BBA 75296

ADSORPTION OF RIBONUCLEASE AT THE AIR–WATER INTERFACE AND ON PHOSPHOLIPID MONOLAYERS

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(Received February 7th, 1969)

SUMMARY

Adsorption of ribonuclease at the air–water interface and on lecithin and cephalin monolayers was followed using a tritiated acetic anhydride-labeled protein.

At the air–water interface, the ribonuclease was adsorbed in two distinctly different and not necessarily monomolecular layers. The first layer several molecules thick seemed to be completely or partly composed of unfolded protein molecules. The second layer seemed to be composed of native ribonuclease molecules in a bi-dimensional crystalline arrangement.

On a condensed phospholipid monolayer, only a one-layer adsorption of native ribonuclease molecules was obtained. When the phospholipid monolayer was expanded, the adsorption characteristics of the ribonuclease on it became similar to those at the air–water interface.

INTRODUCTION

Cytologists and cell physiologists have long recognized the importance of the role of interfaces in biological systems. DANIELLI[1] some twenty five years ago pointed out that many reactions involving the major components of living tissues (lipids, proteins, nucleic acids and carbohydrates) must take place at interfaces; in this respect, the interface between a spread film of lipid and an aqueous solution of protein is of particular significance. Interactions of proteins with phospholipid monolayers seem to be the basis of membrane lipoprotein structure (see for example ref. 2).

Very little has been published in this field, and the only information available from studies involving monolayer techniques comes from a small group of investigators[3–10].

In all these studies was measured the increase of film pressure at a constant area which occurs when lipid monolayers and proteins are brought into contact by various techniques. As pointed out by FRASER[11], this method does not give quantitative results and provides no measure of precisely how much protein is adsorbed on the lipid film. In order to obtain such values, we have introduced a method which involves the direct measurement of the surface concentration by labeling the protein with a tritiated compound.

Ribonuclease was chosen for this investigation since its structure is adequately known[12].

EXPERIMENTAL

Materials

Bovine pancreatic ribonuclease A of puriss. grade, DL-β-γ-dipalmitoyl α-lecithin synth. purum and DL-α-cephalin synth. purum, were all obtained from Fluka (Switzerland). Animal cephalin purchased from the Nutritional Biochemical Corp. (Cleveland, Ohio) has been shown to contain 85% serine and 15% ethanolamine, *plus* traces of inositol and sphingomyelin[13].

Tritiated acetic anhydride in benzene solution and tritiated methyl iodide were obtained from The Radiochemical Centre (Amersham, Great Britain).

5-times distilled water (twice with alkaline permanganate) was used throughout. In all experiments, the aqueous subphase consisted, unless stated otherwise, of 0.2 M phosphate buffer (pH 6.5).

The phospholipids were spread from a hexane–chloroform–isopropanol (3:1:1, by vol.) solution. All organic solvents were of analytical grade and were freshly redistilled. All inorganic reagents were of analytical grade and were used without further purification.

To the acetic anhydride, initially containing 16.6 μmoles/ml of tritiated compound, was added a nontritiated compound. The final concentration was 36.6 μmoles/ml.

Apparatus and procedure

The apparatus employed for measuring the surface radioactivity has been previously described[14]. Measurements were taken commencing immediately from when the surface was cleaned. The phospholipid film was spread on the protein solution by means of an Agla microsyringe from which a drop was allowed to fall on top of a glass rod[15]. The experiments were carried out at room temperature (23° (±1°)).

Acetylation of ribonuclease

Ribonuclease was labeled using tritiated acetic anhydride. Tritium is advantageous for this purpose since it is a low-energy β-emitter with a small range in water (6 μ). Hence using low bulk concentrations, the radioactivity from the surface can be measured without any interference from the radioactivity of the bulk. The acetylation is known to occur on the ten lysyl residues and to be faster on the terminal amino group of the first lysyl residue[16,17]. Two batches were prepared for the experiments described in this study:

