Surface Chemistry of Synthetic Protein Analogues. V. On the Effect of Salt on the Monolayers of the Copolypeptide of L*-Lysine,* L*-Phenylalanine and* L*-Glutamic Acid*

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Introduction

In the previous paper,[1] we have reported on the behaviour of the monolayers of the electrolytic polypeptides, poly-L-glutamic acid and a copolypeptide of L-lysine, L-leucine and L-glutamic acid, in relation to the pH's of substrates. In the present paper, we shall report on the effect of salts on the monolayers of the copolypeptide of L-lysine, L-phenylalanine and L-glutamic acid. The interaction between natural proteins and salts is important in biological systems. The numerous investigations on these interactions have been carried out in bulk solution,[2] but few in interfaces.[3,4] In the recent investigations of protein by spreading monolayer, concentrated solutions of salts such as ammonium sulphate, are generally used as substrates in order to prevent the proteins from passing into substrate water.[5] Since there exist also some interactions between protein and salts at interface, a detailed knowledge of the interaction between protein molecules and salts in the substrate is indispensable for a thorough understanding of the behaviour of protein monolayers on salt solution.

For these reasons we have examined the effect of salt, i.e., potassium chloride, on the

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[1] T. Isemura and K. Hamaguchi, This Bulletin, 27, 339 (1954).

[2] F. Haurowitz, "Chemistry and Biology of Proteins," pp. 66 (1950).

[3] A. Lajtha and E. K. Rideal, *Arch. Biochem. Biophys.*, 33, 252 (1951).

[4] E. Fredericq, *Biochim. et Biophys. Acta*, 9, 601 (1952).

[5] H. B. Bull, *J. Am. Chem. Soc.*, 67, 4, 8, (1945).

monolayers of the amphoteric polypeptide which appears very promising as a protein model.

Experimental

Material and Methods.—The copolypeptide of L-lysine, L-phenylalnine and L-glutamic acid was prepared by Dr. H. Tani and Mr. H. Yuki⁽⁶⁾ and was used without any further purification. This copolypeptide was spread from a solution in N/100 hydrochloric acid to which 10 per cent of isopropyl alcohol was added. Surface pressure and surface viscosity measurements were carried out by the method of hanging plate and of damped oscillatory motion of disc, as reported in the previous papers.[7,8] Potassium chloride was recrystallized three times from water. The pH's of the substrates were adjusted by hydrochloric acid for lower pH values and by potassium bicarbonate or potassium carbonate for higher pH's, which were used by Langmuir and Shaefer for the studies of the effect of salts on the fatty acid monolayers.[9] The areas per amino-acid residue of the copolypeptide were calculated by assuming that the same ratio of L-lysine, L-phenylalanine and L-glutamic acid as the initial monomer concentration, 1 : 3 : 1, was maintained in the copolymer. This assumption turned out to be probably true throughout the analysis of the results.

Results.—The copolypeptide of L-lysine, L-phenylalanine and L-glutamic acid was soluble in the substrates which did not contain any salts. The stable monolayers were obtained when aqueous solutions of potassium chloride were used as substrates. To find out the concentration of potassium chloride, in the substrate solution necessary for giving a stable monolayer, the areas at constant surface pressure (1 dyne/cm.) against the concentration of potassium chloride are plotted as in Fig. 1. From this figure, one per cent of potassium chloride in the substrate was found to be sufficient to obtain a stable monolayer.

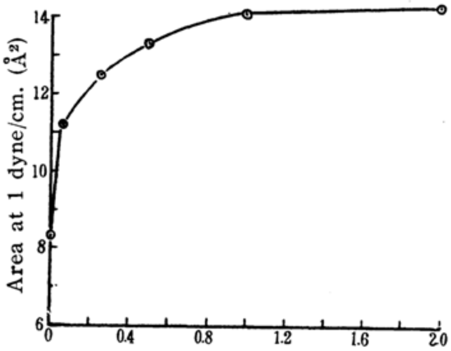

Fig. 1. Effect of the concentration of potassium chloride on the area per residue at 1 dyne/cm (pH 5.6).

The variations of the surface pressure-area (F-A) curves with the pH's of the substrates of constant KCl concentration (0.07 mole/litre), are shown in Fig. 2.

