TABLE I

R_f VALUES AND R_f RATIOS OF *trans*-5-HYDROXY- AND *trans*-4-HYDROXYPIPECOLIC ACIDS BY THIN-LAYER CHROMATOGRAPHY[a]

Compound,[b] pipecolic acids	R_f Solvent[d] 1	2	3	4	R_f ratio[c] Solvent[d] 5	6	7	8
trans-5-Hydroxy-L-, and DL-	0.14	0.26	0.53	0.54	0.67	0.68	0.43	1.06
trans-4-Hydroxy-L-, and DL-	0.11	0.22	0.46	0.47	0.29	0.50	0.24	0.81

[a] Plates coated with silica gel, Brinkmann GF 254. [b] The values of the synthetic and natural compounds are the same. [c] R_f ratio = R_f of cyclic amino acid/R_f of valine. [d] Solvents: 1, benzene–HAc–H_2O (20:20:2); 2, *n*-butyl alcohol–HAc–H_2O (40:10:10); 3, methyl alcohol–pyridine–H_2O (180:8:40); 4, *n*-propyl alcohol–0.2 *N* citrate buffer pH 3.25 (32:18); 5, ethyl alcohol–concentrated ammonium hydroxide (9:1); 6, ethyl acetate–pyridine–H_2O (70:30:10); 7, *t*-butyl alcohol–ethyl alcohol–concentrated ammonium hydroxide (45:45:10); 8, *sec*-butyl alcohol–diethylamine–H_2O (40:5:5).

solvent was removed *in vacuo* and the residue, in 8 ml. of 50% acetic acid, was hydrogenolyzed at room temperature in the presence of 300 mg. of 10% palladium-on-charcoal. At the end of the reaction, the catalyst was removed by filtration, the solution evaporated to dryness, and the oil dried in a desiccator over potassium hydroxide. It weighed 315 mg. (91% from N-carbobenzyloxy-4- and -5-hydroxypipecolic acid methyl ester). The entire sample was dissolved in 5 ml. of citrate buffer pH 2.2 and used for column chromatography.

By use of an automatic amino acid analyzer only two major ninhydrin-positive peaks were observed which corresponded to authentic samples:

Acid	Composition, μmoles/5 ml.	Composition, %
trans-4-Hydroxypipecolic	500	28
trans-5-Hydroxypipecolic	1268	72

Neither before nor after silicic acid column chromatography could the *cis* isomers of 4- and 5-hydroxypipecolic acids be detected on the automatic amino acid analyzer.

Preparative Column Chromatography.—The solution of the reaction mixture in citrate buffer was divided into two equal portions and each portion was submitted to column chromatography on Amberlite IR-120 following the same procedure and apparatus used for the separation of 3-hydroxyprolines.[3,4] When the pump was set to deliver 11.3 ml. of eluate per tube, the *trans*-4-hydroxypipecolic acid was located in tubes 173–200 and the *trans*-5-hydroxypipecolic acid in tubes 213–255. The tubes containing each compound were pooled and the eluate desalted on a column of Dowex-50W in the acid form. The amino acid was eluted from the column with 7.0 *N* ammonium hydroxide and the latter was evaporated to dryness *in vacuo*.

Since neither free amino acid appeared to crystallize from aqueous ethanol, they were converted into their hydrochlorides.

The *trans*-4-hydroxy-DL-pipecolic acid hydrochloride crystallized from 80–90% ethanol. The crystals, dried over phosphorus pentoxide *in vacuo*, weighed 33 mg., m.p. 161–163°.

Anal. Calcd. for $C_6H_{12}NO_3Cl$: C, 39.67; H, 6.66; N, 7.71. Found: C, 38.96; H, 6.77; N, 8.09.

The *trans*-5-hydroxy-DL-pipecolic acid hydrochloride crystallized from 80–90% alcohol. The crystals, dried over phosphorus pentoxide *in vacuo*, weighed 105 mg., m.p. 192–194°.

Anal. Calcd. for $C_6H_{12}NO_3Cl$: C, 39.67; H, 6.66; N, 7.71. Found: C, 39.58; H, 6.84; N, 7.78.

