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Selective Reduction of Peptide-ester Groups in Aqueous Solution III. Valine and Proline Esters

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The selective reduction of peptide-ester groups in aqueous solution with sodium borohydride was examined. For the sterically hindered esters, which resist to the reduction, in particular N-acetylglycyl-L-valine methyl ester and N-acetylglycyl-L-proline methyl ester, the reduction was examined in detail under various conditions. Consequently, these esters could be reduced almost quantitatively to the corresponding alcohols, indicating the potential utility in protein chemistry.

Many attempts have been made to establish the selective reduction of carboxyl groups in peptides and proteins to corresponding alcohols in connection with the study for the determination of carboxyl (C-) terminals of proteins.[2]

In many cases, lithium aluminium hydride and lithium borohydride have been used in tetrahydrofuran, more or less accompanied by the simultanieous reduction of peptide bonds. No reaction conditions for the selective reduction of carboxy or cester groups with lithium aluminium hydride have not yet been established even in small model peptides.[3] On the other hand, the mild reduction of model peptides with lithium borohydride at low temperature was performed without any appreciable side reactions, however more drastic conditions at the boiling temperature of tetrahydrofuran were required for the complete reduction of ester groups in esterified proteins, because they were almost insoluble in the organic solvents.[4] Under these conditions, even in small peptide esters the side reaction was unavoidable.

In the previous paper,[5] we reported that sodium borohydride in aqueous solution, better than in organic solvents,[6] was a good reagent for the selective reduction of ester groups of some model peptides, in which N-acetylglycyl-DL-alanine ethyl ester was reduced almost quantitatively, however the reduction of N-acetylglycyl-L-valine methyl ester was only in about 70%.

In order to improve the reduction condition of sterically hindered peptide esters containing valine ester or proline ester, as well as to establish a useful method for the determination of C-terminal amino acids of proteins, the reduction of some model peptide esters with sodium borohydride was reexamined in detail. Various acetyl dipeptides were subjected to the reduction followed by quantitative analysis. For the analysis of the reduction rate, two methods, trinitrophenylation[7] for the resultant amino alcohols and gas chromatography[8]

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2) J.P. Greenstein and M. Winitz, "Chem. of Amino Acids," John Wiley & Sons, New York, 1960, p. 1586.
3) J.C. Crawhall and D.F. Elliott, *Biochem. J.*, **61**, 264 (1955).
4) A.C. Chibnall and M.W. Rees, *Biochem. J.*, **68**, 105 (1958); A.C. Chibnall, J.M. Mangan and M.W. Rees, *ibid.*, **68**, 111 (1958).
5) O. Yonemitsu, T. Hamada and Y. Kanaoka, *Tetrahedron Letters*, **1968**, 3575; O. Yonemitsu, T. Hamada and Y. Kanaoka, *Chem. Pharm. Bull.* (Tokyo), **17**, 2075 (1969).
6) *cf.* H. Seki, K. Koga, H. Matsuo, S. Ohki, I. Matsuo and S. Yamada, *Chem. Pharm. Bull.* (Tokyo), **13**, 995 (1965); S. Takahashi and L.A. Cohen, *J. Org. Chem.*, **35**, 1505 (1970).
7) T. Okuyama and K. Satake, *J. Biochem.* (Tokyo), **47**, 454 (1960); K. Satake, T. Okuyama, M. Ohashi and T. Shinaoda, *ibid.*, **47**, 654 (1960).
8) W.M. Lamkin and C.W. Gehrke, *Anal. Chem.*, **37**, 383 (1965); D.L. Stalling, G. Gille and C.W. Gehrke, *Anal. Biochem.*, **18**, 118 (1967).

for the recovered amino acids, were applied.

