Monolayers of Some Cyclic Peptides. Fungisporin and Gramicidin J_1

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Chemical structures of many naturally occurring cyclic peptides and their modes of biological action have been investigated and elucidated in recent years. While many informations concerning the chain configuration and other properties of linear polypeptides in spread monolayer are obtained, the studies on cyclic peptides on surface are relatively deficient and stimulate our interest in several respects. Spread linear polypeptides are generally accepted to take the β-configuration[1,2] but spread cyclic peptides, unless the size of their rings is large enough, can not accomodate with this configuration. Thus it is desired to examine the configuration of cyclic peptides in the monolayer. Further, it is noticed that while ordinary linear polypeptides form monolayers of a condensed type owing to the intramolecular hydrogen bonding, the presence of prolyl residue[3] or ionized amino acid residue[4] in amino acid sequence, causes their monolayers to expand more. It is of importance to investigate whether or not this effect is exhibited in the monolayers of cyclic peptides.

Some investigations on the monolayers of cyclic peptides have been performed mainly by Few[5], but their configurations on the surface have so far been discussed only in the cases of tyrocidine A and B and gramicidin SA and SB[6].

From these points of view, the monolayers of two cyclic peptides, i. e., fungisporin and gramicidin J_1, have been studied by measuring surface pressure, potential and viscosity. Fungisporin[7],

1) C. W. N. Cumper and A. E. Alexander, *Trans. Faraday Soc.*, **46**, 235 (1950).
2) J. T. Davies, *Biochim. Biophys. Acta*, **11**, 165 (1953).
3) T. Isemura and S. Ikeda, This Bulletin, **32**, 178 (1959).
4) T. Isemura and K. Hamaguchi, ibid., **27**, 339 (1954); K. Hamaguchi and T. Isemura, ibid., **28**, 9 (1955); T. Isemura, K. Hamaguchi and S. Ikeda, *J. Polymer Sci.*, **23**, 651 (1957).
5) A. V. Few and J. H. Schulman, *Biochem. J.*, **54**, 171 (1953); A. V. Few, *Biochim. Biophys. Acta*, **16**, 137 (1955); idem., *Proc. Second Int. Cong. Surface Activity*, **2**, CZ 168 (1957), Butterworths Scientific Publications, London.
6) A. V. Few, *Trans. Faraday Soc.*, **53**, 848 (1957).
7) Y. Sumiki and K. Miyao, *J. Agr. Chem. Soc. Japan (Nippon Nogei-kagaku Kaishi)* **26**, 27 (1952); K. Miyao, *Bull. Agr. Chem. Soc. Japan*, **19**, 86 (1955).

isolated from spores of some species of *Penicillium* and *Aspergillus*, is an octapeptide, cyclo-(D-Phe-L-Phe-D-Val-L-Val)$_2$[8]. Gramicidin J_1[9] is a heptapeptide, cyclo-(D-Orn-L-Val-L-Orn-D-Phe-D-Leu-L-Phe-L-Pro)[10]. Fungisporin has given a monolayer of condensed type, whereas gramicidin J_1 that of an expanded type. The behavior of these monolayers has been discussed in the light of the configurations of these cyclic peptides.

Experimental

Fungisporin was prepared by Dr. Miyao and was offered to our disposal. It is soluble only in dichloroacetic acid. As its spreading solvent was used a dichloroacetic acid-benzene (1 : 1 v/v) mixture which was found to be the most suitable for the surface potential measurement. Gramicidin J_1 dihydrochloride was a gift of Professor Otani. It was spread from a methanol solution.

Surface pressure, Π, was measured by a balance of a hanging plate type in higher pressure region, but it was measured by a balance of a float type of a better sensitivity in lower pressure or dilute region. The surface potential, ΔV, was measured by the vibrating electrode method and surface viscosity, η, by the damped rotatory oscillation of a disk. From surface potential, the apparent surface moment, μ, was calculated by the Helmholtz equation

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

where A was the area per amino acid residue.