Batch 1: 5.1 mg ribonuclease was dissolved in 0.1 ml of buffer, and 0.013 ml of tritiated acetic anhydride was added. Batch 2: 5.3 mg ribonuclease was dissolved in 0.05 ml of buffer, and 0.02 ml of acetic anhydride was added. The mixture was maintained at 4° for 90 min. Dilute HCl was added, and the sample was dried for 24 h on P_2O_5–KOH *in vacuo*. The dry material was used to prepare a 10-ml solution. The yield of acetylation was measured by scintillation counting:

Batch 1. In the scintillation counter, 0.1 ml gave 29000 counts/min or 153000 disint./min (the efficiency being 19%). This corresponds to $1.53 \cdot 10^7$ disint./min per 10 ml or 6.87 μC. The initial acetic anhydride has an activity of 25 mC/91.7 μmoles. The solution contains then: $(6.87 \cdot 91.7/25000) \cdot 2 = 50.4$ nmoles acetyl residues. These residues are bound to 4.6 mg of ribonuclease (since the water content of the batch

is 10%) or 336 nmoles. Consequently, the yield of acetylation is 15% (0.15 acetyl residue per ribonuclease molecule).

Batch 2. The scintillation results were 65200 counts/min per 0.1 ml or $3.44 \cdot 10^7$ disint./min per 10 ml = 15.5 μC per 10 ml corresponding to: $(15.5 \cdot 91.7/25000) \cdot 2 =$ 114 nmoles acetyl residues. These residues are bound to 4.8 mg ribonuclease or 350 nmoles. The yield of acetylation is then 35% (0.35 acetyl residue per ribonuclease molecule).

The efficiency of our gas flow counter is 1%, so the corresponding counts/min: mg is for the first batch: 4.6 mg ribonuclease = 153000 disint./min or, since the planchet has an area of 5 cm^2: 100 counts/min = $4.6 \cdot 10^4/5 \cdot 1.53 \cdot 10^3$ mg/m^2 = 6 mg/m^2.

On the other hand, the molecular weight of ribonuclease being 13700, 1 m^2/mg corresponds to 2280 $Å^2$/molecule.

The homogeneity of the samples was checked by electrophoresis on a 7.5% acrylamide gel according to the method of REISFIELD *et al.*[18]. One radioactive compound was found which migrates together with the nonacetylated ribonuclease; probably identifiable as monoacetylated ribonuclease.

Preparation of tritiated lecithin

In order to observe the spreading and stability of lecithin monolayers, we prepared a batch of tritiated lecithin. DL-α-Cephalin (phosphatidyl ethanolamine) was allowed to react for 24 h at room temperature with an equivalent amount of tritiated iodide in nitromethane. A large excess of methyl iodide was added, and the reaction was permitted to continue at 60° for 3 days in the presence of excess HCO_3^-. The purity of the labeled lecithin obtained was checked by infrared spectroscopy[19–21], and no traces of ethanolamine or of any intermediary product were detected. The solution concentration was determined on the basis of the phosphorus content.

RESULTS

Two kinds of experiments were performed involving measurement of the adsorption of the protein at the air/water interface and adsorption of the protein when the interface is covered with a spread film of phospholipid. The adsorption was measured by determining the radioactivity emitted from the surface as a function of time.

The determinations were carried out using the two batches of acetylated ribonuclease having different degrees of acetylation. Within the experimental error, the same surface concentration value was obtained in both series of tests.

When nonlabeled ribonuclease was added to a solution containing labeled ribonuclease, a linear relationship was found between the reciprocal of the radioactivity count from the surface ($1/\tau$) and the concentration of the added ribonuclease.

The number of counts from the surface, τ, is proportional to the surface concentration of the labeled ribonuclease. For a constant total surface concentration of ribonuclease if the ratio of the labeled ribonuclease (ribonuclease*) to the nonlabeled ribonuclease is the same in the bulk and in the surface:

$$\tau = \frac{a[\text{ribonuclease*}]^b\,[\text{ribonuclease*} + \text{ribonuclease}]^\sigma}{[\text{ribonuclease*} + \text{ribonuclease}]^b} \qquad (1)$$

where the indices b and σ indicate the bulk phase and the surface phase and where a is the proportionality constant, or:

$$\frac{1}{\tau} = \frac{1}{a[\text{ribonuclease} + \text{ribonuclease*}]^{\sigma}} + \frac{[\text{ribonuclease}]^{b}}{a[\text{ribonuclease*}]^{b}[\text{ribonuclease} + \text{ribonuclease*}]^{\sigma}} \quad (2)$$

