*The Roles of Prolyl Residue in Polypeptide Monolayers. I. On the Chain Configurations Deduced from Surface Pressure and Potential Measurements**

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During the last decade, investigations on synthetic polypeptides as model substances of protein have made great progress. Their monolayer properties have also been studied and clarified by the present authors[1] and many other workers[2,3]. In these studies synthetic polypeptides with non-electrolytic or unionized electrolytic side chains are found to spread as condensed monolayers at the air-water interface. Many globular proteins also give condensed monolayers at the same interface. Generally, monolayers of high polymeric substances are of condensed type under the strong interaction between

* Surface Chemistry of Synthetic Protein Analogus VIII.

1) T. Isemura and K. Hamaguchi, This Bulletin, **26**, 425 (1953); **27**, 125. 339 (1954).
K. Hamaguchi and T. Isemura, ibid., **28**, 9 (1955).
T. Isemura, K. Hamaguchi and S. Ikeda, *J. Polymer Sci.*, **23**, 651 (1957).

2) D. F. Cheesman and J. T. Davies, *Adv. Protein Chem.*, **9**, 439 (1954).

3) C. H. Bamford, A. Elliott and W. E. Hanby, "Synthetic PolyPeptides", Academic Press, New York (1956), p. 355-360.

constituent monomer units and of expanded type under the weak interaction.

Among amino acid residues constituting polypeptide chains in protein, prolyl residue is specific in respect to several properties: (1) it can not be incorporated into the β-configuration of a polypeptide chain because it has a pyrrolidine ring which makes a part of the main chain; (2) it reduces the degree of stertic rigidity because it is inhibited from rotating around some bonds involved; and (3) it can form no hydrogen bond with other carbonyl group because the nitrogen atom of keto-imido bond has no hydrogen atom to participate in the hydrogen bonding.

Synthetic polypeptides, the monolayers of which have so far been studied, contain no prolyl residue. It is of great importance to investigate how these properties of prolyl residue affect the behavior of monolayers of polypeptides and to correlate it with the chain configuration, the chain flexibility and the hydrogen bonding. These studies appear to be useful to interpret monolayer properties of gelatin and collagen.

In this connection, we have investigated the monolayers of synthetic polypetides composed of various proportions and arrangements of prolyl residues, that is, poly-DL-alanine, poly-L-prolyl-L-leucylglycine, copoly-1 : 1 : 1-(L-proline, L-leucine, DL-alanine) and poly-L-proline. Poly-L-hydroxyproline was also examined for comparison with poly-L-proline. In this paper, we will describe the results and some inferences derived from the measurements of surface pressure and potential.

Experimental

Materials.—Poly-DL-alanine is a sample prepared by Dr. Tani[4], the degree of polymerization of which was found to be about 300 by surface pressure measurement. It was spread from the solution in a mixed solvent, dichloroacetic acid-benzene (1 : 9 v/v). Poly-L-prolyl-L-leucylglycine is a sample synthesized by Dr. Sakakibara[5]. Its degree of polymerization was found to be 13 by end-group analysis and surface chemical method. Copoly-1 : 1 : 1-(L-proline, L-leucine, DL-alanine), poly-L-proline II[6] and poly-L-hydroxy-proline are gifts of Dr. Kurtz through Professor Noguchi. The degree of polymerization of copoly-1 : 1 : 1-(L-proline, L-leucine, DL-alanine) was found to be 129 by surface pressure measurement. Poly-L-prolyl-L-leucylglycine and copoly-1 : 1 : 1-(L-proline, L-leucine, DL-alanine) were spread from the solution in a mixed solvent, dichloroaceticacid-benzene (1:4 v/v). The spreading solvent for poly-L-proline II (D. P. 50) and poly-L-hydroxyproline (D. P. unknown) was 50% (v/v) isopropyl alcohol. Because of their hygroscopic nature, poly-L-prolyl-L-leucylglycine, poly-L-proline II and poly-L-hydroxyproline were dried for several hours at the boiling point of toluene in an Abderhalden's pistol, before weighing for preparing the spreading solutions. Poly-L-prolyl-L-leucylglycine contains one molecule of water of crystallization per periodic unit after such drying.

