Surface Chemistry of Synthetic Protein Analogues. IV. On the Monolayers of Electrolytic Synthetic Polypeptides, Poly-L-*Glutamic Acid and the Copolypeptide of* L-*Lysine,* L-*Leucine and* L-*Glutamic Acid*

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Introduction

In the preceding three papers of this series[1-3], we have reported on the properties of the monolayers of some synthetic polypeptides, all of which have non-electrolytic side chains. It was found that the polypeptides having sidechains which are less than four carbon atoms in length, such as poly-DL-norleucine, gave the surface pressure-area curves which closely resembled in shape those of natural proteins. In relation to only the length of side chains, poly-DL-norleucine may be a suitable one as a model of proteins. Strictly speaking, however, this polypeptide may be inadequate because of the lack of electrolytic nature.

The copolypeptide of L-lysine, L-leucine and L-glutamic acid which is described in the present paper should be a more promising protein model for the following three reasons: namely, (1)this copolypeptide has amino groups, carboxyl groups and non-polar hydrocarbon groups in side chains, (2) plausible length of side chain and (3) the configuration of L-type. All of these properties are found in natural proteins in general.

This polypeptide films gave the different surface pressure-area (F—A) and surface viscosity-area (η—A) curves on the substrates of various *p*H values and the results obtained are interpreted in terms of the ionization degree of its amino and carboxyl groups. In addition, further simplified, non-amphoteric, poly-L-glutamic acid monolayers on the substrates of different hydrogen ion concentrations were also examined.

The interest about these electrolytic polypeptides concentrate not only upon the protein model but also upon this polyelectrolytic behaviour of which investigations are recently developed by Kuhn and Katchalsky[4,5] We have investigated the properties of these polypeptides monolayers from the viewpoint of polyelectrolyte in addition to the comparison of these polypeptides with natural proteins.

Experimental

Materials and Methods. The copolypeptide of

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[1] T. Isemura and K. Hamaguchi, This Bulletin, **25**, 40 (1952).

[2] T. Isemura and K. Hamaguchi, ibid., **26**, 425 (1953).

[3] T. Isemura and K. Hamaguchi, ibid., **27**, 125 (1954).

[4] W. Kuhn, O. Kunzel and A. Katchalsky, *Helv. Chim. Acta*, **31**, 1994 (1948).

[5] A. Katchalsky, *J. Polymer Sci.*, **7**, 393 (1951).

L-lysine, L-leucine, and L-glutamic acid and poly-L-glutamic acid were prepared by Dr. H. Tani and Mr. H. Yuki⁽⁶⁾ and were used without any further purification. This copolypeptide was spread from its methanol solution and poly-L-glutamic acid from a solution in N/100 sodium hydroxide containing 1 percent isopropyl alcohol. The film of the polyelectrolyte is profoundly affected by the existence of a small amount of salt in the substrate In order to minimize the effect of various salt ions the *p*H of the substrates was adjusted inevitably only with hydrochloric acid or sodium hydroxide without using the buffer. The value of *p*H was measured by using the Beckman glass electrode. The change of the *p*H values before and after experiment, however, did not exceed 0.2 unit at most.

Surface pressure and surface viscosity measurements were carried out by the method of a hanging plate and of damped oscillatory motion of a disc, as reported in the previous papers.[1,3] The area per amino acid residue of the copolypeptide was calculated by assuming that the same ratio of L-lysine, L-leucine and L-glutamic acid as the initial monomer concentration ratio of this polymerization, 1:2:1, was maintained in the copolymer.

Results. The F—A and η—A curves of the copolypeptide on the substrates of different *p*H values were shown in Figs. 1 and 2. It was found from these figures that the F—A and η—A curves were varied with the substrate *p*H and that the F—A curves on the substrate of which *p*H values lie between 5.8 and 9.4 were of condensed type, but the more acidic or the more alkaline in the outsides of this *p*H range the substrate becomes, the more the F—A curves expand. Furthermore, while in this *p*H range the surface viscosity was very large even at lower pressure region—this is the analogous fact with the case of non-polar polypeptides[3]—it was not sensitive until the surface pressure became considerably high on the either side of this *p*H range. The surface viscosities decrease with the rise of temperature of the substrate at any *p*H value excepting at *p*H 7, where the effect of the temperature was reverse, i.e., the higher the temperature the larger was the viscosity. (Fig 3).