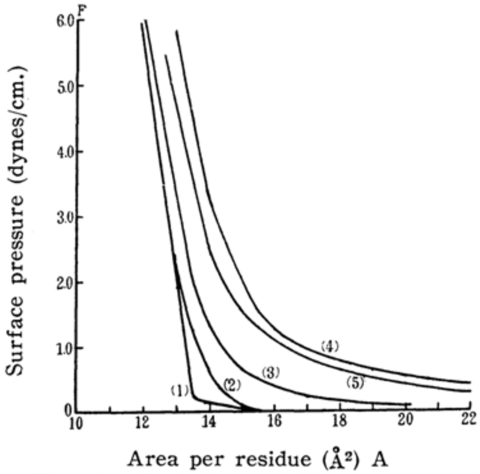

Fig. 2. Variations of F-A curves with the pH's of substrates; (1) pH 5.6—8.0, (2) pH 5.0, (3) pH 3.8, (4) pH 2.2, (5) pH 10.3 (0.07 mol. of KCl/litre).

A single F-A curve of condensed type was obtained in the pH range between 5.6 and 8.0. Changing the pH values of the substrate out of this pH range, F-A curves expanded gradually. The areas per residue at 1 dyne/cm. against pH's of the substrates are plotted in Fig. 3.

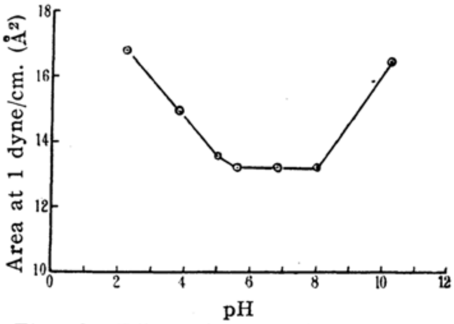

Fig. 3. Effect of pH of the substrate on the area per residue at 1 dyne/cm. (0.07 M KCl).

In this figure the minimum area region which is parallel to the pH axis, appears between pH 5.6 and 8.0.

The variations of F-A curves as the function of the concentration of potassium chloride in the substrate at constant pH (pH 2.2) are shown in Fig. 4.

It would be clear from this figure that the film expands with the decrease in the concentration of potassium chloride.

The surface viscosity-area (η-A) curves of the copolypeptide on the substrates of different concentrations of potassium chloride (at pH 5.6) are shown in Fig. 5. Fig. 6 shows the curve of η at constant area (at 17 Å²/residue) plotted against the concentration of potassium chloride.

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

7) T. Isemura and K. Hamaguchi, This Bulletin, **25**, 40 (1952).

8) T. Isemura and K. Hamaguchi, ibid., **27**, 125 (1954).

9) I. Langmuir and V. J. Shaefer, *J. Am. Chem. Soc.*, **59**, 2400 (1937).

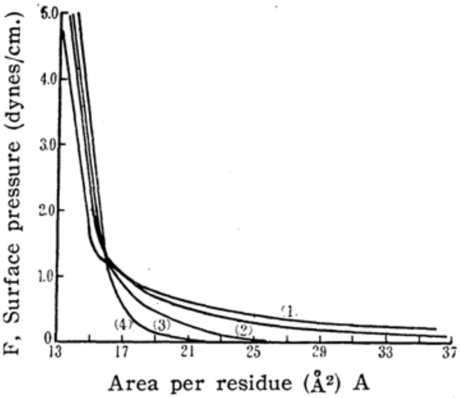

Fig. 4. Variations of F-A curves with concentration of KCl. (1) 0.035 M, (2) 0.07 M, (3) 0.13 M, (4) 0.40 M KCl. (pH 2.2)

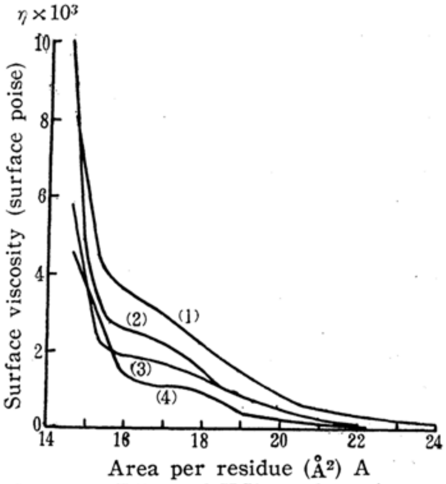

Fig. 5. Effect of KCl on the η-A curves of the copolypeptide. (1) 0.07 M, (2) 0.14 M, (3) 0.28 M, (4) 0.56 M KCl. (pH 5.6)

