O⟶*N*-Acetyl Shift of *O*-Acetylserine and *O*-Acetylthreonine. An Approach to the Separation of Serine and Threonine Fraction from Amino Acid Mixture

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In relation to the modification of proteins, the *N*→*O*-peptidyl shift of serine and threonine residues in proteins through the influence of concentrated sulfuric acid was intensively investigated by Elliott[1-3]. This technique was applied to silk fibroin[1,4], lysozyme[2], wheat proteins[5,6] and clupein[7] in order to split *N*-seryl and *N*-threonyl bonds. Anhydrous formic acid was also shown to be useful as the same purpose of the modification of proteins by Josefsson and Edman[8], but a main reaction of anhydrous formic acid with proteins was demonstrated to be *O*-formylation of serine and threonine residues[9-11].

It was reported that of the β-hydroxy-α-amino acid residues in proteins *N*→*O*-peptidyl shift which resulted with concentrated sulfuric acid or concentrated phosphoric acid, mainly occurred at the serine residues[3,4]. However no experiment on the *N*→*O*-acyl shift has been performed with synthetic *N*-acylthreonine. The kinetic studies on the reverse reaction, *O*→*N*-acyl shift, have been reported for *O*-acetylserine and *O*-acetylserylleucine[8], but that for *O*-acylthreonine or its peptide has not been investigated. In the present communication, the rate of *O*→*N*-acetyl shift of *O*-acetylserine measured by the ninhydrin reaction was compared with that of *O*-acetylthreonine at several pH's, at several temperatures and in the presence and absence of inorganic salts. The present results show that *O*-acetylthreonine is more labile than *O*-acetylserine and that it shifts to the *N*-acetyl derivative even at neutral pH. In addition, an approach to the separation of β-hydroxy-α-amino acids, serine and threonine, from the hydrolyzate of casein is described. The principle of the present method is based on the facts that only serine and threonine react with acetic acid and hydrogen chloride to give their *O*-acetyl derivatives, and that the *N*-acetyl derivatives which were derived from the *O*-acetyl derivatives by the *O*→*N*-acetyl shift can be easily separated from free amino acids by the aid of ion exchange resin.

Experimentals

***O*-Acetyl-L-serine.**—This compound was synthesized by the method of Sheehan and his coworkers[12]. M. p. 167~168°C (with decomp.). Found: C, 40.5; H, 6.22; N, 9.40. Calcd. for $C_5H_9O_4N$: C, 40.8; H, 6.17; N, 9.52%.

***O*-Acetyl-DL-threonine and *O*-Acetyl-D-threonine.**—A solution of 10 g. of DL-threonine in 100 ml. of acetic acid was saturated with hydrogen chloride at 0°C and it was kept overnight at room temperature. The solvent was removed in vacuo and the above procedures were repeated. The residues were crystallized by the addition of ether under scratching the wall of the flask. Upon recrystallization from ethanol-ether, 15 g. of *O*-acetyl-DL-threonine hydrochloride were obtained (78.4%). M. p. 155~157°C (with decomp.). On addition of 3.3 g. of triethylamine into a solution of 6.5 g. of the hydrochloride in 150 ml. of ethanol, *O*-acetyl-DL-threonine was crystallized. It war recrystallized twice from water-ethanol. Yield, 4.7 g. (87.4%). M. p. 144~145°C (with decomp.) (reported m. p.[13] 146~149°C). Found: C, 44.66; H, 6.98; N, 8.59. Calcd. for $C_6H_{11}O_4N$: C, 44.71; H, 6.88; N, 8.69%.

Similarly *O*-acetyl-D-threonine was prepared from D-threonine through its hydrochloride (m. p. 155~156°C, with decomp.) with over-all yield of 68.3%. M. p. 145~146°C (with decomp.). Found; C, 44.68; H, 7.08; N, 8.68%. $[\alpha]^{20}_{D}=-21.25$ (*c* 2, in 2 N HCl).