The synthetic *trans*-4-hydroxy-DL-pipecolic acid behaved exactly as the known L-compound in color reactions. On paper both gave a yellow color with ninhydrin which turned to grayish brown on standing. When viewed under an ultraviolet light the ninhydrin spots exhibited a dull, brick red fluorescence. Both gave a blue color with isatin and a reddish orange color with sodium 1,2-naphthoquinone-4-sulfonate[8] characteristic of the cyclic amino acids. The synthetic and natural compounds cochromatographed on the automatic amino acid analyzer, exhibiting a unique and characteristic spectral ratio, *i.e.*, the yellow (440 mμ) and the red (570 mμ) curves were practically coincident (Fig. 1). The racemic and the natural 4-hydroxypipecolic acids cochromatographed in eight solvent systems (Table I).

The synthetic *trans*-5-hydroxy-DL-pipecolic acid and the natural amino acid likewise gave a blue color with isatin and a reddish orange with sodium 1,2-naphthoquinone-4-sulfonate. With ninhydrin on paper the 5-isomer gave a brilliant purple color which fluoresced under an ultraviolet light as bright red. The compounds cochromatographed on the amino acid analyzer giving a maximum absorption with ninhydrin at 440 mμ, following the spectral pattern of most cyclic amino acids. They also cochromatographed in eight solvent systems (Table I).

Acknowledgments.—We are greatly indebted to Dr. J. W. Clark-Lewis for samples of *cis*- and *trans*-4-hydroxy-L-pipecolic acid. The technical assistance of Mr. Edwin Wilson is gratefully acknowledged.

(8) D. Müting, *Naturwiss.*, **29**, 303 (1942); K. V. Giri and A. Nagabhushanam, *ibid.*, **39**, 548 (1952).

[CONTRIBUTION FROM THE NATIONAL INSTITUTE OF ARTHRITIS AND METABOLIC DISEASES, NATIONAL INSTITUTES OF HEALTH, PUBLIC HEALTH SERVICE, U. S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE, BETHESDA 14, MARYLAND]

Differential Oxidation of Protein-Bound Tryptophan and Tyrosine by N-Bromosuccinimide in Urea Solution

BY M. FUNATSU,[1] N. M. GREEN,[2] AND B. WITKOP

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Spectrophotometric evidence is provided for the existence of N-bromourea arising from the action of N-bromosuccinimide on 8.0 *M* solutions of urea in water. N-bromourea reacts with tyrosine or its analog at a rate >200 times more slowly than does N-bromosuccinimide in aqueous systems. This difference in reactivity permits the cleavage of tryptophan peptide bonds in protein without cleavage of tyrosine bonds.

N-Bromosuccinimide (NBS) has been widely used for cleavage of both tryptophyl and tyrosyl peptide bonds in proteins and for the approximate estimation of tryptophan by spectrophotometric titration.[3] The reactions have been carried out frequently in a solution of 8.0 *M* urea in order to increase the accessibility of the amino acid residues and to improve the yield of cleavage products. The effect of urea on the reaction of NBS with model peptides has not been studied, and the following experiments with tyrosine and tryptophan analogs demonstrate their differential reactivity under such conditions.

(1) Visiting Scientist of the U. S. Public Health Service, summer, 1963.
(2) Visiting Scientist of the U. S. Public Health Service, 1963–1964.
(3) *Cf.* B. Witkop, *Advan. Protein Chem.*, **16**, 221 (1961).

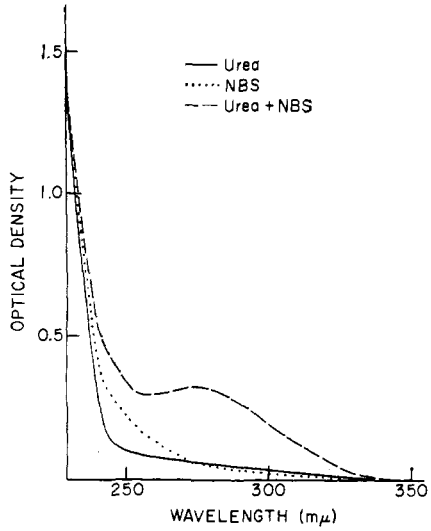

Fig. 1.—Ultraviolet absorption (- - - -) of NBS (1.2 mmoles) in 8.0 *M* urea–0.2 *M* sodium acetate buffer at pH 4.5 compared with that of urea (———) and NBS (. . . .).

