TABLE I

COMPOSITION AND CHROMATOGRAPHIC CHARACTERISTICS OF XAD AND ITS DERIVATIVES

Compound	Ribose mole/mole base 15 min.	Ribose mole/mole base 75 min.	Phosphate mole/mole base 10 min.	Phosphate mole/mole base Total	R_f in solvents[a] Iso[b]	Eth[c]	Ph[d]	But[e]
XAD	1.50	2.05		2.04[i]	0.21	0.10	0.55–.65	
XRP[f]	0.56	0.95	0.10	1.02	.21	.18	.42–.49	
XR[f]		1.16	.00	0.00	0.40–.45	.62		
X[g]		0.00	.00	.00	.62			
Adenine[f,h]	.00	.00	.00	.00	.90(.90)			0.41(.41)
5′-AMP	1.00	1.00						

[a] X derivatives were located with a Mineralight lamp (Max. emission 253 mμ). [b] Isobutyric acid, NH_3, H_2O (66:1:33). [c] Ethanol, 1 *M* ammonium acetate *p*H 7.5 (7:3). [d] Phenol, H_2O (8:2 v:v). [e] Butanol, acetic acid, H_2O (4:1:5). [f] Obtained from XAD after 10 min. hydrolysis in 1 *N* HCl at 100°. [g] Obtained from XAD after 1 hour hydrolysis with 1 *N* HCl at 100°. [h] Values in parentheses correspond to adenine isolated from 5′ AMP. [i] Initial inorganic phosphate was zero.

X passes from *p*H 6 to 12 the 366 mμ band shifts to 405 mμ (Am at *p*H 12 = 11.2 × 10^3). At *p*H above 9, XRP has another band with maximum at 854 mμ (Am = 2.87 × 10^3).

The Am values for XRP were calculated assuming the ratio X:ribose (or X:phosphate) = 1:1. When these values were subtracted from the spectrum of XAD, the differential spectrum corresponded to 1 mole of adenosine monophosphate per mole of XRP (Am at 257 mμ for AMP in XAD, calcd. = 14.9 × 10^3; reference value[3] for 5′ AMP Am = 15 × 10^3).

XAD was obtained from the Ba soluble fraction of rabbit muscle extract, which was absorbed on Dowex 1 (formate form), eluted with 4 *M* formic acid and purified by paper chromatography and electrophoresis. The average yield was 1 μmole per kg. of muscle.

(3) R. M. Bock, *et al.*, *Arch. Biochem. and Biophys.*, **62**, 253 (1956).

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SELECTIVE CLEAVAGE OF PEPTIDE BONDS. II. THE TRYPTOPHYL PEPTIDE BOND AND THE CLEAVAGE OF GLUCAGON[1]

Sir:

When the action of N-bromosuccinimide on indole derivatives such as carbobenzyloxy(Cbz)-tryptophan, acetyltryptophan, Cbz-tryptophylglycine and tryptophan-containing peptides and proteins, such as gramicidin D, chymotrypsin(ogen) and lysozyme, in dilute aqueous solutions (2 × 10^{-4} *M*) is followed *in situ* with a self-recording ultraviolet spectrophotometer, one notices the disappearance of the indole absorption at 280 mμ and the concomitant appearance of a new band at 240-250 mμ and a low-intensity band at 307 mμ. The effect of added N-bromosuccinimide on the indole spectrum is instantaneous and linear up to the consumption of *ca.* 1.5 moles/mole of tryptophan with optimal conditions at *p*H 4 in aqueous acetate buffer. Multiplication of the decrease in optical density at 280 mμ by an empirical factor (1.31) gives the extinction due to tryptophan in the peptide or protein. The "titration" of tryptophan in representative proteins yielded 5.7% for chymotrypsin,[2] 5.7% for chymotrypsinogen[3] and 7.8% for lysozyme.[4] The use of differential ultraviolet spectrophotometric recording allows the detection