Distilled water was used as aqueous subphases, unless otherwise stated. The pH of subphases was adjusted by 0.01 N hydrochloric acid or by 0.01 M potassium carbonate, if necessary. To certify the minimum solubility of the sample into the aqueous phase, potassium chloride was often added, which was recrystallized from water after removing surface active impurities by using active charcoal as adsorbent and then baked.

Methods.—Surface pressure was measured by a surface balance of float type with a mica float and a double torque system. Its sensitivity was better than 0.01 dyne per cm. Surface potential was measured by the vibrating electrode method. The electrode was made of gold of 2 cm. in diameter. The signal picked up by the electrode was amplified by a three stage A. C. amplifier and detected by an oscilloscope. The potential was determined by the compensation method with a potentiometer. The trough was made of polymethyl methacrylate, the rim of which was coated by a thin paraffin layer to ensure a high contact angle.

All the measurements were carried out at room temperature, but the fluctuation of temperature never exceeded 1° during the experiments. The solution was spread by means of a micrometer syringe. Fifteen minutes after spreading, the compression of film was started. The films of all polypeptides other than poly-L-proline and poly-L-hydroxyproline showed no time effect.

Results

Surface pressure-area (Π-A) and surface potential-area (ΔV-A) curves of poly-DL-alanine, poly-L-prolyl-L-leucylglycine and copoly-1 : 1 : 1-(L-proline, L-leucine, DL-alanine) are shown in Figs. 1, 2, and 3, respectively. For comparison with poly-L-prolyl-L-leucylglycine, area of copoly-1:1:1-(L-proline, L-leucine, DL-alanine) in Fig. 3 was denoted in the unit of A^2 per three amino acid residues. In Fig. 4 the Π-A and ΔV-A curves of poly-L-proline together with the Π-A curve of poly-L-hydroxyproline both on 1 M potassium chloride are shown. The Π-A and ΔV-A curves of all these polypeptides other than the last

4) H. Tani and C. Ooizumi, Symposium on Synthetic Polypeptids, Osaka, Oct. 28, 1957.
5) H. Kitaoka, S. Sakakibara and H. Tani, This Bulletin, **31**, 802 (1958).
6) A. Berger, J. Kurtz and E. Katchalski, *J. Chem. Soc.*, **76**, 5552 (1954).

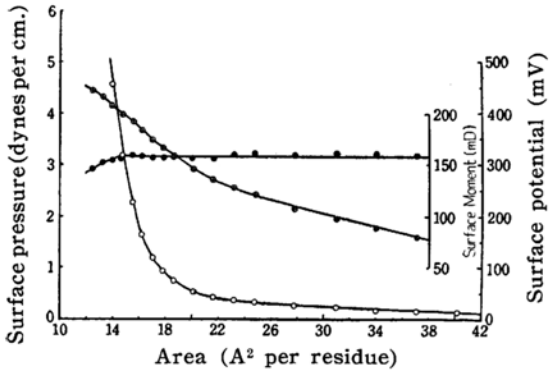


Fig. 1. Surface pressure (○), potential (⊕) and moment (●) *vs.* area curves of poly-DL-alanine at 18°C.

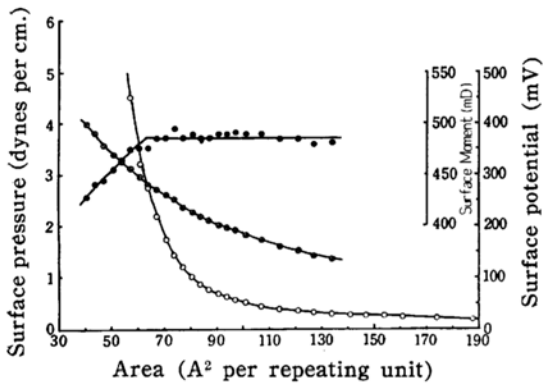


Fig. 2. Surface pressure (○), potential (⊕) and moment (●) *vs.* area curves of poly-L-prolyl-L-leucyglycine at 27°C.