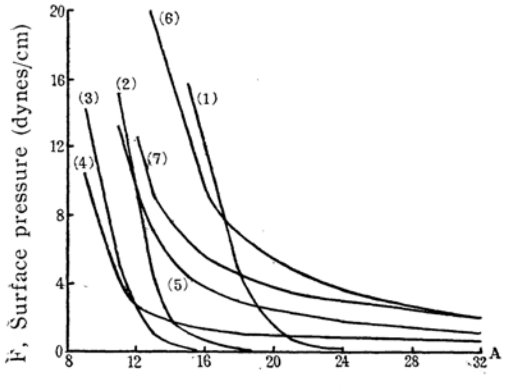

Fig. 1. Surface pressure-area curves for monolayers of the copolypeptide on the substrates of different *p*H values. Curve (1), *p*H 7, (2), *p*H 9.4, (3), *p*H 5.8, (4), *p*H 4.4, (5), *p*H 3.0, (6), *p*H 12.3, and (7) *p*H 1.6. (8°C).

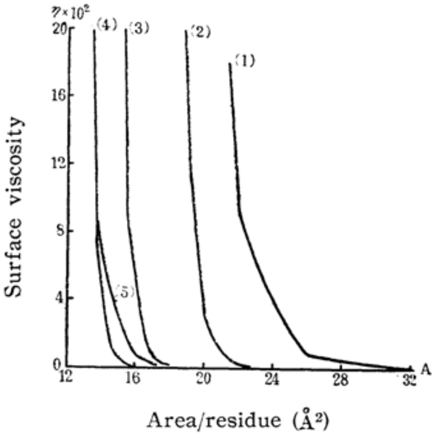

Fig. 2. Surface viscosity-area curves for monolayers of the copolypeptide on the substrate of different *p*H values. Curve (1) *p*H 7, (2) *p*H 8.3, (3) *p*H 9.2, (4) *p*H 5.8 and (5) *p*H 1.6. (7°C)

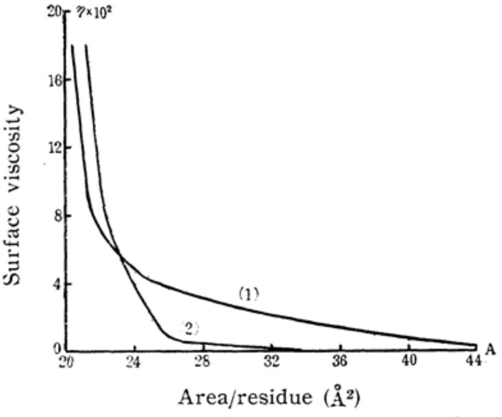

Fig. 3. Effect of temperature on surface viscosity-area curve for monolayer of the copolypeptide at *p*H 7. Curve (1) 14°C, (2) 7°C.

The F—A and η—A curves of poly-L-glutamic acid were shown in Figs. 4 and 5. Fig. 6 shows the relations of *p*H-area (at constant pressure), and *p*H-surface pressure (at constant area), namely curve (I) shows the plot of the area at 1 dyne/cm. against *p*H (ca. 10°C), curve (II) the same as curve (I) but at the temperature 21°C., curve (III) the surface pressure at 15 Å²/residue against *p*H, and curve (IV) the area at $\eta=10^{-2}$ surface poises

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6) H. Tani, H. Yuki, K. Okawa, S. Sakakibara and C. Oizumi, *Seni Kagaku Kenkyusho Nenpo* (*Mem. Inst. Fiber Research*) **7**. 98 (1953).

against pH. It was noted that the area or surface pressure decreased steeply at about pH 4.6.