Results

Fungisporin.—The $\Pi-A$, $\Delta V-A$, $\mu-A$ and $\eta-A$ curves of fungisporin on 0.01 M potassium chloride are shown in Fig. 1. The monolayer was found to be of a condensed type. The condensation of the film may be ascribed mainly to the hydrogen bonding and partly to the cohesion between hydrocarbon side chains. At the region of rise of the $\Pi-A$ curve the surface pressure declined gradually with time after each compression. In Fig. 1 the values at 1 min. per interval are plotted. The delay for attaining a steady surface pressure after each compression has been noticed in some condensed films of long chain substances and of high polymers with long side chain and is, therefore, closely related to the structure of fungisporin. The surface potential was reproducible only at the areas smaller than about 40 Å^2. This is also commonly observed for condensed films of low molecular weight substances, and consequently, consistent with the fact that fungisporin is a small molecule, an octapeptide. The surface viscosity was manifest at the areas below

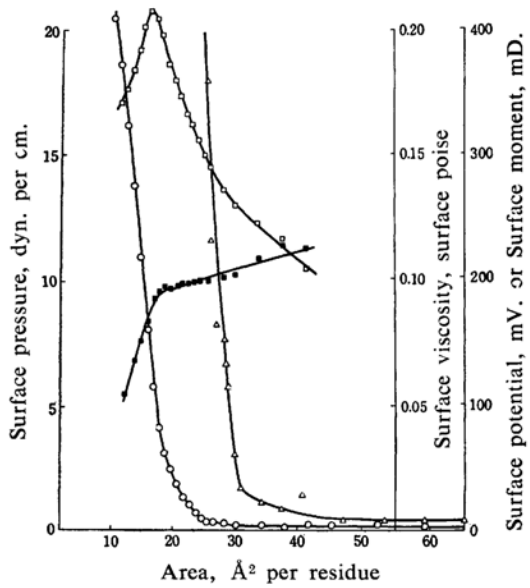


Fig. 1. Monolayer of fungisporin on 0.01 M potassium chloride (14°C). ○ surface pressure; □ surface potential; ■ surface moment; △ surface viscosity.

40 Å^2, where a surface potential was found to be reproducible.

The area, A_μ, where the slope of $\mu-A$ curve suddenly changed was found to be 17.5 Å^2 and the area, A_Π, of the minimum surface compressibility was 16.0 Å^2. These areas are nearly identical with each other and will represent the area occupied by an amino acid residue. The surface moment, μ_c, at the area, A_μ, was 200 mD. Both the area and surface moment are larger than those found for linear polypeptide monolayers.

Gramicidin J_1.—Since gramicidin J_1 contains two ornithyl residues in a molecule, it forms an ionized monolayer on distilled water and an unionized monolayer on aqueous solution at pH higher than 10.5.

The unionized monolayer of gramicidin J_1 on 0.01 M potassium carbonate at pH 11.2 showed a characteristic feature, when a neutral salt such as potassium chloride was added into the aqueous subphase. The effect of the concentration of potassium chloride on the $\Pi-A$ curve is illustrated in Fig. 2. The $\Pi-A$ curve in the absence of neutral salt was smooth, but a break point appeared when potassium chloride was present. The break point was found to be independent of salt concentration and was 27 Å^2 and 5.6 dyn. per cm. In the absence of salt or on 0.001 M potassium chloride, slight time effects were observed in the region of surface pressure higher than the break point. The $\Pi-A$ curves were not altered by salt concentration between 0.01 M and 1 M. In Fig. 3 are

8) K. Miyao, ibid., **24**, 23 (1960).
9) S. Otani and Y. Saito, *Proc. Japan Acad.*, **30**, 991 (1954).
10) S. Otani, "Chemistry of Proteins" ed. by S. Akabori and S. Mizushima, Vol. 5, Kyoritsu Shuppan Co., Ltd., Tokyo (1957), p. 297.

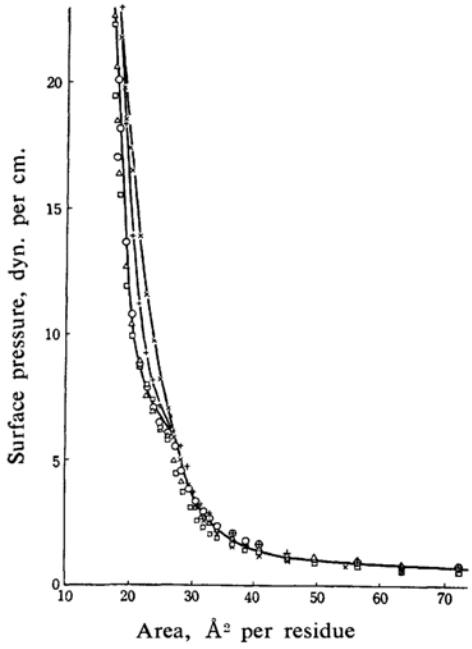


Fig. 2. Effect of potassium chloride on the surface pressure-area curve of gramicidin J_1 on 0.01 M potassium carbonate (15°C). × no salt; + 0.001 M; ○ 0.01 M; △ 0.1 M; □ 1 M.

shown the $\Pi-A$, $\Delta V-A$, $\mu-A$ and $\eta-A$ curves on 0.01 M potassium carbonate when 0.01 M potassium chloride are added. Surface potential was scarcely affected by salt concentration and the corresponding break point such as observed in the $\Pi-A$ curve was not found in both $\Delta V-A$ and $\mu-A$ curves.

