*Synthetic Amphoteric Polypeptide. II. Synthesis and Some Properties of a Reversibly Contractile Amphoteric Polypeptide**

By Heimei YUKI, Shumpei SAKAKIBARA, Toshio TAKI and Hisaya TANI

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K. H. Meyer[1] has suggested that the contraction and relaxation of muscle may be attributed to the electrostatic attraction and repulsion of ionised ammonium and/or carboxyl groups in the molecule of myosin. Recently W. Kuhn and A. Katchalsky et al.[2] have synthesized vinyl-type polyanions as such mechanochemical systems. Studies concerning these substances are, of course, very useful and instructive, but a study of polypeptide-type polyampholyte would be more desirable. In this paper the synthesis of a three-dimensional amphoteric polypeptide network composed of L-glutamic acid, L-lysine and DL-cystine residues, and some properties concerning its reversible contractility are described.

Preparation

A mixture of N-carboxy anhydrides of ε-N-carbobenzoxy-L-lysine, γ-benzyl L-glutamate and DL-cystine, the molar ratio of which was 26 : 26 : 1, was polymerized in dry chlorobenzene-pyridine mixture. Reduction of the polymer by phosphonium iodide gave the hydriodide of a linear polypeptide consisting of L-glutamic acid, L-lysine and DL-cysteine residues. The amino acid composition of this polypeptide was approximately the same as that derived from the starting monomer mixture. This linear polypeptide hydriodide was soluble in water, methanol and ethanol, and gave positive biuret and nitroprusside reactions. The free polypeptide had an isoelectric point at pH 7.4 and was insoluble in the pH range between about 5.0 and 10.0.

When the foil, made on a glass plate from the methanol solution of this linear polypeptide hydriodide, was soaked in commercial (not purified) ether overnight, it turned insoluble in water and yellowish-brown in color, due to the liberation of iodine; this color vanished by soaking in dilute alkali. This insoluble foil may be considered to be a network polypeptide, in which cysteine residues were converted into cystine ones.

* A preliminary report of the part of this paper was published in *J. Am. Chem. Soc.*, **75**, 3042 (1953).

1) K.H. Meyer, *Biochem. Z.*, **214**, 253 (1929); *Experientia* **7**, 361 (1951).

2) W. Kuhn, B. Hargitay, A. Katchalsky and H. Eisenberg, *Nature*, **165**, 514 (1950); A. Katchalsky and H. Eisenberg, ibid., **166**, 267 (1950); W. Kuhn and B. Hargitay, *Experientia*, **7**, 1 (1951); A. Katchalsky, *J. Polymer Sci.*, **7**, 392 (1951).

Measurements of the Contractility

A piece of this foil, which was swollen in distilled water, became greatly extended in dilute acids and in dilute alkalis, and then contracted to its initial length in pure water (see Table I).

TABLE I

	Parallel to the orientation		Perpendicular to the orientation	
Immersion medium*	Length (×55, cm.)	Ratio	Length (×55, cm.)	Ratio
dry sample	1.77	1.00	4.00	1.00
in water	2.07	1.17	4.70	1.18
in 0.01 N NaOH	2.99	1.69	6.80	1.70
in water	2.30	1.30	5.42	1.36
in 0.01 N HCl	2.75	1.55	6.42	1.61
in water	2.10	1.19	4.78	1.20

* The foil was immersed in the media in descending order.

An attempt was made to give an orientation to the foil by allowing the methanol solution to flow in one direction when casting it on a glass plate, but practically no effect on the swelling was observed.

A strip, cut from the foil, was fastened at one end and a load attached to the other end. It was then immersed in various media and its length measured as a function of time.

The elongation of the strip in dilute hydrochloric acid and in dilute aqueous sodium hydroxide and its contraction in a neutral medium are shown in Fig. 1. The strip was elongated more in the alkaline than in the acidic medium, and the time required for contraction by neutralising from the alkaline solution was longer than that from the acidic solution. This time of contracting from the alkaline solution was shortened by using dilute aqueous sodium chloride as a neutral medium instead of pure water and by slightly agitating the medium. This behavior suggests that sodium ions were more strongly adsorbed on the strip than chloride ions. When the strip, which was elongated in alkaline (or acidic) medium, was soaked in an acidic (or alkaline) one, it contracted strongly momentarily and then elongated in the usual manner in the latter medium.

