Synthetic Amphoteric Polypeptide. III.
A Water-Soluble Amphoteric Polypeptide

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In the preceding paper[1] it was reported that the synthetic polypeptides, copoly-1:1:3-(DL-glutamic acid, DL-lysine, DL-phenylalanine) and copoly-1:1:1.9-(L-glutamic acid, L-lysine, L-leucine), are insoluble in water in the neutral pH region. On the other hand, in view of the great solubility of polyserine[2] in water, it may be considered that the water-solubility of a number of proteins, such as albumins, should be contributed largely by the serine and threonine residues in these proteins. To confirm this, it is desired to obtain an amphoteric polypeptide which contains any amount of serine residue as a component and is soluble in water over a whole pH range. In order to know how many serine residues are required to make the polypeptide freely soluble in water, four copolymers composed of various compositions of L-glutamic acid and DL-serine were synthesized and their solubilities were tested. While α-poly-L-glutamic acid is sparingly soluble in water in its free acid form, it was found that the copoly-(L-glutamic acid, DL-serine) was freely soluble in water, if the content of serine residue becomes more than about 50 mole per cent; hence the synthesis of copoly-1:1:2-(L-glutamic acid, L-lysine, DL-serine), as a water-soluble amphoteric polypeptide, was investigated.

The four copolymers composed of L-glutamic acid and DL-serine, the molar ratios of which were 4:1, 3:2, 2:3 and 1:4, and copoly-1:1:2-(L-glutamic acid, L-lysine, DL-serine) were obtained from four corresponding copoly-(γ-benzyl L-glutamate, *O*-benzyl-DL-serine) and copoly-1:1:2-(γ-benzyl L-glutamate, ε-*N*-carbobenzoxy-L-lysine, *O*-benzyl-DL-serine), respectively, which were prepared by the *N*-carboxy anhydride method as described in the preceding paper[1]. In the debenzylation reaction of these copolymers some attention should be paid to the occurrence of side reaction, because in most cases some degree of ester linkages may be formed during the reaction between hydroxyl group of serine residue and carboxyl group of either glutamic acid residue or of an acid used as solvent.

The debenzylation of copoly-(γ-benzyl L-glutamate, *O*-benzyl-DL-serine) was carried out in dioxane with dry hydrogen bromide, and in acetic acid with phosphonium iodide. The reaction with hydrogen bromide in dioxane gave the products in theoretical amount all insoluble, but swellable, even in hot aqueous potassium hydrogen carbonate. Poly-L-glutamic acid and poly-DL-serine, prepared under similar conditions from poly-γ-benzyl-L-glutamate and poly-*O*-benzyl-DL-serine, were readily soluble in aqueous potassium hydrogen carbonate and in water respectively. Those insoluble products obtained from the copolymers could be converted into soluble forms by treating with aqueous triethylamine (pH 10) at room temperature overnight. Under this condition, no change in the content of amino-nitrogen of poly-DL-serine and in the value of specific rotation of poly-L-glutamic acid was observed. These results indicate clearly that the inter- and/or intra-chain ester linkages were broken by a fairly mild condition, such as aqueous triethylamine, without accompanying any hydrolysis of the peptide bond or racemization.

The reaction of phosphonium iodide on the four copolymers in glacial acetic acid gave the products, the yields of which somewhat exceeded the theoretical amounts, partly insoluble in aqueous potassium hydrogen carbonate. These products could also be converted into perfectly soluble forms by treating with aqueous triethylamine, and may be considered as polymers the hydroxyl groups of which had been partly esterified both by acetic acid used as solvent and by the carboxyl groups of glutamic acid residues.

The final products obtained by these

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[1] H. Yuki, S. Sakakibara and H. Tani, This Bulletin, **29**, 654 (1956).

[2] K. Okawa and H. Tani, *J. Chem. Soc. Japan, Pure Chem. Sec.* (*Nippon Kagaku Zassi*), **75**, 1199 (1954).

two different processes were all soluble in aqueous potassium hydrogen carbonate and copoly-2 : 3- and copoly-1 : 4-(L-glutamic acid, DL-serine) were soluble even in water. When dried in vacuo at an elevated temperature, these polypeptides showed a slight tendency to become insoluble in aqueous potassium hydrogen carbonate, but could readily be returned soluble by heating in this medium.

It was found that a pyrrolidone ring may be formed by the ring-closure of glutamyl residue, existing at the amino-end of these polymer molecules, because no free amino-nitrogen was detected in poly-L-glutamic acid prepared by this method.

