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SURFACE FILMS OF PROTEIN AND A PROTEIN–LIPOPOLYSACCHARIDE COMPLEX EXTRACTED FROM *PSEUDOMONAS AERUGINOSA*

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SUMMARY

Protein and a protein–lipopolysaccharide complex were extracted from the cell envelopes of *Pseudomonas aeruginosa* as fractions of endotoxin and spread on aqueous subphases as monomolecular layers. The surface properties of these materials, *i.e.* surface pressure, surface viscosity and surface potential, were measured as a function of the surface area.

The protein molecule was partially unfolded at the air–water interface and had a limiting area of 0.54 m^2/mg protein in the state of minimum compressibility. Lipopolysaccharide alone did not form a surface film on water. As a complex with protein, lipopolysaccharide did not dissolve completely in the aqueous phase but was attached to the protein film. This protein aggregates or dissociates in the bulk phase as a function of the pH of the solution so the effect of pH on its surface properties was examined. The effects of NaCl and KCl in the subphase on the protein film differed from their effects on a film of the complex.

The film of the complex is mainly formed from protein and this protein film is stiffened and stabilized by lipopolysaccharide attached to the lower surface of the film.

INTRODUCTION

Recently, a number of biological phenomena have been explained on a molecular basis. In addition, the ultrastructure of biological systems has been studied by electron microscopy. From these studies the unit membrane theory has been proposed to explain the fundamental structure of membranes and layers in biological systems[1–3]. It seems to be possible to elucidate the ultrastructure of biological systems and their functions on the basis of molecular schemes[4–8]. Moreover, it may be possible to interpret the mechanism of development of an organism on the basis of various biological structures.

In the present work, the surface chemistry of monomolecular films of materials isolated from *Pseudomonas aeruginosa* was studied at an air–water interface. The films examined were far from being models of biological membranes and the conditions under which they were examined were rather different from those of biological

systems. However, studies on monolayers of biological macromolecules seem useful in elucidating the role of protein and lipid in the structure of membranes.

Usually protein molecules are denatured by surface tension and stretch out covering a limiting area of 0.8–1.0 m^2/mg protein. This area seems to be due to unfolded polypeptide chains at an air–water interface. However, the protein studied in this work tends to resist unfolding due to surface tension. This may be a characteristic of protein from the cell envelope, which is a kind of structural protein. It seems that the film formed by a complex of protein and lipopolysaccharide mainly consists of a protein film with lipopolysaccharide at the lower surface of the protein which stabilizes it.

EXPERIMENTAL

Materials

Materials were extracted from the cell envelopes of *Pseudomonas aeruginosa* by the method of HOMMA *et al.*[9]. *Ps. aeruginosa* was cultured in a tank. Cells were harvested and autolyzed by addition of toluene. Then $ZnCl_2$ was added and a precipitate was obtained. This was dissolved in a small amount of sodium phosphate solution. The zinc was removed as zinc phosphate and the toxicant was condensed from the supernatant. The resulting preparation was named the "original endotoxin". The original endotoxin was separated into two components (Component I and Component II) by zone electrophoresis. Chemical analysis showed that Component I was a complex of protein and lipopolysaccharide while Component II was composed of nucleic acids. Component I was separated into protein (Protein P) and lipopolysaccharide (Lipopolysaccharide LS) with "lower phase solvent"[10]. Protein P was successively purified by ultracentrifugation, fractional precipitation using cold alkaline ethanol and zone electrophoresis. The resulting preparation appeared homogeneous by disc electrophoresis. Lipopolysaccharide LS was purified by extraction with phenol from the supernatant of the "lower phase solvent" solution.

All salts used for the preparation of buffers were recrystallized twice from aqueous solution. NaCl and KCl were heated for about 1 h at their partial-melting temperatures. Organic reagents used for preparation of monolayers were "A" grade for spectroscopy. Water which had been distilled in a copper distillation assembly and re-distilled in an all glass assembly was used as the subphase.

Procedure

Sample materials, *i.e.* Protein P, Lipopolysaccharide LS and Component I were spread at an air–water interface using a conventional Langmuir trough (50.0 cm × 14.5 cm × 0.9 cm). Sample materials were each dissolved at a concentration of 0.3 mg/ml in aqueous solution containing 0.1 M NaOH and isoamyl alcohol (1:50, by vol.) or isopropyl alcohol (1:5, by vol.). These samples were usually spread on the water surface using a micrometer syringe (Alfa, Muromachi) at the rate of 0.03 ml/min. The plunger of the syringe was driven by a motor. TRURNIT's method[11] was also employed to charge protein on a water surface. In this case, the solution of protein was delivered from a micrometer syringe at the rate of 0.06 ml/min onto the hemispherical top of a glass rod (0.8 cm diameter, 8 cm length) placed on the trough. The surface area covered with the sample was reduced automatically 30 min after spreading the sample.