The lengths of the strip at various pH values from 2 to 11 are shown in Fig. 2. The minimum length was at pH 3–4 in Clark-Lubs' buffer and at pH 5 in acetate buffer. It is noticeable that the strip contracted more strongly in phthalate

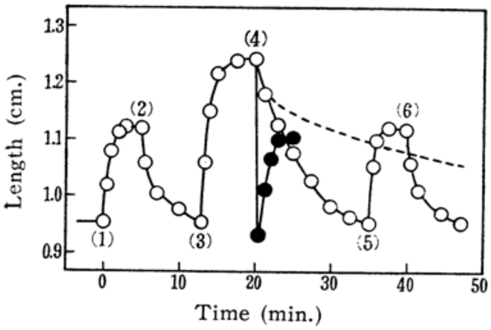

Fig. 1. Reversible lifting and lowering of a load by a strip.

Dry strip: $0.764\times0.038\times0.003$ cm^3, 0.065 mg. Load: 5.07 mg. Medium: (1) and (5), 0.005 N NaCl (15 ml.)+0.01 N HCl (3 ml.); (2) and (6), 0.005 N NaCl (18 ml.); (3), 0.005 N NaCl (15 ml.)+0.01 N NaOH (3 ml.); (4) ○, 0.005 N NaCl (slight agitation); (4) ●, 0.005 N NaCl (15 ml.)+0.01 N HCl (3 ml.); (4) dotted line, H_2O.

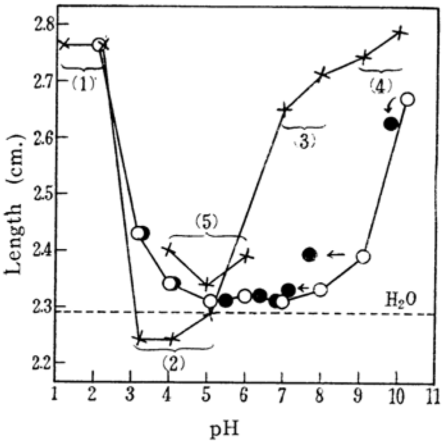

Fig. 2. Relationship between pH of the medium and length of a strip.

Dry strip: length, 1.974 cm.; weight, 0.4 mg. Load: 18 mg. Medium (70 ml.): ×, buffer solutions —— (1), HCl–KCl; (2), potassium biphthalate-NaOH; (3), KH_2PO_4-NaOH; (4), H_3BO_4-KCl-NaOH; (5), AcOH-AcONa. ○, NaOH-HCl mixtures (ionic strength 0.01). ●, After immersion of the strip.

buffer than in pure water. This behavior is supposed to be attributable to the large bulk of the bivalent phthalate ions.

In the solutions made by mixing various proportions of 0.01 N hydrochloric acid and 0.01 N sodium hydroxide, the length of the strip was a minimum at pH from 5 to 7 and the pH of the media shifted towards pH 6.5 after immersion of the strip. These results demonstrate the adsorption of sodium and chloride ions on the strip, i. e., the ion-exchange ability of the strip. The maximum pH shift was found in solution of pH 9, in which the excess of the number of sodium ions over the number of chloride ions was approximately equal to the number of carboxyl groups in the strip used, and the length of the strip reached the equilibrium value only after a very long time.

The elongation of the strip was constant in the media so acidic (i. e., pH below 2) that no dissociation of the carboxyl groups in the strip could take place.

The elongation of the strip by a load up to about two thousand times the dry weight of the strip per 1 cm. of length was measured in 0.01 N hydrochloric acid and in 0.01 N aqueous sodium chloride. The results are shown in Fig. 3. In

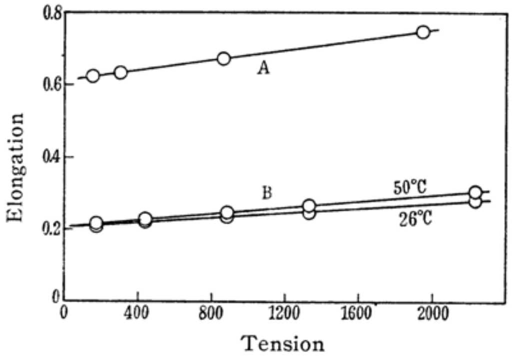

Fig. 3. Relationship between tension and elongation of a strip.