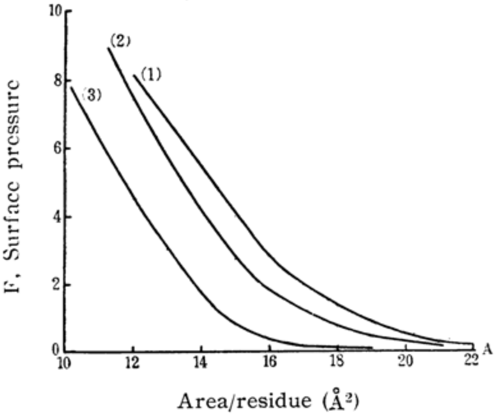

Fig. 4. Surface pressure-area curves for monolayers of poly-L-glutamic acid. Curve (1) pH 1.6∼4.6, (2) pH 5.1 and (3) pH 5.6. (10°C).

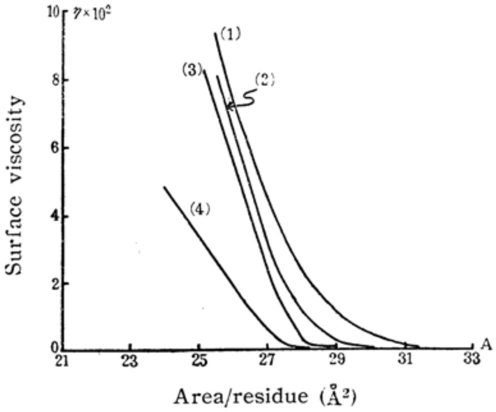

Fig. 5. Surface viscosity-area curves for monolayers of poly-L-glutamic acid. Curve (1) pH 1.6, (2) pH 3.0, (3) pH 4.4 and (4) pH 4.8. (22°C).

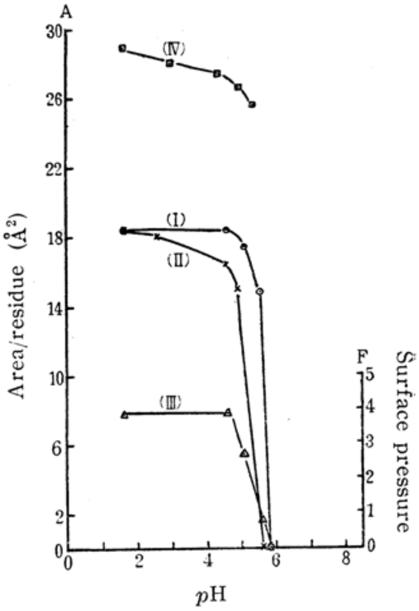

Fig. 6. Monolayers of poly-L-glutamic acid. Curve (I) Area at 1 dyne/cm.—pH curve at 10°C. (II) Area at 1 dyne/cm.—pH curve at 21°C. (III) Surface pressure at 15 Å²/residue—pH curve at 10°C. (IV) Area at $\eta=10^{-2}$ surface poise—pH curve at 22°C.

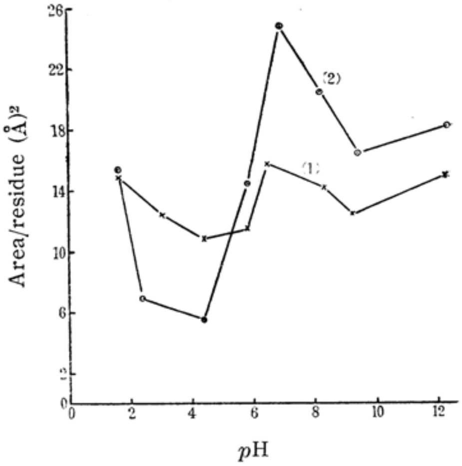

Fig. 7. Monolayers of the copolypeptide. (1) Area at compressibility minimum against pH curve, (2) Area at $\eta=2\times10^{-2}$ surface poise against pH curve.

Discussion

Compressibility δ was calculated from the F—A curves at various pH by the equation

$$\delta=-\frac{1}{A}\,\frac{\partial A}{\partial F} \qquad (1)$$

and was plotted against the area. In Fig. 7 the area of the minimum compressibility ($\delta_{\text{min.}}$) appearing in the δ—A curves is plotted against the pH of the substrates.