***N*-Acetyl-DL-threonine and *N*-Acetyl-D-threonine.**—Both the compounds were prepared by similar

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methods for the preparation of *N*-acetylserine reported previously[9]. 2.7 g. of *O*-acetyl-DL-threonine were dissolved in 50 ml. of 0.5 N ammonium hydroxide and the solution was kept at room temperature for 3 hr. It was concentrated in vacuo at 60°C and the ammonium salt of *N*-acetyl-DL-threonine was obtained as crystals. The above salt was dissolved in 100 ml. of water. The solution was placed on an acid form of Amberlite IR-120 column (7 cm^2×10 cm.) and the column was washed with 150 ml. of water until the effluent became neutral. On concentration of the effluent to a syrup and on addition of ethyl acetate, 2.5 g. of *N*-acetyl-DL-threonine were obtained. 100 ml. of ethyl acetate were added to a solution of the crude material in 4 ml. of ethanol and 2.2 g. (81.5%) of the purified material were obtained as needles. M. p. 130~132°C (reported m. p.[14] 131~133°C). Found: C, 44.62; H, 6.91; N, 8.60. Calcd. for $C_6H_{11}O_4N$: C, 44.71; H, 6.88; N, 8.69%.

N-Acetyl-D-threonine was similarly prepared. M. p. 103~104°C. $[\alpha]_D^{18}=+1.5$ (*c* 2, in water). Found: C, 43.89; H, 6.87; N, 8.34%.

Measurement of the Rate of *O*→*N*-Acetyl Shift. —The *O*-acetyl compound was dissolved in a desired buffer solution with the concentration of 0.1%. One milliliter of the solution was pipetted at scheduled intervals into a 100 ml. of volumetric flask containing 1 ml. of 1 N hydrochloric acid to stop the reaction and filled with water to the mark. The amount of the *O*-acetyl compound that remained was analyzed by the ninhydrin method of Moore and Stein[15] using 1 ml. of the diluted solution.

Isolation of Serine and Threonine from the Acid Hydrolyzate of Casein. — Twenty-five grams of casein (commercial preparation) were hydrolyzed with 250 ml. of 20% hydrochloric acid at 100°C for 24 hr. and it was concentrated to a syrup. The residue was dissolved in 300 ml. of hot water and treated with 5 g. of charcoal. The filtrate was applied to a column of Amberlite IR-120 (acid form, 9 cm^2×35 cm.) and the column was washed with 700 ml. of water to remove hydrochloric acid. The adsorbed amino acids were eluted with 500 ml. of 1.5 N ammonium hydroxide from the column and the column was washed with 300 ml. of water. The eluate was evaporated in vacuo and was dried in an evacuated desiccator. Twenty-three grams of acid free hydrolyzate were obtained. The results of amino acid analysis of the hydrolyzate by the Levy's dinitrophenyl technique[16] are listed in Table I. The hydrolyzate was suspended in 250 ml. of acetic acid and saturated with hydrogen chloride at room temperature for 48 hr., acetic acid was removed in vacuo and the above *O*-esterification procedures were repeated. The residues were dried in a desiccator over sodium hydroxide pellet for 24 hr. in vacuo and then it was dissolved in 200 ml. of water. The pH of the solution was adjusted to 8.0 with about 175 ml. of 2 N sodium hydroxide and it was diluted to 500 ml. with water. The solution was kept at room temperature for 4 hr. with an occasional addition of 2 N sodium hydroxide to keep the pH at above 8. Then the reaction mixture was treated with a column of Amberlite IR-120 (acid form, 20 cm^2× 32 cm.) and the column was washed with 1.5 l. of water to separate *N*-acetylserine and *N*-acetylthreonine from the other amino acids. The effluent and washings which contained the *N*-acetylamino acids were combined and concentrated to a syrup. It was hydrolyzed with 200 ml. of the boiling 20% hydrochloric acid for 10 hr. The concentrated hydrolyzate was dissolved in 100 ml. of water and treated with a column of Amberlite IR-120 (acid from, 7 cm^2×15 cm.) to remove hydrochloric acid as was described previously. The adsorbed amino acids were eluted with 200 ml. of 2 N ammonium hydroxide and the column was washed with 200 ml. of water. The eluate and washings were combined and it was concentrated in vacuo and decolorized with 300 mg. of charcoal. Amino acid analysis of the dried residues which amounted to 2.08 g. (9.0% of the original hydrolyzate) by the Levy's method showed that they contained 0.105 g. of aspartic acid, 0.925 g. of serine (4.0% of the original hydrolyzate), 0.836 g. of threonine (3.6%) and no other amino acids. The recoveries of serine and threonine were 80.5 and 88.8%, respectively. Aspartic acid might have been eluted with the *N*-acetylhydroxyamino acids from the second column, but could be easily removed by the treatment of a weakly basic ion exchange