It was noticed that the $\Pi-A$ curves of gramicidin SA and SB likewise exhibited the same kind of break points on 1 M potassium chloride at pH 12.5, and the break was attributed to the reorientation of side chains of ornithyl residues[6]. However, no break on the $\Delta V-A$ and $\mu-A$ curves for gramicidin J_1 suggests that the break on the $\Pi-A$ curve would not come from the reorientation of ornithyl residues but presumably from the micelle formation as proposed for fatty acid monolayers[11].

At pH 11.2 where they were unionized, the monolayers of gramicidin J_1 were of an expanded type, as shown in Fig. 2. Correspondingly, the surface viscosity was manifest first in the region of high surface pressures[4,12] as shown in Fig. 3. A constant value of the surface moment was observed over a wide area. The smallest area, A_μ, of constant surface moment was found to be 19.0 Å², the area, A_Π, of minimum surface compressibility to be 20.5 Å² and the gelling area, A_η, to be 20.0 Å². All these areas were scarcely dependent on salt concentrations not less than 0.01 M and nearly identical

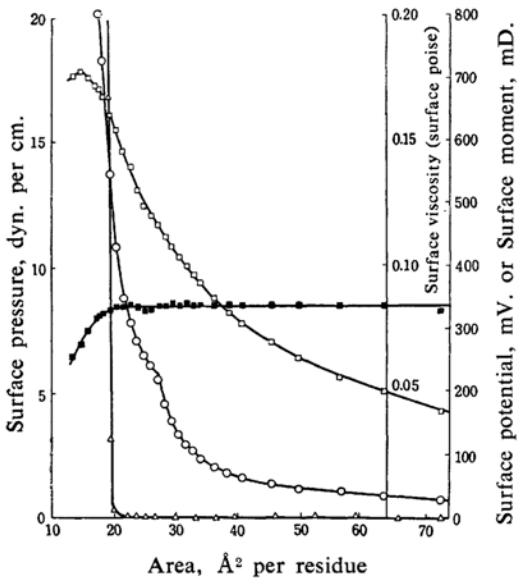


Fig. 3. Monolayer of gramicidin J_1 on 0.01 M potassium carbonate in the presence of 0.01 M potassium chloride (15°C). ○ surface pressure; □ surface potential; ■ surface moment; △ surface viscosity.

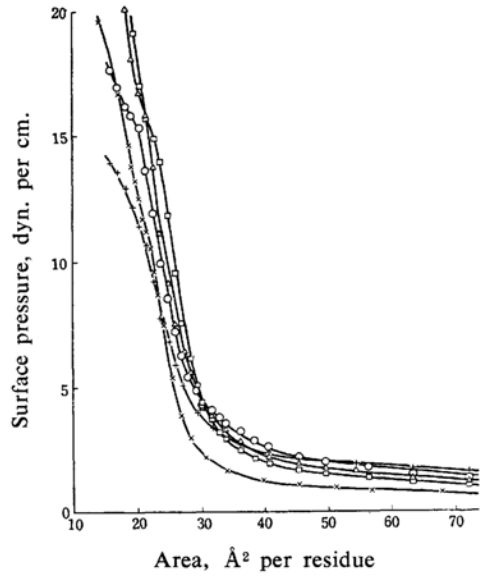


Fig. 4. Effect of potassium chloride on the surface pressure-area curve of gramicidin J_1 on distilled water (15°C). × no salt; + 0.001 M; ○ 0.01 M; △ 0.1 M; □ 1 M.

11) I. Langmuir, *J. Chem. Phys.*, 1, 756 (1933).
12) S. Ikeda and T. Isemura, This Bulletin, 32, 659 (1959).

with one another. Consequently, they represent the area occupied by an amino acid residue. The constant surface moment, μ_c, was about 340 mD.

On distilled water at pH 5.6 gramicidin J_1 forms an ionized monolayer. In this case, the monolayer behaves unlike the unionized monolayer but in a quite specific manner, as the salt concentration is varied. The variation of the $\Pi-A$ curves with the concentration of patassium chloride is shown in Fig. 4. In the absence of salt or on 0.001 M potassium chloride, the monolayers were somewhat unstable and had a tendency to dissolve into aqueous subphases on compression. On potassium chloride of the concentrations of 0.01 M to 1 M, however, the monolayers exhibited a typical behavior of ionized monolayer. Monolayers were more condensed at higher salt concentrations and more expanded at lower concentations. The $\Pi-A$ curves pass a reversal point[13], 30.5 Å^2 and 4.3 dyn. per cm.