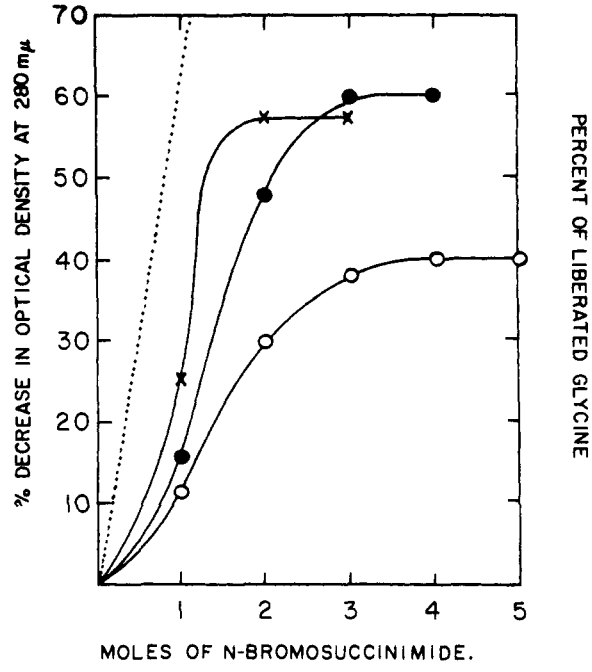

Fig 1. The liberation of glycine from N-benzoyltryptophylglycine (●), indole-3-propionylglycine (×) and carbobenzyloxytryptophylglycine (○) as a function of the addition of N-bromosuccinimide to the solution of the peptides in acetate formate buffer at *p*H 4. The decrease in optical density at 280 mμ (.....) reaches 100% after the addition of 1.53 moles of NBS.

and determination of tryptophan bound in protein on a micro scale and offers advantages over known spectral methods.[5]

After determination of the tryptophan content

(1) Presented in part before the Division of Biological Chemistry at the 134th ACS Meeting in Chicago, Sept. 7–12, 1958.

(2) Reported 5.7%: J. L. Weil and A. R. Buchert, *Arch. Biochem. Biophys.*, **46**, 266 (1953).

(3) Reported 5.6%: 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.

(4) Reported 7.1 and 9.1%: C. Fromageot and M. Privat de Garilhe, *Biochim. et Biophys. Acta*, **4**, 509 (1950), and J. C. Lewis, N. S. Snell, D. J. Hirschmann and H. Fraenkel-Conrat, *J. Biol. Chem.*, **186**, 23 (1950). *Cf.* the decrease of ε at 280 mμ in the oxidation of lysozyme with periodate: K. Maekawa and M. Kushibe, *Bull. Agricultural Chemical Society of Japan*, **19**, 28 (1955).

(5) *Cf.* W. L. Bencze and K. Schmid, *Anal. Chem.*, **29**, 1193 (1957).

of a peptide or protein by N-bromosuccinimide "titration," an additional 1-2 moles of N-bromosuccinimide per mole of tryptophan is added for the controlled cleavage[6] of the C-tryptophyl bonds. Figure 1 summarizes experiments with model peptides. The general usefulness of the method was demonstrated with glucagon,[7,8] the crystalline hyperglycemic-glycogenolytic peptide from pancreas, containing only one tryptophan among 29 amino acids.[9] N-Bromosuccinimide leads to the liberation of a major new ninhydrin-positive peptide, giving positive platinic chloride reaction for methionine[10] and negative reactions for histidine and arginine. Its hydrolysis yielded aspartic acid, threonine, methionine and leucine. This tetrapeptide, which arises from the C-terminal sequence TRY-LEU-MET-ASP-THR, has been obtained by the action of chymotrypsin[11] and trypsin[12] on glucagon. However, the cleavage of glucagon by N-bromosuccinimide is more rapid (<1 min.) and more selective than that by any known peptidase. The new method is being applied to other proteins and peptides.

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

(7) A. Staub, L. Sinn and O. K. Behrens, *J. Biol. Chem.*, **214**, 619 (1955).

(8) We are greatly indebted to Dr. O. Behrens, The Lilly Research Laboratories, for his interest and a liberal sample.

(9) W. W. Bromer, L. G. Sinn and O. K. Behrens, THIS JOURNAL, **79**, 2807 (1957).

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

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

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

(13) Visiting Scientist at the National Institutes of Health on leave of absence from the Weizmann Institute, Rehovoth, Israel.

