Studies on the Denaturation of Lysozyme. III. Comparative Study of Lysozyme Denaturation on Surface and in Solutions

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(Received September 17, 1957)

Previously, the author has reported the surface-[1-4], heat-[5], and urea-denaturation[6] of lysozyme. The properties of the monolayers of lysozyme are closely related to the state of the molecules in spreading solutions. Therefore, the studies on the denaturation in solutions throw light on the mechanism of the surface denaturation. In this paper will be shown the correlation between the denaturation of lysozyme on surface and that in solutions.

Experimental

Lysozyme was prepared from hens' egg white by direct crystallization method, recrystallized four times and finally lyophilized.

Surface pressure was measured by the hanging plate method of Wilhelmy type. Simultaneously surface potential was measured by the vibrating electrode method. Surface viscosity was measured by the damping of the oscillatory rotation of a disc on the surface covered with monolayers.

The precipitation method to investigate the heat denaturation was as follows: a lysozyme solution in a test-tube was heated in a constant-temperature bath. After each time-interval the test-tube was taken out from the bath. When borate buffer of pH 11.0 (i.e., the isoelectric point of lysozyme) was added, the denatured protein was precipitated. The amounts of the precipitates thus formed were measured by micro-Kjeldahl method. The viscosity of lysozyme solutions was measured by means of Ostwald viscometer. The other experimental details should be consulted for the previous papers[1-6].

Results and Discussion

Surface Chemical Method to Investigate Protein Denaturation[1] — Bull[7] reported that the monolayer of lysozyme is not formed on water surface. However, it was found that the stable monolayers are formed on the substrate of which pH lies near its isoelectric point. When lysozyme was spread from 0.001 N hydrochloric acid solution, the surface pressure-area (F–A) curve had much smaller limiting area than that of the usual proteins, such as ovalbumin. However, the F–A curves obtained with the films spread from 8 or 10 M urea solutions began to develop from the area of about 14.5 Å²/residue. This value was also found with the films of other proteins or synthetic polypeptides.

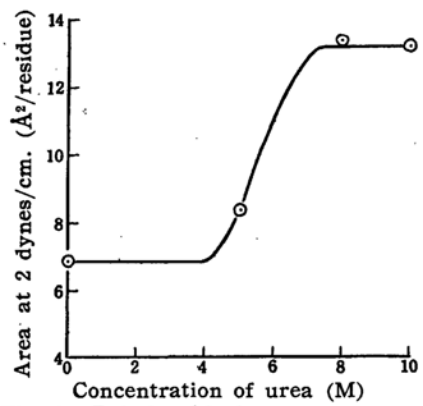

Fig. 1. Relationship between areas at 2 dynes/cm. and urea concentrations in spreading solutions.

Fig. 1 shows the relation between the areas at 2 dynes/cm. and the urea concentrations in the spreading solutions. From Fig. 1 it was expected that the expansion of lysozyme monolayers may reflect the degree of denaturation in urea solutions. This fact means that the denaturation of lysozyme in solutions can be investigated by the surface chemical method.

"Surface Heat Denaturation" of Lysozyme[2] — As described above, the F–A curve of lysozyme monolayer was affected by the kind of spreading solution. On the other hand, it was found that the properties of lysozyme monolayers were also affected by the temperature of the substrate. The effect of temperature of the substrate on the area at 2 dynes/cm. is shown in Fig. 2. As shown in this figure the area became larger with the rise of temperature of the substrate.

1) K. Hamaguchi, *J. Biochem.*, **42**, 449 (1955).
2) K. Hamaguchi, ibid., **42**, 705 (1955).
3) K. Hamaguchi, ibid., **43**, 83 (1956).
4) K. Hamaguchi, ibid., **43**, 355 (1956).
5) K. Hamaguchi, ibid., **44**, 695 (1957).
6) K. Hamaguchi, ibid., **45**, No. 2 (1958).
7) H. B. Bull, *J. Biol. Chem.*, **185**, 27 (1950).