The minima appear at pH 4.4 and 9.6 and the maximum at pH 7 in this curve. The area at $\eta=2\times10^{-2}$ surface poise was plotted against pH in this figure and this relation is also of W-shape similarly. This relation resembles that found by Gorter, van Ormondt and Dorn,⁷⁾ who pointed out that the spreading was maximum at the isoelectric point of the protein. Later, the same relation was also recognized by Bateman and Chambers⁸⁾

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7) E. Gorter, J. van Ormondt and F. P. Dorn, *Proc. Kon. Akad.*, Amsterdam, **35**, 838 (1932), cited by McBain, "Colloid Science," (1950).

8) J. B. Bateman and L. A. Chambers, *J. Chem. Phys.*, **7** 244 (1939).

and others. Dervichian,[9] however, suggested that this phenomenon was ascribed to the maximum insolubility of proteins at the isoelectric point. In our case, however, the dependence of the area upon *p*H of the substrate may not be ascribed to the difference of solubility, because the solvent used for spreading is methanol and the area becomes again larger when the *p*H is sufficiently higher or lower.

When the copolypeptide was spread on a large area of the substrate, the polar as well as non-polar side chains and —CO—NH— groups in the main chain would be laid on the surface. With the compression of the film, the non-polar side chains tend to stand up perpendicularly to the surface in air, remaining the main chain on the surface of the substrate. Consequently, the long linear polypeptide would assume more or less extended structure in the spread monolayer.

Now, we shall consider on which *p*H lies the isoelectric point of this copolypeptide. At *p*H 7, some peculiar phenomena were observed, that is, (a), the maximum appeared in the A (at compressibility minimum)-*p*H curve (Fig. 7), (b) the surface viscosity is very high even in larger area and (c) the temperature dependence of the viscosity is opposite to that at the other *p*H values (Fig. 3). Since the number of $—NH_3^+$ ions might be the same as that of $—COO^-$ ions at the isoelectric point, it is probable that the number of salt-linkages between these ions is at its maximum and that the large network structure of the film may be completed. In such a state the resistance against shear becomes very large and the surface viscosity is expected to be high in a larger area. If the salt-linkages between these ions is possible, the degree of hydration and also the hydrophilic tendency of these groups decrease and so it is considered that the F—A curve at the isoelectric point develops from the larger area. With increasing or decreasing the *p*H of substrates from the isoelectric point, either positive or negative charge of the molecules exceeds the other and the number of salt-linkages between NH_3^+ and $—COO^-$ ions decreases. The decrease of the number of salt-linkages results in the lowering of surface viscosity, for the resistance against shear becomes necessarily smaller. All the results and considerations above mentioned lead us to the conclusion that the isoelectric point of this copolypeptide may lie at *p*H 7.

The surface viscosity of the protein monolayer is often discussed in terms of molecular shape in an analogous manner with the viscosity of its bulk solution.[10],[11] In our opinion, the surface viscosity may be entirely different from the bulk viscosity in nature. Therefore, it cannot be considered similarly.

It is well known that the polyampholyte, natural[12],[13] and synthetic,[14],[15] shows a minimum viscosity at its isoelectric point in the bulk solution. This is caused by the coiling of the polyelectrolyte chains by the intramolecular neutralization and no effective free charge in the molecule. On the other hand, the polyampholyte molecule in the monolayer is extended by the surface force. The number of positive and negative ionic groups is maximum in total even though the number of either ionic groups is equal to each other at isoelectric point. As a result, these ionic groups tend to interact intermolecularly forming salt-linkage. This is the reason of maximum surface viscosity and minimum bulk viscosity at isoelectric point.

Surface viscosity depends not only on the interaction between the molecules but also on the degree of molecular packing. There exist two extreme cases of orientation, one of which is that the side chains are directed perpendicular and the other, parallel to the water surface. Between these two extremes the forms or orientations which the polypeptide molecules may prefer on the water surface should be different from the substrate *p*H, depending on the degree of ionization of amino and carboxyl groups. Quantitative analysis of F—A and η—A curves is, therefore, very difficult.

As mentioned above, the F—A curves were of condensed type on the substrates of which *p*H values lie in the range between 5.8 and 9.4, but changed to expanded type if the substrate becomes more alkaline or more acidic than either end of this *p*H range. These facts also relate to the degree of ionization of carboxyl or amino groups; when the number of either positive or negative ions increases, the film expands by the electrical repulsion between the free charges.