TABLE I. AMINO ACID ANALYSIS OF THE HYDROLYZATE OF CASEIN

Amino acid	g. amino acid per 100 g. of hydrolyzate
Glycine	1.52
Alanine	2.77
Valine	6.57
Leucine+Isoleucine	15.92
Serine	5.01
Threonine	4.09
Methionine	3.42
Phenylalanine	4.72
Tyrosine*	4.10
Proline	10.19
Histidine**	2.69
Lysine	7.82
Arginine***	2.05
Aspartic acid	8.40
Glutamic acid	19.02
Total	98.29

* Tyrosine was estimated by the 1-nitroso-2-naphthol method[17].

** Histidine was estimated by the Knoop reaction[18].

*** Arginine was estimated by the Sakaguchi reaction[19].

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resin. In another experiment, a mixture of serine and threonine was isolated with the yield of 12.8%. Amino acid analysis of the isolated material showed 83.0% and 88.3% recoveries of serine and threonine, respectively.

Results and Discussion

Stein et al.[20] reported a simple isolation method of serine from the acid hydrolyzate of proteins by the specific precipitating reagent, *p*-hydroxyazobenzene-*p'*-sulfonate. However this reagent also forms sparingly soluble salts with the other amino acids, especially basic ones. Therefore when an attempt is made to isolate serine from usual protein hydrolyzates by the above technique, interfering amino acids have to be removed previously. For this purpose the oxazoline method reported by Elliott[21] is useful. The esters of serine and threonine form the oxazoline derivatives by the action of benziminoethyl ether. However the other amino acids give the aminoether derivatives and the former can be separated from the latter materials by vaccum distillation.

The present method seemed to be simpler than the oxazoline method and was applied to the acid hydrolyzate of casein. Eighty to ninety per cent of serine and threonine were recovered as was described in the experimental part. Sometimes β-hydroxy-α-amino acid fractions thus obtained were contaminated with small amounts of aspartic acid, but the latter amino acid was easily removed by a weak basic ion exchanger such as Amberlite IR-4B.

It is believed that the $O \rightarrow N$-acyl shift of *O*-acetyl-β-hydroxy-α-amino acids proceeds

TABLE II. OPTICAL ROTATION OF SERINE AND THREONINE OBTAINED BY THE $O \rightarrow N$-ACETYL SHIFT OF THE *O*-ACETYL DERIVATIVES*

	Before the reaction	After the reaction	Literature[22]
L-Serine	+15.5	+15.5	+15.1
D-Threonine	+16.5	+16.5	+15.0

* The specific rotation was measured in 2% solution in 2 N HCl at 25°C.