Elongation: $(l-l_0)/l_0$, where l and l_0 are the lengths of the immersed and the dry strip respectively. Tension: $W/(w_0/l_0)$, where W and w_0 are the weights of load and the dry strip respectively. A: In 0.01 N HCl at room temperature; $l_0=$ 1.46 cm., $w_0=0.30$ mg. B: In 0.01 N NaCl at 26°C and 50°C; $l_0=1.985$ cm., $w_0=0.23$ mg.

these media the elongation was proportional to the weight of load; but after removing the load a slight hysteresis was observed, which vanished on prolonged standing.

The length of the strip increased slightly on raising the temperature. The relation of temperature *versus* tension, for a constant length of the strip, is shown in Fig. 4. This figure was ob-

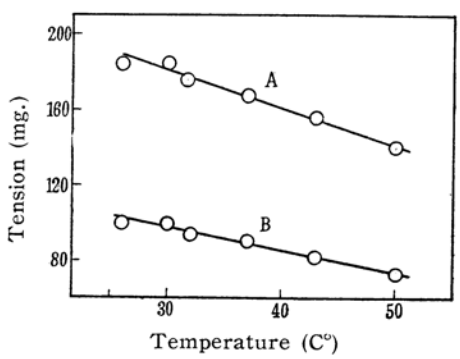

Fig. 4. Relationship between temperature and tension at constant lengths of a strip.

Dry strip: length, 1.985 cm.; weight, 0.23 mg. A: length=2.500 cm. B: length =2.450 cm.

tained graphically from the data of the measurements of load-elongation relationships at various temperatures from 20 to 50°C. It was found that the strip tends to decrease its tensile strength with increasing temperature.

It is interesting to study the effects of inorganic salts on the contractility of this strip compared with that of actomyosin.

The strip, immersed in water, was elongated by the addition of sodium chloride; the higher its concentration, the greater the elongation. The sodium chloride concentration was increased stepwise from 0.1 to 4 N and then decreased reversely. As shown in Fig. 5, the elongation

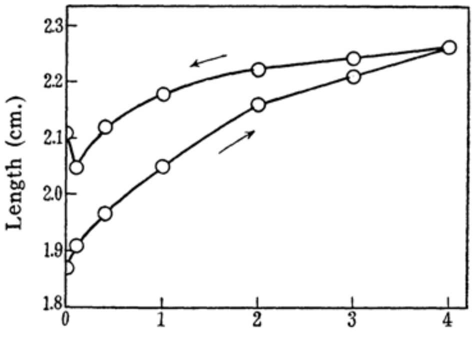

Fig. 5. Effect of concentration of NaCl on length of a strip.
Dry strip: length, 1.594 cm.; weight, 0.3 mg. Load: 18 mg.

of the strip was not reversible and its length could not return completely to its initial value. Upon immersing this strip in distilled water it was elongated. Subsequent immersion in dilute hydrochloric acid then caused it to contract rapidly, suggesting that the adsorption of sodium ions in the strip is rather strong.

The behavior of the strip in concentrated sodium chloride solution was somewhat complicated. When it was transferred directly into 4 N aqueous sodium chloride from distilled water, its length increased instantly; this was followed by a slow additional elongation proportional to the time, until the equilibrium length was approached. This elongated strip contracted now reversely by replacing it in distilled water; the rate of contraction was rapid at first and then slow (see Fig. 6).

Very striking and interesting phenomena were observed in the following series of experiments (see Fig. 6). The strip, which was elongated previously in 4 N aqueous sodium chloride as described above, contracted sharply on the addition of a small amount of dilute hydrochloric acid to the medium, and regained its original length in distilled water. When it was soaked again in 4 N aqueous sodium chloride, no change in length was observed, but on subsequent immersion in distilled water an instantaneous strong elongation and contraction was observed. On insertion of the strip into 4 N aqueous sodium chloride, it was lengthened in a manner similar to that when first immersed in this medium.

