

THE DENATURATION OF PROTEIN UNDER HIGH PRESSURE

I. The Reversal from the Pressure Denaturation of
Ovalbumin and Horse Serum Albumin

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From the measurements of the turbidities of ovalbumin and horse serum albumin (HSA) under high pressure by means of the high pressure apparatus with optical windows, the pressure denaturations and the reversals to their native states by the release of pressure were investigated. Ovalbumin solutions were turbid more or less, though the degrees depended upon the pH and the salt content, and the turbidity did not reverse at all from the denatured states to the native ones in the case of the release of pressure. And also, the ovalbumin compressed at the pressures above about 4,000 kg/cm² precipitated at the isoelectric point. On the other hand, the turbidity and the solubility in water of HSA depended very sensitively on the salt content. The pH 6.0 and salt-free HSA solution became more easily turbid than in the isoelectric and salt-free solution by compression, and the reversal to the native state by the release of pressure was found, but not complete. The pH 4.8 and 6.8 salt-free HSA solutions did not become turbid under high pressure. The pH 4.8 and 6.8 buffer containing HSA solution became considerably turbid under high pressure, and then completely and rapidly reversed by releasing the pressure. The HSA solution which perfectly reversed as to turbidity was equivalent to the native solution in the solubility in water at the isoelectric point, the optical rotation, the ultraviolet absorption spectra and the reactivity of sulfhydryl groups, but different in the solubility in the 10% solution of sodium sulfate and the susceptibility to proteolysis. Therefore, HSA reverses partially from the pressure denaturation by the release of pressure as well as the urea denaturation by the dialysis.

Introduction

The denaturation reactions of protein solutions have been studied by many workers since many years ago, for instance, heat denaturation, alkali or acid denaturation, urea denaturation, and so on. And also it is well known that proteins denature due to compression at pressures in the range of about 3,000 to 10,000 kg/cm² and at lower temperatures where the heat denaturation does not occur¹⁾. We call it the pressure denaturation of proteins. Compared with the extensive data that have been accumulated with respect to the heat denaturation, there are a few studies on the pressure denaturation. On the other hand, the retardations of the heat denaturations of human serum albumin, serum globulin and tobacco mosaic virus under the pressures below 1,000 kg/cm² were investigated kinetically¹⁾. Thus, it is interesting to mention that the effect of the pressure below 1,000 kg/cm² on the denaturation is contrary to that of the pressure above 3,000 kg/cm².

(Received January 15, 1964)

1) F. H. Johnson, H. Eyring and M. J. Polissar, "The Kinetic Basis of Molecular Biology" John Wiley & Sons, Inc., New York, Chapter 9, p. 286 (1954)

The studies of the pressure denaturation for the investigation of the protein structure have advantages as well as disadvantages. One of the advantages is that the system is very simple in the pressure denaturation, since it is composed of only protein and water molecules and does not contain any denaturing reagent such as urea. Moreover, the study of the pressure denaturation is analogous to the study of the structural alteration of protein molecules by exchange of the solvents, since high pressure alters the water structure and then results in the change of the solvation surrounding the ionizing groups. (The high pressure promotes the ionization of protein molecules.) However, the pressure denaturation is excellent in the point of the view that it includes only the changes of physical properties of the solvent without any changes of chemical properties of the solvent. On the contrary, the measurements of the various physico-chemical properties under high pressure are difficult in the technique because of the distortion of the optical window and cell, and trouble-some from the need of the measurement of compressibility. We can use two kinds of apparatus, one of which is only able to compress the solution, and the other is the apparatus with optical windows which is able to measure the turbidity under high pressure.