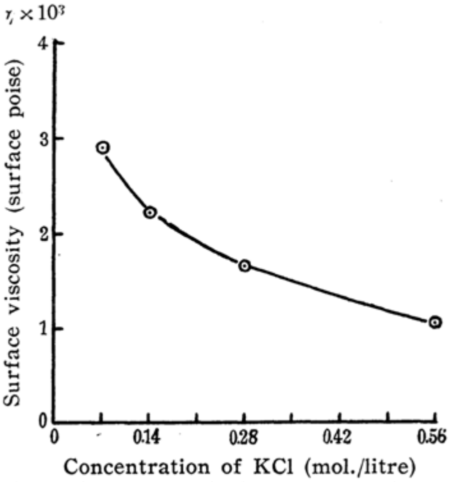

Fig. 6. Effect of the concentration of KCl on the surface viscosity (at 17Å²/residue). (pH 5.6)

The greater the concentration of potassium chloride becomes, the lower is the surface viscosity at constant area. Thus, the effect of potassium chloride on the η-A curves could be detected markedly while no effect on the F-A curves was observed when more than 1% potassium chloride was present in the substrates (Fig. 1). The fact recognized in common with all these η-A curves was that the surface viscosity begins to increase steeply at the area of 15.9 Å²/residue, irrespective of the concentration of potassium chloride.

The η-A curves of the copolypeptide on the substrates of different pH values, maintaining the concentration of KCl to be constant (0.28 M/litre), are shown in Fig. 7. Fig. 8 shows the surface viscosities at constant area (17 Å²/residue) against pH curve.

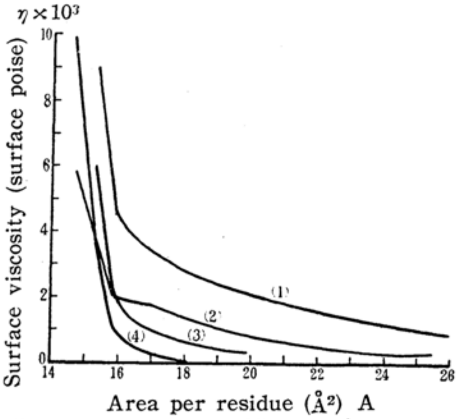

Fig. 7. Effect of pH on η-A curves of the copolypeptide (1) pH 6.8, (2) pH 5.6, (3) pH 8.0, (4) pH 2.2 (0.28 M KCl).

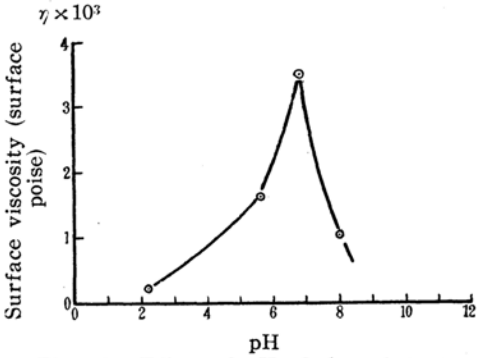

Fig. 8. Effect of pH of the substrate on surface viscosity (at 17Å²/residue). (0.28 M KCl)

It has been found from these figures that the surface viscosity becomes maximum at pH 6.8. On a more acidic substrate, the value of surface viscosity rather fluctuated and reproducible η-A curves could not be obtained in the pH range between 5.0 and 3.8.