Surface properties, *i.e.* surface pressure (π), surface viscosity (η) and surface potential (ΔV), were measured with automatic recording systems as functions of the surface area. The rate of reduction of the surface area was checked for each material in order to find suitable conditions to give the film enough time to stabilize and equilibrate. Technical details are described in a previous paper[12]. All measurements were carried out at 20°. The temperature was controlled by circulating water at constant temperature through a coiled glass pipe at the bottom of the trough. For surface viscosity measurements the disk was rotated at a constant speed of 0.21 rad/sec.

RESULTS

The surface pressure and surface viscosity of a film of Protein P on distilled water at 20° are shown in Fig. 1. Surface pressure is first detected at about 0.75 m^2/mg Protein P. On decreasing the surface area, the surface pressure rises gradually reaching a maximum of 38 dyne/cm. No further increase of surface pressure was observed on further compression of the film. It is concluded from the characteristics of the surface pressure–area curve that Protein P spreads as an expanded film. The minimum compressibility was found at an area of 0.54 m^2/mg Protein P, where the surface viscosity is first detectable. The area 0.54 m^2/mg is a limiting area representing the area occupied by 1 mg protein spread in two dimensions. The limiting area of Protein P is smaller than that of an extended protein reported previously (0.8–1.0 m^2/mg)[13].

To examine the extent of unfolding of the protein during spreading, Protein P was spread by TRURNIT's method[11]. The results obtained using this method were identical to those obtained using the usual micrometer syringe method. This method seems to be useful for spreading a protein perfectly only when the protein tends to unfold on the surface. A similar result was reported by YAMASHITA AND BULL[14].

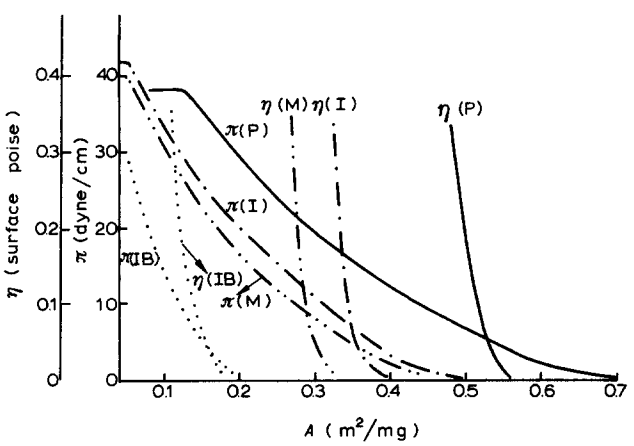

Fig. 1. Surface pressure and surface viscosity–area curves of films of components of endotoxin from *Ps. aeruginosa* on water at 20°. Surface area (A) (m^2/mg) is calculated assuming that all the material spreads on the surface. The curves for the pressure π (dyne/cm) and surface viscosity η (surface poise) of Protein P are shown as: ———, π (P); ———, η (P). The other curves are: –·—·– Component I (protein–lipopolysaccharide complex); ·····, Component IB; –··—··–, Mixture M (protein–lipopolysaccharide mixture).

The surface pressure and viscosity–area curves for Component I spread on water at 20° are also shown in Fig. 1. These curves indicate that Component I has the same characteristics as Protein P. Surface pressure is first observed at 0.5 m^2/mg and increases gradually to a maximum value of 42 dyne/cm. This value is higher than that for Protein P. Its minimum compressibility was found at 0.43 m^2/mg, and surface viscosity also became detectable at this area. Since the surface pressure–area and surface viscosity–area curves are at smaller areas than those of Protein P, the limiting area of Component I is smaller than that of Protein P.