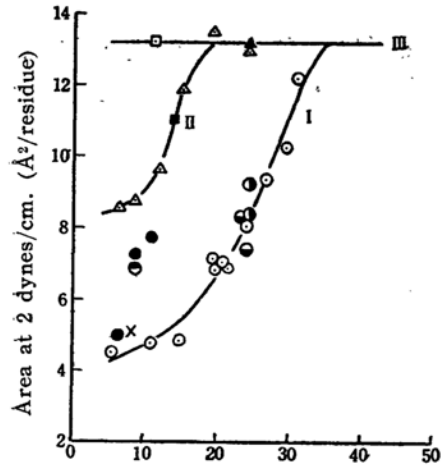

Fig. 2. Relationship between areas at 2 dynes/cm. and temperatures of substrate. Spreading solutions: ⊙, 0.001 N hydrochloric acid (fresh); ◒, 5 M urea solution; ◑, 0.001 N hydrochloric acid heated at 65° for two hours prior to spreading; △, 8 M urea solution; ▣, 8 M urea solution heated at 55° for two hours prior to spreading; ▲, 10 M urea solution; ■, 0.02 N sodium hydroxide (old); ●, 0.001 N hydrochloric acid (old); ◓, 0.04 N sodium hydroxide; ×, 0.02 N sodium hydroxide (fresh).

Curve I in Fig. 2 was obtained for the films spread from 0.001 N hydrochloric acid. The films spread from 5 M urea solution and from 0.001 N hydrochloric acid solution which was heated to 65°C. for two hours beforehand, occupied the areas on this curve. This fact shows that lysozyme molecules are not denatured in these solutions.

Curve II was obtained for the films spread from 8 M urea solution. Although curve II shifted to larger areas than curve I, the dependence of the areas on the temperature was also observed. The films spread from strong alkaline solutions also belong to this curve. These facts show that lysozyme is denatured in these solutions.

When the temperature of the substrate was sufficiently high, the films occupied the areas on curve III, irrespective of spreading solutions. That is, curves I and II tend to approach this straight line above 35° and 18°, respectively. The areas occupied by the films spread from 10 M urea solution also fall on curve III. Therefore, this straight line represents the maximum expansion beyond which the films of lysozyme do not expand.

The facts described above should be designated as "the surface heat denaturation" of lysozyme. Such a phenomenon has not yet been observed. It is generally believed that the temperature coefficient is unity for surface denaturation[8]. The direct examination of the denaturation in solutions can clarify the dependence of the monolayer properties on the spreading solutions and further can make our surface chemical method to investigate the denaturation more certain, details of which will be described in the later part of this paper.

Mechanism of Surface Denaturation of Lysozyme[2]—In order to analyze the results shown in Fig. 2, we make the following two assumptions: (a) The maximum expansion represented by curve III in Fig. 2 corresponds to that of the films which consist of the completely unfolded molecules of lysozyme and the films having smaller areas than this maximum expansion consist of two different kinds of molecular configuration, one of which is in a state of complete surface denaturation and the other, globular or partially altered configuration. (b) The latter configuration was assumed by the molecules existing in the adsorbed layer and would contribute neither to surface pressure nor to surface potential.

These assumptions were confirmed by the fact that the surface potential of the films spread from various solutions was a function of surface pressure and not of the area[4]. By these assumptions, C_n in Eq. (1) represents the concentration of the "globular" molecules in adsorbed layer.

$$\frac{1}{A}-\frac{1}{A_d}=C_n \qquad (1),$$

where A is the area at any given expansion and A_d the area represented by curve III in Fig. 2. The globular molecules present in adsorbed layer gradually suffer surface denaturation with time, giving rise to completely unfolded films. Because the curves shown in Fig. 2 were obtained for the films all of which were stood for 30 minutes before measurements, the relations indicate that the amounts of reactant, suffered surface denaturation, are different according to the temperature of substrate.

Therefore, the rate constant of surface denaturation must be obtained, when the F-A curves of the films are measured on the substrate at a given temperature at

8) H. B. Bull and H. Neurath, ibid., **125**, 113 (1938).

different ages after spreading. In fact, the F–A curves on the substrate at a given temperature shifted to larger areas with time after spreading.

The logarithms of the concentrations of "globular" molecules in adsorbed layer, C_n, which are calculated from Eq. (1) by the use of the areas at 2 dynes/cm., are plotted against time in Fig. 3 and linear relations are obtained.

