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Spreadability of Ovalbumin Monolayers at Air-water Interface

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The spreading processes of ovalbumin have been studied quantitatively at air-water interface with either no additive or a small amount of hydrochloric acid for controlling subphase pH. Limiting area which is obtained by extrapolating force(F)-area(A) curve to $F=0$, increases with time, t, which has elapsed after the application of protein solution at the surface of subphase. Near the isoelectric point, the spreading processes are completed instantaneously to give the equilibrated value of limiting area which is found to be 0.83 m²/mg. The processes are much slower at both sides of the point, but the limiting area in equilibrium is found to be the same as in the case of isoelectric pH. It has also been shown that the processes are expressed in terms of two first-order reactions.

Work has been done by many investigators[1-3] on the nature of protein monolayers at air-water interfaces, in relation to the mechanism of their denaturation processes. In many cases, however, the experiments were carried out under the tacit assumption that the protein films were in the equilibrated state in which surface-chemical processes could be considered to reflect thermodynamical properties, though the assumption could hardly be verified. It is practically difficult to confirm the equilibrium state of protein monolayer at the air-water interface. In many cases, therefore, it has been assumed that the equilibrated state is attained if a monolayer gives no time effects upon the quality to be studied. This was indeed the case in determining the thermodynamical properties of monolayers of various proteins to be compared.

On the other hand, the denaturation processes are considered to take place during the course of monolayer formation by applying a protein solution to the surface of subphase water. The course is conveniently defined[4-6] to involve (1) dissolution of protein molecules into the bulk of subphase and their return to the surface by diffusion, (2) their spreading at the surface and (3) their uncoiling to form the structures of denatured protein. Thus the denaturation processes seem to be studied conveniently by elucidating the course of monolayer formation with elimination of processes (1) and (2) as much as possible. This has been achieved to some extent by the addition of a large amount of salt into subphase,[1] or of surface active, volatile solvent to spreading solution.[7] The former involves the difficulty to eliminate surface-chemical impurities from subphase,[8] while the latter gives little effects on eliminating the process (1). None of these methods always reflects the true nature of surface denaturation of protein molecules, because process (3) may be much affected by the addition of salt or solvent. Any additives should be avoided for quantitative studies on the spontaneous processes of protein denaturation. It has been pointed out[4] that the decreased contribution of processes (1) and (2) to the whole spreading course can be expected by applying dilute protein solution without additives on the top of the glass rod standing in the trough which contains water alone.[9] This has proven to be effective for decreasing the conditions of (1) and (2), making it easy to understand the surface denaturation of protein molecules in terms of unfolding processes of their polypeptide chains.[4-6] The technique has been extended in the present investigation to elucidate the kinetic processes of protein denaturation at air-water interface.

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Experimental

A sample of ovalbumin was recrystallized seven times from aqueous ammonium sulfate and its purity was confirmed by paper electrophoresis and by microscopic observation of crystalline shape. Its aqueous solution was treated with Sephadex-G25 to remove inorganic materials and made the desired concentration (0.0859 mg/ml) which was confirmed spectrometrically. The water both for the spreading solution and for the subphase was distilled twice from alkaline permanganate in Hysil glass ware. Hydrochloric acid for controlling pH was distilled at its azeotropic point.

A glass tray, 48 cm × 15 cm in area and 0.5 cm in depth, had a well for a glass plate to be dipped for measuring surface pressure, F (dyn/cm). The plate, 2.4 cm × 3.2 cm × 0.015 cm, was cleaned by soaking it into a hot chromic acid mixture or alkali-alcohol solution prior to each measurement. The displacement of the plate was magnified by a lamp and scale system to give the surface pressure with an accuracy of 0.05 dyn/cm. The whole apparatus was thermostated at 20± 0.5°C.

The protein solution was applied onto the glass rod (0.5 cm dia. × 10 cm above the water level), which stood vertically in the subphase with pH 2.0—8.2 at such a dropping rate as to permit less than 0.1% of protein molecules to get into the subphase, the figure being based on the theoretical calculation by Trurnit.[9] Surface contamination was low enough to give $F<5$ dyn/cm when the aqueous surface was kept standing for 24 hr and then compressed to 1/20 its area.

The surface pressure was measured as a function of area, A (m²/mg), at given time, t, which had elapsed from the application of spreading solution to the surface of subphase. The limiting area, A^0, obtained by extrapolating the straight part of F-A curve was taken as a measure of the extent of the denaturation processes at given time, t.