Ten mM aqueous solution of N-acetylglycyldipeptide esters (I, II, III and IV) were treated with ten fold excess of sodium brohydride at 20°. Each aliquots were subjected to the acid hydrolysis, and the recovered amino acids were converted to the corresponding N-trifluoroacetyl amino acid *n*-butyl esters and analyzed by gas chromatography.[8] The results are shown in Fig. 1. Non-terminal glycine was recovered almost quantitatively in all cases, which shows the high selectivity in the reduction process. Phenylalanine and alanine in the C-terminal were disappeared over 95% within a few hours, however, 20% of valine and 50% of proline remined unaffected, presumably because of their steric effects.[9]

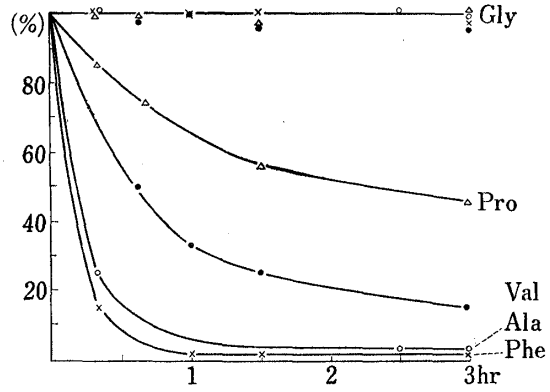

Fig. 1. Recovery of Amino Acids in the Reduction of N-Acetylpeptide-esters with Sodium Borohydride at 20°: —●— N-Acetylglycyl-L-phenylalanine ethyl ester (I); —○— N-Acetylglycyl-DL-alanine ethyl ester (II); —×— N-Acetylglycyl-L-valine methyl ester (III); —△— N-Acetylglycyl-L-proline methyl ester (IV)

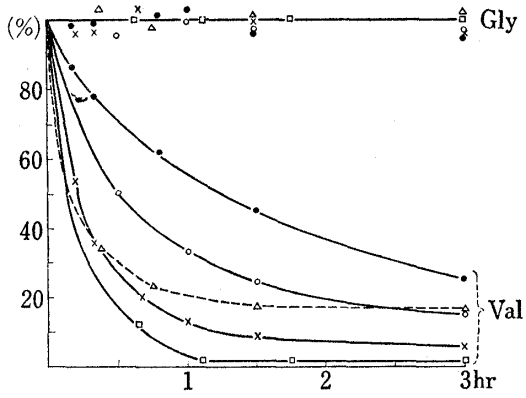

Fig. 2. Recovery of Valine and Glycine in the Reduction of III under Various Conditions: Temperature, Molar equivalents of sodium borohydride: —●— 4°, 10; —○— 20°, 10; —△— 40°, 10; —×— 30°, 10; —□— 30°, 25

In order to improve the reduction yields, the effects of the reaction temperature and the amout of the reducing agent were examined in the reduction of N-acetylglycyl-L-valine methyl ester (III).[5] As shown in Fig. 2, although the rate of the reduction of III at 40° was much faster than that at 20°, no improvement in yield was observed. The higher tem-

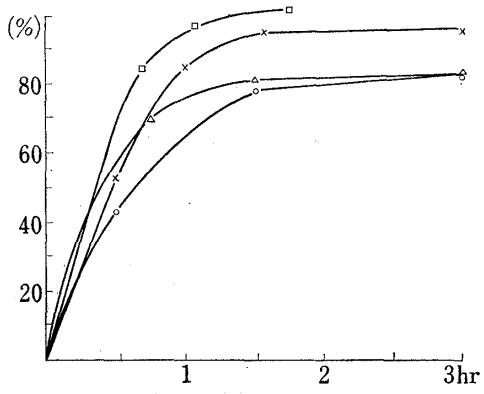

Fig. 3. Yield of Valinol Determined by Trinitrophenylvalinol: Temperature, molar equivalents of sodium borohydride: —○— 20°, 10: —×— 30°,10: —△— 40°,10; —□— 30°,25