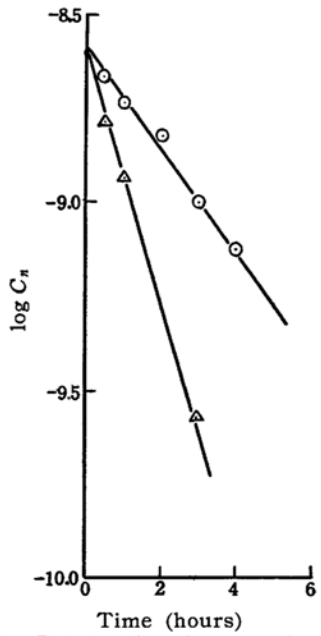

Fig. 3. Relationship between log C_n and elapsed time. Temperature of substrate: ⊙, 11°; △, 21°C.

This is the indication that the surface denaturation obeys the first order kinetics. Therefore, the first order rate constant can be calculated. By means of Arrhenius equation the apparent activation energy can be calculated from the rate constants at 11° and 21°C. and is found to be 15 kcal./mole.

From these considerations, it is found that the activation energy of 15 kcal./mole is necessary in order to obtain the monolayers consisting of completely unfolded molecules when native lysozyme is spread at an air-water interface. This is the reason why the F–A curves of lysozyme films are profoundly affected by the temperature of substrate. When the proteins such as ovalbumin and serum albumin, which have been so far investigated in detail, are spread as monolayers, the activation energy to pass from globular molecules to completely unfolded ones would be very low and then the completely unfolded films are formed instantaneously. Therefore, in the case of these protein films, neither the effect of temperature nor the time effect has been observed. The mechanism of surface denaturation can be clarified by using the proteins such as lysozyme which are very difficult to be denatured by surface.

The free energy, enthalpy and entropy activation, which are calculated from the methods according to Eyring and Stearn, are as follows: $\Delta F^*=22.4$ kcal./mole, $\Delta H^*=14.4$ kcal./mole, and $\Delta S^*=-28$ e. u. at 11°. With protein denaturation, in general, there are great increases in entropy and heat during the activation process. In this case, however, there is a decrease in entropy and a small increase in heat. The reason for this is not clear. There are two possibilities to explain the negative value of entropy: (a) that involved in a decrease in degree of freedom at interfaces compared with that in solutions and (b) that involved in freezing water molecules to the protein molecules.

Urea Denaturation of Lysozyme[6]—As described above, the properties of lysozyme monolayers depend on the concentration of urea in the spreading solutions. It was expected that this fact would reflect the denaturation in urea solutions and so the denaturation can be examined by the surface chemical method. It is an interesting problem whether the results obtained by a direct examination of the behavior of lysozyme in urea solutions

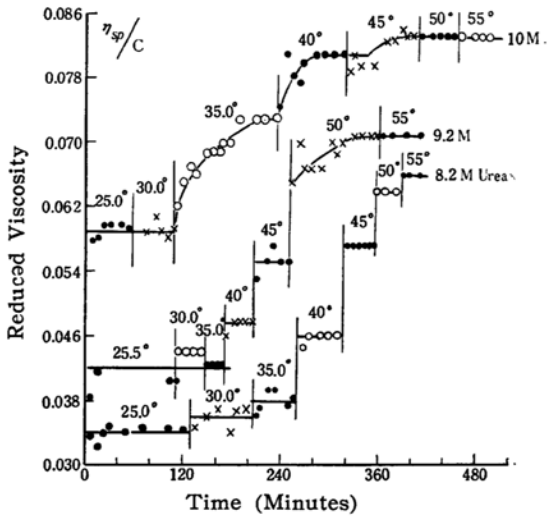

Fig. 4. Change with time of reduced viscosities of lysozyme in urea solutions. Concentration of lysozyme: 0.41 g./100 ml. pH 5.4-5.8.

correspond to those by the surface chemical method or not. Furthermore, the mechanism of surface denaturation will be made more clear when the denaturation in solutions is studied by other physicochemical methods. The urea denaturation of lysozyme was examined by viscosity measurements. The change with time of the reduced viscosity of lysozyme in the solutions at various urea concentrations over a rather wide temperature range are shown in Fig. 4.