The dependence of the degree of ionization (α) of the polyelectrolyte on *p*H may be expressed theoretically by the following equation,[5]

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[9] D. Dervichian, *Nature*, **144**, 629 (1939).

[10] J. T. Davies, *Biochim. et Biophys. Acta*, **11**, 165 (1953).

[11] K. Imahori, This Bulletin, **27**, 146 (1954).

[12] E. K. Rideal, "Introduction to Surface Chemistry," p. 431, (1930).

[13] H. B. Bull, *Trans. Faraday Soc.*, **36**, 80 (1940).

[14] T. Alfrey Jr. and H. Morawetz, *J. Am. Chem. Soc.*, **74**, 436 (1952).

[15] T. Alfrey Jr., R. M. Fuoss, H. Morawetz and H. Pinner, ibid., 438.

$$pH-pK_o=\log\frac{\alpha}{1-\alpha}+\frac{0.4343}{kT}\frac{\partial F(\nu)}{\partial\nu} \quad (2),$$

Where $F(\nu)$ is the free energy of electrostatic field of a molecule having ν ionized groups and K_0 the intrinsic dissociation constant. We cannot find out the relation of pH and α from equation (2), because of the difficult calculation of $F(\nu)$. However, according to Arnold and Overbeek,[16] pK_0 of polymeric acid obtained from extrapolation of titration data to a very low degree of ionization is practically equal to the dissociation constant of a monobasic acid of the corresponding monomeric acid composition. So, the degree of ionization α_L of amino groups and α_G of carboxyl group can be evaluated to a first approximation by assuming the dissociation constant of $-NH_2$ group of lysine (pK_{0L}) and of $-COOH$ group of glutamic acid (pK_{0G}) to be 10.5 and 4.3, respectively, using the following simple equation,

$$pH-pK_0=\log\frac{\alpha}{1-\alpha} \quad (3).$$

Havinga and M. den Hertog-Polak[17] suggested that the value of pH of the electrolytic surface film may be different from that of the bulk solution. On the other hand, Glazer and Dogan[18] showed that there exists little effect with the surface film of polyampholyte such as proteins. In the present calculation, the pH value of the bulk solution was used as that of the surface phase.

If we assume that the polymerization ratio of lysine and glutamic acid of this copolypeptide is 1 : 1 (this assumption is valid when the isoelectric point lies near pH 7 as mentioned above), equation:

$$|\varDelta\alpha|=\alpha_L-\alpha_G \quad (4)$$

may be considered to give a measure of effective free charges. On the other hand, if the inter or intramolecular salt-linkages are possible, the probability of its linkage may be proportional to the product of α_L and α_G. Fig. 8 represents the relation of $|\varDelta\alpha|$ and α_L—α_G against pH.

As found from Fig. 8 the pH range in which F—A curves are of condensed type correponds to $\varDelta\alpha$ of at most 5%. (In this pH range eqn. (3) may well be applied.) This copolypeptide has, however, the residues of lysine, leucine and glutamic acid in the ratio of 1:2:1 as described above. So, this value of $|\varDelta\alpha|$ corresponds to only about 1% ionization of the total number of side chains. It is unfortunate, however, that the pH range which gives the F—A curves of condensed type could not be examined in detail, because of the small amount of the sample. The films which gave the F—A curves of condensed type are characterized by the high viscosity even at low surface pressures (Fig. 9) and this fact corresponds to the cases of non-polar polypeptides. From these results it is considered that in the pH range which gave the F—A curves of condensed type the molecules forming the film are in such a state that few free charges in molecules exist.

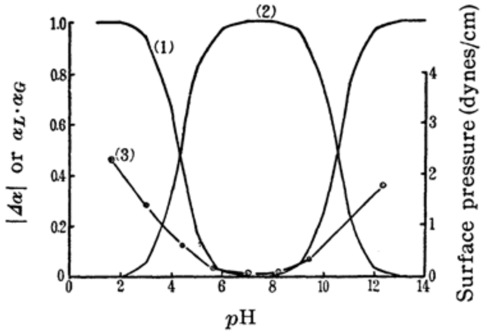

Fig. 8. (1) $|\varDelta\alpha|$—pH curve, (2) $\alpha_L\cdot\alpha_G$—pH curve, (3) surface pressure at 30 Å^2/residue—pH curve.