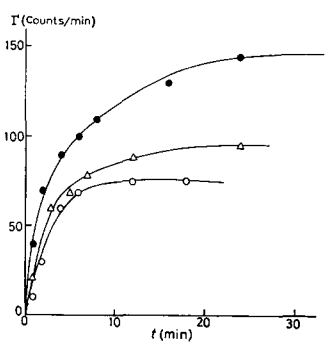
Eqn. 2 shows that at saturation when $[\text{ribonuclease*} + \text{ribonuclease}]^{\sigma}$ does not vary with the amount of ribonuclease added to the bulk and when the ratios of the labeled to the nonlabeled protein in the bulk and in the surface are the same, $1/\tau$ is linear with respect to $[\text{ribonuclease}]^{b}$. It can be seen that this proportionality would not hold if, for instance, ribonuclease* would tend to accumulate preferentially in the surface.

We investigated the dependence of the surface concentration of the ribonuclease (Γ) on the following parameters: bulk concentration (c_b); amount of phospholipid spread; pH; and ionic strength (I).

Influence of the bulk concentration

The Γ *vs.* t curves have the shape of adsorption curves with a saturation plateau which is stable for a few hours (Fig. 1). The influence of bulk concentration was investigated for the case of a free air–water interface and in the presence of a fully compressed phospholipid monolayer. In the first case, we can only have adsorption of protein at the air–water interface. In the second case, because of the high pressure, we may assume that only adsorption on to the film takes place. Penetration into and solution in the film which might have been expected to occur seems to be prevented by the compression in such close packed films.

As can be seen from Fig. 2, the curves Γ *vs.* c_b at the air–water interface display two steps, corresponding to the adsorption of two layers. Corresponding to the first plateau, the area occupied by the ribonuclease molecule at the air–water interface



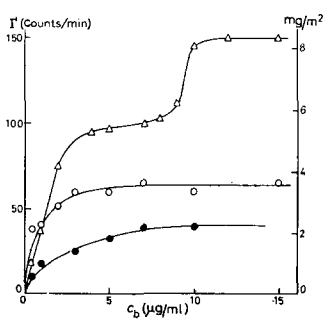


Fig. 1. Adsorption of ribonuclease at the air–water interface. 0.2 M phosphate buffer (pH 6.5). Variation of surface concentration with time for: ○, c_b = 2 μg/ml; △, c_b = 4 μg/ml; ●, c_b = 10 μg/ml.

Fig. 2. Variation of surface concentration with bulk concentration. 0.2 M phosphate buffer (pH 6.5). Γ was taken after 50 min. △, air–water interface; ●, interface covered with a closely packed (37 Å²/molecule) lecithin monolayer; ○, interface covered with a closely packed (0.27 m²/mg) cephalin monolayer.

is $A = 425$ Å^2. For the second layer, the area is 800 Å^2/molecule. The first step represents adsorption at the air–water interface (high-energy surface) and the second adsorption on a film of protein (low-energy surface) rather similar to adsorption on a film of lecithin ($A = 1020$ Å^2/molecule) or of cephalin ($A = 640$ Å^2/molecule).

The two-step adsorption isotherm is rather difficult to explain; it is similar in appearance to a gas adsorption isotherm where ordinary adsorption is followed by capillary condensation[22]. It is very likely, in the present case, that the second step in adsorption is enhanced by the interaction between the molecules adsorbed in the second layer; the adsorption energy in this layer might be proportional to the ribonuclease concentration there.

Assuming the adsorption to be diffusion-controlled (probably true only for short adsorption times and for low bulk concentrations at the air–water interface), $\Gamma = 2c_b(Dt/\pi)^{1/2}$ where D is the diffusion coefficient and t the time. We found that Γ is proportional to c_b (Fig. 2) and to $\sqrt{t}$ (Fig. 3). The diffusion coefficients calculated from the slopes of the plots Γ *vs.* c_b and Γ *vs.* $\sqrt{t}$ were respectively $6.3 \cdot 10^{-7}$ ($\pm 15\%$) cm^2/sec and $6 \cdot 10^{-7}$ ($\pm 15\%$)cm^2/sec, values which are in good agreement with the diffusion coefficient measured by ultracentrifugation $7.75 \cdot 10^{-7}$ ($\pm 5\%$) cm^2/sec.