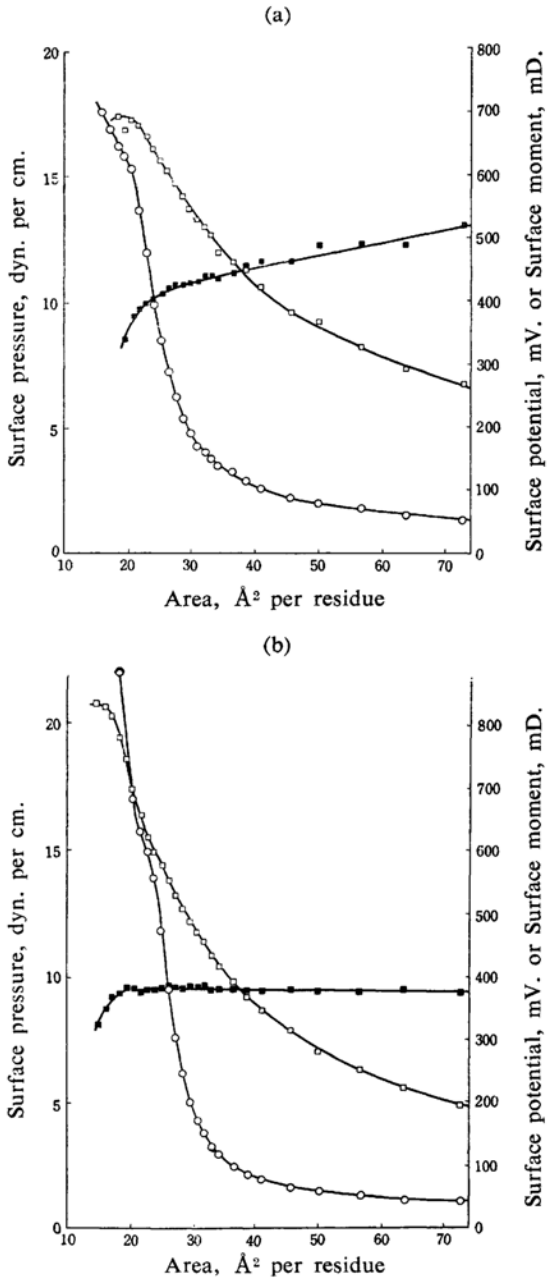


Fig. 5. Monolayers of gramicidin J_1 on distilled water in the presence of potassium chloride (15°C). a, 0.01 M; b, 1 M. ○ surface pressure; □ surface potential; ■ surface moment.

In Fig. 5 are illustrated some of the $\Delta V-A$ and $\mu-A$ curves together with the $\Pi-A$ curves. The surface moment gave no constant value on 0.01 M potassium chloride but a constant value, 380 mD, on 1 M potassium chloride. On the $\Delta V-A$ and $\mu-A$ curves no significant change was observed at the area corresponding to the reversal point on the $\Pi-A$ curve. The surface viscosity was very low and not detected even at an area as small as 10 Å^2 on 0.01 M potassium chloride.

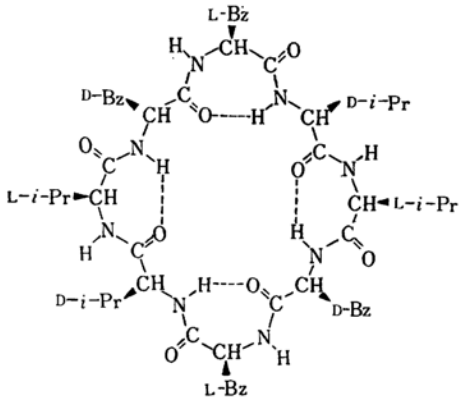
In the presence of 0.01 M potassium chloride, the ionized monolayer of gramicidin J_1 on neutral subphase was more expanded than the unionized monolayer on 0.01 M potassium carbonate and the surface potential of the former was higher than that of the latter.