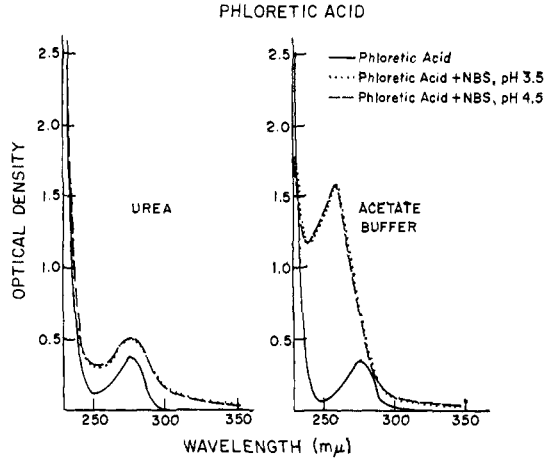

Fig. 2.—Action of NBS on phloretic acid in the presence and in the absence of urea (8.0 *M*). The slight increase in absorbance accompanying the addition of NBS in urea is due largely to the formation of N-bromourea. It was easily reversed by addition of a reducing agent, *e.g.*, mercaptoethanol.

Spectrophotometric titration of skatole or N-acetyltryptophanamide with NBS in 3.0 *M* urea at pH 4.6, 6, and 8 showed an appreciable reduction in the rate of the reaction, but no change in the spectrum of the product relative to that obtained in the absence of urea. The slower reaction suggested the possibility that the NBS had reacted with the urea to give an N-bromourea, which then replaced the NBS as the oxidizing agent. Examination of the absorption spectrum of NBS in urea supported this conclusion (Fig. 1). A new absorption band, λ_{max} 275 mμ (ε 265), appeared rapidly when solutions of NBS and urea (8.0 *M*) were mixed. At pH 3.5 the half-time for the reaction was 30 sec., above pH 5 it was too fast for measurement.

When phloretic acid, as a representative of a tyrosine derivative, was treated with NBS in 8.0 *M* urea, the reaction was much slower than in an aqueous buffer system (Fig. 2). The reaction was followed by measuring the appearance of the dibromodienone absorption band at 260 mμ.[4] With NBS in the absence of urea the reaction went rapidly to completion after a lag period during which the concentration of dibromophloretic acid

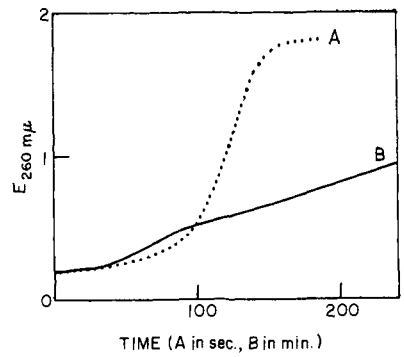

Fig. 3.—The reaction between phloretic acid (0.2 mmole) and NBS (1 mmole) in sodium acetate buffer (0.2 *M*, pH 3.5) (A, · · · ·, time scale in sec.) and in the same buffer containing 8.0 *M* urea (B, ———, time scale in min.).