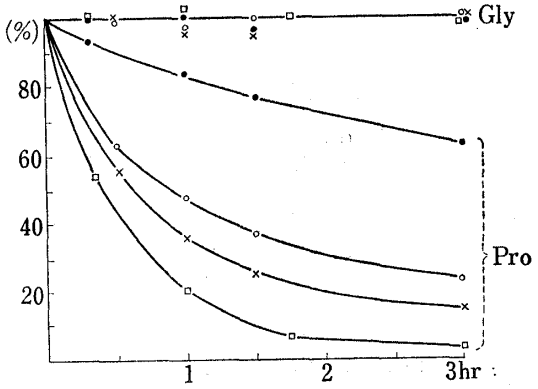

Fig. 4. Recovery of Proline and Glycine in the Reduction of IV: Temperature, molar equivalents of sodium borohydride: —●— 4°, 20; —○— 20°, 20; —×— 30°, 20; —△— 30°, 30

9) H. Seki, K. Koga and S. Yamada, *Chem. Pharm. Bull.* (Tokyo), **15**, 1948 (1967).

perature must have also accelerated the ester hydrolysis, which is the only side reaction in the reduction with sodium borohydride. The best result (98%) was obtained at 30° with twenty fold excess of the reducing agent. At the same time, the valinol formed by the reduction of III was treated with sodium 2,4,6-trinitrobenzene-1-sulfonate to yield N-(2,4,6-trinitrophenyl)valinol, which was analyzed quantitatively by its optical density at 350 nm.[7] The results in Fig. 3 are in good agreement with that in Fig. 2.

Acetylglycyl-L-proline methyl ester (IV) was also subjected to the reduction. As shown in Fig. 4, IV resisted more than III to the reduction, *e.g.*, the yield with ten fold excess of the reagent at 30° was only 50%. As the amount of sodium borohydride was increased, the reduction yield also increased remarkably, and the best result (95%) was obtained at 30° with thirty fold excess of the reagent.

Since sodium borohydride is a fairly strong base, there is a fear that the higher concentration of sodium borohydride may result in the more accerelation of the ester hydrolysis, however, this is not the case as described above. Figure 5 shows the pH change during the reduction proceeded in 0.2 and 0.3M solution of sodium borohydride, showing no difference between the two cases.

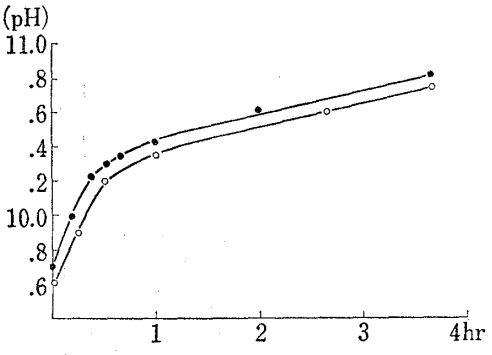

Fig. 5. pH Change during the Reduction of IV: —●— with 0.2 M solution, —○— with 0.3M solution of sodium borohydride

The results presented in this paper, in which we have shown that the large excess of sodium borohydride in aqueous solution at 30° reduced peptide esters without any side reactions, suggest a potential usefulness of this simple method for protein chemistry, especially for the determination of C-terminal amino acids of proteins. In fact, recently, we have applied this method to the C-terminal analysis of some proteins such as lysozyme and insuline, analyzed the C-terminal amino acids in almost quantitative yields. An application in the protein chemistry will be published soon.

Experimental

N-Acetylglycyl-L-phenylalanine Ethyl Ester (I)——A solution of 1.17 g of acetylglycine and 1.01 g of triethylamine in 40 ml of anhydrous acetonitrile was chilled to −5° and treated with 1.09 g of ethyl chloroformate. After stirring for 10 min at −5°, a cold solution of L-phenylalanine ethyl ester prepared from 2.3 g of L-phenylalanine ethyl ester hydrochloride and 1.01 g of triethylamine in 20 ml of acetonitrile was added. The mixture was stirred for 1 hr at 0° and then overnight at room temperature. The precipitated triethylamine hydrochloride was removed by filtration and the solvent was replaced by 50 ml of anhydrous tetrahydrofuran. The tetrahydrofuran solution was allowed to stand for 3 hr in a refrigerator. The precipitate was removed again by filtration and the filtrate was evaporated *in vacuo*. The crude product was chromatographed on a column of 20 g of silica gel. Elution with ethyl acetate gave 1.9 g of a colorless solid, which was recrystallized from benzene–*n*-hexane to give 1.5 g (51%) of colorless needles, mp 80—82°. *Anal.* Calcd. for $C_{15}H_{20}O_4N_2$: C, 61.63; H, 6.90; N, 9.58. Found: C, 61.50; H, 6.98; N, 9.47.

