromoacetamide in 70% acetic acid, dinitrophenylleucine was obtained as the sole ether-soluble dinitrophenyl derivative in 6% yield. There was no cleavage of the two tyrosyl peptide bonds.[25] In a mixture of tyrosyl and tryptophyl peptides the first 2–3 equivalents of N-bromosuccinimide liberate exclusively the amino acid next to tryptophan.[26]

Experimental[27]

All peptide esters and the *p*-nitroanilide (Table II) were prepared by the carbodiimide method. The free peptides were obtained by the controlled action of 1.5 equivalents of base. Representative examples are given for the ester and free peptide of indole-3-propionylglycine. The data for the remaining peptides are summarized in Table II.

Ethyl Indole-3-propionylglycinate (XI).—A solution of 1.14 g. of dicyclohexylcarbodiimide (5.56 mmoles) in 20 ml. of methylene chloride was added to a solution of 740 mg. (5.29 mmoles) of glycine ethyl ester hydrochloride, 0.74 ml. (5.29 mmoles) of triethylamine and 1.0 g. (5.29 mmoles) of indole-3-propionic acid in 40 ml. of methylene chloride. After 5 hr. at room temperature (magnetic stirring) the dicyclohexylurea (857 mg.) was removed by filtration, and the solution extracted with 20-ml. portions of 1.0 *N* hydrochloric acid, 1.0 *N* potassium bicarbonate and saturated sodium chloride solution. After evaporation of the methylene chloride the residue was dissolved in 35 ml. of warm benzene. After filtration from dicyclohexylurea (113 mg.; total urea = 970 mg. equal to 78% of theor.), addition of cyclohexane induced crystallization of 960 mg. (67%) of ethyl indole-3-propionylglycinate, m.p. 77–78°.

Anal. Calcd. for $C_{15}H_{18}NO_3$: C, 65.67; H, 6.61; N, 10.21. Found: C, 65.76; H, 6.52; N, 10.12.

Indole-3-propionylglycine (V).—A solution of 800 mg. (2.92 mmoles) of ethyl indole-3-propionylglycinate in a mixture of 10 ml. of methanol, 10 ml. of water and 4.4 ml. (4.08 mmoles, 50% excess) of 1.0 *N* sodium hydroxide was allowed to stand for 2 hr. at room temperature. The aqueous solution obtained by evaporation of the methanol was extracted with chloroform to remove any unchanged starting material and, after acidification, extracted with ethyl acetate. The dried (sodium sulfate) ethyl acetate extract was evaporated to give an oil which crystallized from ethanol–water. The yield of colorless needles of indole-3-propionylglycine, m.p. 159–160°, after drying at 100° *in vacuo* for 2 hr., was 510 mg. (71%).

Anal. Calcd. for $C_{13}H_{14}N_2O_3$: C, 63.40; H, 5.73; N, 11.38. Found: C, 63.39; H, 5.60; N, 11.21.

Indole-3-propionyl-L-proline (IX).—A solution of 1.89 g. (10 mmoles) of indole-3-propionic acid and 1.01 g. (10 mmoles) of triethylamine in 50 ml. of anhydrous tetrahydrofuran was cooled to −15°, and 1.08 g. (10 mmoles) of ethyl chlorocarbonate was added with shaking. After 30 min. at −15°, a solution of 1.15 g. (10 mmoles) of L-proline in 5 ml. of 2.0 *N* sodium hydroxide was added, and the flask was shaken vigorously for 5 minutes. Following removal of the tetrahydrofuran *in vacuo* at room temperature, the residue was diluted to 50 ml. with water, and the resulting solution was extracted three times with 10-ml. portions of ether. Acidification with 2.0 *N* sulfuric acid to *p*H 4 gave an oil which solidified upon scratching and cooling. The crude product was filtered, washed with ice-cold water and dried over sulfuric acid *in vacuo* at room temperature to give 2.6 g. (91%) of indole-3-propionyl-L-proline, m.p. 205°. Two recrystallizations from hot water raised the melting point to 207°.