The denaturation reaction of proteins is one of the complex classes of reactions and therefore, at the present time there is no general agreement of the word "denaturation". Formerly, some workers defined denaturation in terms of a change of the solubility in water at the isoelectric point, but the definition faced serious disadvantage, for instance, serum albumin is unfolded both in salt-free acid and in strong urea solutions, but when the acid or urea is removed the protein returns to the form in which the protein is soluble at the isoelectric point²⁾³⁾. If the denaturations were studied merely in terms of solubility in water at the isoelectric point, the changes undergone by serum albumin in urea and in acid would be overlooked. On the other hand, ovalbumin is well known as one of the proteins which are difficult to return to the soluble form at the isoelectric point.

This paper will report that in the investigation of the pressure denaturation of serum albumin it is not proper to measure only the changes of the following properties after releasing the pressure: the optical rotation, the ultraviolet absorption spectra, the reactivity of the sulfhydryl groups and the solubility in water at the isoelectric point, and also that the pressure denaturation and the reversibility of horse serum albumin can not be discussed without specifying the salt concentration in the solution.

Experimentals

Materials Ovalbumin and horse serum albumin (abbreviation, HSA) were prepared by the method of Sørensen and Høyrup⁴⁾ from hen's egg white and horse blood, respectively. Their solution were dialyzed until salt were removed. Especially, the solution of HSA was carefully treated, since the pressure denaturation and the reversal sensitively depended upon the salt concentration presenting in the solution.

2) J. Roche and M. S. Chonaiech, *Soc. Biol.*, **133**, 171 (1940)

3) H. Neurath, G. R. Cooper and J. O. Erickson, *J. Biol. Chem.*, **142**, 249 (1942)

4) S. P. Sørensen and M. Høyrup, *Compt. rend. trav. lab. Carlsberg*, **12**, 12 (1915)

High pressure apparatus and procedures The schematic layout of the high pressure apparatus I is shown in Fig. 1. This apparatus was used to compress samples at pressures up to 10,000 kg/cm², and the properties of the compressed samples were measured after releasing pressures. A sample solution charged in a polyvinylchloride sack was placed in the high pressure chamber A, which was then filled up with pressure transmitting medium (water). The pressure vessel was held in the oil-press B, and the sample was hydrostatically compressed. The pressure in chamber A was estimated from the product of the pressure gauge C reading by the ratio of the cross-sectional area of piston J₂ to that of J₁. The temperature was kept constant by circulating water from the thermostat through the jacket I surrounding the vessel, but owing to large heat capacity and conductance of the apparatus, the temperature in the vessel was often found to be appreciably different from the thermostat. So the temperature in the vessel was measured directly by the thermister K in a small side-chamber of the vessel.

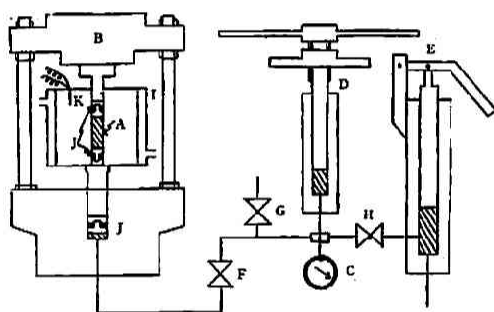


Fig. 1 Schematic layout of high pressure apparatus I

- A: high pressure chamber
(Ni-Cr-2, HRC 40-42)
- B: oil-press
- C: Bourdon type pressure gauge
- D: injection pump
- E: hand pump
- F, G, and H: high pressure valves
- I: water jacket
- J: piston of Bridgman type
(Ni-Cr-2, HRC 42-45)
- K: thermister

Fig. 2 shows the high pressure apparatus II with optical windows constructed after Fishman and Drickamer⁵⁾. This apparatus was used for the direct measurement of the turbidity of protein solution under high pressure. The synthetic sapphire windows F (12 mm × 10 mm) are cemented to the plugs D₁ with epoxy resin. The pressure in the pressure chamber H is estimated from the product of the reading of the pressure gauge by the ratio of the piston B₁ and B₂ areas. A sample solution charged in an injection type test-tube was placed in the chamber H, which was then filled up with water, and hydrostatically compressed by sending the transmitting medium (machine oil) from the injection pump connected at J. The light of a tungsten lamp strikes a photoelectric cell after passing through a glass filter and the protein solution. The light intensity was measured by an ammeter (a full scale, 50 μA). The decrease of the light intensity is in proportion to the turbidity. The pressure was increased in steps to about 6,000 kg/cm², and then decreased in steps down to the atmospheric pressure. The light intensity was measured at each step.