Component I is composed of protein and lipopolysaccharide. Lipopolysaccharide LS also did not form a film on water. The lipopolysaccharide in Component I may dissolve in the aqueous phase and the film formed by Component I may be due to the protein only. The surface area on Fig. 1 was calculated from the amount of Component I spread, assuming that all the Component I spread was adsorbed at the water surface. If the surface pressure and viscosity of Component I are plotted against an area calculated assuming that only the protein of Component I remained on the water surface, the surface pressure–area curve is similar to that of Protein P itself, although it does not coincide with the latter, as shown in Fig. 2. The lipopolysaccharide in Component I is not entirely dissolved in the aqueous phase, as discussed later.

To examine the properties of Component I, a mixture (Mixture M) of Protein P and Lipopolysaccharide LS with the same composition as Component I was spread on water. The surface behavior of Mixture M was essentially the same as that of Component I, as shown in Figs. 1 and 2.

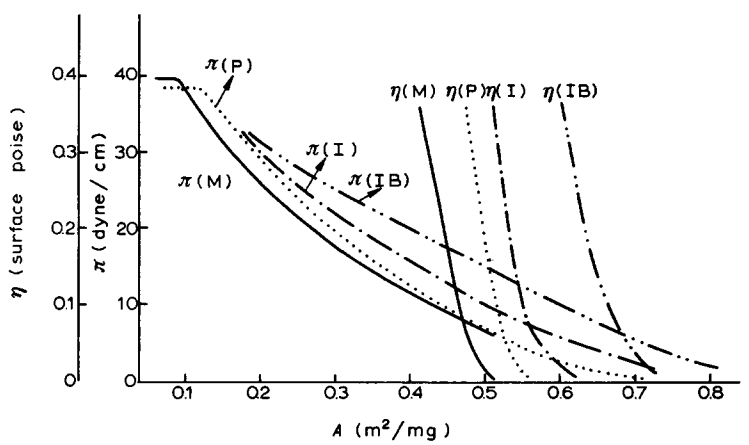

Fig. 2. Surface pressure and surface viscosity–area curves plotted against the protein contents of Protein P, Component I, Component IB and Mixture M. The surface areas (A) are calculated assuming that the films are constructed entirely of the protein in the samples (protein content: Component I and Mixture M, 66 wt.%; Component IB, 20 wt.%) ·····, Protein P; –·—·–, Component I; –··—··–, Component IB; ———, Mixture M.

Component IB, which contains only 20 % protein, was spread on water to examine the role of protein in formation of the surface film. Results on Component IB are also shown in Fig. 1. Its surface pressure and viscosity–area curves were at very small areas.

Effect of pH

One of the authors (GOTO[17]) found that Protein P molecules in aqueous solution aggregate or dissociate as a function of the pH of the solution. Accordingly, to examine the effect of pH on the properties of films, the pH of the aqueous phase was varied using 0.01 M phosphate buffer of pH 6.2 or 8.0 as the aqueous phase. The results are shown in Figs. 3 and 4. Aqueous solutions of 0.033 M KOH or NaOH were used to examine the effects of extreme pH values. The pH of the aqueous phase was re-measured after each experiment. The surface pressure of Protein P at a given surface area was higher on an alkaline subphase than on water in the region of low pressure. The surface viscosity was also detectable on alkaline solution at a smaller area than on water. The effect of the alkaline pH value may be to cause the protein to dissolve because electrolytic dissociation increases.

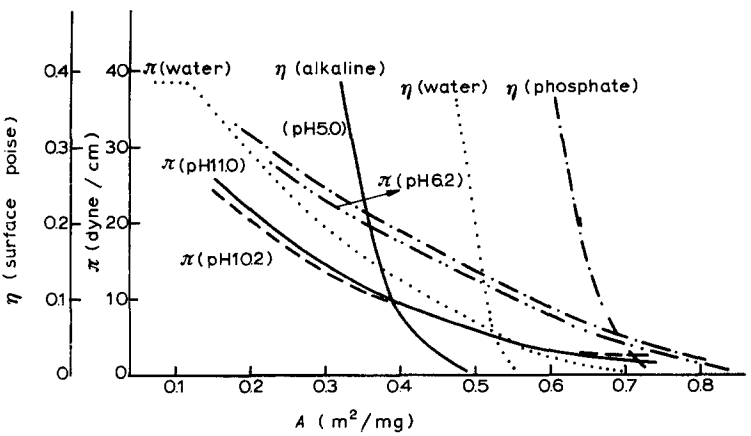

Fig. 3. Effect of pH on the Protein P monolayer. Surface pressure π and surface viscosity η are plotted against the surface area (A) of Protein P on water (· · · · ·, $\pi(w)$ and $\eta(w)$); 0.01 M phosphate buffer (–·—·–, pH 8.0; –· ·—· ·–, pH 6.2); 0.033 M alkaline solution (———, KOH and NaOH, pH 11.0) at 20°.