When the surface was covered by a fully packed lecithin monolayer, the concentration of ribonuclease in the surface at saturation was about one third of the surface concentration without film. DOTY AND SCHULMAN[3] could not detect by surface-pressure measurements any interaction between protein and lecithin at a pressure of 15 dynes/cm.

Influence of the amount of phospholipid spread

The amount of lecithin spread is indicated in Fig. 4 in which the area per molecule in the monolayer is shown. At both high (above 60 Å^2) and low (under 40 Å^2) areas per molecule, the surface concentration is nearly independent of the amount spread. Between these two extreme values, there is a steep increase in adsorbed ribonuclease with the area per spread lecithin molecule. Cephalin gives a

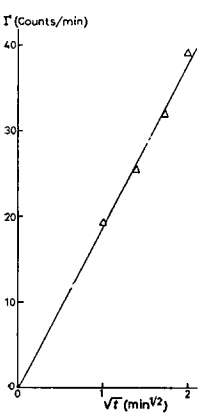


Fig. 3. Variation of surface concentration with $\sqrt{t}$, demonstrating diffusion controlled adsorption; $c_b = 1$ μg/ml.

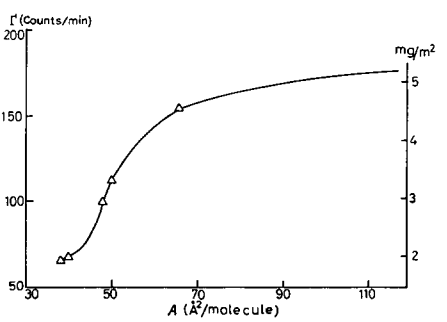
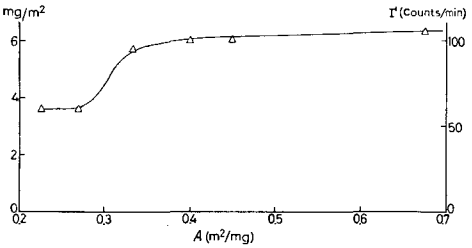


Fig. 4. Variation of surface concentration with the amount of lecithin spread for a bulk concentration of 7 μg/ml, after 90 min and in 0.2 M phosphate buffer (pH 6.5).

curve (Fig. 5) very similar to that obtained with lecithin and shows a transition for an area of 0.31 m^2/mg.

By increasing the area per phospholipid molecule, the hydrocarbon chains are revealed so that hydrophobic interactions with the nonpolar residues of the protein in the monolayer can occur. These interaction forces seem to enhance adsorption as well as conformational changes in the adsorbing protein molecules. Alternatively, the adsorption forces can be related to the surface energy of the adsorbing surface.



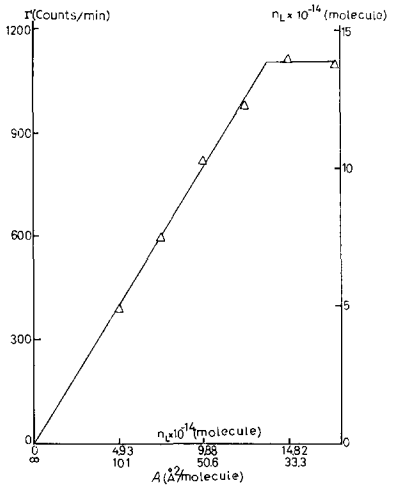


Fig. 5. Variation of surface concentration with the amount of animal cephalin spread for a bulk concentration of 7 μg/ml, after 90 min and in 0.2 M phosphate buffer (pH 6.5).

Fig. 6. Variation of the amount of lecithin present on the surface as a function of the amount spread. Subphase: 0.2 M phosphate buffer (pH 6.5).

Increasing the area per lecithin molecule (from 42 to 50 $Å^2$) results in a rise in surface tension from 30 to 50 dynes/cm, which corresponds to an appreciable increase in the surface energy of the adsorbing surface.