At pH 4.4, $\alpha_L-\alpha_G\doteqdot0.5$ (at this pH eqn. (3) cannot be applied but simply qualitatively), namely the number of $-NH_3^+$ ions is greater than that of $-COO^-$ ions by about 50% and the F—A curve is of expanded type. This value of $|\varDelta\alpha|$ corresponds to about 10% ionization of the total number of side chains. The film which gives the F—A curves of expanded type is not sensitive for surface viscosity until the film pressure is rather high (Fig. 9). The plots of surface viscosity against surface pressure are shown in Fig. 9. The films which do not bear free charges, for instance, those of non-electrolytic or electrolytic polypeptides which are in the isoelectric state, show the steep curve (I) and increasing the free charges makes the curve flattened for the pressure axis (curve II). Joly[19] measured the surface viscosity against film pressure relations of natural proteins on the aqueous solution of 0.01 N hydrochloric acid. It is found from his results that the more alkaline the isoelectric point is, the more flattened are the η—F curves as shown in curve (II). Consequently,

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[16] R. Arnold and J. Th. G. Overbeek, *Rec. trav. chim.*, **64**, 190 (1950).

[17] E. Havinga and M. den Hertog-Polak, *Rec. trav. chim.*, **71**, 64 (1952).

[18] J. Glazer and M. Z. Dogan, *Trans. Faraday Soc.*, **49**, 449 (1953).

[19] M. Joly, "Surface Chemistry." *Research* Supplement, Butterworth, p. 157 (1949).

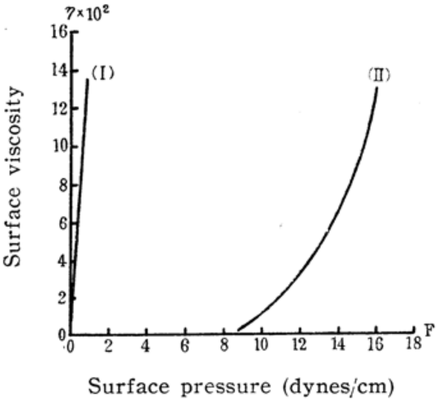

Fig. 9. Surface viscosity-surface pressure curves for monolayer of the copolypeptide. Curve (I) *p*H 7, (II) *p*H 1.6.

the change of the F—A curves to the expanded type must be ascribed to the electrical repulsion of the free charges in polypeptide molecules.

In Fig. 8 surface pressures at constant area are also plotted against the *p*H of the substrate. If the surface pressures exerted at constant area depend markedly on the electrical repulsion between free charges of the molecules, this F—*p*H curve must resemble the $|\Delta\alpha|$ *p*H—curve. This is in fact the case if F—*p*H curve is plotted at a larger area, for instance, 30 Å^2/residue; with the lowering of $\Delta\alpha$, F becomes smaller and a minimum point appears at *p*H 7. This fact also supports the theory that the isoelectric point of this copolypeptide lies at *p*H 7. When the surface pressures at smaller area than 25 Å^2/residue are plotted against *p*H, there exists discontinuity between *p*H 6 and *p*H 7 and the curve like $|\Delta\alpha|$—*p*H curve could not be obtained, since at such an area region surface pressure depends also on the form or orientation of the molecules.

As is shown in Fig. 7, surface area at constant pressure or at constant viscosity against *p*H curves is of W-shape, and now this curve will be examined in relation to the $|\Delta\alpha|$—*p*H and $\alpha_L\cdot\alpha_G$—*p*H curves. The $\alpha_L\cdot\alpha_G$—*p*H curve shows a maximum at the isoelectric point, *p*H 7, in other words, there exists maximum number of salt-linkages between $—NH_3^+$ and $—COO^-$ ions, the A—*p*H curve shows also a maximum at this *p*H. When the substrate becomes more acidic or alkaline from the isoelectric point, the area becomes smaller, and this region corresponds to the droop of the $\alpha_L\cdot\alpha_G$—*p*H curve. In this region the effect of $\alpha_L\cdot\alpha_G$ is more predominant than that of $|\Delta\alpha|$, i.e., the condensing effect of salt-linkages is more pronounced than the expanding effect of electrical repulsion by free charges. When the substrate becomes still more acidic or alkaline, the area becomes larger again and these regions correspond to the rise of the $|\Delta\alpha|$—*p*H curve, i.e., the effect of electrical repulsion ($|\Delta\alpha|$) is more predominant than that of salt-linkages ($\alpha_L\cdot\alpha_G$). It is of interest that two minima appearing in the curve of W-shape correspond to about pK_0 values of lysine and glutamic acid, respectively.