It is very remarkable that the reduced viscosity was increased instantaneously with the rise of temperature and did not change with time at each temperature. Most of the proteins show the time effect on the viscosity in urea solution. In the case of lysozyme, however, the time effect was only observed in 9.2 and 10 M urea. Fig. 5 shows the maximum values of the reduced viscosities at each temperature.

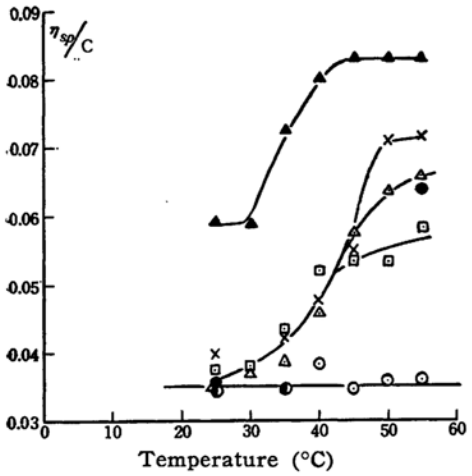

Fig. 5. Relationship between maximum reduced viscosities and temperatures. ◐, 0 M; ⊙, 4.2 M; ◙, 6.3 M; ●, 7.4 M; △, 8.2 M; ×, 9.2 M; ▲, 10 M urea.

It was found that the reduced viscosity in 4.2 M urea did not change between 25° and 55°C. and was the same as that of native lysozyme. In above 6.3 M urea solution the reduced viscosity increased from about 30° and tends to approach the constant value from 45°. Although the viscosity-temperature curves for 6.3, 8.2 and 9.2 M urea were almost the same at lower temperatures, the viscosity became greater with the increase in concentration of urea at higher temperatures. The reduced viscosity in 10 M urea was greater than that in 9.2 M urea even at 25°. In this case the viscosity was also increased from 30° and became constant from 45°. In order to examine the reversibility of the urea denaturation, after the lysozyme solutions once heated up to 55° had been kept at room temperature overnight, the viscosity was measured again at 25°. In 10 M urea, the reduced viscosity thus obtained was the same as that at 55°. The viscosity in 9.2 M urea returned to a smaller extent and the reversibility was nearly complete in 8.2 M urea.

With urea denaturation of proteins, in general, the reversibility is studied on the solutions after removal of urea by dialysis. In the case of lysozyme, however, the reversibility was observed merely by the lowering of temperature. This is a very remarkable fact. It might be more appropriate to term these phenomena as the "heat denaturation of lysozyme in urea solutions" rather than "urea denaturation". From the facts described above, it is assumed that at each temperature native lysozyme molecules are in equilibrium with those of another modification which are stable above 50° in 8 M urea. The equilibrium constant (K) at each temperature may be calculated from Eq. (2).

$$K = \frac{\eta - \eta_0}{\eta_\infty - \eta} \qquad (2),$$

where η_0 is the reduced viscosity of native lysozyme and η_∞ the viscosity above 50°.

When the logarithms of the equilibrium

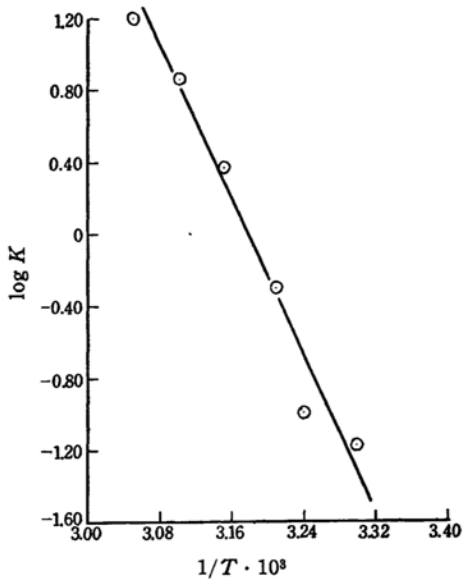

Fig. 6. Relationship between logarithms of equilibrium constants (K) and reciprocals of absolute temperatures.