Discussion

The Relationships between F-A Curves and pH's of Substrates (at Constant Con-

centration of KCl).—As shown in Fig. 2, the monolayers of the copolypeptide at pH between 5.6 and 8.0 of the substrates gave the same F-A curve of condensed type and in both the outsides of this pH range the F-A curves were of expanded type. The minimum area region appearing in the area (at constant pressure) against pH's of substrates curve (Fig. 3), corresponds to the region in which F-A curves have condensed type. This region between pH 5.6 and 8.0 corresponds to the isoelectric region and would be designated as the "isoelectric band" of the copolypeptide at this salt concentration (0.07 M/litre). When salts are contained in the substrate the isoelectric "band" appears rather than a "point" and the width of this band is the function of the salt concentration; an increase in the concentration of salt in the substrate widened the band. Even at pH 2.2, therefore, the F-A curves were of condensed type and it was expected that the F-A curves would represent the isoelectric property throughout all the pH's of the substrates, if potassium chloride was present in the concentration of more than 0.42 M/litre. This is due to the effect that an increase in ionic strength would reduce the coulombic force between the ionized groups of the copolypeptide. The fact that the film expands at each side outer than the isoelectric region is due to the electrostatic repulsion of -COO$^-$ or -NH$_3^+$ ions themselves when the one group exceeds the other.

In the previous paper,¹⁾ it was pointed out that in the case of the copolypeptide of L-lysine, L-leucine and L-glutamic acid, the area at constant pressure against pH curve is of W-shape in the absence of salts in the substrate and that this fact can be interpreted in terms of free charge ($\Delta\alpha=|\alpha_L-\alpha_G|$) and of salt linkage ($\alpha_L\cdot\alpha_G$), if α_L and α_G represent the ionization degree of amino and carboxyl groups. Referring to the present results, salts would have the effect of depressing the maximum point appearing in the $(A)_F$-pH curve. Detailed considerations about this effect will be present in the later part of this paper.

The Relationships between F–A Curves and Ionic Strength of Substrates (at Constant pH).—The effect of potassium chloride on the F–A curves of this copolypeptide is shown in Fig. 4. It may be found from this figure that the films are more expanded at low ionic strength in the substrates. This fact can be interpreted by the increment of the surface pressure due to the free energy of the electrical double layer which is formed between the ionized groups of the monolayer (in this case, -NH$_3^+$ ions of lysine residues) and the counter ions (Cl$^-$).

We can calculate the electrical potential difference of the double layer underneath the film from the F–A curves by the procedure adopted by J. T. Davies.[10,11]

According to Verwey and Overbeek,[12] the free energy of the double layer is represented by

$$G_s=-\frac{8nkT}{\kappa}\left(cosh\ \frac{ze\psi_0}{2kT}-1\right), \tag{1}$$

where k is the Boltzmann constant, T the absolute temperature, n the number of ions in 1 cc. of bulk solution, i.e., $n=NC/1000$ (C: mol./litre, N: Avogadro number), z the valency of salt, e the electronic charge, ψ_0 the surface potential and $\kappa=\sqrt{\frac{8\pi nz^2e^2}{\varepsilon kT}}$ (ε: dielectric constant).

The surface tension is equivalent to the surface free energy per unit area. When the electrical double layer is formed, the surface tension is decreased by G_s (because of the negative sign of equation (1)) and correspondingly the surface pressure is increased by ΔF, namely

$$\Delta F=-G_s=\frac{8nkT}{\kappa}\left(\cosh\frac{ze\psi_0}{2kT}-1\right). \tag{2}$$

After numerical substitution with $k=1.38\times10^{-16}$ erg/°K, $T=293$°K, $\varepsilon=80$, $e=4.8\times10^{-10}$ e.s.u., $z=1$, the following relation is obtained:

$$\Delta F=6.1\sqrt{c}\left(\cosh\frac{ze\psi_0}{2kT}-1\right). \tag{3}$$

From Eq. (3)

$$\psi_0=51.2\ \cosh^{-1}\left(1+\frac{\Delta F}{6.1\sqrt{c}}\right) \tag{4}$$

is obtained, where ψ_0 is expressed in millivolt units.

The surface pressure increment (ΔF) due to the presence of the electrical double layer is written

$$\Delta F=F^+-F^\circ, \tag{5}$$

where F^+ is the observed pressure and F° the pressure if the film had no charged groups. F° is a negative quantity, as shown in the later part, so it might be recognized as the cohesional force between film molecules.

On the other hand, the Gouy potential ψ_G, which is at a uniformly charged surface when uni-univalent salts are present, is expressed as

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[10] J. T. Davies, *Proc. Roy. Soc.*, A208, 224 (1951).