It is clear from the following reasons that the W-shaped A—*p*H curve is not due to the solubility of this copolypeptide into the underlying water as reported by Dervichian. In the case of poly-L-glutamic acid film, the increase of *p*H shifts the F—A curves to smaller area region and this is clearly due to the increasing solubility of the film substance. A sudden decrease in area and in pressure occurs at *p*H 4.6. The α value at this *p*H calculated from eqn. (3) is approximately equal to 0.6. In the case of the copolypeptide $|\Delta\alpha| \doteqdot 0.5$ at *p*H 4.4, based on eqn. (3). In this case, however, there exist the other residues of hydrophobic leucine and non-ionized glutamic acid and if the ratio of lysine, leucine and glutamic acid residues in the molecule is 1:2:1 as described above, this value of $|\Delta\alpha|$ corresponds to about 10% ionization of the total number of the side chains. In such a state, solubility effect cannot be considered in the light of our results of poly-L-glutamic acid.

Next, let us consider about the F—A and η—A curves of poly-L-glutamic acid. The displacement of the F—A curves to a smaller area with the rise of the *p*H of the substrate may be due to the increasing solubility of this polypeptide into the substrate by the ionization of carboxyl groups. It is an interesting fact that both the pressure at constant area and the area at constant pressure or surface viscosity decreased rapidly if the *p*H of the substrate exceeds 4.6. This value of *p*H corresponds to the pK value of glutamic acid.

Berger and Katchalski(20) showed that the reduced viscosity of poly-aspartic acid solution increases rapidly at about *p*H 4.6. Although there have been no experiments on the viscometric behaviour of poly-glutamic acid to be compared with, our present monolayer results may correspond approximately to the bulk data. According to Katchalsky,(5) the rapid increase

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20) A. Berger and E. Katchalski, *J. Am. Chem. Soc.*, **73**, 4084 (1951).

of reduced viscosity is due to the elongation of a polypeptide molecule by the electrical repulsion of ionized groups. If the elongation of a polymer molecule into a rod-like filament occurs, the film-forming molecules cannot remain on the surface and dissolve into the underlying water[21]. Our results represent the correspondence between the bulk and monolayer behaviour.

Summary

The surface pressure and surface viscosity of the spread monolayers of the copolypeptide of L-lysine, L-leucine and L-glutamic acid and of poly-L-glutamic acid were measured in relation to substrate *p*H.

The copolypeptide of L-lysine, L-leucine and L-glutamic acid.—The F—A curves in the range of *p*H 5.8 and 9.4 were of condensed type, but at both outsides of this *p*H range they were of expanded type. The area at constant pressure or surface viscosity against *p*H curves were of W-shape and a maximum appeared at *p*H 7. That the isoelectric point of the copolypeptide may lie at *p*H 7 was concluded from the observations of the monolayer properties. The W-shaped curve could be interpreted in terms of the ionization degree of amino and carboxyl groups. At the isoelectric point, *p*H 7, the surface viscosity became maximum. This is in contrast to the bulk viscometric behaviour of polyampholytes.

Poly-L-glutamic acid.—The area at constant pressure or surface viscosity and the pressure at constant area decreases rapidly if the *p*H of the substrate exceeds 4.6. This fact corresponds to the viscometric behaviour of electrolytic polypeptides in solution, i. e., when the elongation of a molecule by the electrical repulsion of ionized groups occurs, the rapid increase in viscosity and in solubility appears in solution and at monolayer, respectively.

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[21] T. Isemura, H. Hotta and S. Otsuka,, This Bulletin, **27**, 93 (1954).