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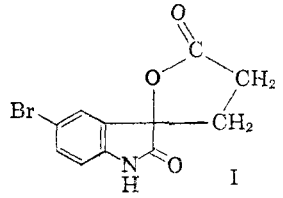
THE USE OF NEIGHBORING GROUP EFFECTS FOR THE SELECTIVE CLEAVAGE OF PEPTIDE BONDS. I. ON THE MECHANISM OF OXIDATION OF β-SUBSTITUTED INDOLES WITH N-BROMOSUCCINIMIDE[1]

Sir:

When indole-3-propionic acid was treated with 3 moles of N-Bromosuccinimide in methanolic acetate buffer of *p*H 4.0, a neutral compound was obtained as colorless needles from methanol–water, m.p. 199.5-200.5°, $C_{11}H_8NO_3Br$ (calcd.: C, 46.83; H, 2.86; N, 4.97; Br, 28.33. Found: C, 46.70; H, 2.92; N, 4.92; Br, 28.46); $\lambda\lambda_{max}^{EtOH}$ 308, 260 mμ; $\lambda\lambda_{max}^{KBr}$ 5.62 (five membered lactone), 5.76 μ (oxindole). The data suggest the structure I of a spiro lactone of a dioxindole-3-propionic acid carrying a bromine, presumably in the 5-position. When the same reaction was carried out with indole-3-propionylglycine ($C_{13}H_{14}N_2O_3$, m.p. 159-160°; found: C, 63.39; H, 5.60; N, 11.21) up to 60% of glycine was liberated.[2] Table I summarizes the yields of liberated amines from the parent peptides or amides as a function of the length of the indole-β-side chain and of the nature and substitution of the amine and shows that optimal cleavage

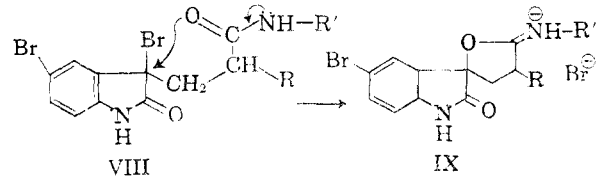


I

No.	Compound	M.p., °C.	Yield of amine	
II	Ethyl indole-3-acetylglycinate	88	3	*a*
III	Ethyl indole-3-propionylglycinate	77–78	55	*a*
IV	Ethyl indole-3-butyrylglycinate	115–116	17	*a*
V	Indole-3-propion-*p*-nitroanilide	216–219	..	*b*
VI	Ethyl N-carbobenzyloxyoxytryptophylglycinate	170–173	13	*a*
VIII	Ethyl N-carbobenzyloxytryptophylglycinate	117	39	*a*

[a] Measured colorimetrically in a Beckman Model B spectrophotometer at 570 mμ with a glycine ethyl ester standard; lactone I does not interfere in this determination. Independent chromatographic analysis proved the presence of only one ninhydrin-positive material corresponding to the liberated amine. [b] No *p*-nitroaniline detected in the ultraviolet.

occurs with the propionic acid side chain where 1,5-interaction VIII and formation of a cyclic imino



VIII IX

ether IX and hydrolysis[3] to a γ-lactone are possible. The imidole contribution is suppressed in the *p*-nitroanilide V and no cleavage occurs. 1,5-[4] and 1,6-interactions[5] have been observed in displacement reactions caused by participating amide groups and a close analogy exists in the reaction of N-bromosuccinimide with β-benzamidopropene.[6] 1,4-Interaction in indole-3-acetyl derivatives is negligible, while 1,6-interaction (IV) leads to less than 1/3 of free amine compared with III. The failure of N-bromosuccinimide to liberate much ethyl glycinate from the 2-hydroxytryptophan derivative VI, in contrast to the tryptophan derivative VII, points to a compound other than VIII as the true intermediate, possibly a β-bromoindolenine or β-bromoindolinol.[7]

The concept of selective activation of inert peptide groups by making them participants in intramolecular displacement reactions raises the

(1) Presented in part at the Fourth International Congress of Biochemistry in Vienna, Sept. 1–6, 1958.

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

(3) *Cf.* R. Kuhn and D. Weiser, *Angew. Chemie*, **69**, 371 (1957).

(4) *Cf.* O¹,2'-cyclouridine: D. M. Brown, A. Todd and S. Varadarajan, *J. Chem. Soc.*, 2388 (1956).

(5) *Cf.* O²,5'-cyclouridine: D. M. Brown, A. Todd and S. Varadarajan, *ibid.*, 868 (1957).

(6) L. Goodman and S. Winstein, THIS JOURNAL, **79**, 4788 (1957).

(7) α-Bromination is observed in non-aqueous systems: F. Troxler and A. Hofmann, *Helv. Chim. Acta*, **40**, 2161 (1957).