[11] K. Hamaguchi, *Mem. Inst. Sci. Ind. Res., Osaka University*, 11, 175 (1954).

[12] H. R. Kruyt, "Colloid Science," Elsevier, Vol. I, pp. 140 (1952).

$$\psi_G = 2.303\frac{kT}{e}\log\left(\frac{134^2\times 4}{A^2C}\right), \qquad (6)$$

where A is the area per ion of film-forming substance in Å² unit.

If we assume that ψ_G is the same as ψ_0, we can calculate ψ_0 by Eq. (6). However, the following two assumptions are necessary: (a) At pH 2.2 all the -NH_2 groups of lysine residues are ionized to -NH_3^+ ions. (b) It is difficult to find out the area per ion in the case of polyampholytes. In the present case, we assume in regard to the first approximation as described above, that the initial monomer concentration ratio of lysine, phenylalanine and glutamic acid residues, i.e., 1:3:1, is maintained in the copolymer produced, so that the area per -NH_3^+ ion may be five times the area per residue. By these assumptions ψ_0 can be calculated by Eq. (6) for the case of 20 Å²/residue, namely 100 Å²/-NH_3^+ ion. ψ_0 becomes 115 mv. when $c=0.075$ mol./litre. Substituting this figure in Eq. (4) and then in Eq. (5) we obtain $F^\circ = -5.8$ dynes/cm. Using this F° value ψ_0 can be calculated by Eq. (4) at different salt concentrations. The results are summarized in Table I.

TABLE I

c (mol./litre)	F (dynes/cm.) at 20 Å²/residue	ψ_0 (mv.) calculated from eq. (4)	ψ_0 (mv.) calculated from eq. (6)
0.04	0.65	130	131
0.075	0.55	115	115
0.135	0.30	102	100
0.405	0.05	80.4	72.8

The values of ψ_0 calculated from Eq. (4) are in close agreement with those of ψ_G from (6). Therefore, the following equation, which is readily derived from Eq. (6), is also applied for ψ_0 values calculated from the F–A curves.

$$\left(\frac{\partial\psi_0}{\partial\log c}\right)_A = -59\,mv. \qquad (7)$$

This relation was found for the monolayers of α-brompalmitic acid[13] and of octadecyltrimethylammonium chloride[10] on the substrates containing sodium chloride.

The Relationships between η-A Curves and Ionic Strength or pH's of Substrates.—As shown in Figs. 5 and 6 an increase of the ionic strength in the substrate accompanied the decrease of the surface viscosity and the surface viscosity began to increase steeply at the area of about 15.9 Å²/residue. This area corresponds to the area per residue calculated with the X-ray data for close packed arrangement of peptide of β-configuration.[7]

Cumper and Alexander[14,15] showed that although the effect of the ionic strength of salts could not be detected for the spreading monolayers of proteins, in the case of adsorbed protein films an increase in ionic strength decreased the surface viscosities. We could find out the same effect for the spreading monolayers of the copolypeptide as for the adsorbed films found by them.

There are two possibilities to explain why the surface viscosity decreased by an increase in ionic strength of salts in the substrate.

(a) In the case of polyelectrolyte solutions, the viscosity is generally decreased by an increase in salt concentration, and this might attributed to the coiling of polyelectrolyte molecules, for salt reduces the electrostatic repulsion between the intramolecular charged groups.[16] The relation between the surface viscosities and the salt concentration which was found in the present experiments (Fig. 6), is analogous to the bulk viscometric behaviour of polyelectrolytes just mentioned. The surface viscometric behaviour would be, therefore, explained by the same reason as for bulk viscosities.

(b) There exist many salt-linkages between -NH_3^+ and -COO^- ions at pH 5.6, for this pH value lies in the isoelectric band as described previously. The high surface viscosities would be the result of the great resistance due to these inter- or intramolecular salt-linkages. By the presence of salts, however, the coulombic force between -NH_3^+ and -COO^- ions is minimized. Therefore, the effect of salt on the surface viscosities would be interpreted in terms of this reducing effect of electrostatic interaction.