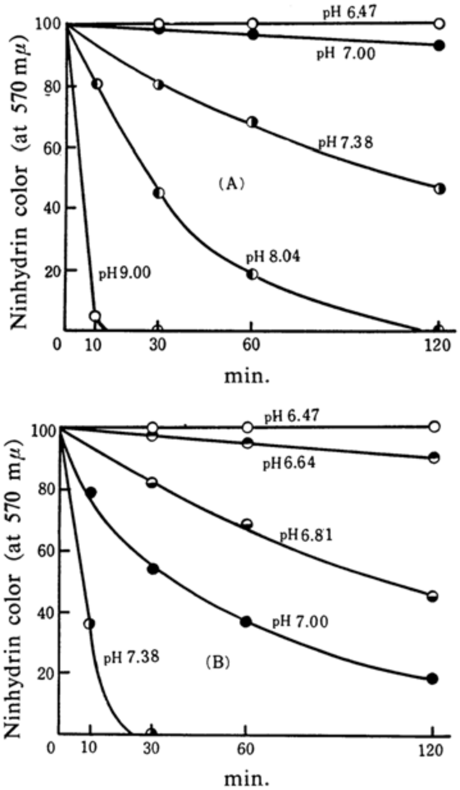

Fig. 1. $O \rightarrow N$-Acetyl shift of *O*-acetyl derivatives of L-serine (A) and DL-threonine (B) in 0.2 M phosphate buffer of various pH's at 20°C.

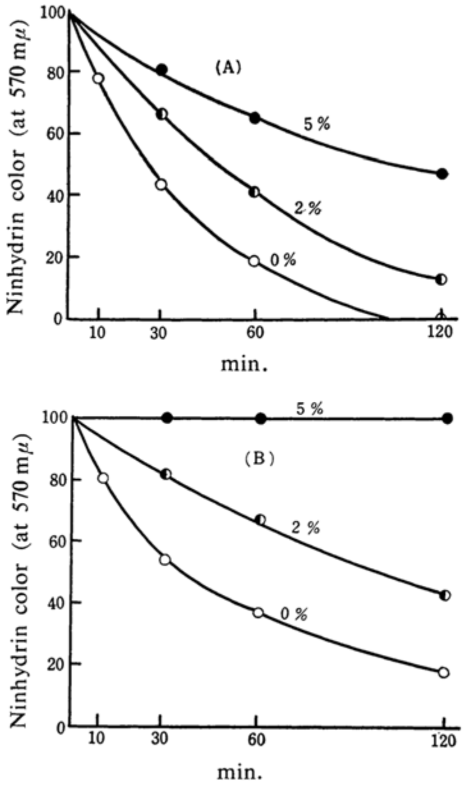

Fig. 2. Effect of sodium chloride on the $O \rightarrow N$-acetyl shift at 20°C. 0.2 M phosphate buffers of pH 8.04 and 7.00 were used for *O*-acetyl-L-serine (A) and -DL-threonine (B) respectively.

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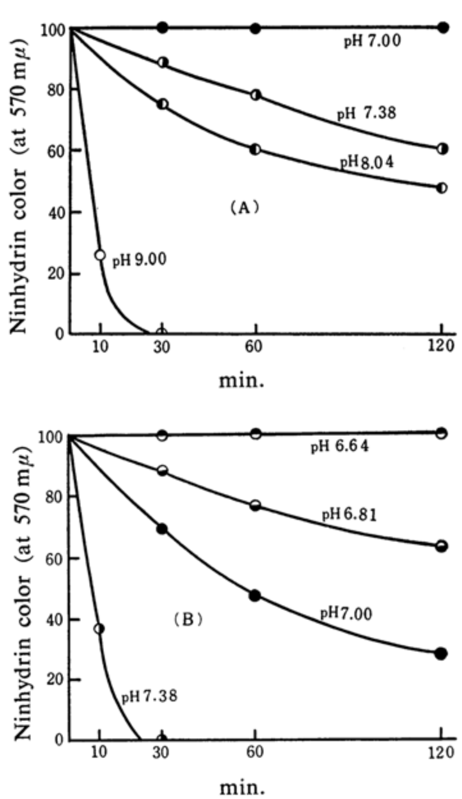

Fig. 3. *O*→*N*-Acetyl shift of *O*-acetyl-L-serine (A) and -DL-threonine (B) in 0.05 M phosphate buffer at 20°C.