The optical rotations of these copoly-(L-glutamic acid, DL-serine) were measured in their anionic forms and the intrinsic rotation of L-glutamic acid residue was calculated for each polypeptide, as the serine residues might be regarded to have no contribution to the optical rotation of the polymer since they were racemic. These values were almost constant (−50° to −57°) and differed from that of poly-L-glutamic acid (−88°).

When the debenzylation of copoly-1 : 1 : 2-(γ-benzyl L-glutamate, ε-*N*-carbobenzoxy-L-lysine, *O*-benzyl-DL-serine) was carried out in glacial acetic acid with phosphonium iodide, it gave a cross-linked product insoluble, but swellable, in various aqueous and organic media, and this product could not be turned soluble by treating with triethylamine. The debenzylation of this copolymer was effected in dichloroacetic acid by using the theoretical amount of phosphonium iodide satisfactorily without formation of any cross-linkage. From the resulting polypeptide hydriodide, free copoly-1 : 1 : 2-(L-glutamic acid, L-lysine, DL-serine) was obtained by treating repeatedly with triethylamine.

This amphoteric polypeptide was found to be soluble in water over the whole pH range. Intrinsic viscosity of its solution in water was 0.04, and such small [η] value may reflect rather low molecular weight. In view of the known lability of serine peptide to acid, the molecular weight depression, if any, might be due to the action of hydrogen iodide during the debenzylation process.

Dependence of specific viscosity on pH is shown in Fig. 1. The viscosity was minimum at the isoelectric point (pH 7), and increased gradually on both sides. This behavior is typical of amphoteric polyelectrolyte.

Dependence of specific rotation on pH was shown in Fig. 2. The specific rotation was *laevo*-rotatory over whole pH range; the value was constant (−32°) from pH 3.6 to pH 11.3, and increased rapidly on both sides. This specific rotation *vs* pH curve is very similar to that of serum albumin[3] and of ovalbumin[4].

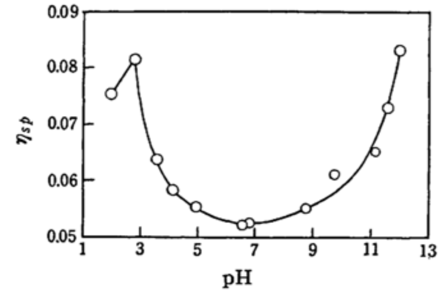

Fig. 1. Relation between specific viscosity of copoly-1:1:2-(L-glutamic acid, L-lysine, DL-serine) and pH.

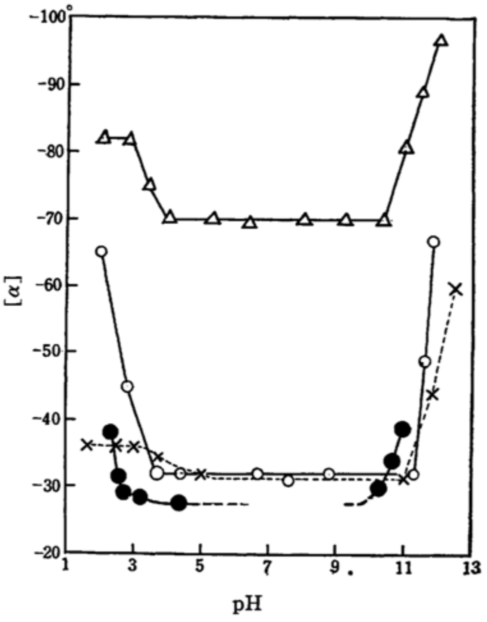

Fig. 2. Relation between specific rotation and pH.
○, Copoly-1:1:2-(L-glutamic acid, L-lysine, DL-serine); ●, Copoly-1:1:1.9-(L-glutamic acid, L-lysine, L-leucine)[1]; △, Serum albumin[3]; ×, Ovalbumin[4].

The optical rotatory behavior of synthetic polypeptide was discussed recently

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3) M. A. Colub and E. E. Pickett, *J. Polymer Sci.*, **13**, 427 (1954).

4) J. T. Edsall and J. F. Foster, *J. Am. Chem. Soc.*, **70**, 1860 (1948).

both theoretically[5] and experimentally[6-8], and the workers concerned concluded that the chain configuration of the polymer contributes a great deal to this behavior. In the polypeptides obtained here, serine residue is DL-form and hence the problem of chain configuration would be rather complex[9], but it is very interesting that the behavior concerning optical rotation of this synthetic amphoteric polypeptide is quite similar to that of albumins; their specific rotations are *laevo*-rotatory in the whole pH range and have minimum constant values over wide ranges of pH around their isoelectric points. This behavior may correspond to the observation of P. Doty and his collaborators[8], who found that the specific rotation of poly-L-glutamic acid is constant until about 40% of the side-chain carboxyl group are dissociated. The reason for the very interesting result that the intrinsic rotation of glutamic acid residue in copoly-(L-glutamic acid, DL-serine) is almost constant in spite of the great difference of glutamic acid content and differs from that of poly-L-glutamic acid, remained obscure.