Discussion

Configurations of Cyclic Peptides in Monolayer.—The molecular configuration of cyclic peptide has so far been investigated by a polarized infrared spectra[14] and an X-ray diffraction[15] on gramicidin S crystal. The infrared study suggested, that a gramicidin S molecule consists of ten seven-membered rings, each with a hydrogen bond between the imino and carbonyl groups. Few[6] has proposed the configurations of tyrocidines and gramicidin SA in a monolayer in such a way, as the maximum number seven-membered of rings can be formed in a plane and the other peptide bonds can take the β-configuration. We can deduce the configurations of fungisporin and gramicidin J_1 in monolayer in the same way.

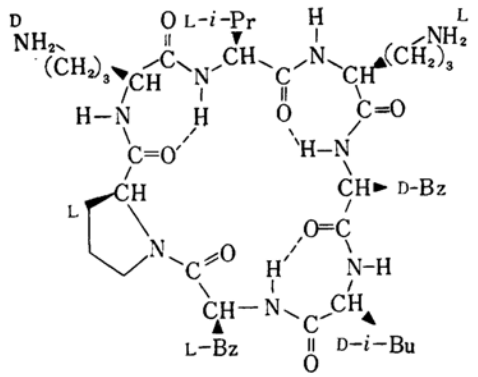
In accordance with the above conditions, the configurations of fungisporin in a monolayer are confined to be square-like ones. One of them is illustrated as follows:

13) J. N. Philips and E. Rideal, *Proc. Roy. Soc.*, **A232**, 159 (1955).
14) N. B. Abbott and E. J. Ambrose, ibid., **A219**, 17 (1953).
15) D. Crowfoot-Hodgkin, *Cold Spring Harbor Symp. Quant. Biol.*, **14**, 65 (1950).



An alternative configuration is obtained by shifting each side chain one by one, in either sence, along the perimeter of this configuration, the orientation of side chains being altered. The other possibilities can be obtained by turning each of these two configurations over. It can not be distinguished which side of the molecular plane is exposed to the air or to the aqueous phase, when the molecule is put on surfaces, and consequently all of these four configurations are equally probable.

The unionized gramicidin J_1 molecule can take only one confiugration in the monolayer, subject to the foregoing requirements:



An alternative is obtained by turning of this configuration. In considering the difference of D-leucyl and L-valyl residues in hydrophilic nature, it is a little more favorable to choose the latter configuration (if the air is above the plane).

In all these cases of fungisporin and gramicidin, the areas occupied by a residue are consistent with those obtained experimentally. It is remarkable to note that the side chains of all the D-amino acid residues orientate oppositely to these of all the L-residues with respect to the plane of a molecule. Thus, if the side chain of the L-ornithyl residue in gramicidin J_1 tends towards the aqueous phase, that of the D-ornithyl residue is directed to the air.

Since the formation of a seven-membered ring will force the carbonyl groups to direct towards the aqueous phase, the surface moment is expected to be higher than that of the β-configuration. The surface moment of fungisporin is mainly composed of two different orientations of the carbonyl groups, one hydrogen bonded intramolecularly and the other not participating in hydrogen bonding. It was found to be about 200 mD, which is actually higher than that of the β-configuration, 160 mD[3]. On the other hand, the surface moment of gramicidin J_1 is a mean value of three orientations of carbonyl groups, hydrogen bonded, not hydrogen bonded and belonging to L-phenylalanyl residue, and two amino groups of D- and L-ornithyl residues. The experimental value was very high and 340 mD. It is much higher than 273 mD and 274 mD for gramicidin SA and SB[6]. If the contribution of each carbonyl group is assumed to be equally 200 mD, as in fungisporin, the two amino groups must contribute about 980 mD or, in the average, an amino group about 490 mD. This value would be compared with the surface moments of long chain amines, 300 to 400 mD[16]. However, this value is too high if the opposite directions of two ornithyl residues are taken into account. The reason for this and, accordingly, for the large values of the surface moment, as compared with those of gramicidin SA is not clear.

Condensation and Expansion of the Monolayers. — Fungisporin gave a monolayer of

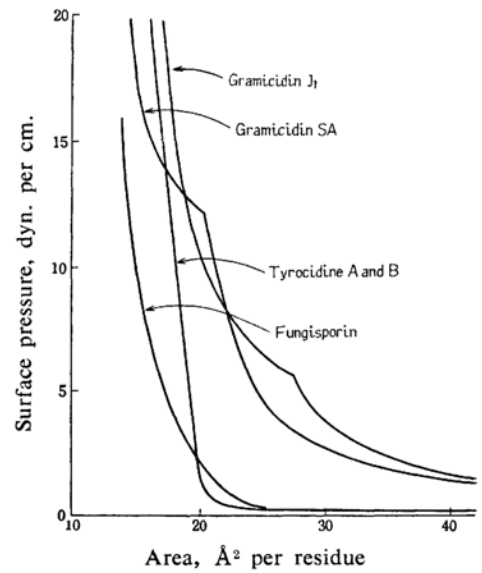


Fig. 6. Surface pressure-area curves of some cyclic peptides. Gramicidin SB gives a nearly identical curve with gramicidin SA.