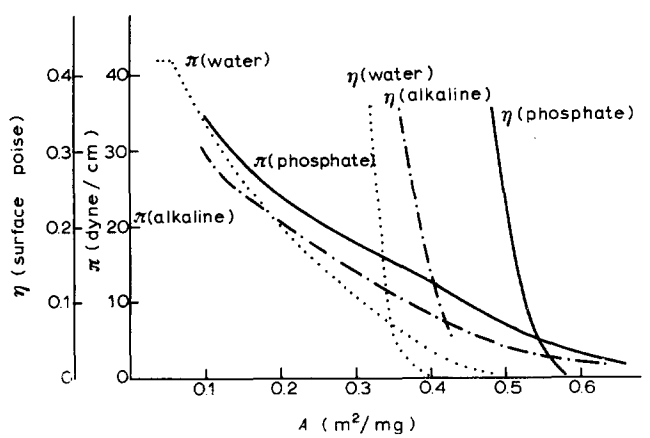

Fig. 4. Effect of pH on the Component I monolayer. The surface pressure π and surface viscosity η are plotted for Component I on water (· · · · ·, $\pi(w)$ and $\eta(w)$); 0.01 M phosphate buffer (———, pH 8.0); 0.033 M alkaline solution (–·—·–, KOH and NaOH, pH 11.0) at 20°. The surface area is calculated assuming that all the material spreads on the surface.

The effect of pH on Component I was similar, but the difference between the film on an alkaline solution and that on water was greater, as shown in Fig. 4.

Effects of salts

The effects of salts on the films formed by the protein and complex were examined using 0.1 M aqueous solutions of KCl or NaCl. The results are shown in Figs. 5 and 6.

With Protein P, the surface pressure at a given surface area was higher for a film on salt solution than on water in the region of lower surface pressure. The surface pressure gradient of the film on salt solution was lower than that on water. The area where the maximum pressure of the film was first observed was also smaller on salt

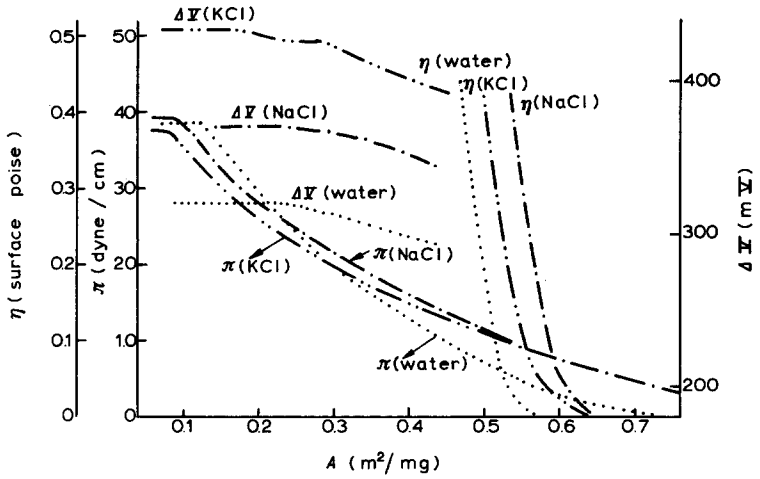

Fig. 5. Effect of salt on the Protein P monolayer. Protein P was spread on 0.1 M KCl and 0.1 M NaCl at 20° and the surface pressure π, surface viscosity η and surface potential ΔV are shown in comparison with values of a film on water. –··—··–, KCl; –·—·–, NaCl; ·····, water.