Although the viscosity and optical rotation could be measured on copoly-1 : 1 : 1.9-(L-glutamic acid, L-lysine, L-leucine)[1] only at both sides of the neutral pH region, because of its water-insolubility in the neutral pH region, the object of the measurements of these properties could now be attained completely on copoly-1 : 1 : 2-(L-glutamic acid, L-lysine, DL-serine) over the whole pH range. Some physicochemical properties of these synthetic amphoteric polypeptides are very similar to those of some natural proteins, and the latter, copoly-1 : 1 : 2-L-glutamic acid, L-lysine, DL-serine), may be regarded as the first member of 'model albumin', if the former, copoly-1 : 1 : 1.9-(L-glutamic acid, L-lysine, L-leucine)[1], together with copoly-1 : 1 : 3-(DL-glutamic acid, DL-lysine, DL-phenylalanine)[1], might be called 'model globulin'.

Experimental

Poly-γ-benzyl L-glutamate, poly-*O*-benzyl-DL-serine, and copoly-(γ-benzyl L-glutamate, *O*-benzyl-DL-serine).—Six different mixtures of *N*-carboxy-γ-benzyl L-glutamate anhydride[1] and *N*-carboxy-*O*-benzyl-DL-serine anhydride[2] were prepared: their molar ratios were 1:0, 4:1, 3:2, 2:3, 1:4 and 0:1, and the total amount was 10 m mol. in each mixture. Ten percent solution of each mixture in chlorobenzene was heated in a sealed glass tube for 2 hours at 90°C and then for one week at 40°C. The polymer mas precipitated by adding petroleum ether, washed with methanol and ether and dried. The results are shown in Table I.

Polymer G was soluble in dioxane, tetrahydrofurane, chloroform, nitrobenzene, pyridine and dichloroacetic acid, and was insoluble in water, ethanol and glacial acetic acid. While the solubility of polymer I was similar to that of polymer G polymer, II was soluble only in dichloroacetic acid and in hot dioxane, and polymer III, IV and S were soluble only in dichloroacetic acid.

TABLE I

Polymer	Molar ratio A/B	Appearance of polymerizing solution	Yield %		Anal. %			Formula*
					C	H	N	
G	1 : 0	viscous solution	97	Calcd.			6.5	$[A]_n$
				Found			6.4	
I	4 : 1	viscous solution	97	Calcd.	66.1	6.0	6.6	$[4A, B]_n$
				Found	65.7	6.4	6.6	
II	3 : 2	transparent gel	93	Calcd.	66.4	6.1	6.9	$[3A, 2B]_n$
				Found	65.9	6.4	7.0	
III	2 : 3	opalescent gel	94	Calcd.	66.8	6.1	7.2	$[2A, 3B]_n$
				Found	65.8	6.2	7.2	
IV	1 : 4	opalescent gel	92	Calcd.	67.3	6.2	7.6	$[A, 4B]_n$
				Found	66.4	6.2	7.6	
S	0 : 1	opalescent gel	91	Calcd.			7.9	$[B]_n$
				Found			8.0	

*A : γ-Benzyl L-glutamate residue, $C_{12}H_{13}NO_3$.
B : *O*-Benzyl-DL-serine residue, $C_{10}H_{11}NO_2$.

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5) D. D. Fitts and J. G. Kirkwood, ibid., **78**, 2650 (1950); *Proc. Natl. Acad. Sci.*, **42**, 33 (1956).

6) E. R. Blout and M. Idelson, *J. Am. Chem. Soc.*, **78**, 497 (1956).

7) P. Doty and J. T. Yang, ibid., **78**, 500 (1956).

8) P. Doty, A. Wada, J. T. Yang and E. R. Blout, *J. Polymer Sci.*, **23**, 851 (1957).

9) A. Elliott, W. R. Hanby and B. R. Malcolm, *Nature*, **178**, 1170 (1956); E. R. Blout, P. Doty and J. T. Yang, *J. Am. Chem. Soc.*, **79**, 759 (1957).