16) E. F. Porter, *J. Am. Chem. Soc.*, **59**, 1883 (1937); J. Glazer and M. Z. Dogan, *Trans. Faraday Soc.*, **49**, 448 (1953).

condensed type but gramicidin J_1 gave an unionized monolayer of expanded type. It was noticed[6] that tyrocidine A and B formed condensed films on aqueous subphases at the ionic strength 1.0, irrespective of pH, but gramicidin SA and SB formed expanded films under the same condition. These results are summarized in Fig. 6. It was concluded by Few[6] that the expansion of films of cyclic peptides is closely related to the content of ornithyl residues. It is, however, known in the case of unionized linear polypeptides[4] that the L-lysyl residue, which has a longer side chain than the ornithyl residue only by one methylene group, does not influence the type of monolayers but remains to be of a condensed type. We, therefore, no longer regard his proposed explanation as adequate. We have previously observed that the monolayers of synthetic linear polypeptides are transformed gradually from a condensed type to an expanded type as the content of prolyl residue is increased[3,12] and further, this effect is virtually demonstrated in the case of protein films such as gelatin[17]. It can be seen that even for the monolayers of cyclic peptide the prolyl residue plays the same role as for linear polypeptides. In comparing the amino acid composition, fungisporin contains no prolyl residue in a heptapeptide[8], gramicidin J_1 one in an octapeptide[10], both tyrocidine A and B one in a decapeptide[18,19] and gramicidin SA two in a decapeptide[20]. The proportion of prolyl residues in gramicidins are higher than in fungisporin and tyrocidines. Thus the former peptides give more expanded monolayers than the latter. On the contrary, gramicidin SB, whose complete structure is still unknown, but whose monolayer expands to the same extent as gramicidin SA, will have two prolyl residues if it is a decapeptide.

Ionization of Dilute Gramicidin J_1 Monolayer.—Because of the expanded nature of gramicidin J_1 monolayers, it was possible to measure accurately the surface pressure and potential at the region of very large areas. Then the behavior of dilute ionized monolayers of gramicidin J_1 when spread on neutral salt solution can be treated in terms of the theory of the ionized monolayer so far presented.

In general, the surface pressure of an ionized monolayer can be divided into two parts:

$$\Pi = \Pi_0 + \Pi_i$$

where Π_0 is the surface pressure of an unionized monolayer, which is independent of salt concentration, when the other effect is ignored. In this equation the contribution of deformation due to the electric repulsion is not included. The surface pressure due to ionization, Π_i, is, according to the theory of the electric double layer[21,22], given by

$$\Pi_i = 2kT\left\{-\frac{2n}{\kappa}+\sqrt{\left(\frac{2n}{\kappa}\right)^2+\frac{1}{A_i^2}}\right\}$$

where k is the Boltzmann constant, T the absolute temperature, n the salt concentration (molecules per cm^3) and κ the Debye-Hückel parameter. Here A_i is the area per ionized group. Evidently, the surface pressure is lower, as neutral salt is more concentrated in an aqueous phase. However, values of the surface

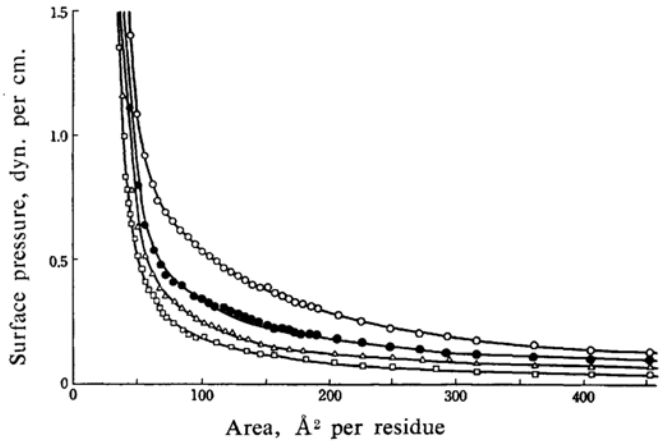


Fig. 7. Effect of potassium chloride on the surface pressure-area curve of dilute gramicidin J_1 monolayer on distilled water (15°C). ○ 0.01 M; ● 0.025 M; △ 0.1 M; □ 1 M.