WATKINS[23] has pointed out that the maximum pressure obtainable by adding an excess of dipalmitoyl lecithin to a saline surface of a fixed area is not greater than 50 dynes/cm. The excess evidently does not contribute to the surface pressure and disappears in the subphase. In order to correlate the amount of lecithin spread and the actual concentration of the surface layer, we prepared a batch of tritiated lecithin. As can be seen in Fig. 6, the amount of lecithin present on the surface of a salt solution having an ionic strength of one is proportional to the amount spread untill an area of 37 $Å^2$/molecule is reached. Thereafter, a saturation plateau appears; the added excess of lecithin disappears in the subphase and does not contribute to the count. This value of 37 $Å^2$/molecule corresponds to the area of maximal compression found in the π–A curve of lecithin[23–25].

The minimum area a hydrocarbon chain can occupy in the crystalline state is known[26] to be 18.5 $Å^2$. Therefore, dipalmitoyl lecithin in the crystalline state can occupy a minimum area of 37 $Å^2$/molecule. It seems, thus, that dipalmitoyl-lecithin films reach a crystalline state when spread in these amounts.

Influence of the pH

The influence of pH in the range 2–11.5 on the adsorption of ribonuclease was investigated in a phosphate buffer at an ionic strength equal to 1 (Figs. 7 and 8). The shapes of the curves Γ *vs.* pH with and without lecithin are very similar; there is a shift toward higher pH values in the former, probably due to the presence of the phosphate ions of lecithin on the surface. The maximum at neutral pH may correspond to the isoelectric point on the surface under the given conditions. This maximum occurs at pH 6.7; from extrapolating the data of BARNETT AND BULL[27], the isoelectric point of ribonuclease at this ionic strength is found to be 6.8 in the solution.

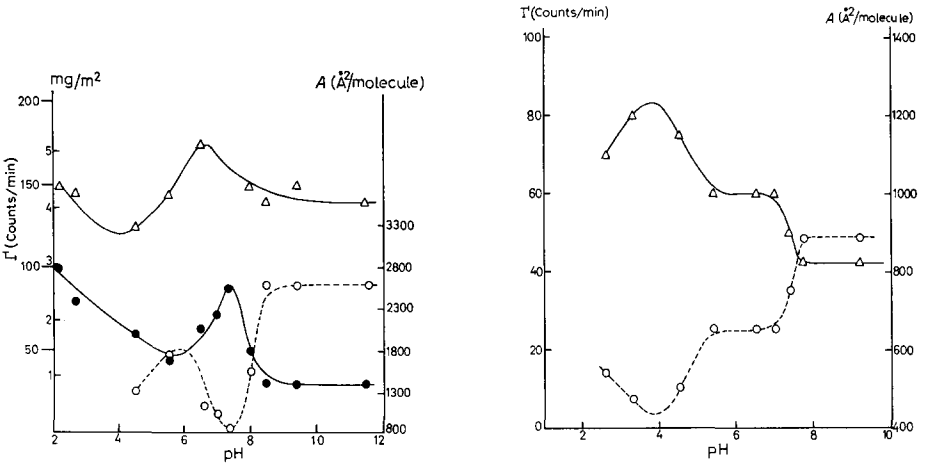


Fig. 7. Variation of surface concentration with pH of the solution for a bulk concentration of 7 μg/ml, after 90 min. 0.2 M phosphate buffer. △, air–water interface; ●, interface covered with a closely packed (37 Å²/molecule) lecithin monolayer. – – – –, variation of the area per ribonuclease molecule with pH, the interface being covered with the lecithin monolayer.

Fig. 8. Variation of surface concentration with pH of the solution for a bulk concentration of 7 μg/ml, after 90 min in 0.2 M phosphate buffer. The interface is covered with a closely packed cephalin monolayer (0.27 m²/mg). – – – –, variation of the area per ribonuclease molecule with pH.

However, other unknown factors related to the interactions between different groups and to conformational changes at different pH's may be involved. The broken line in Fig. 7 represents the areas per ribonuclease molecule at different pH's. At higher pH's, the area varies between 870 (pH 7.4) and 2600 Å² (above pH 9).