The former possibility (a) cannot be admitted for the following reasons. The areas where the effect of salts appears, are smaller than 21 Å²/residue and this smaller area region corresponds to the state in which polymer molecules are very close to each other. In such a state it could not be considered that the surface viscosity depends on the shape of each polymer molecule. Furthermore, if the increase in surface viscosity by the decrease in the concentration of salt would be due to the elongation of a polymer molecule, the reason why a maximum point appears in the $(\eta)_{\overline{A}}$ pH curve (Fig. 8), cannot be ex-

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[13] D. J. Crisp, Surface Chemistry (*Research Supplement*), Butterworth, (1949) p. 65.

[14] C. W. N. Cumper and A. E. Alexander, *Trans. Faraday Soc.*, **46**, 235 (1950).

[15] C. W. N. Cumper and A. E. Alexander, *Revs. Pure and Applied Chem.* (Australia), **1**, 121 (1951).

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

plained. These facts support the possibility (b). Davies,[17] in his paper on the shape of protein molecules at interfaces, considered that the surface viscosity of protein at oil-water interface is very much higher than at air-water interface, is one of the evidences that the protein molecules are to a greater extent unfolded into long chains of considerable flexibility at the former than at the latter. He compared, however, the surface viscosity at oil-water interface with that at air-water interface in a region of very small area. Furthermore, he referred to Joly's data[18] of surface viscosities at air-water interface in which the substrate was acidified by N/100 HCl and also to the data of Cumper and Alexander[14] at oil-water interface in which the pH's of the aqueous phase were not described explicitly. However, in view of our results mentioned above, we cannot agree with his opinion. Inokuchi[19] pointed out that the area at which the instantaneous elasticity of egg albumin films appears on 5% ammonium sulphate solution at pH 5.5, was three times the area as on 0.1 N HCl. This would also be interpreted in terms of salt-linkages when we take into account that the isoelectric point of the protein lies at pH 4.8.

The decrease in surface viscosities at constant area is approximately proportional to the square root of the ionic strength in the substrate. This fact corresponds to the bulk viscosity or rigidity of very concentrated gelatin solutions.

The Relationships between $(A)_F$-pH, $(\eta)_A$-pH, $\Delta\alpha$-pH and $\alpha_L \cdot \alpha_G$-pH Curves.—In the previous paper,[1] it was pointed out that the $(A)_{F or \eta}$ (at constant F or at constant η)-pH curve which was obtained with the films of the copolypeptide of L-lysine, L-leucine and L-glutamic acid on the substrate which did not contain any salts, was of W-shape and the maximum point in this curve corresponded to its isoelectric point. We calculated the ionization degrees of $-NH_2$ groups of lysine residue (α_L) and of $-COOH$ groups of glutamic acid residue (α_G) at each pH by the following simple approximate equation

$$\mathrm{pH}-\mathrm{p}K_0=\log\frac{\alpha}{1-\alpha}, \tag{8}$$

where K_0 is the intrinsic dissociation constant which is assumed to be 10.5 and 4.3 for $-NH_2$ and $-COOH$ groups, respectively. $\Delta\alpha=|\alpha_L-\alpha_G|$ might be assumed to give a measure of effective free charges and $\alpha_L \cdot \alpha_G$ the probability of salt-linkages. This assumption might be justified if the polymerization ratio of lysine and glutamic acid is 1:1. The W-shaped $(A)_{F or \eta}$-pH curve could be interpreted as follows in terms of the $\Delta\alpha$-pH and $\alpha_L \cdot \alpha_G$-pH curves. $\alpha_L \cdot \alpha_G$-pH curve showed a maximum at pH 7.0 and the $(A)_{F or \eta}$-pH curve also showed a maximum at this pH. When the substrate becomes more acidic or alkaline from this isoelectric point, the area becomes smaller and this region corresponds to the droop $\alpha_L \cdot \alpha_G$-pH curve. In this region the effect of $\alpha_L \cdot \alpha_G$ is more predominant than that of $\Delta\alpha$, i.e., the effect of salt-linkages is more pronounced than that 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 $\Delta\alpha$-pH curve, i.e., the effect of electrical repulsion is more predominant than that of salt-linkages.