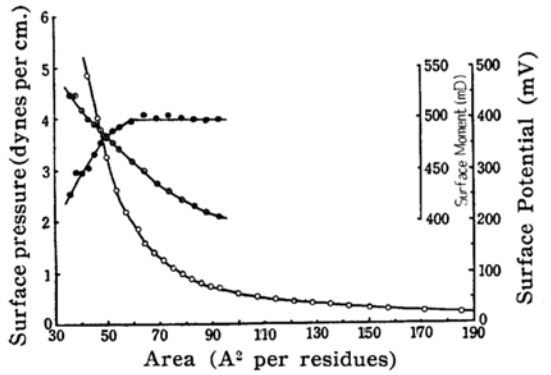


Fig. 3. Surface pressure (○), potential (⊕) and moment (●) *vs.* area curves of copoly-1 : 1 : 1-(L-proline, L-leucine, DL-alanine) at 16°C.

two were not affected by the addition of potassium chloride in the concentration of 0.1 M into the aqueous subphase. Therefore, they are considered to be spread completely as monomolecular films. The surface moment-area (μ-A) curves are also shown in these figures, which were calculated by the Helmholtz equation,

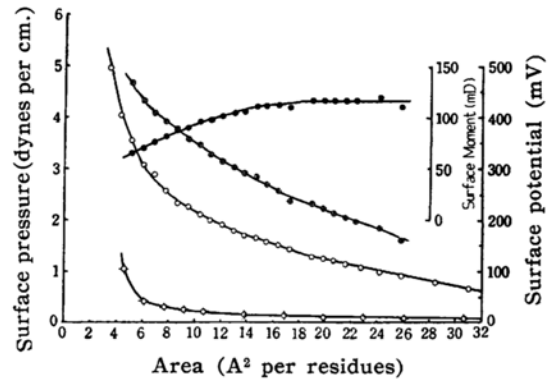


Fig. 4. Surface pressure (○), potential (⊕) and moment (●) *vs.* area curves of poly-L-proline and surface pressure-(◇) area curve of poly-L-hydroxyproline on 1 M potassium chloride at 29°C.

$$\Delta V = \frac{4\pi\mu}{A}$$

In the Π-A curve there appears an area, A_Π, where the surface compressibility

$$\delta = -\frac{1}{A}\frac{\partial A}{\partial \Pi}$$

is a minimum. This area corresponds to that occupied by a repeating unit in the close-packed monolayer. The surface moment is constant, namely, μ_c, over certain regions of area. When the monolayer is compressed, the surface moment begins to decrease at a certain definite area, A_μ. This area is that where the electric dipole of repeating unit begins to change its orientation. Since the dipole is considered to be contributed mainly by the carbonyl groups of peptide bonds in the polypeptides studied (except for poly-L-hydroxyproline), the values of surface moment are concerned with the orientation of carbonyl groups in the monolayer. All these characteristics are summarized in Table I.

TABLE I
MONOLAYER CHARACTERISTICS OF POLYPEPTIDES

Polypeptides	A_Π A² per repeating unit	A_μ A² per repeating unit	μ_c mD
Poly-DL-alanine	14.9	14.8	158
Poly-L-prolyl-L-leucyl-glycine	57	63	162×3
Copoly-1 : 1 : 1-(L-proline, L-leucine, DL-alanine)	46*	60*	166×3

* A² per three amino acid residues.

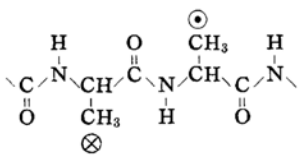
In spite of the marked difference in surface pressure among three polypeptides completely spread as monolayer, that is,

poly-DL-alanine, poly-L-prolyl-L-leucylglycine and copoly-1 : 1 : 1-(L-proline, L-leucine, DL-alanine), the values of surface moment were found to be nearly equal to one another, μ_c being about 160 mD per amino acid residue, as seen in Table I. This means that the orientation of the carbonyl groups of all these polypeptides are almost the same. Taking the electric moment of carbonyl group to be 360 mD, following Davies[7], it may be directed to 30° downwards to the aqueous surface.