With animal cephalin (Fig. 8) the curve Γ *vs.* pH displays two maxima which may correspond to the isoionic points of the phospholipid (pH 3.8) and of the protein (pH 6.7).

Influence of the ionic strength

At the air–water interface, the surface concentration increases with ionic strength; there is a "salting out" effect. On a lecithin monolayer spread from an excess of added lecithin, the opposite tendency was found (Fig. 9). Postulating that the equilibrium concentration of a monolayer spread from an excess of lecithin may depend on the subphase composition, we investigated, using labeled lecithin, the lecithin spread on the surface as a function of ionic strength (Fig. 10).

The saturation concentration of the spread lecithin monolayer is proportional to the ionic strength, until an ionic strength equal to one is reached; in pure water, only 65% remains on the surface.

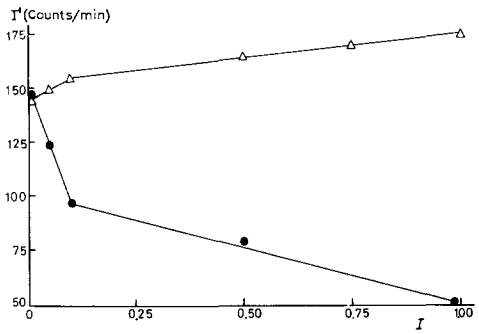


Fig. 9. Variation of surface concentration with ionic strength. c_b = 7 μg/ml; t = 90 min; pH 6.5. △, air–water interface; ●, interface covered with a closely packed lecithin monolayer.

If we compare this curve with those of Γ *vs.* A (Fig. 4) and of Γ *vs.* I (Fig. 9), it can be seen that dissolution of the film is partially responsible for the increase in the surface concentration of ribonuclease when the ionic strength decreases. Below I = 0.1, the surface area per lecithin molecule begins to exceed the value 48 Å^2 (Figs. 6 and 10) at which point a major change in ribonuclease adsorption occurs (Fig. 4).

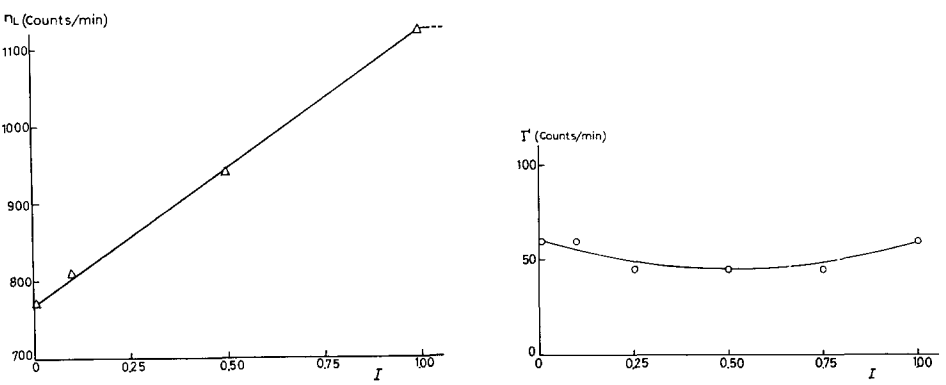


Fig. 10. Variation of amounts of lecithin present on the surface as a function of the ionic strength of the subsolution.

Fig. 11. Variation of surface concentration with ionic strength, when the interface is covered with a closely packed animal cephalin monolayer. c_b = 7 μg/ml; t = 90 min; pH 6.5.

When an excess of animal cephalin is spread on the surface, the influence of the ionic strength on Γ is very small; a "salting in" effect occurs below I = 0.5 and a "salting out" effect above (Fig. 11). It seems that the interactions between ribonuclease and phospholipids are hydrophobic rather than ionic.

DISCUSSION

Since SCHULMAN and co-workers[3,4] showed that the interaction between proteins and lipids is slight at a surface pressure of 15 dynes/cm and is not measurable with lecithin, ELEY and co-workers[5–8] and other investigators subsequently worked with initial surface pressures of 2 dynes/cm. However, as we have shown, in this pressure region the surface concentration is practically the same as that without a film. Former investigators[3–10] attempted to study the penetration of the phospholipid monolayers by proteins. Even light penetration would have a marked effect on the surface pressure of the already compressed monolayer, where a small decrease in area results in a large increase in pressure. These workers did not consider, however, the possibility of subsequent layer formations without any penetration of the spread phospholipid monolayers.