17) S. Ikeda and T. Isemura, This Bulletin, 33, 137 (1960).
18) A. Paladini and L. C. Craig, *J. Am. Chem. Soc.*, 76, 688 (1954).
19) T. P. King and L. C. Craig, ibid., 77, 6627 (1955).
20) R. Consden, A. H. Gordon, A. J. P. Martin and R. L. M. Synge, *Biochem. J.*, 41, 596 (1947); A. R. Battersby and L. C. Craig, *J. Am. Chem. Soc.*, 73, 1887 (1951).
21) J. T. Davies, *Proc. Roy. Soc.*, A208, 224 (1951).
22) S. Ikeda and T. Isemura, This Bulletin, 33, 131 (1960).

TABLE I. SURFACE PRESSURE DUE TO IONIZATION AT 200 Å² PER RESIDUE

n	Π	(A) an ionized residue		(B) two ionized residues	
		Π_i	Π_0	Π_i	Π_0
mol./l.	dyn./cm.	dyn./cm.		dyn./cm.	
0.01	0.29	0.230	0.06	0.699	−0.41
0.025	0.19	0.159	0.03	0.550	−0.35
0.1	0.12	0.087	0.03	0.326	−0.21
1	0.07	0.032	0.04	0.111	−0.04

pressure, Π_0, at a constant area, which are obtained by subtracting the calculated values of Π_i from the experimental values of Π, should be independent of salt concentration, even if the salt concentration is varied.

The $\Pi-A$ curves of dilute ionized gramicidin J_1 monolayers on potassium chloride solutions of various concentrations are shown in Fig. 7. The surface pressure is virtually lower at higher salt concentrations. In the monolayer of gramicidin J_1 in an unionized state the two amino groups of ornithyl residues, which are responsible for the ionization of the molecule, are assumed to be oppositely directed to each other. If this configration is still retained in the state of ionization, the electric double layer will be concerned with only one amino group, probably of the L-ornithyl residue. Taking account of this, we have estimated the values of Π_0 at an area, $A=200$ Å², by the foregoing procedure for both cases, that only one ionized amino group ($A_i=7A$) and two ionized groups ($A_i=(7/2)A$) are assumed to concern the electric double layer. The results are given in Table I. In the former case the obtained values of Π_0 are nearly constant and positive, unlike in the latter case. Thus it is likely, that the electric double layer is formed by the ionization of only one amino group and not related to that of the other. This might support the proposed configuration of gramicidin J_1 in monolayer.

According to Schulman and Hughes[23], the surface potential of an ionized monolayer can be expressed by

$$\Delta V=\frac{4\pi\mu_M}{A_i}+\psi_M$$

where μ_M is the surface moment of an ionized group (one or two seventh of the moment per amino acid residue according as one or two ionized ornithyl residues) and ψ_M is the potential of the monolayer. In this equation the first term assumes the ordinary Helmholtz equation and the second term is, according to the theory of electric double layer[21,22], expressed by

$$\psi_M=\frac{2kT}{e}\sinh^{-1}\frac{\kappa}{4nA_i}$$

23) J. H. Schulman and A. H. Hughes, *Proc. Roy. Soc.*, A138, 430 (1932).

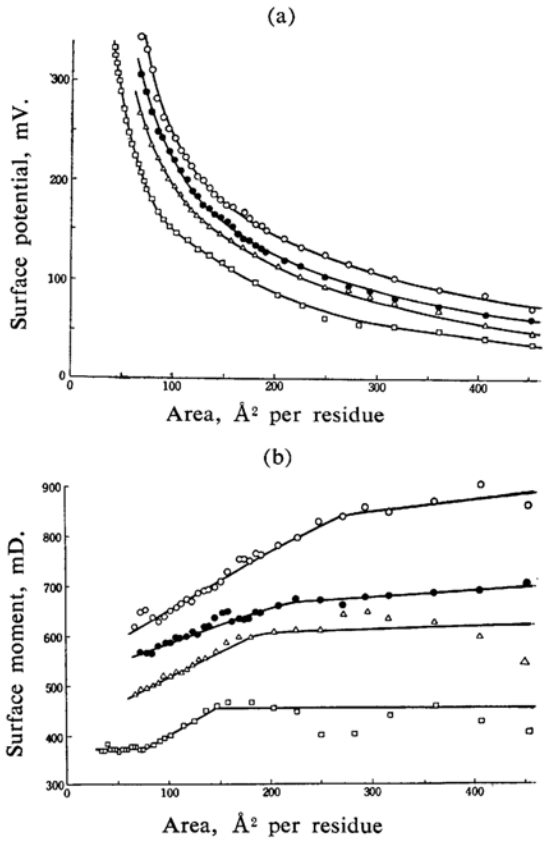


Fig. 8. Effect of potassium chloride on dilute monolayer of gramicidin J_1 (15°C). a, surface potential; b, apparent surface moment. ○ 0.01 M; ● 0.025 M; △ 0.1 M; □ 1 M.