Discussion

Chain Configuration in Polypeptide Films. — *a) Poly-DL-alanine.* — Poly-DL-alanine gives a monolayer of condensed type. Its Π-A and ΔV-A curves have already been reported by Glazer and Dogan[8], and its μ-A curve has been measured by Davies[7]. The Π-A curve in Fig. 1 shifts somewhat to larger area than that of Glazer and Dogan's, but the ΔV-A curve is in good agreement. The A_μ value found from the μ-A curve is much less than Davies'. The monolayer of poly-DL-alanine is much more condensed than some vinyl polymers with side-chains of the same order of length, such as polyvinyl acetate[9-12] and polyethyl acrylate[9]. This is attributed to the fact that the polypeptide chain is held together rigidly by the intrachain hydrogen bonds and is less flexible by the partial double bond nature of peptide bonds. The minor dependence of surface pressure on temperature also supports this view.

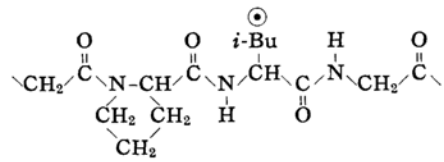
From the data in Table I, it appears probable that the β-configuration with the alternation of side-chains, methyl groups, up and down to the surface,



⊙ above the interface
⊗ below the interface

makes most parts of the chain, as Cumper and Alexander[13] and Davies[7,14] suggested from the surface viscosity and surface potential measurements, respectively.

b) Poly-L-prolyl-L-leucylglycine.—Recently, Leung and Marsh[15] determined the crystal structure of the tripeptide, L-leucyl-L-prolylglycine, which is similar to the repeating unit of poly-L-prolyl-L-leucylglycine. They found that all the peptide bonds are planar-*trans* and the tripeptide is in a highly extended configuration. Such a configuration appears to be probable also in this polypeptide. On the basis of these requirements and data in Table I, a possible configuration at the interface may be suggested as follows:



⊙ above the interface

in which the main chain assumes a β-configuration except for the glycyl-L-prolyl bond, and the side-chain of L-leucyl residue stands up out of the aqueous surface. While at the area larger than A_μ all pyrrolidine rings of L-prolyl residues lie flat on the aqueous surface, at the area less than A_μ some of them rise on the surface and at the area equal to A_Π all of them rise and are closely packed.

c) Copoly-1 : 1 : 1-(L-proline, L-leucine, DL-alanine). — Although copoly-1 : 1 : 1-(L-proline, L-leucine, DL-alanine) has the same proline content as poly-L-prolyl-L-leucylglycine, the arrangements of prolyl and the other residues in the amino acid sequences might be different from each other. As seen in Table I, whereas the values of A_μ are equal for these two polypeptides, A_Π for the former is much less than that of the latter. The former monolayer is more expanded. At the area larger than A_μ, their chain configurations appear to be similar to each other, and they should, however, be different at the area less than A_μ. The amino acid sequence in the former polypeptide cannot, therefore, be entirely periodic as in the latter. On the other hand, if it would contain any successive prolyl residues in some parts of the chain, no prolyl residues could always

7) J. T. Davies, *Biochim. Biophys. Acta*, **111**, 165 (1953).
8) J. Glazer and M. Z. Dogan, *Trans. Faraday Soc.*, **49**, 448 (1953).
9) D. J. Grisp, *J. Colloid Sci.*, **1**, 49 (1946).
10) G. C. Benson and R. L. McIntosh, ibid., **3**, 223 (1948).
11) T. Isemura, H. Hotta and T. Miwa, This Bulltetin, **26**, 380 (1953).
12) M. J. Shick, *J. Polymer Sci.*, **25**, 465 (1957).
13) C. W. N. Cumper and A. E. Alexander, *Trans. Faraday Soc.*, **46**, 235 (1950).
14) J. T. Davies, ibid., **49**, 949 (1953).
15) Y. C. Leung and R. F. Marsh, *Acta Cryst.*, **10**, 815 (1957); **11**, 17 (1958).

exist at the interface in an equivalent manner, orientating the pyrrolidine rings and carbonyl groups irregularly. Then the $A_{//}$ and μ_c values would be less than those observed. Thus the copolypeptide should contain the considerable amounts of L-prolyl-R-L-prolyl sequences, where R is either L-leucyl or DL-alanyl residue. Such parts of the chain will be folded by the steric hindrance and rigidity, submerging a part of the main chain into the aqueous subphase, instead of raising the pyrrolidine rings out of the surface, when the monolayer is compressed to the area less than A_{μ}. The other parts of the chain will remain the same as in poly-L-prolyl-L-leucylglycine. Hence, $A_{//}$ is much less than A_{μ}. It may, therefore, be considered that copoly-1:1:1-(L-proline, L-leucine, DL-alanine) is composed of a polypeptide with no successive but irregular arrangements of prolyl residues.