When salt is present in the substrate, however, the maximum in the W-shaped $(A)_F$-pH curve disappeared and the curve had a minimum $(A)_F$ region between pH 5.6 and 8.0 which is parallel to the pH axis (Fig. 3). As described above, salt ions have the effect of reducing the electrostatic interaction between $-NH_3^+$ and $-COO^-$ ions. Therefore, it is very probable that a maximum point which should appear in the $(A)_F$-pH curve, is depressed by salt ions and so the portion corresponding to the $\alpha_L \cdot \alpha_G$-pH curve which

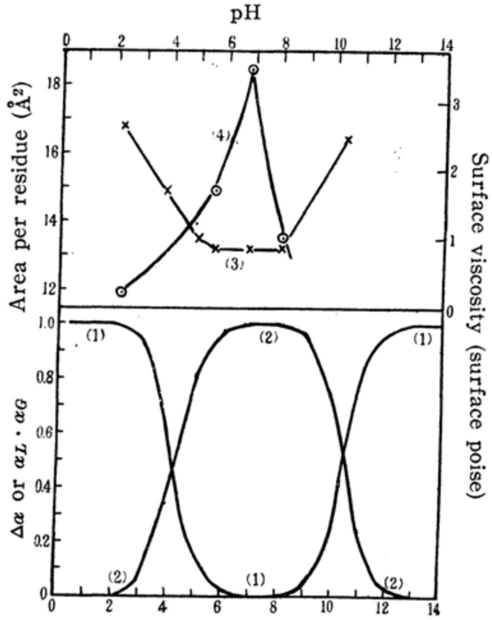

Fig. 9. (1) $\Delta\alpha$-pH, (2) $\alpha_L\,\alpha_G$-pH, (3) $(A)_F$-pH, (4) $(\eta)_A$-pH curves.

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

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

[19] K. Inokuchi, This Bulletin, **26**, 500 (1953).

represents the term of salt-linkages, disappears. Then as shown in Fig. 3 the $(A)_F$-pH curve has only the portion corresponding to the $\Delta\alpha$-pH curve. Ellis and Pankhurst[20] showed that the $(F)_A$-pH curve obtained with collagen films, showed a maximum at pH 2.2 and sodium chloride in the substrate caused the maximum to be depressed and finally to disappear. The $(\eta)_A$-pH curve has a maximum point at pH 6.8 and corresponds to the $\alpha_L \cdot \alpha_G$-pH curve. This fact also supports the consideration that the surface viscosities depend markedly on the number of inter- or intra- molecular salt likages. The $(A)_F$-pH, $(\eta)_A$-pH, $\Delta\alpha$-pH and $\alpha_L \cdot \alpha_G$-pH curves are shown together in Fig. 9.
These relationships show that the isoelectric point of the copolypeptide lies near pH 6.8.

Summary

By studying the surface pressure-area and surface viscosity-area curves of the copolypeptide of L-lysine, L-phenylalanine and L-glutamic acid over a range of pH values and salt contents in the substrate, the following four distinct results were obtained:

(1) When the concentration of potassium chloride in the substrate was kept constant (0.07 M/litre), the $(A)_F$-pH curve has a minimum area region between pH 5.6 and 8.0 which is parallel to the pH axis and this region corresponds to the isoelectric band of this copolypeptide at this salt concentration. The width of this band was the function of the salt concentration; when the salt content was increased this band became wider.

(2) When the pH's of the substrates was kept constant (pH 2.2), the F-A curves were more expanded at low salt concentrations. This fact could be interpreted by the increment of surface pressure due to the free energy of the electrical double layer which is formed between the ionized groups of the monolayer (NH_3^+ ions) and the counter ions (Cl^- ions). The values of surface potentials calculated from the F-A curves and from Gouy's equation agreed with each other.

(3) The surface viscosities became higher when the salt concentrations in the substrate at constant pH's were decreased and became maximum at its isoelectric point. The surface viscosity depends markedly on the number of inter- or intra-salt-linkages between -NH_3^+ and COO^- ions and not on the shape of each polymer molecule.

(4) The $(A)_F$-pH and $(\eta)_A$-pH curves could be interpreted in terms of effective free charges and the number of salt-linkages.

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[20] S. C. Ellis and K. G. A. Pankhurst, *Nature*, **163**, 600 (1949); *Trans. Faraday Soc.*, **50**, 82 (1954).