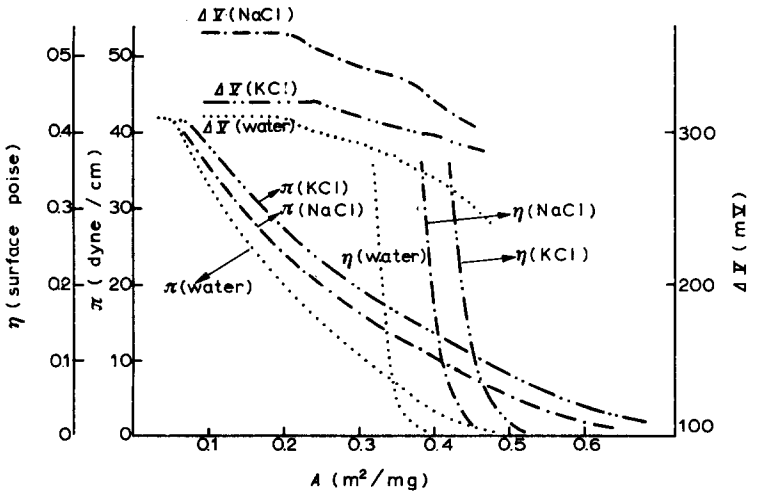

Fig. 6. Effect of salt on the Component I monolayer. Component I was spread on 0.1 M KCl and 0.1 M NaCl at 20°. –··—··–, KCl; –·—·–, NaCl; ·····, water.

solution than on water. The surface potential was affected by the type of salt and a larger variation in surface potential was observed with KCl than with NaCl.

With Component I, the properties of the film were also affected by the type of salt in the subphase and the effects of NaCl and KCl were the reverse of those with Protein P.

DISCUSSION

It has been reported that the characteristics of a monolayer are due to the fact that the individual molecules in an expanded film move independently and slide around one another[15,16]. However, the characteristics of the expanded film observed in this work may be due to the structure of the film, for the molecules of protein and lipopolysaccharide are chain-like macromolecules and the side-chains of protein and phosphate groups of lipid can become ionized. In the film on an aqueous subphase, the molecules interact with each other laterally and electrostatic interactions occur between oppositely charged groups, so that the molecules become entangled with one another. Molecules of Protein P tend to aggregate in solution[17] and in films. Moreover, the irregular movement of talcum powder put on the film and the fluctuation of the surface potential of a film of large area suggest that the molecules in the film are partially aggregated.

Various models have been proposed for concentrated films of protein[18-21]. AUGENSTEIN AND RAY[20] suggested that a film has a mosaic form with folded and unfolded molecules of protein. BATEMAN AND CHAMBERS[21] considered it as a mosaic of molecules having different degrees of unfolding. The molecules of Protein P on an aqueous phase must have a folded portion and an extended peptide chain because Protein P molecules are thought to be partially unfolded[17] in solution. Moreover, the limiting area of a film of Protein P on water was found to be so small that it can not be the limiting area of an entirely unfolded peptide chain. Even using the method of TRURNIT[11] the limiting area did not increase further by unfolding the molecule of Protein P during spreading. The scheme for the film of Protein P described above does not conflict with previous schemes proposed for films of proteins. This is because previous schemes were given to interpret the properties of ordinary proteins which tend to unfold at an interface, while the present scheme is for a kind of structural protein which has a special conformation in solution and at an interface.

The film of protein and lipopolysaccharide consists of molecules of protein on the surface of the aqueous subphase with the lipopolysaccharide molecules attached beneath the protein in the aqueous phase. The network formed by Protein P alone may be changed by addition of lipopolysaccharide, the change affecting the conformation and/or the role of the protein. The character of an expanded film of Protein P of Component I may be elucidated in terms of the structure of the network in the film.

At the area where the minimum compressibility is observed, the network structure is closely packed. In an area smaller than that showing the minimum compressibility, the network structure of the film may be destroyed by diminishing the surface area. After the surface pressure has reached a maximum, the film collapses and is squeezed from the surface into the subphase with further compression of the

film. As reported by MACRITCHIE[22], the collapse of a protein monolayer under a surface pressure of over 30 dyne/cm is irreversible and a coagulum is formed.

To compare the properties of Protein P, Component I, Component IB and Mixture M, the surface pressure and viscosity are plotted against the area calculated to be occupied by only the protein in these materials (Fig. 2). It is evident that the films of Component I and Component IB, which are composed of protein and lipopolysaccharide, are more expanded than that of Protein P and, when plotted against the amount of protein, the extent of expansion is a function of the lipopolysaccharide content. These results support the structure of the film proposed above. There are two possible explanations of the role of lipopolysaccharide in the monolayer of Component I; (1) the lipopolysaccharide molecules may be inserted into the monolayer of protein in some way which is facilitated by their association with protein and (2) the protein molecules may be unfolded or rearranged under the influence of the lipopolysaccharide adsorbed beneath them, so that they occupy a greater area.