d) Poly-L-proline. — Poly-L-proline exists in two forms[16]. Poly-L-proline I is prepared by polymerization of N-carboxy-L-proline anhydride in pyridine and precipitated by adding ether to the reaction mixture. It is partly soluble in water and dextro-rotatory, $[\alpha]_D^{25} = +40°$. When it is dissolved in formic acid and precipitated by ether, it is transformed into poly-L-proline II, which is soluble in water and highly levo-rotatory, $[\alpha]_D^{25} = -540°$. But poly-L-proline I is regenerated when poly-L-proline II is dissolved into higher alcohols[17].

The poly-L-proline sample given to us was poly-L-proline II. However, at least, its most part is probably transformed into poly-L-proline I in the present spreading solution, that is, in 50% (v/v) isopropyl alcohol solution. The Π-A curves of poly-L-proline on various aqueous subphases are shown in Fig. 5. It is unstable on salt-free subphases, and the higher the pH of the aqueous subphase, the more stable and the more expanded the film. The addition of potassium chloride into the water phase also aids the spreading of film. In any cases examined, films are of expanded type and pressure-soluble.

According to the study of X-ray diffraction by Cowan and McGavin[18], poly-L-proline II in the crystalline state exists in a left-handed helix with 3 residues per

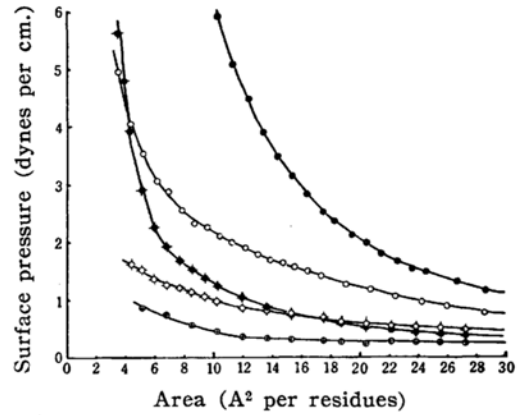


Fig. 5. Surface pressure-area curves of poly-L-proline on various aqueous subphases at 29°C: ⊕, 0.01 N HCl; ◇, H_2O; ○, 1 M KCl; ◆, 0.01 M K_2CO_3; ●, 0.01 M K_2CO_3; 1 M KCl.

turn, the keto-imido bonds being planar-*trans*. On the other hand, though no data on the structure of poly-L-proline I is available, Katchalski[17] inferred it to be a right-handed helix with *cis* bonds, and actually Crick and Rich[19] have deduced it to have approximately $3^1/_8$ residues per turn. Both structures have a high degree of steric rigidities, only permissible to the *cis-trans* transformation. Harrington and Sela[20] have demonstrated this for aqueous solutions. It will be found that no proline residues of poly-L-proline can always exist in an equivalent manner, when it is spread at the interface. Then no carbonyl groups can always orientate to the same direction, and the surface moment is, therefore, relatively low, owing to the intrachain cancellation of dipole moments. In fact, the values of μ_c did not exceed 120 mD in the cases of various subphases examined. The results obtained in the present investigation suggest that the *trans* peptide bonds in poly-L-proline dissolve into the aqueous subphase and the *cis* bonds remain on the surface, after being subjected to the *cis-trans* transformation, as soon as poly-L-proline I is spread. The lower the pH of subphase, the more feasible the transformation will be. The dependence of film behavior on the pH appears to have some correlation with the mutarotation of poly-L-proline I in formic acid, acetic acid or water. The addition of potassium chloride will support the *trans-cis* transformation, reducing the water

16) J. Kurtz, A. Berger and E. Katchalski, *Nature*, **178**, 1066 (1956).
17) E. Katchalski, Symposium on Synthetic Polypeptides, Osaka, Oct. 28, 1957.
18) P. M. Cowan and S. McGavin, *Nature*, **176**, 501 (1955).
19) F. H. C. Crick and A. Rich, cited in ref. (20).
20) W. F. Harrington and M. Sela, *Biochim. Biophys. Acta*, **27**, 24 (1958).

activity. The expanded nature of poly-L-proline films may be attributed to their being devoid of hydrogen bond, as shown later.