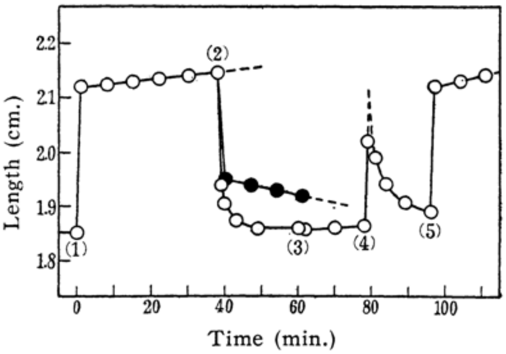

Fig. 6. Contractility of a strip in concentrated NaCl solution. I.
Dry strip: length, 1.594 cm.; weight, 0.30 mg. Load: 18.0 mg. Medium: (1), (3) and (5), 4 N NaCl; (4), H_2O; (2) ○, 4 N NaCl (15 ml.) +0.01 N HCl (3 ml.); (2) ●, H_2O.

This series of experiments was repeated by using 2 N aqueous sodium chloride (see Fig. 7).

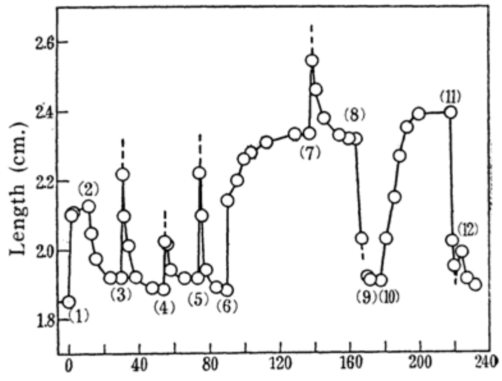

Fig. 7. Contractility of a strip in concentrated NaCl solution. II.
Dry strip: length, 1.594 cm.; weight, 0.30 mg. Load: 18.0 mg. Medium: (1), 2 N NaCl; (2) and (4), 2 N NaCl (16.5 ml.) +0.01 N HCl (1.5 ml.); (3), (5), (7), (9) and (12), H_2O; (6), 2 N NaCl (16.5 ml.)+0.01 N NaOH (1.5 ml.); (8) and (11), H_2O (16.5 ml.) +0.01 N HCl (1.5 ml.); (10), H_2O (16.5 ml.) +0.01 N NaOH (1.5 ml.).

When the strip was transferred from distilled water into 2 N aqueous sodium chloride solution containing a small amount of hydrochloric acid, an instantaneous elongation and contraction was observed. This phenomenon appeared to be an overlap of the elongation by the sodium chloride and the following contraction by the acidic sodium chloride solution.

The effect of the addition of a small amount of sodium hydroxide to aqueous sodium chloride was contrary to that of hydrochloric acid. The strip was elongated further stronger in the alkaline sodium chloride solution than was elongated in 2 N aqueous sodium chloride. Then, on soaking in distilled water, a strong elongation and contraction was observed; but the final length of the strip was the same as that in the preceeding medium. Soaking the strip in dilute hydrochloric acid then caused it to return to the original length which it had had in distilled water. This process was similar to the contraction observed on the transfer of the strip into dilute hydrochloric acid from dilute aqueous sodium hydroxide (see Fig. 7).

A series of immersion media was prepared by dissolving various amounts of sodium chloride in 0.005 N hydrochloric acid, and the length of the strip was measured in these media (see Fig. 8). Though at the lower concentration (i.e., below 0.1 N) of sodium chloride the length of the strip was somewhat longer than that in hydrochloric acid alone, the strip contracted with an increase in the concentration of salt, and at the higher concentration (i.e., above 0.1 N) the extent of contraction was proportional to the logarithm of the concentration. This behavior is contrary to that in neutral sodium chloride solution.

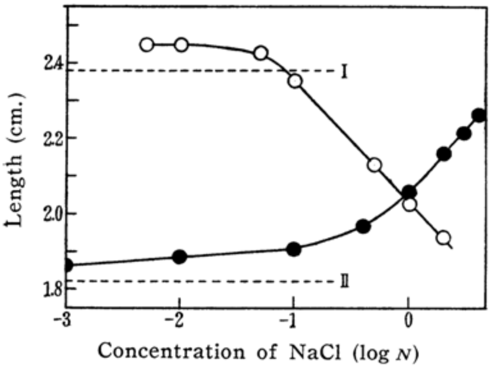

Fig. 8. Length of a strip in acidic and neutral NaCl solution.