TABLE II

Product	Reaction condition		Yield** %		Anal.** %			Formula***
	Polymer/ Dioxane mg./ml.	Reaction time* min.			C	H	N	
G′	500/15	60	101	Found			10.5	$[A']_n$
				Calcd.			10.9	
I′	400/15	25	100	Found	44.5	5.9	11.3	$[4A', B']_n$
				Calcd.	45.8	5.5	11.6	
II′	180/12	20	106	Found	41.3	5.8	11.8	$[3A', 2B']_n$
				Calcd.	44.9	5.6	12.5	
III′	300/13	15	100	Found	41.2	6.0	12.7	$[2A', 3B']_n$
				Calcd.	43.9	5.6	13.5	
IV′	400/15	25	101	Found	40.0	5.5	13.7	$[A', 4B']_n$
				Calcd.	42.8	5.7	14.7	
S′	400/10	20	94	Found			15.2	$[B']_n$
				Calcd.			16.1	

* The time hydrogen bromide passed through; during this period the polymer was completely precipitated.

** Each sample was dried over phosphorus pentoxide at 108°C/2 mmHg for five hours, but the data show that even after this treatment, the polymer may contain about 3～6% of water.

*** A′; Glutamic acid residue, $C_5H_7O_3N$. B′: Serine residue, $C_3H_5O_2N$.

TABLE III

Polypeptide	Intrinsic rotation of glutamic acid residue $[\alpha_{glu}]_D$	Insoluble pH range	Amino-nitrogen %
G′	−88°*(at pH 8.3, 10.4)	below 3.9	0.00
I′	−50° (at pH 9.6)	below 3.5	0.04
II′	−56° (at pH 9.8)	below 1.1	0.18
III′	−57° (at pH 9.8)	none	0.21
IV′	−51° (at pH 10.1)	none	0.18
S′	0°	none	0.19 (at pH 6 and 10.1)

* Reported value6): −83°.

Poly-L-glutamic acid, poly-DL-serine and copoly-(L-glutamic acid, DL-serine).—(*a*) *Debenzylation with hydrogen bromide in dioxane.* To a mixture of the polymer and dioxane, dry hydrogen bromide was passed at room temperature, the solvent was distilled off in vacuo, and the residue was washed successively with anhydrous ether, methanol, and ether. After drying, white powder was obtained. The results are shown in Table II.

While polypeptide G′ was soluble in aqueous potassium hydrogen carbonate and polypeptide S′ was soluble in water, the others were insoluble but swellable in aqueous potassium hydrogen carbonate. A suspension of each insoluble copolymer in water containing an equivalent amount of potassium hydrogen carbonate was changed to a clear solution by adding a few drops of triethylamine and on standing overnight at room temperature. The optical rotation, pH and amino-nitrogen value were determined on each solution. No change in these values was observed even after standing the solution for two weeks. In Table III the value of $[\alpha_{glu}]_D$, the intrinsic rotation of L-glutamic acid residue, was calculated based on the cencentration of glutamic acid residue contained in the solution, but not on the concentration of polymer itself.

(*b*) *Debenzylation with phosphonium iodide in glacial acetic acid.*—About 1.4 g. of weighed polymer sample (I, II, III or IV) was suspended in 75 ml. of glacial acetic acid. To this mixture 4～5 g. phosphonium iodide was added in portions at 65～65°C during 4.5 hours with passing through dry hydrogen. The solvent was evaporated in vacuo under a stream of dry hydrogen, and the remaining precipitate was washed thoroughly with dry ether and ethanol, and dried. A white powder was obtained on each polymer, the yield of which was 106% for I″; 117% for II″, 117% for III″, and 138% for IV″, where I″, II″, III″ and IV″ are the corresponding products from the polymers I, II, III and IV, respectively. These were all insoluble in water and in methanol, and I″ and IV″ are mostly, while II″ and III‴ are partly soluble in aqueous potassium hydrogen carbonate. After dissolution was attained by standing in aqueous triethylamine for several hours, each solution was concentrated in a vacuum desiccator over conc. sulfuric acid. To the syrups thus obtained, acetone and a few drops of acetic acid were added and the precipitated polymers were washed with acetone, methanol and ether. After drying, the purified polymers were obtained in 85~95% of the theoretical yields. The final products of I'' and II'' were insoluble in water and soluble in aqueous potassium hydrogen carbonate, and III'' and IV'' were soluble in water. All the solutions gave strong positive biuret reactions. After drying at 108°C/2 mm.Hg over phosphorus pentoxide for five hours, the samples became insoluble, but could readily be turned soluble by heating in aqueous potassium hydrogen carbonate. The observed analytical data were as follows— I'': C, 44.5; H, 5.9; N, 11.3. II'': C, 43.7; H, 6.3; N, 12.1. III'': C, 43.2; H, 6.4; N, 13.2. IV'': C, 44.8; H, 5.7; N, 14.5%.