constants are plotted against the reciprocals of absolute temperatures, a straight line is obtained as shown in Fig. 6. Therefore, the change in enthalpy ($\Delta H°$) can be evaluated from van't Hoff's equation. The change in standard free energy ($\Delta F°$) and entropy ($\Delta S°$) can be also obtained by the usual thermodynamic calculations. These thermodynamic parameters are as follows: $\Delta F° = -530$ cal./mole, $\Delta H° = 4{,}950$ cal./mole, and $\Delta S° = 17.2$ e. u. at 45°. The values of $\Delta H°$ and $\Delta S°$ are much smaller than those generally accepted for protein denaturation. This fact suggests that the structural change of lysozyme molecules in 8 M urea is only minor.

The investigations on urea denaturation have been mainly concerned with the structural change of protein molecules by urea and not with the interaction of urea with protein molecules. The interaction is inferred easily by monolayer technique[3]. Because urea is not a surface active substance, protein monolayers should not be profoundly affected unless urea molecules interact with protein monolayers. The F–A curves of lysozyme monolayers were shifted to larger areas with the increase in urea concentration in the substrate. The increase in area proceeded stepwise with the increase in urea concentration and was smaller at lower temperatures. These phenomena could be explained by the cleavage of bonds by urea attack which remain intact by spreading and by the adsorption of urea on the bonds emerged by the cleavage. It was also inferred from these experiments that urea molecules are scarcely adsorbed on lysozyme molecules in urea solutions.

Relationship between Monolayer and Bulk Properties of Lysozyme — The concern, here, is with the comparison of the results on the urea denaturation obtained by the surface chemical method with those by viscosity measurements. The behavior of lysozyme monolayer spread from 5 M urea solution was the same as that of native lysozyme. In 4 M urea the reduced viscosity did not change between 25° and 55° and was the same as that of native lysozyme. It is concluded, therefore, that lysozyme is not denatured in the urea solutions of which concentrations are below 4 or 5 M. The area of lysozyme monolayer was increased from 5 M urea (Fig. 1). The reduced viscosity also became sensitive to the temperature from the same urea concentration.

The expansion of lysozyme monolayer spread from 10 M urea was maximum and the areas scarcely depend on the temperature of the substrate. The reduced viscosity in 10 M urea was greater than that in 9.2 M urea. In addition, the effect of time on the reduced viscosity was observed and the viscosity did not reverse to the original value by cooling. These facts suggest that the secondary structure of lysozyme molecules may be destroyed in 10 M urea.

While the areas occupied by the lysozyme monolayers spread from 8 M urea was greater than that spread from native lysozyme, the temperature dependence of the expansion was the same as that of native lysozyme. On the other hand, it was found from the viscosity measurements that there exists an equilibrium between native and reversibly denatured molecules in 8 M urea. Therefore, the facts shown in Fig. 2 will be well convinced when the latter molecules are considered to be easily denatured by surface owing to a decrease in the activation energy for surface denaturation. The denaturation in 8 M urea may be caused by a rather slight change in the tertiary structure of the lysozyme molecule. The foregoing considerations show the remarkable correspondence between monolayer and bulk properties of lysozyme.

Heat Denaturation of Lysozyme[5] — In this section will be described the heat denaturation of lysozyme. As a matter of course, the heat denaturation in

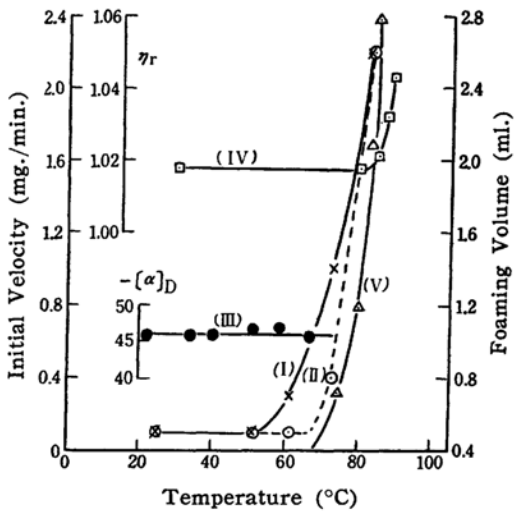

Fig. 7. Foaming volume (curves I and II), optical rotation (curve III), relative viscosity (curve IV) and initial velocity (curve V) as a function of temperature.

solutions is detected at much higher temperatures than "the heat denaturation on surface" owing to the absence of interface.