Anal. Calcd. for $C_{16}H_{18}N_2O_3$: C, 67.11; H, 6.34; N, 9.78. Found: C, 66.82; H, 6.21; N, 9.77.

Cleavage of Peptides.—A formate–acetate buffer was prepared by adjusting the *p*H of a solution, 0.2 *M* in acetic acid and 0.2 *M* in formic acid, to 4.0. The peptide was dissolved in ethanol, and the solution was mixed with an equal quantity of formate–acetate buffer so as to give a final concentration of peptide equal to 0.01 *M*. One ml. of peptide solution was treated with the desired volume of 0.01 *M* aqueous solution of N-bromosuccinimide, and the reaction mixture was diluted to 50 ml. To ascertain the extent of cleavage, 1 ml. of the diluted solution was mixed with 1 ml. of Moore–Stein reagent,[28] the mixture was heated for 20 min. in a boiling water-bath, and after dilution with propanol–water (1:1), the developed color was read at 570 mμ in a Beckman model B spectrophotometer. The expected products (amino acids or amino acid esters) were carried through the same procedure (without N-bromosuccinimide) to determine the theoretical color yields. Peptide and reagent blanks were also run. Usually, 2 or 3 ml. of N-bromosuccinimide (corresponding to 2 or 3 moles of N-bromosuccinimide per mole of peptide) was sufficient to cause maximum cleavage. To ensure accuracy of the Moore–Stein assay it was established in all cases by paper chromatography that the expected product was the sole ninhydrin-positive product from the reaction. The

(24) H. Fraenkel-Conrat, J. J. Harris and A. L. Levy in Biochemical Methods of Analysis," Vol. 2, D. Glick, Ed., Interscience Publishers, Inc., New York, N. Y., 1955.

(25) G. L. Schmir, L. A. Cohen and B. Witkop, THIS JOURNAL, **81**, 2228 (1959).

(26) G. L. Schmir and L. A. Cohen, *ibid.*, in press.

(27) All melting points are corrected. The microanalyses were performed by Dr. W. C. Alford and associates of the Microanalytical Services Unit of this Laboratory.

(28) S. Moore and W. H. Stein, *J. Biol. Chem.*, **176**, 374 (1959).

results of the cleavage of the various peptides are summarized in Table I. Cleavages were carried out under other conditions by substitution of the appropriate medium for *p*H 4 acetate–formate buffer.

"Titration" of Tryptophan in Peptides and Proteins with N-Bromosuccinimide.—To 3 ml. of a solution of the peptide or protein (O.D. = 0.50–1.90 at 280 mμ) in acetate buffer of *p*H 4.0 (or a mixture of the buffer with alcohol or acetonitrile, if necessary for dissolution of the peptide) in a quartz cuvette was added a freshly prepared solution of N-bromosuccinimide (0.001–0.002 *M*) in water. The oxidant, in 0.10-ml. portions, was added rapidly and with thorough mixing, by the use of a Nylon "adder-mixer,"[29] and the decrease in absorbance at 280 mμ was measured after each addition. The optical density decreased with increasing amounts of N-bromosuccinimide, and increased slightly after the end-point. The maximum decrease in optical density, after correction for dilution, was multiplied by an empirical factor, 1.31, to give the optical density corresponding to the tryptophan content. The use of the factor is required because the oxidation products of tryptophan[10,11] absorb to a certain extent at 280 mμ. In the calculation of the tryptophan content, an extinction coefficient of 5,500 was used. The results of the application of this method to several proteins are shown in Table III.

TABLE III

DETERMINATION OF TRYPTOPHAN IN VARIOUS PROTEINS BY "TITRATION" WITH N-BROMOSUCCINIMIDE

Protein	Tryptophan, % Found	Reported	Reference
Chymotrypsin	5.7	5.7	31
Chymotrypsinogen	5.7	5.6	32
Lysozyme	8.3[11]	7.1 and 9.1	33
Trypsinogen	3.4[30]	3.7	34
Trypsin	3.3[30]	4.5 and 1.0	35

(29) P. D. Boyer and H. L. Segal in "The Mechanism of Enzyme Action," W. D. McElroy and B. Glass, Eds., The Johns Hopkins University Press, Baltimore, Md., 1954, p. 523.