Analysis of other properties of protein solution

(1) Solubility test in water: After the solution was compressed with the high pressure apparatus I, the pH of the sample solution was brought to the isoelectric point, 4.8 with acetate buffer (final conc.,

5) E. Fishman and H. G. Drickamer, *Anal. Chem.*, **28**, 804 (1956)

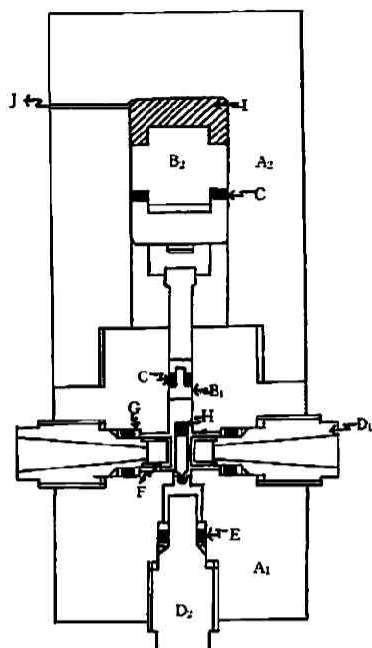


Fig. 2 Schematic layout of high pressure apparatus II

- A₁: cylinder of high pressure part
(AISI-4340, H_{RC} 48-50)
- A₂: cylinder of low-pressure part
(mild steel)
- B₁: piston of Bridgman type in high
pressure part (SKD-3, H_{RC} 58-60)
- B₂: piston of Bridgman type in low-
pressure part (mild steel)
- C: rubber packing
- D₁: plug with optical window
(SKD-3, H_{RC} 50)
- D₂: plug (SKD-3, H_{RC} 50)
- E: packing composed of copper, lead,
and steel
- F: optical window
(12mm ϕ $\times$ 10mm, sapphire)
- G: cap to support optical window
(18-8, Ni-Cr)
- H: glass tube to charge sample
solution
- I: pressure transmitting medium
(machine oil)
- J: to oil-injection pump

0.1M). And after filtration, the amount of the remaining protein molecules in the filtrate was colorimetrically measured at wave length of 520m μ by the Biuret reaction⁶⁾.

(2) Solubility test in sodium sulfate solution: After compression, sodium sulfate (final conc., 10%) and pH 4.8 acetate buffer (final conc., 0.1M) was added to the compressed solution, and then the mixture was kept for 30 min. in a thermostat at 40°C. After filtration, the amount of the remaining protein molecules in filtrate was measured by the same method as the above solubility test in water.

(3) Measurement of reactive sulfhydryl groups: Reactive sulfhydryl groups of protein were oxidized by potassium ferricyanide (abbreviation, PFC) at pH 6.8, where the oxidation reaction was specific, and found by measuring the absorbance of PFC⁶⁾. The pH of the sample solution was adjusted with phosphate buffer (final conc., 0.1M). PFC was added in excess of the amount needed to react with all of the sulfhydryl groups (final conc., 1/1,000 to 1/2,000M). The compression was done with the high pressure apparatus I. Two cases A and B were examined. PFC was added to a compressed protein solution in case A, and a mixture of protein solution and PFC was compressed in case B. The diminished value of absorbance at 420m μ , where the absorption maximum of PFC is located but the absorption of potassium ferrocyanide is negligible, is in proportion to the amount of oxidized sulfhydryl groups. The linear relationship was ascertained in the oxidation of sulfhydryl groups in cysteine molecules by PFC.