General Behavior of Polypeptide Monolayers.—From the Π-A curves in Figs. 1, 2, 3 and 4, it can be noticed that the polypeptide films change from the condensed type to the expanded type in the following order:

poly-DL-alanine;

poly-L-prolyl-L-leucylglycine;

copoly-1 : 1 : 1-(L-proline, L-leucine, DL-alanine);

poly-L-proline.

This order suggests that the higher the prolyl content, the more expanded the film, although the extent of the expansion of film depends also on the arrangement of prolyl residues in the polypeptide sequence. Even poly-DL-alanine, which has the shortest side-chain among polypeptides to be spread as monolayer, gives a very condensed film. The film of poly-L-proline is one of the most expanded.

To distinguish which factor of prolyl residue mentioned earlier is the most responsible for the expansion of film, the film of poly-L-hydroxyproline was studied which differs from poly-L-proline only in the presence of hydroxyl groups on pyrrolidine rings capable of hydrogen bonding. Its Π-A curve on 1 M potassium chloride solution is shown in Fig. 4. The film was unstable and spread incompletely even on such a concentrated salt solution. It is, however, of condensed type. Therefore, the expanded nature of poly-L-proline film may be attributed to the absence of hydrogen bond. The order of expansion of polypeptide films will mainly come from the fact that the prolyl residues involved reduce the number of hydrogen bonds in the film state. The hydrogen bonds in film hold the polypeptide chain together and make it rigid, and, conversely, their decrease in number will make the chain extended and flexible. Accordingly, it is believed that the chain flexibility caused by the reduced number of intrachain hydrogen bonds is generally more effective than the steric rigidity in the monolayer of polypeptide containing prolyl residues.

It is found from Table I that while the condensed poly-DL-alanine monolayer has equal values of A_{Π} and A_{μ}, the expanded monolayer of the other two polypeptides has A_{Π} values less than A_{μ} values. Such a difference between monolayers of condensed and expanded type is extensively observed in other polymers so far reported. Since the condensation and expansion of monolayers of polymers are related to the intrachain interaction, this property depends on the chain configuration in monolayer. The condensed monolayer will collapse if it is compressed beyond the close-packed area, because the molecular chain is held together rigidly. In the expanded monolayer, however, the molecular chain can readily reorientate or fold itself on compression, as it is flexible. The difference in chain configuration or intrachain interaction is considered to be manifested in the monolayer property mentioned above.

Summary

To elucidate the roles of prolyl residue in polypeptide monolayers, the surface films of poly-DL-alanine, poly-L-prolyl-L-leucylglycine, copoly-1 : 1 : 1-(L-proline, L-leucine, DL-alanine), poly-L-proline and poly-L-hydroxyproline were studied by the surface pressure and potential measurements.

The area of minimum surface compressibility and the smallest area of constant surface moment were assigned to each of the first three polypeptides which were found to form monomolecular films. In the region of large area their surface moments were equal to one another and, therefore, the orientations of carbonyl groups might be the same. From these data their chain configurations at the interface were deduced. Poly-DL-alanine exists in the β-configuration and the other two have the similar configuration except for the bonds attached to prolyl residues. Based on the departure in surface pressure behavior of copoly-1 : 1 : 1-(L-proline, L-leucine, DL-alanine) from that of poly-L-prolyl-L-leucylglycine, the arrangement of prolyl residues in the sequence of the former polypeptides was discussed. In the poly-L-proline film it is likely that the parts of *cis* peptide bond form the film and the parts of *trans* bond dissolve into the aqueous subphase.

The polypeptide films were found to be more expanded as the prolyl content was higher, although the arrangement of prolyl residues also affected the expansion of film. From the comparison of poly-L-proline film with poly-L-hydroxyproline,

the evidence was obtained that the expansion of film comes from the decrease in number of hydrogen bonds because of the presence of prolyl residues. The condensation or expansion and its related properties of the film were interpreted in terms of the chain rigidity or flexibility and the intrachain interaction.

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