To see whether the film of Component I contained both protein and lipopolysaccharide, materials in the film were chemically analyzed as follows. The spread film was collected with a small amount of subphase by the thread method. Then a solution of film was condensed under reduced pressure. Protein was analyzed by the method of LOWRY *et al.*[23] and lipopolysaccharide was estimated by the phenol–H_2SO_4 method[24]. It was found that the film contained both protein and lipopolysaccharide.

The surface pressure–area curve of Mixture M was at a smaller area than that of Protein P and the collapse pressure of Mixture M of 40 dyne/cm is intermediate between those of Protein P and Component I. It seems that a complex of protein and lipopolysaccharide is formed in the film of Mixture M but that this complex is not identical with the complex of Component I, for the surface properties of Mixture M differ from those of either Protein P or Component I, as shown in Fig. 2. Moreover, the maximum pressure, which is related to the structure of the film, is intermediate between those of Protein P and Component I.

The results obtained using phosphate buffer cannot be explained as being merely due to the effect of pH, because the properties of Protein P in solution suggest that the surface properties of a film on a subphase of pH 6.2 should be identical with those on water and different from those on a subphase of pH 8.0. However, the results obtained on phosphate buffers of pH 6.2 and 8.0 were essentially the same, as shown in Fig. 3. GERSHFELD AND PAK[25] reported that bridges of hydrogen bonds between phosphate groups are formed in a monolayer of alkyl phosphate. Thus, with Protein P the effect of pH may be reduced to the interaction of divalent phosphate ions in the phosphate buffer with molecules of Protein P.

The compressibilities of films of Protein P and Component I on water and on an alkaline solution, respectively, are plotted against the surface area in Fig. 7. The area of the film of Protein P giving the minimum compressibility was 0.64 m^2/mg on water and 0.28 m^2/mg on alkaline solutions. The minimum compressibility of films of Component I on both water and alkaline solution was observed at an area of 0.43 m^2/mg. The area at which the minimum compressibility is observed corresponds to the limiting area. The limiting area may be changed by changing the amount of material spread at the interface by dissolution of part of the material or by changing the conformation of the molecules due to variation in the surroundings. The difference between the effects of alkaline solution on the films of Protein P and Component I

may be due to an effect of lipopolysaccharide in preventing the protein from dissolving.

The effect of salt is mainly due to salting-out or to its effect in changing the structure of the film. Based on the view of the structure of the film mentioned above, ions probably combine with the film and make its structure more rigid by changing the electrostatic field around the protein or lipopolysaccharide. The higher surface potential of films on salt solution is probably due to binding of ion with the film. The difference between the effects of K^+ and Na^+ on the surface potential may be related to a difference of the numbers of ions bound, a difference in the structure of hydrated water and/or a difference in the binding site.

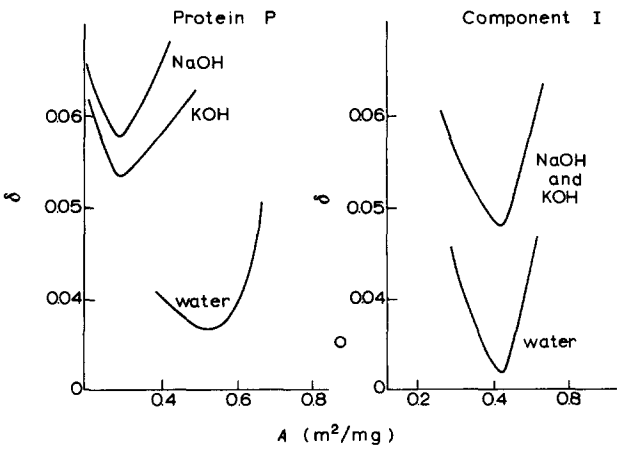

Fig. 7. Surface compressibility–area curves for Protein P and Component I on water and on alkaline solution, respectively, at 20°. The compressibility $\delta = -(1/A)(dA/d\pi)$ is plotted against the surface area. The compressibility minimum is observed when the film is in a close-packed state with a limiting area.

With regard to the nature of the interaction of protein and lipopolysaccharide to form a complex, ELEY AND HEDGE[26] reported, from studies on monolayers, that hydrogen bonds are the most important in formation of the cephalin–protein complex while ionic interactions are the most important in formation of lecithin–protein complex. Moreover, PAYENS[27] showed that the complex of lactoglobulin and phosphatide was due to an ionic interaction at pH 3.9, where the two materials have opposite charges. However, NICKERSON *et al.*[28] found that protein and saccharide are bound with ester bonds in the complex of yeast walls. Moreover, nonpolar penetration may be involved in formation of the complex since the nonpolar side chains of protein penetrate into the lipophilic portion of lipopolysaccharide.