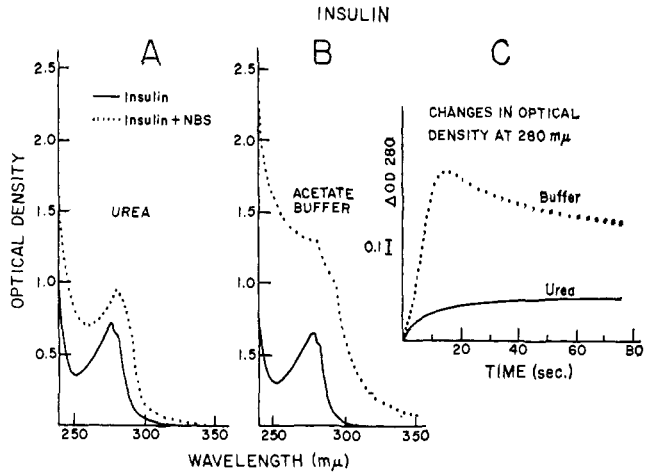

Fig. 4.—(A) The only change in optical density observed on addition of NBS to insulin in 8.0 *M* urea is due to the formation of N-bromourea, whereas in acetate buffer (B), the tyrosine residues of insulin are readily oxidized. The changes in optical density at 280 mμ with time (C) in urea (solid line) are small and correspond to the formation of an equivalent amount of N-bromourea, while in acetate buffer (dotted line) oxidation of tyrosine is instantaneous and rapid.

was building up.[5] In 8.0 *M* urea there was a similar lag period and after 10 min. the dibromodienone spectrum slowly increased, reaching 80% of the maximal value in 18 hr. There was a 250-fold difference in the maximum rate in the two experiments (Fig. 3).

As mentioned above, the rate of reaction with indole derivatives was only slightly reduced in urea; it was impossible to measure the magnitude of the effect by ordinary spectrophotometric methods since the rates were so high.

The results in Table I illustrate this difference between the behavior of tyrosine and tryptophan and show that the same difference is observable when they are present in proteins. The conversion of indoles to oxindoles is accompanied by a decrease in absorbance at 280 mμ and a slight increase at 260 mμ (line 1). If the oxindole is brominated by an excess of NBS (line 2), there is a smaller decrease at 280 and a larger increase at 260 mμ, due to an intensification and red shift of the oxindole absorption band as a result of bromination. Conversion of phloretic acid to the corresponding dibromodienone by 3 moles of NBS produced little change in absorbance at 280 but a large increase at 260 mμ. The effects observed with lysozyme and insulin confirm that

(4) G. L. Schmir and L. A. Cohen, *J. Am. Chem. Soc.*, **83**, 723 (1961).

(5) J. G. Wilson and L. A. Cohen, *ibid.*, **85**, 569 (1963).

TABLE I[a]

	Indole, μmole	Phenol, μmole	NBS, μmoles	$\Delta\epsilon_{280} \times 10^{-3}$ No urea	$\Delta\epsilon_{280} \times 10^{-3}$ 8.0 *M* urea	$\Delta\epsilon_{260} \times 10^{-3}$ No urea	$\Delta\epsilon_{260} \times 10^{-3}$ 8.0 *M* urea
N-Acetyltryptophanamide	0.5	0	0.8	−4.0	−4.0	0	0.5
Skatole	0.5	0	2.5	−2.7	−2.5	4.5	3.2
Phloretic acid	0	0.6	2.5	0.05	0	5.5	0
Lysozyme[b]	0.6	0.3	2.5	−3.4	−3.9	3.9	1.4
Insulin	0	1.0	2.5	0.1	0	2.0	0

[a] NBS, in 8.0 *M* urea or in water, was added to the appropriate compound in 2 ml. of 0.2 *M* sodium acetate buffer, pH 3.5, with or without urea. The spectra were scanned immediately and subsequent changes in absorbance at 260 and 280 mμ were followed. The values quoted in the table were those observed after 15 min. Phloretic acid in urea showed slow changes after this time (Fig. 2), but the other compounds gave steady readings. Owing to the varying ratio of NBS to tyrosine and tryptophan in the different compounds, the absorbance changes observed are only comparable on a qualitative basis. [b] Primary structure of lysozyme (Try_6, Tyr_3): R. E. Canfield, *J. Biol. Chem.*, **238**, 2698 (1963); P. Jollès, *Angew. Chem.*, **76**, 20 (1964).

urea had little effect on the decrease in absorbance at 280 but greatly reduced the increase at 260 mμ. Even after several hours, insulin showed no increase in absorbance at 260 mμ (Fig. 4), in contrast to phloretic acid, presumably because the N-bromourea is consumed by other reducing groups (-SS-, R-SR) present in the protein.