Dry strip: length, 1.594 cm.; weight, 0.30 mg. Load: 18.0 mg. ○, Solution of NaCl in 0.005 N HCl. ●, Aqueous solution of NaCl. Dotted lines I and II represent the lengths of the strip in 0.005 N HCl and water, respectively.

The effects of neutral inorganic salts containing bivalent ions are shown in Fig. 9. The length-concentration relationships were similar for $CaCl_2$, $MgCl_2$, Na_2SO_4 and $(NH_4)_2SO_4$ solutions to that for aqueous sodium chloride. On the contrary, the strip contracted in aqueous $CuSO_4$ and in aqueous $ZnCl_2$ at the concentrations below and above 0.1 N respectively, and very strongly in aqueous $HgCl_2$ at the whole concentration tested. Since it has been known that these metal ions (Hg^{++}, Cu^{++} and Zn^{++}) are capable of forming complexes with amino groups and particularly that Hg^{++} ion can combine strongly with sulfur atoms, the contraction of the strip in these solutions appears to be due to the formation of cross-linkages of these complexes. In aqueous $KHCO_3$ the strip was elongated strongly as a result of its alkalinity. The strip was also slightly elongated with increasing temperature in all these salt solutions.

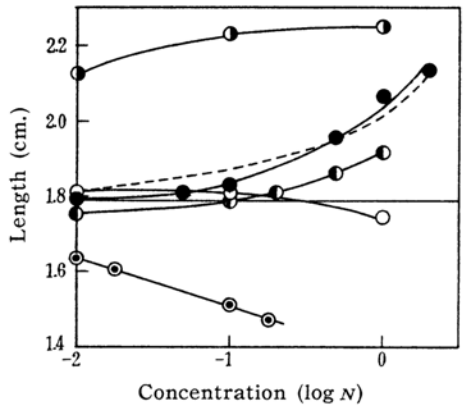

Fig. 9. Effects of various salts on length of a strip.

Dry strip: length, 1.47 cm.; weight, 0.30 mg. Load: 19.5 mg. ●, $CaCl_2$; ○, $ZnCl_2$; ◐, $CuSO_4$; ⊙, $HgCl_2$; ◑, $KHCO_3$; dotted line, NaCl. $MgCl_2$, Na_2SO_4, $(NH_4)_2 \cdot SO_4$ gave curves similar to that for $CaCl_2$.

Discussion

W. Kuhn and A. Katchalsky et al.[2] reported with their three-dimensional polyanion network that a statistical consideration concerning the entropy of a three-dimensional network and considerations concerning the electrostatic energy of the ionised system are required for the theoretical treatment of the elongation and contraction phenomena. S. Asakura[3] and H. Noguchi[4] stated that the entropy force of counter-ions bound by the system plays a predominant role, tending to expand the system against the rubber-like elastic force.

In this experiment the phenomena shown by the strip-form polyampholyte system was, as expected, more complicated than that shown by the polyanion system. Contrary to the polyanion system, the strip of this amphoteric polypeptide decreased its restoring force with increasing temperature. This fact shows that at a given temperature the internal energy and the entropy increase with the elongation of the strip: that is,

$$(\partial E/\partial l)_T > 0 \quad \text{and} \quad (\partial S/\partial l)_T > 0$$

in the relation

$$\begin{aligned} K &= (\partial E/\partial l)_T - T(\partial S/\partial l)_T \\ &= (\partial E/\partial l)_T + T(\partial K/\partial T)_l \end{aligned}$$

in which K, E, S, l, and T represent restoring force, internal energy, entropy, length and absolute temperature respectively.

It appears that there are some cross-linkages in the foil in addition to the cystine -S-S- linkages and that the number of the

3) S. Asakura, *Busseiron Kenkyu*, **62**, 39 (1953).
4) H. Noguchi, ibid., **62**, 30 (1953).

former changes with the increase and decrease of temperature by their rupture and reformation. Such linkages might include the salt linkages between carboxyl and ammonium ions and the hydrogen bonds between two amide linkages. This consideration is supported also by the strong elongation of this strip in concentrated salt solutions and by both the viscosity increase and the insolubility of synthetic linear polypeptide, reported in the preceeding paper[5], in neutral region. Furthermore, it was found that synthetic high molecular copoly-1 : 1-(L-glutamic acid, L-lysine)[6], which was insoluble in distilled water, could be dissolved in concentrated sodium chloride solution. The solubility and the viscosity behavior of these linear amphoteric polypeptides is closely related to the contractility of this strip and qualitatively coincide with it.