A subsequent layer can be formed by adsorption of the protein at the interface between the spread monolayer and the aqueous solution. The surface energy of this interface cannot be easily assessed from the surface tension of the monolayer-covered surface. The total surface energy of the latter can be considered to be the sum of the interfacial energies monolayer–air and monolayer–water. If we assume that the lowest energy of the phospholipid–air interface is equal to that of an air–oil interface, approx. 20 ergs/cm^2, close to zero surface tensions[23] measured for fully compressed lecithin monolayers would imply a negative interfacial energy. This means essentially that the water molecules are more firmly attracted to the interface than to each other. Even if we regard the close to zero surface-tension measurements as experimental artifacts, close to zero interfacial tensions between monolayer and water have certainly been found in bilayers[28].

There still remains the possibility that macromolecules adsorb on a strongly hydrophilic surface. If no specific chemical interactions are involved between certain functional groups, the adsorption forces acting on each residue are very small. Under these conditions, it has been shown that there is no appreciable variation in surface tension with adsorption, that the number of macromolecular anchor points in the surface is relatively small and that its bulk conformation is preserved[29].

The ribonuclease molecule in solution (pH 7.7) has been shown[30] to be oblong and ellipsoidal with principal axes of 65 and 27 Å. The molecules are assumed to bind about 0.6 g of water per g of protein. The circular cross-sectional area perpendicular to the long axis is about 580 Å^2. For hexagonal packing, the area required for a native molecule oriented perpendicularly to the surface would be 630 Å^2; for rectangular packing it would be 730 Å. These values are well within the limits for the area determined for an ribonuclease molecule in a saturated adsorbed layer on the phospholipid monolayers (Figs. 7 and 8, broken lines). Electrostatic repulsion, swelling of the molecules and conformational changes could explain the much higher areas per molecule (1600 and 2600 Å^2, respectively) on both sides of the isoelectric pH.

Although the saturation surface areas per ribonuclease molecule in the second layer adsorbed at the air–water interface are only slightly lower than the areas of the molecules adsorbed on the lecithin monolayer, the adsorption isotherms are distinctly different. The second layer at the air–water interface does not start to form right away after completion of formation of the first layer at a bulk concentration of about 4 μg/ml. Only when a bulk concentration of 9 μg/ml is reached does a steep adsorption

step appear. This adsorption step indicates that an approximately constant chemical potential of the ribonuclease in the surface is maintained during the build up of the second layer. In this respect, there is a resemblance to capillary condensation in gas adsorption[22]. Here, however, the probable cause of the constant chemical potential in the surface during the second adsorption step is bidimensional crystallization of the ribonuclease molecules in the surface. The alignment of the adsorbed ellipsoidal molecules during this surface crystallization process is again with the long axis lying vertical to the surface.

To summarize these adsorption experiments, the following pattern for the ribonuclease adsorbed at the air–water interface and phospholipid monolayers are suggested.

(i) At the air–water interface, the ribonuclease is adsorbed in two distinctly different, not necessarily monomolecular, layers. The first layer, probably several molecules thick, seems to be composed of completely or partly unfolded protein molecules. The present impression is that the first layer varies little with the composition of the subphase, but this point is still being clarified by running a series of detailed adsorption isotherms at different pH's and ionic strengths. The second layer seems to be composed of native ellipsoidal ribonuclease molecules in a bidimensional crystalline arrangement oriented with their longer axis perpendicular to the surface.

(ii) On a condensed phospholipid monolayer, only a one-layer adsorption of native ribonuclease molecules is obtained. The adsorbed molecules are preferentially oriented with their long axis vertical to the surface. When the phospholipid monolayer is expanded, the adsorption characteristics of the ribonuclease on it become similar to those at the air–water interface.

ACKNOWLEDGEMENTS

This work was supported by Délégation Génerale à La Recherche Scientifique et Technique No. 67-01-031-00212-75-01 and by the National Institutes of Health under Research Agreement No. 615134.

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