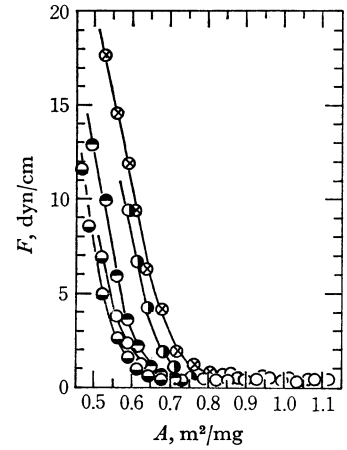

Fig. 1. Time dependence of F-A curves of ovalbumin monolayer spread on subphase of pH 5.2—6.0; —◒—, t=15 min; —○—, 1 hr; —◓—, 4 hr; —◑—, 24 hr; —⊗—, 42 hr

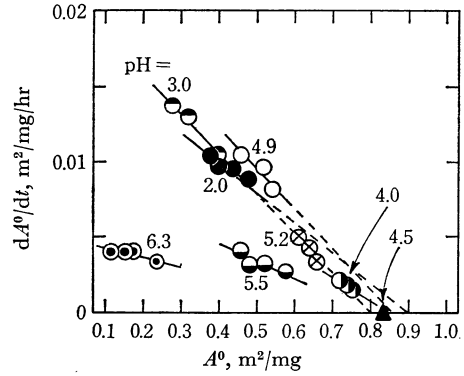

Fig. 2. The relationship of dA^0/dt and A^0 for ovalbumin nomolayers on subphases of various pH.

Results and Discussions

Figure 1 gives the F-A curves as a function of t obtained with monomolecular film of ovalbumin spread on the subphase of pH 5.2. It was inevitable to find a gradual change of pH during the elapsed time, giving the final pH of 6.0 at t=42 hr. It can be seen that the F-A curve is shifted to the right, showing an increase of limiting area with increasing time. The shift suggests an asymptotic tendency to reach the definite A^0 value at $t=\infty$. This is indeed the case of the linear relationship of $\log A^0$ with t, suggesting the validity of the equation

$$dA^0/dt = k_2(A_\infty{}^0 - A^0), \qquad (1)$$

where $A_\infty{}^0$ is A^0 at $t=\infty$ and k_2 the constant. The relationship holds throughout the long period of t >2—3 hr.

In Fig. 2 is shown the dA^0/dt as a function of A^0 for the monolayers on subphases varying in pH values. The slope of the linear part gives the rate constant, k_2, of the first order denaturation process of ovalbumin approaching the equilibrated state, in which the monomolecular film consists of completely unfolded molecules of protein. If k_2 is plotted against pH of subphase, one finds the maximum value, as seen in Fig. 3, near the isoelectric point of protein. In both sides from the isoelectric point, k_2 decreases with increasing departure from the point. It is thus concluded that the spreading processes in the period sufficiently long after the initial spreading are expressed by

$$(A_\infty{}^0 - A^0) = (A_\infty{}^0 - A_2{}^0)\exp(-k_2 t) \qquad (2)$$

where $A_2{}^0$ is A^0 value at $t=0$.

Rigorously speaking, however, Eq. (2) does not hold in the small t region, namely, shortly after the spreading, as can be seen in Fig. 4, which gives considerable deviation of the empirical values from the calculated slope (solid lines) based on Eq. (2). If, however, we plot the deviation against t on the same figure, we have the dotted lines which show fairly linear relationships. Therefore, the whole processes could better be expressed by

$$(A_\infty{}^0 - A^0) = (A_\infty{}^0 - A_2{}^0)\exp(-k_2 t) + (A_2{}^0 - A_1{}^0)\exp(-k_1 t) \qquad (3)$$

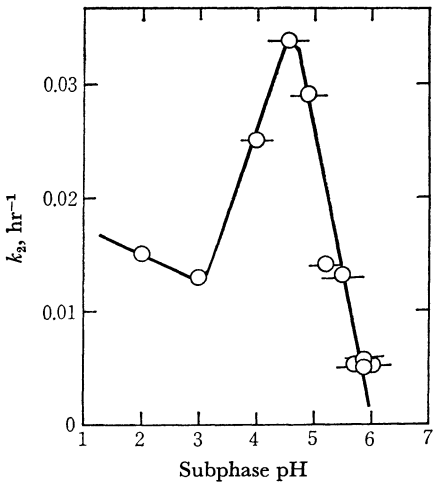

Fig. 3. k_2 as a function of subphase pH. The circles are given for the average pH from the initial and final values, which are shown as the horizontal bars.