The temperature where lysozyme begins to be denatured by heat depends on the method to be used. This temperature was determined by the measurements of foaminess, optical rotation, viscosity, and of solubility. The results are shown in Fig. 7. It is evident that a change appears in foaming volume at the lowest temperature, 50°. Curve I was obtained for the solutions immediately after heating for a quarter of an hour at each temperature. This fact suggests that lysozyme molecules are slightly denatured and changed to a state susceptible to surface denaturation by vigorous shaking. As described above, native lysozyme is not easily denatured by surface, but readily undergoes surface denaturation when the protein is denatured in solutions beforehand. The foaming volume, however, decreased reversibly by standing at room temperature after heating (curve II). The surface chemical method could not distinguish the behavior of the monolayers spread from a solution of native lysozyme and those spread from a solution heated at 65° beforehand. These facts suggest that lysozyme might be reversibly denatured in the vicinity of 50°. The optical rotation did not change up to about 70° (curve III). This is the indication that the reversible denaturation might not result from a greater change in the secondary structure but from a change in the tertiary structure. It is interesting that the optimum temperature of the enzymatic activity of lysozyme (55°)[9] corresponds to the temperature from which the foaminess increases. That is, the enzymatic activity may be decreased by a minor change in the tertiary structure.

The viscosity began to increase from about 85° (curve VI). The increase in viscosity, however, accompanied the increase in turbidity. Therefore, this is not due to the effect of the unfolding of each lysozyme molecule. The solubility at the isoelectric point began to change from 70°. That is, the denaturation could be detected by the precipitation method, nevertheless the viscosity did not change. Therefore, the heat denaturation of lysozyme might be caused by the aggregation of the molecules rather than by the unfolding.

The effects of temperature, lysozyme concentration, and of pH on the heat denaturation were determined by the precipitation method in detail. Increase in the concentration of lysozyme had the effect of speeding up the rate of its denaturation. The reaction order of the heat denaturation was determined by the differential, integral, and half-life method. As a result, the initial velocity of the heat denaturation (v_0) was expressed approximately by the following equation:

$$v_0 = k\frac{[P_0]^2}{[H^+]} \qquad (3),$$

where $[P_0]$ is the initial concentration of lysozyme and $[H^+]$ the hydrogen ion concentration. However, the reaction order increases as the reaction proceeds.

The apparent activation energy calculated from Arrhenius equation using the initial velocities was found to be 50.3 kcal./mole, which was independent of the initial concentration.

Role of Disulfide Bonds in Denaturation of Lysozyme[4,6].—One of the most striking structural features of lysozyme molecule is that five intrapeptide disulfide bridges exist within its rather small molecule. Accordingly, it is important to investigate the role played by these disulfide bridges. Sodium sulfite was used as a splitting agent for these bonds. In Fig. 8 is shown the effect of sodium sulfite (0.03 M) on the reduced viscosity of lysozyme in different urea concentrations[6].

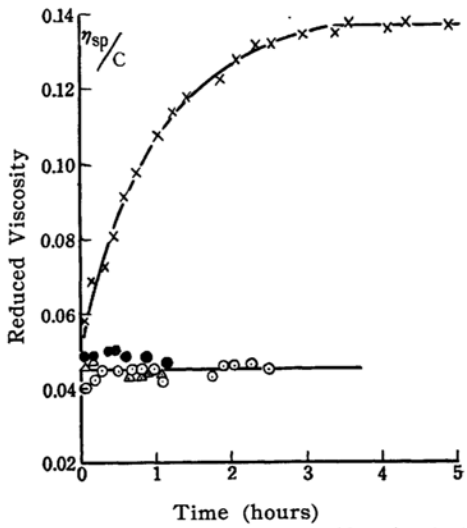

Fig. 8. Effect of sodium sulfite (0.03 M) on reduced viscosity at different urea concentrations. (30.0°, pH 9.6). ●, 6.3 M urea; ⊙, 6.3 M urea, 0.03 M Na_2SO_3; △, 8.2 M urea; ×, 8.2 M urea, 0.03 M Na_2SO_3.