Copoly-1 : 1 : 2-(γ-benzyl L-glutamate, ε-N-carbobenzoxy-L-lysine, *O*-benzyl-DL-serine) (V).—A mixture of 1.315 g. (5 m mol.) or γ-benzyl N-carboxy-L-glutamate anhydride, 1.530 g. (5 m mol.) of α-*N*-carboxy-ε-*N*-carbobenzoxy-L-lysine anhydride and 2.210 g. (10 m mol.) of *N*-carboxy-*O*-benzyl-DL-serine anhydride in a mixture of 47 ml. of chlorobenzene and 3 ml. of pyridine was sealed in a glass tube. The mixture was heated at 100°C until dissolved, and then allowed to stand for several days at 40°C. The solution gradually increased in viscosity and finally became opalescent gel. The polymer, precipitated on addition of petroleum ether, was thoroughly washed with methanol. After being reprecipitated from chloroform and methanol, the polymer was obtained as a white powder. Yield 3.85 g. (92% of the theor.)

Anal. Found: C, 65.4; H, 6.6; N, 8.3. Calcd. for $[(C_{12}H_{13}NO_3), (C_{14}H_{18}N_2O_3), 2(C_{10}H_{11}NO_2)]_n$: C, 66.1; H, 6.4; N, 8.4%.

The polymer was soluble in chloroform, dioxane, tetrahydrofuran, pyridine and dichloroacetic acid, partially soluble in acetone, and was insoluble in alcohol, ether, petroleum ether and water. It was partially soluble, with swelling, in hot acetic acid.

Copoly-1 : 1 : 2-(L-glutamic acid, L-lysine, DL-serine).—A solution of 3.00 g. of polymer V in 24 ml. of dichloroacetic acid was treated with 5 g. of phosphonium iodide. The reaction product was precipitated by the addition of about 100 ml. of dry ether, and the precipitate was washed repeatedly with dry ether, and then 2.4 g. of a yellowish powder was obtained. This powder was dissolved in methanol and a small amount of insoluble orange-brownish precipitate, included in the product, was almost all removed by centrifugation, and further, the trace of this matter remaing in the solution was precipitated by the addition of a small amount of ether. To the almost colorless clear solution, thus obtained, was added a solution of 0.9 g. of triethylamine in methanol; the resulting precipitate was washed repeatedly with methanol containing a small amount of triethylamine until no iodide ion was detected in the polymer by Beilstein test and by Volhardt method. The free polypeptide was obtained as a white powder. Yield 1.07 g. (68% of the theor.)

Anal. Found: C, 46.4; H, 6.1; N, 15.4; Amino-N, 3.0. Calcd. for $[(C_5H_7NO_3), (C_6H_{12}N_2O), 2(C_3H_5NO_2)]_n$: C, 47.3; H, 6.8; N, 16.2; Amino-N, 3.2%.

This free polypeptide was soluble in water, dilute mineral acids and glacial acetic acid, slightly soluble in methanol and ethanol, and insoluble in acetone and ether. The aqueous solution showed a strong biuret reaction. The viscosities at 25°C and the optical rotations were measured on the solutions having the same concentration of polymers, i.e. 0.464 g./100 ml., and various pH values made by appropriate amounts of hydrochloric acid or sodium hydroxide.

Summary

1. Preparation of four copoly-(L-glutamic acid, DL-serine), the molar ratios of the two amino acid residues were 4 : 1, 3 : 2, 2 : 3 and 1 : 4, by debenzylation of the corresponding four copoly-(γ-benzyl L-glutamate, *O*-benzyl DL-serine), was attempted.

2. The inter- and/or intra-molecular ester-linkages, formed during the debenzylation reaction between hydroxyl group of serine residue and carboxyl group of glutamic acid residue, was broken by treating with aqueous triethylamine without accompanying racemisation or hydrolysis of the peptide bond.

3. Copoly-(L-glutamic acid, DL-serine) became freely soluble in water when the content of serine residue was more than about 50 mole per cent.

4. Copoly-1 : 1 : 2-(L-glutamic acid, L-lysine, DL-serine), prepared by debenzylation from the parent copolymer, was soluble in water over a whole pH range, and the pH dependence of viscosity and of optical rotation was measured on its solutions. It was found that these properties are very similar to those of natural albumins.

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