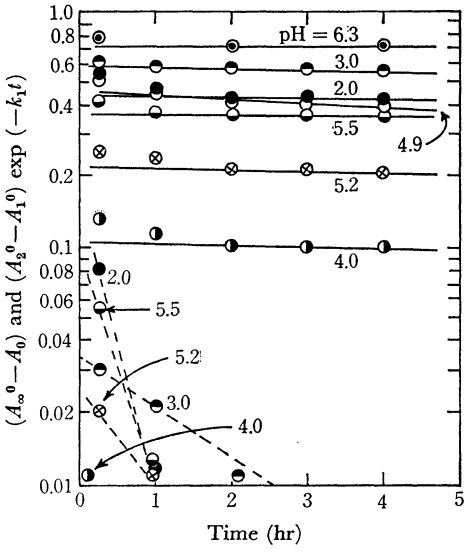

Fig. 4. Time dependences of $A_\infty{}^0-A^0$ (solid lines) and $(A_2{}^0-A_1{}^0)\exp(-k_1t)$ (dotted).

where $A_1{}^0$ is the extrapolated value of the dotted lines to $t=0$ and k_1 the rate constant for the process shown as the dotted lines in Fig. 4. Equation (3) suggests that the spreading processes are expressed as the sum of two independent processes, one is fast and the other slow, and that, if we assume the first order reaction for one of them, the other should give the same order in its mode.

The above results suggest that the uncoiling of protein molecules at air-water interface involves the two processes; the fast process starts from $A_1{}^0$, while the slow process covers the range $A_2{}^0$ to $A_\infty{}^0$. These values are plotted in Fig. 5 against the pH of subphase on which the protein is spread.

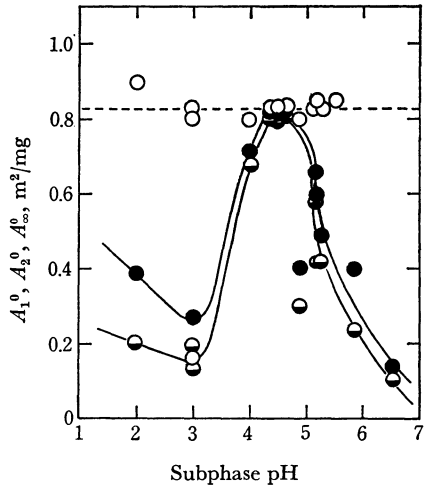

Fig. 5. $A_1{}^0$ (semiclosed), $A_2{}^0$ (closed) and $A_\infty{}^0$ (open) as a function of subphase pH.

It is clear from Fig. 5 that the A^0 value (0.83 m^2/mg) is independent of pH, as mentioned previously, while $A_1{}^0$ and $A_2{}^0$ give almost the same tendency to each other, giving the maximum value near the isoelectric point, where the spreading is completed instantaneously. It should be noted that the two solid curves in the figure resemble in shape the pH dependences of limiting area reported by many investigators[1-3] who paid little attention to the time-dependent nature of protein monolayers. This means that, when subphase pH is at the isoelectric point, the spreading of protein molecules at air-water interface and the unfolding of their polypeptide chains are completed immediately after their arrival at the interface, and that, at any other pH, the protein molecules shrink to give smaller $A_1{}^0$ and $A_2{}^0$ values and then gradually uncoil to reach finally the same $A_\infty{}^0$ value. This process is characterized by the slower rate of uncoiling for the more deviated pH value from the isoelectric point, as can be seen in Fig. 3.

It has been claimed by many investigators[1-3] that the limiting area of protein molecules gives the maximum value when they are spread on subphase of isoelectric pH value. It seems to be more reasonable to treat the spreading of protein at air-water interface as a reflection of kinetic processes to be characterized by the unfolding of protein molecules concerned. The subphase pH does not influence the limiting area, $A_\infty{}^0$, in the equilibrium state, but affects markedly the kinetic processes with which the protein molecules uncoil to approach the equilibrated state.

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