N-Acetylglycyl-L-proline Methyl Ester (IV)— N-Acetylglycyl-L-proline methyl ester (IV) was synthesized similarly from 1.17 g of acetylglycine and 1.98 g of L-proline methyl ester by the mixed anhydride method. Twice recrystallization from anhydrous ether gave 770 mg (34%) of colorless needles, mp 77°. *Anal.* Calcd. for $C_{10}H_{16}O_4N_2$: C, 52.62; H, 7.07; N, 12.27. Found: C, 52.71; H, 7.05; N, 12.36.

N-Acetylglycyl-DL-alanine Ethyl Ester (II) and N-Acetylglycyl-L-valine Methyl Ester (III)——Compounds II and III were synthesized as described in the previous paper.[5]

Reduction of Acetyldipeptide Esters with Sodium Borohydride. A. Analysis of the Recovered Amino Acids——A solution of 0.1 mmole of N-acetyldipeptide esters in 10 ml of water was placed in a test tube at various temperature, 0—40°, solid sodium borohydride (38—114 mg, 1—3 mmole) was added in one portion. At appropriate time intervals, 1 ml aliquots were transfered into test tubes for the analysis and excess of sodium borohydride was decomposed by the addition of 1 drop of 6N hydrochloric acid. And then 1 ml of 12N hydrochloric acid was added to each aliquot in a test tube, which was sealed with a glass stopper and

heated at 110° for 5 hr. After evaporation of hydrochloric acid, the residue was dried in an alkaline desiccator overnight.

N-Trifluoroacetyl amino acid *n*-butyl esters from the dried hydrolyzate were synthesized essentially according to the method reported by Gherke, *et al.*[8] To the dried hydrolyzate was added 2 ml of 1.2N hydrogen chloride in anhydrous methanol, and the solution was stirred for 30 min at room temperature. After removal of the solvent *in vacuo* at 60°, the residue was transesterified with 2 ml of 1.2N hydrogen chloride in anhydrous *n*-butanol at 100° for 2 hr. The butanol was removed *in vacuo* at 60°, and the residue was treated with 0.2 ml of trifluoroacetic anhydride in 2 ml of methylene chloride for 2 hr at room temperature. The trifluoroacetic anhydride and solvent were removed *in vacuo* at room temperature, the residue was dissolved in 1 ml of anhydrous tetrahydrofuran, insoluble precipitates were removed by filtration, the solvent was evaporated to dryness *in vacuo*, and finally the residue was dissolved again in 0.3 ml of anhydrous tetrahydrofuran to afford the sample solution for the analysis by gas chromatography.

Analyses were performed on a Shimazu gas chromatograph 4APF using a stainless column (300 × 0.4 cm) packed with 1.5% (w/w) neopentylglycolsuccinate on 60—80 mesh acid-washed and hexamethyldisilasane-treated chromosorb W.

B. Analysis of the Amino Alcohols——The above dried hydrolyzate was dissolved in 10 ml of water, 1 ml of the solution was trasfered into another test tube, and the water was evaporated to dryness. The residue was dissolved in 2 ml of 2% sodium bicarbonate and 1 ml of 0.7% sodium 2,4,6-trinitrophenyl-1-sulfonate, and allowed to stand at room temperature in a dark place for 3 hr. The reaction mixture was extracted with five portions of 3 ml of ether, the combined ether extracts were washed with water, dried, and the solvent was removed *in vacuo* at room temperature. The residue was dissolved in 5 ml of ethanol and 5 ml of 2N hydrochloric acid, and assayed by its absorbance at 350 nm on a Hitachi Recording Spectrophotometer EPS-3T.