In the absence of free SH groups, NBS reacts much more rapidly with tryptophan than with any of the other oxidizable amino acid side chains. Under these circumstances NBS is specific for tryptophan, provided that minimal amounts of reagent are added and the tryptophan is not buried deeply in the protein. However, when NBS is used for cleavage of tryptophyl peptide bonds, it is necessary to use a considerable excess of reagent to obtain adequate yields of cleavage products.[6] Under these circumstances there may be some danger that tyrosyl bonds will also be cleaved. It is therefore advantageous to perform such experiments in urea solutions, both to improve the yield of cleavage products by unfolding the molecule and to reduce nonspecific cleavage of tyrosyl bonds.[7]

(6) L. K. Ramachandran and B. Witkop, *J. Am. Chem. Soc.*, **81**, 4028 (1959).

(7) Bromocarbamide (N-bromourea) has recently been introduced as a highly selective oxidant for the conversion of aldose derivatives to aldonic acid derivatives: J. Kiss, *Chem. Ind.* (London), 73 (1964).

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

Gramicidin A. II. Preparation and Properties of "*seco*-Gramicidin A"

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RECEIVED NOVEMBER 25, 1963

Treatment of "gramicidin A" with 1.5 *N* anhydrous hydrogen chloride in absolute methanol at room temperature for 1 hr. selectively cleaved one peptide bond and led to the liberation of two new NH_2-terminal residues in the same Val:Ileu ratio as the starting material which consisted of valine-gramicidin and isoleucine-gramicidin. "*seco*-Gramicidin A" has no free carboxyl. Further methanolysis of DNP-"*seco*-gramicidin A" at 75° gave a mixture of DNP-L-valylglycine and DNP-L-isoleucylglycine dipeptides.

The absence of free $-NH_2$ and -COOH groups and the mode of incorporation of aminoethanol have been the delaying factors in the structural elucidation of gramicidin A. Synge and James[2] carefully examined partial hydrolysis of gramicidin, assuming that "*ortho*-amide" bonds were involved in the binding of aminoethanol and that they would be more labile to hydrolysis than normal peptide bonds. By the action of a mixture of aqueous 10.0 *N* hydrochloric acid and dioxane on gramicidin at low temperature, various peptide fragments were obtained, most of which were recognized to have NH_2-terminal tryptophan residues. The marked heterogeneity of the products, however, permitted no definite conclusion.

We have now found that "gramicidin A," partially purified by countercurrent distribution, on treatment with anhydrous hydrogen chloride (1.5 *N*) in methanol at room temperature led to rapid and specific cleavage of only one linkage in the molecule. The product, provisionally called "*seco*-gramicidin A" contains all of the building stones of gramicidin A in addition to a new NH_2-terminal group.

(1) Associate in the Visiting Program of the USPHS, 1960–1963.

(2) R. L. M. Synge, *Biochem. J.*, **39**, 355 (1945); A. T. James and R. L. M. Synge, *ibid.*, **60**, 109 (1951).

The Cleavage Reaction.—The cleavage of gramicidin A with methanolic hydrogen chloride at room temperature was followed both by the increase in optical rotation and by the liberation of terminal amino groups. The results are summarized in Fig. 1. The specific rotation of gramicidin increased rapidly and reached a plateau after 2 hr. The concomitant liberation of primary amino groups reached 1 mole of amino group per mole of gramicidin after 80 min. and thereafter proceeded at a markedly slower rate. The free amino groups were determined before and after treatment of the cleavage products with base. As shown in Fig. 1 the NH_2 values were somewhat lower after base treatment, but the difference was insignificant. The free amino groups were determined colorimetrically after trinitrophenylation[3] of the cleavage products under slightly basic condition (pH 7.66).

If "*seco*-gramicidin" arises through acid-catalyzed N → O acyl migration, the possibility must be considered that the basic conditions of the trinitrophenylation method might reverse this process and lead to O → N acyl migration. The detection of free amino

(3) K. Satake, T. Okuyama, M. Ohashi, and T. Shinoda, *J. Biochem.* (Tokyo), **47**, 654 (1960).