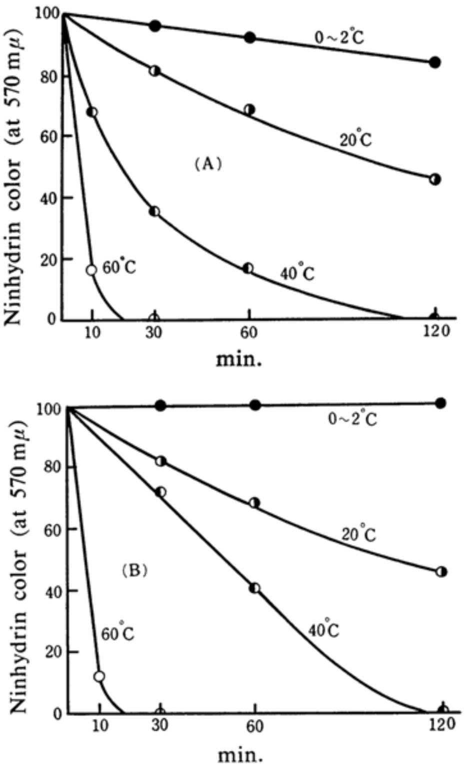

Fig. 4. Effect of temperature on the *O*→*N*-acetyl shift. 0.2 M phosphate buffers of pH 7.38 and 6.81 were used for *O*-acetyl-L-serine (A) and -DL-threonine (B) respectively.

through oxazoline derivatives as intermediates[1,3]. Consequently a possibility of racemization during the reaction must be considered. Synthetic *O*-acetyl-L-serine and -D-threonine were converted to their *N*-acetyl derivatives by incubating them at pH 8 or above, respectively, and then the acetyl group was removed by acid hydrolysis. It is clear from Table II, that no racemization occurred during *O*→*N*-acetyl shift reaction.

If the difference between the rate of the *O*→*N*-acetyl shift of *O*-acetylserine and that of *O*-acetylthreonine were remarkable, both the amino acids might be separable from each other. The rates of *O*→*N*-acetyl shift of *O*-acetylserine and *O*-acetylthreonine at various pH's were measured. As is shown in Fig. 1, the rate of the reaction of *O*-acetylthreonine is greater than that of the serine derivative under the conditions used. The above results seemed to indicate that the mutual separation of serine and threonine would be possible. Therefore a mixture of serine and threonine was treated with acetic acid and hydrogen chloride in order to convert them to their *O*-acetyl derivatives. The hydrochlorides of the *O*-acetyl derivatives thus obtained were incubated at room temperature keeping the pH at 7.0 with sodium hydroxide solution. In this case it was observed that the reaction was quite slow. The difference between the conditions of this reaction and those of model reaction shown in Fig. 1 is the presence of sodium chloride which was formed by the neutralization of the hydrochloride of the *O*-acetylamino acids with sodium hydroxide in the former experiment. Thus the effect of sodium chloride on the shift reaction was examined. As is shown in Fig. 2, sodium chloride inhibits the *O*→*N*-acetyl shift. When Fig. 3, which records the reaction rate of the shift in a low ionic strength of the buffer, 0.05 M phosphate, is compared with Fig. 1, it is clear that the increase of the ionic strength of the buffer has the accelerating effect on the reaction rate contrary to sodium chloride. Temperature increase accelerates the reaction rate and the temperature dependence is remarkable as is shown in Fig. 4. Therefore the mutual separation of serine and threonine from the mixture by the above reaction is affected by the many factors described above. The best conditions could not be found thus far.

In any rate the present experiments demonstrated that *O*-acetylthreonine was more labile than *O*-acetylserine, and even at pH 7 acetyl group of *O*-acetylthreonine migrated to its amino group. Therefore, if the shift reaction occurred at threonine bonds in proteins by the influence of concentrated sulfuric acid, it is difficult to estimate the number of the migrated threonine bond quantitatively, and precautions have to be taken to discuss the results for the modified proteins by this technique.

Summary

The reaction rate of *O*→*N*-acetyl shift of *O*-acetylserine was compared with that of *O*-acetylthreonine, and it was found that the latter was greater than the former. By utilizing the difference of the rates of the reaction of these amino acid derivatives, an attempt to separate serine and threonine from their mixture was unsuccessful owing to the fact that the shift reaction was affected by many factors, i.e., the ionic strength of inorganic salts present, pH of the reaction medium and the reaction temperature. Serine and threonine were isolated from the acid hydrolyzate of casein through their *O*-acetylation and subsequent *O*→*N*-acetyl migration with 80~90% yield.

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