where e is the elementary charge. These equations indicate that the surface potential is lower, as neutral salt is more concentrated in the aqueous phase. In Fig. 8a is shown the variation of the $\Delta V-A$ curves with concentration of potassium chloride in the aqueous phase, which is in accord with the above indication. We have found, however, that the same procedure as done for the surface pressure is not suitable to distinguish whether one or two ornithyl residues concern the electric double layer, since the surface potential can not be measured with sufficient accuracy so as to allow it. Instead of doing so, we have tested the above equations by another method. These

equations show that the apparent surface moment, μ, calculated from $A \cdot \Delta V/4\pi$, generally falls on compression and its value is lower at higher salt concentrations. On highly concentrated salt solutions the apparent surface moment should be constant over a wide region of areas, In Fig. 8b is shown the $\mu-A$ curves for gramicidin J_1 on potassium chloride solutions. Although a minor error in surface potential measurement causes a marked scatter of the surface moment at large areas, it can be seen that the general trend of the $\mu-A$ curves is consistent with the prediction. On such a concentrated salt solution as 1 M, however, the Helmholtz equation appears to be obeyed by the ionized monolayer at areas smaller than 90 Å^2 but fails to hold at larger areas.

In addition, it was found that only at moderate areas the ionized monolayer obeys the electrochemical equation[24]

$$\left(\frac{\partial \Delta V}{\partial \ln n}\right)_A = -\frac{kT}{e}$$

as illustrated in Fig. 9.

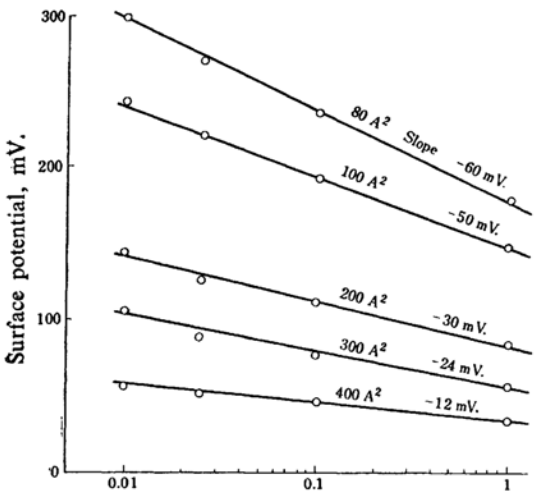


Fig. 9. Surface potential plotted against logarithm of concentration of potassium chloride. Theoretical slope at moderate areas, −57 mV. at 15°C.

Summary

Monolayers of two naturally occurring cyclic peptides, fungisporin and gramicidin J_1, were investigated by the measurements of surface pressure, potential, and viscosity. Fungisporin, an octapeptide composed of phenylalanyl and valyl residues, gave a monolayer of a condensed type. Gramicidin J_1, an electrolytic heptapeptide containing a prolyl and two ornithyl residues, gave an unionized monolayer of an expanded type on an alkaline subphase. It behaved in a somewhat specific manner when neutral salt such as potassium chloride was present.

The configurations of these cyclic peptides in monolayer were suggested in such a way as intramolecular hydrogen bonding could cause a maximum number of seven-membered rings. Then fungisporin can take four possible square-like configurations, which are equally probable, and gramicidin J_1 can take two possible configurations. When these configurations of both peptides are put on surfaces, side chains of all the D-residues always orientate oppositely to those of all the L-residues with respect to the surfaces.

In taking account of the condensation and expansion of these monolayers, together with those of tyrocidine A and B and gramicidin SA, it was concluded that cyclic peptides give more condensed monolayers as the contents of prolyl residue are higher. Thus a prolyl residue plays the same role in cyclic peptides as in linear polypeptides.

Gramicidin J_1 gave a monolayer of more expanded type on a neutral subphase, and exhibited a characteristic behavior common with ionized monolayers when neutral salt such as potassium chloride was added. At very large areas the the behavior was adequately explained by the theory of the electric double layer, when only one ionized residue was assumed to concern the double layer.

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24) D. J. Crisp, Research, "Surface Chemistry", Butterworths Scientific Publications, London (1949), p. 65.