As found in most instances, the strip adsorbed cations more strongly than anions. This difference in adsorption appears to be caused by reasons such as the decrease in the number of amino groups during the formation of the foil, but the true reason remained unknown. The difference in the hydrations of cations and anions is also considered to be concerned with the above behavior of the foil. Although no satisfactory conclusion could be obtained, since the phenomena are too complicated, the contractility of this foil, e. g. the rapid elongation and contraction of the strip found in some instances, is very interesting as compared with the behavior of actomyosin.

Experimental

N, N′-Dicarbobenzoxy-cystine.—This was synthesized from L-cystine according to the Bergmann's method[7], but showed no optical rotation in acetic acid probably due to the racemisation during the reaction. Recrystallized from acetone-chloroform, it combined 1/4 mol. of chloroform m. p. 101–103° (sintered from 80°C).

Anal. Found: C, 49.83; H, 4.71; N, 5.14; SO_4 and Cl, 40.53. Calcd. for $C_{22}H_{24}N_2O_8S_2 \cdot 1/4\ CHCl_3$; C, 49.63; H, 4.54; N, 5.20; SO_4 and Cl, 40.63% (S and Cl were measured by Stragand's method[8] and obtained as the sum of converted SO_4 and Cl).

DL-Cystine Bis-(N-carboxy-anhydride). — To the solution of 2 g. of dicarbobenzoxy-cystine in 12 ml. of dry dioxane 4.4 g. of phosphorus pentachloride was added. After shaking for 15 min. at 10°C, the mixture was filtered and heated for one hour at 40°C. The crystallized anhydride was filtered and washed with ethyl acetate. Yield 1.0 g., colorless needles. m. p. 134° (decomp.). It contained 1 mol. of combined dioxane.

Anal. Found: C, 38.04; H, 4.18; N, 7.31; S, 16.65. Calcd. for $C_8H_8N_2O_2S_2 \cdot C_4H_8O_2$: C, 37.90; H, 4.24; N, 7.37; S, 16.84%.

Copolymerization.—A mixture of 2.104 g. (8.0 m mol.) of γ-benzyl N-carboxy-L-glutamate anhydride[5], 2,448 g. (8.0 m mol.) of ε-N-carbobenzoxy-L-lysine anhydride[5] and 0.117 g. (0.308 m mol.) of DL-cystine bis-(N-carboxy-anhydride) in 42 ml. of chlorobenzene and 3 ml. of dry pyridine was heated rapidly to 100°C in a sealed glass tube until in solution, and then kept for ninety hours at 50°C and for ten days at room temperature. The solution gradually increased in viscosity and finally became an opalescent gel. The polymer, precipitated by adding petroleum ether, was allowed to swell in acetone, and after reprecipitating by petroleum ether, it was obtained as a white powder. Yield 3.890 g. (99% of the theor.).

Anal. Found*: C, 63.86; H, 6.54; N, 8.70. Calcd. for $[26(C_{12}H_{13}NO_3), 26(C_{14}H_{18}N_2O_3), (C_3H_8N_2O_2S_2)]n$: C, 64.37; H, 6.45; N, 8.81%.

The polymer swelled in chloroform, acetone, pyridine, dioxane, tetrahydrofuran, and hot glacial acetic acid, but could not be dissolved.

Reduction of the Polymer.—After 0.7 g. of the polymer was swelled by heating in 50 ml. of glacial acetic acid, 1.7 g. of phosphonium iodide was added in portions during five hours at 55°C, with dry hydrogen bubbling through the solution. The supernatant liquid was separated from the resultant precipitate by decantation, and a small amount of absolute alcohol was added. This dissolved the major part of the precipitate, but some swollen material remained. The polymer was reprecipitated from the solution by freshly distilled ether and was then extracted repeatedly with water. The extracts were combined and were concentrated in vacuo at 40°C. The polypeptide hydriodide was precipitated from the residual syrup by adding ethanol and ether, washed with ether and dried. White powder; yield 0.27 g. (48% of the theor.). It was soluble in water, methanol, and ethanol, and gave positive biuret and nitroprusside test.