In conclusion, this work shows that the film of Component I of endotoxin extracted from *Ps. aeruginosa* on an aqueous subphase is mainly formed by protein. The structure of this protein film is stiffened by lipopolysaccharide attached to the underneath of the protein film. Thus the film of protein is stabilized by lipopolysaccharide, as reported by MACRITCHIE AND ALEXANDER[29]. A similar conclusion was reached from examination of electron micrographs of films of Protein P and Component I spread on aqueous solutions[30].

REFERENCES

1 J. F. DANIELLI AND H. DAVSON, *J. Cell Comp. Physiol.*, 5 (1935) 495.
2 H. DAVSON, *Circulation*, 26 (1962) 1022.
3 J. D. ROBERTSON, in J. M. ALLEN, *Molecular Organization and Biological Function*, Harper, New York, 1967, p. 65.
4 J. L. KAVANAU, *Structure and Function in Biological Membranes*, Holden, San Francisco, 1965.
5 L. K. JAMES AND L. G. AUGENSTEIN, *Adv. Enzymol.*, 28 (1966) 1.
6 P. MUELLER, D. O. RUDIN, H. T. TIEN AND W. C. WESCOTT, in J. F. DANIELLI, K. G. A. PANKHURST AND A. C. RIDDIFORD, *Recent Progress in Surface Science*, Vol. 1, Academic Press, New York, 1964, p. 379.
7 J. B. FINEAN, *Prog. Biophys. Mol. Biol.*, 16 (1966) 143.
8 L. ROTHFIELD AND W. HOENE, *J. Bacteriol.*, 93 (1967) 1705.
9 Y. HOMMA, K. YAMAMURA, F. EGAMI AND M. NAOI, *Bull. Soc. Chim. Biol.*, 40 (1958) 647.
10 W. F. GOEBEL, G. T. BARRY AND T. SHEDLOVSKY, *J. Exp. Med.*, 103 (1956) 557.
11 H. J. TRURNIT, *J. Colloid Sci.*, 15 (1960) 1.
12 A. SUZUKI AND T. ISEMURA, *Annu. Rep. Biol. Works, Osaka Univ.*, 15 (1967) 85.
13 D. F. CHEESMAN AND J. T. DAVIES, *Adv. Protein Chem.*, 9 (1954) 439.
14 T. YAMASHITA AND H. B. BULL, *J. Colloid Interface Sci.*, 24 (1967) 310.
15 M. JOLY, *J. Colloid Sci.*, 5 (1950) 49.
16 H. E. RIES, JR. AND D. C. WALKER, *J. Colloid Sci.*, 16 (1961) 361.
17 S. GOTO, manuscript in preparation.
18 D. F. CHEESMAN AND H. SCHULLER, *J. Colloid Sci.*, 9 (1954) 113.
19 H. B. BULL, *Adv. Protein Chem.*, 3 (1947) 95.
20 L. G. AUGENSTEIN AND B. R. RAY, *J. Phys. Chem.*, 61 (1957) 1380.
21 J. BATEMAN AND L. CHAMBERS, *J. Phys. Chem.*, 45 (1941) 209.
22 F. MACRITCHIE, *J. Colloid Sci.*, 18 (1963) 555.
23 O. H. LOWRY, N. J. ROSEBROUGH, A. L. FARR AND R. J. RANDALL, *J. Biol. Chem.*, 193 (1951) 265.
24 M. DUBOIS, K. A. GILLES, J. K. HAMILTON, P. A. REBERS AND F. SMITH, *Anal. Chem.*, 28 (1956) 350
25 N. L. GERSHFELD AND C. Y. C. PAK, *J. Colloid Sci.*, 23 (1967) 215.
26 D. D. ELEY AND D. G. HEDGE, *J. Colloid Sci.*, 11 (1956) 445.
27 T. A. J. PAYENS, *Biochim. Biophys. Acta*, 38 (1960) 539.
28 W. J. NICKERSON, G. FALCONE AND G. KESSLER, in M. V. EDDS, Jr. *Macromolecular Complexes*, Ronald, New York, 1959.
29 F. MACRITCHIE AND A. E. ALEXANDER, *J.Colloid Sci.*, 16 (1961) 57.
30 S. GOTO, manuscript in preparation.