(30) These values are taken from T. Viswanatha, W. B. Lawson and B. Witkop, *Biochim. Biophys. Acta*, **40**, 216 (1960).

(31) J. L. Weil and A. R. Buckert, *Arch. Biochem. Biophys.*, **46**, 266 (1953).

(32) Cited in J. H. Northrop, M. Kunitz and R. M. Herriott, "Crystalline Enzymes," 2nd Ed., Columbia University Press, New York, N. Y., 1948, p. 26.

(33) C. Fromageot and M. Privat de Garilhe, *Biochim. Biophys. Acta*, **4**, 509 (1950); J. C. Lewis, N. S. Snell, D. J. Hirschmann and H. Fraenkel-Conrat, *J. Biol. Chem.*, **186**, 23 (1950).

(34) B. Keil and F. Sorm, *Chem. Listy*, **48**, 735 (1954); *C. A.*, 13747 (1954).

(35) R. J. Block and D. Bolling, "The Amino Acid Composition of Proteins and Foods," 2nd Ed., Thomas, Springfield, Ill., 1951, p. 158; R. J. Block and K. W. Weiss, "The Amino Acid Handbook," C. C Thomas, Publisher, Springfield, Ill., 1956, p. 294.

The Cleavage of Glucagon.—A solution of 9 mg. (2.56 μmoles) of crystalline glucagon[20] in 25 ml. of 0.08 *M* ammonium formate–acetate buffer of *p*H 4 was prepared, and 3 ml. of the solution was "titrated" with 2×10^{-3} *M* N-bromosuccinimide. The amount of N-bromosuccinimide required to extinguish the tryptophan absorption at 280 mμ was 0.4 ml., corresponding to 2.6 moles of N-bromosuccinimide per mole of glucagon. The solutions were combined, and 6.27 ml. of N-bromosuccinimide (total N-bromosuccinimide was 5 moles per mole glucagon) was added. Solvent and buffer were removed by lyophilization, and the residue was chromatographed as a band on one sheet of Whatman No. 3 paper, using the system 1-butanol–acetic acid–water (4:1:5). Two principal bands were visible after spraying and developing with ninhydrin; the band corresponding to the expected tetrapeptide, having an R_f of about 0.27, and a fainter and thinner band, having an R_f of about 0.39, corresponding to a large peptide, possibly oxidized uncleaved glucagon, since on hydrolysis it gave a complex chromatogram of amino acids. The band containing the small peptide was eluted and hydrolyzed overnight in 6 *N* hydrochloric acid at 105°. A part of the hydrolysate (together with reference amino acids) was subjected to two-dimensional paper chromatography [phenol–water (5:1) and butanol–acetic acid], which, after development with ninhydrin, made visible spots for aspartic acid, threonine, leucine, methionine and its sulfoxide. Methionine sulfoxide was probably formed by autoxidation during the drying of the phenolic chromatogram, since unidimensional chromatograms of similar hydrolysates made with butanol–acetic acid showed practically no oxidized methionine. An amino acid analysis of the remainder of the hydrolysate by the method of Moore and Stein[28] showed that aspartic acid, threonine, methionine and leucine were present in equimolar proportions with methionine being slightly lower. From the color yields obtained in the Moore–Stein chromatography it was estimated that the yield of tetrapeptide in the glucagon cleavage was about 8%.

In separate experiments the yield of tetrapeptide was estimated by dinitrophenylation of the reaction mixture, followed by hydrolysis and paper chromatography of the resulting dinitrophenyl (DNP) amino acids. In one experiment, in which glucagon was treated with 3.5 moles of N-bromosuccinimide in aqueous formate–acetate buffer, a 14% yield of DNP-leucine corresponding to the N-terminus of the tetrapeptide, was obtained, in addition to 0.1% of DNP-Thr.[36] In a second experiment, glucagon was treated with 4.0 moles of N-bromoacetamide in 70% acetic acid, and DNP-Leu was obtained as the sole ether-soluble DNP-amino acid in 6% yield.

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