Anal. Found: C, 33.77; H, 5.25; N, 10.83; I, 32.1; amino-N, 3.46. Calcd. for $[26(C_5H_7NO_3), 26\ C_3H_{12}N_2O \cdot HI), 2(C_3H_5NOS)]_n$: C, 34.25; H, 5.23; N, 10.96; I, 32.3; amino-N, 3.56%.

Preparation of the Foil.—The foil was made on a parraffin-treated glass plate by casting a 2 % methanol solution of the above polypeptide hydriodide. After immersing overnight in commercial ether, the foil became orange-yellow in color and turned insoluble, but swelled in water. This foil gave no nitroprusside test, and was quite flexible and strongly elastic in water. It was decolorised by soaking in dilute alkali, and, after washing with dilute hydrochloric acid and water, it was contracted easily by alcohol and ether, and dried.

The powdered polypeptide hydriodide was treated with commercial ether as before and then

5) H. Yuki, S. Sakakibara and H. Tani, This Bulletin, 29, 654 (1956).
6) H. Yuki and H. Tani, Unpublished.
7) M. Bergmann and L. Zervas, *Ber.*, 65, 1192 (1932).
8) G. L. Stragand and H. W. Safford, *Anal. Chem.*, 21, 625 (1949).

with 0.001 N sodium hydroxide, washed with water, ethanol and ether, and dried. This substance contained a small amount of combined sodium.

Anal. Found: C, 49.99; H, 7.12; N, 14.77; Na, 3.92. Calcd. for $[16(C_5H_7NO_3), 10(C_5H_6NO_3\cdot Na), 26(C_9H_{12}N_2O), (C_6H_8N_2O_2S_2)]_n$: C, 49.28; H, 6.98; N, 15.75; Na, 3.24%.

Swelling of the Foil (Table I).—A piece of the foil was immersed in the media and its length was measured by photographs (magnification 55×), taken after one hour of immersion.

Elongation and Contraction of the Strip.—The strips used for measurements were 1.5–2 cm. in length, 0.04–0.07 cm. in width, and 0.2–0.5 mg. in weight. Assuming the specific gravity to be approximately equal to unity its thickness was about 0.003 cm.

Glass clips were attached to the two ends of the strip. The upper clip was fastened to the hook of a glass rod. The lower clip was used itself as a load, and, if necessary, also used for hanging platinum rings, varied according to the desired additional load. The strip was immersed in the medium in a large test tube, held in a thermostat. The upper end of the strip was fixed and the change of the length was followed with a microscope.

To obtain the reproducibility the measurements were made after training the strip to elongate and contract, using successively dilute aqueous NaOH, water, dilute HCl and water. By this training the strip came to a constant length in a given medium. In most cases either the length of the strip in distilled water or that in dilute aqueous sodium chloride was used as a standard, from which each experiment was started. The strip was used in each experiment after recovering its standard length by washing it with dilute hydrochloric acid and then with water in order to remove any of the rather strongly adsorbed cations. The measurements were made at 25°C unless otherwise stated.

Summary

1. A reversibly contractile amphoteric polypeptide, copoly-26 : 26 : 1-(L-glutamic acid, L-lysine, DL-cystine), was synthesised.

2. The foil of this polypeptide contracted at its isoelectric region and extended in both acidic and basic media. This extension and contraction was pH-dependent and fully reversible.

3. The load-elongation relation of the strip of this foil was measured in aqueous media using the load up to about 2,000 times the dry weight per 1 cm. of length of the strip. This relation was linear in this range.

4. The temperature-elongation relation was measured in aqueous media at 20–50°C. From the data it was concluded that, at a given temperature, the internal energy and entropy increased with the elongation of the strip.

5. The change of length of the strip in aqueous media, containing hydrochloric acid, sodium hydroxide, sodium chloride and some other neutral salts individually or in pairs, was measured. Some peculiar and interesting phenomena were observed.

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Department of Chemistry, Faculty of Science, Osaka University, Osaka