9) C. Fromageot, *Bull. soc. chim. biol.* Supple. **11-12**, 63 (1948).

Fig. 8 clearly shows that the effect of sodium sulfite was not observed in 6.3 M urea but was very striking in 8.2 M urea. In the absence of sulfite, however, the reduced viscosity was almost the same irrespective of the urea concentration. In 8.2 M urea at pH 9.6 containing 0.03 M sodium sulfite, the reduced viscosity was increased rapidly with time and the final value was much higher than that in the absence of sodium sulfite at 30°. These facts show that the disulfide bridges might be masked in the interior of the molecules and that the degree of denaturation in 6.3 M urea is so small that the disulfide bonds can not react with sulfite. Thus, the easiness of the splitting of the disulfide bonds gives a measure of denaturation by urea. The marked increase in viscosity by the splitting of disulfide bonds in 8 M urea suggests that the five intrapeptide bonds of lysozyme must be incorporated into widely separated parts of polypeptide chains and they do not neighbor with each other along the chain.

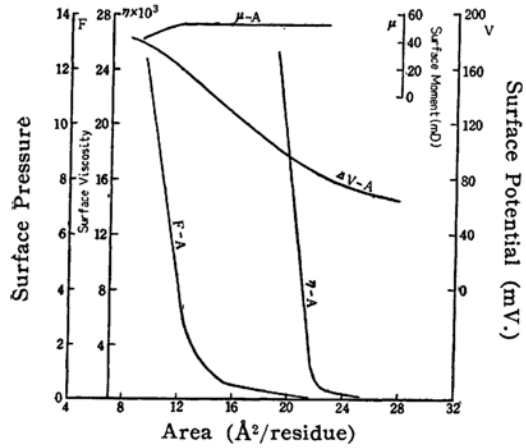

Fig. 9. F-A, η-A, ΔV-A and μ-A curves of lysozyme monolayer spread from 8 M urea solution. Substrate, 10^{-2} M potassium carbonate (pH 10.5), 19°.

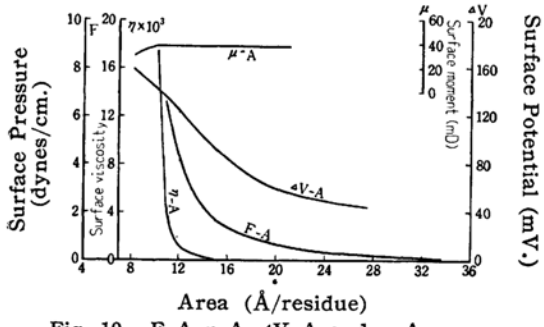

Fig. 10. F-A, η-A, ΔV-A and μ-A curves of lysozyme monolayer spread from 8 M urea solution. Substrate, 10^{-2} M K_2CO_3 $+10^{-2}$ M Na_2SO_3.

Fig. 9 shows the surface pressure-area, surface viscosity-area (η-A), surface potential-area (ΔV-A) and surface moment-area (μ-A) curves of lysozyme monolayers spread from 8 M urea[4].

In Fig. 10 are shown the effect of sodium sulfite in the substrate on the properties of the monolayer spread from 8 M urea[4]. From the comparison of the results shown in Figs. 9 and 10, it was found that the F-A, η-A and ΔV-A curves are greatly changed by the presence of sulfite in the substrate. The changes are explained by the increase in ionizable groups due to splitting of disulfide bonds.

Summary

In order to clarify the correlation between the denaturation of lysozyme on surface and that in solutions, the surface-, heat-, and urea-denaturation which were so far reported by the author were summarized.

The properties of lysozyme monolayers depend not only on the temperature of substrate but also on the state of lysozyme molecules in spreading solutions. The mechanism of the formation of the protein monolayer was clarified by the examination of the denaturation in solutions. Because of the very high content of disulfide bonds in lysozyme molecules, the role played by these bridges in the denaturation on surface and in solutions was also examined and its significance was made clear.

The author expresses his hearty thanks to Professor T. Isemura for his kind guidance throughout the present work.

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