(4) Measurement of optical rotation: The optical rotation was measured at room temperature and atmospheric pressure with the polarimeter attached to a Hitachi photoelectric spectrophotometer.

6) S. Nagaoka, T. Kaminaga and S. Araya, *J. Biochem.*, **41**, 37 (1954)

(5) Measurement of susceptibility to proteolysis: The crystalline bacterial alkaline proteinase extracted from *Bacillus subtilis* was used as proteolytic enzyme⁷⁾. Two volumes of the proteinase solution (enzyme conc., 0.05%) adjusted with pH 7.4 phosphate buffer (buffer conc., 0.1M) were mixed with one volume of the sample solution (protein conc., 0.6%). The digestion was carried out for 10min. at 30°C and stopped by mixing two volumes of *M* trichloroacetic acid. After an hour, the resulting suspension was filtered off, and the concentration of the trichloroacetic acid soluble products of the digestion in the filtrate was measured by Folin's method⁸⁾.

(6) Measurement of difference spectra of ultraviolet absorption: The sample solution was diluted to a proper concentration and the difference spectra of the ultraviolet absorption were measured in reference to the native solution. The measurement was carried out with a Hitachi photoelectric spectrophotometer (type EPU 2A).

Results

Solubility test after releasing pressure As shown in Fig. 3, when the pH 4.8 and 6.8 ovalbumin solutions adjusted with buffer solution, acid or alkaline solution were compressed at the pressure above about 4,000kg/cm², the pH being brought to 4.8, the ovalbumin solutions were denatured and precipitated. Moreover, if salt-free ovalbumin solution adjusted at any pH with alkali or acid was denatured by compression, the pH being brought to 4.8, ovalbumin precipitated. The amount of precipitate was

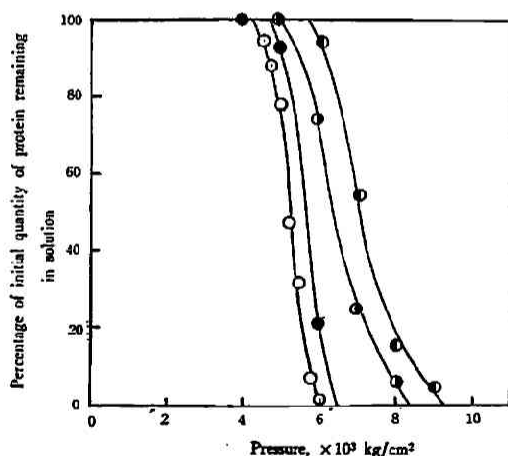


Fig. 3 Solubility test of ovalbumin solutions in water at the isoelectric point
1% ovalbumin solutions were compressed for 10min. at 30°C at various pressures, and after the protein molecules precipitated by bringing the pH to 4.8 were filtered off, the protein concentration in the filtrate was colorimetrically measured by Bieuret reaction.
—○—: pH 4.8 solution containing 0.1M acetate buffer solution
—●—: pH 4.8 salt-free solution adjusted with 0.1N NaOH
—○—: pH 6.8 solution containing 0.2M phosphate buffer solution
—●—: pH 6.8 salt-free solution adjusted with 0.1N sodium hydroxide

much dependent on the pH of the solution at the compression as illustrated in Fig. 4. Thus, the pressure-treated ovalbumin precipitated at the isoelectric point without serious dependence on the salt concentration. These results were compared with those of HSA solution. That is, when the isoelectric HSA adjusted with acetate buffer solution (buffer conc., 0.1M) was compressed for 60 min. at 10,000

7) Purchased from Nagase Sangyo Comp. Ltd., Japan.

8) "Koso Kenkyuho" Edited by Akabori, Asakura-Shoten, Tokyo, Vol. 1, p. 165 (1955)

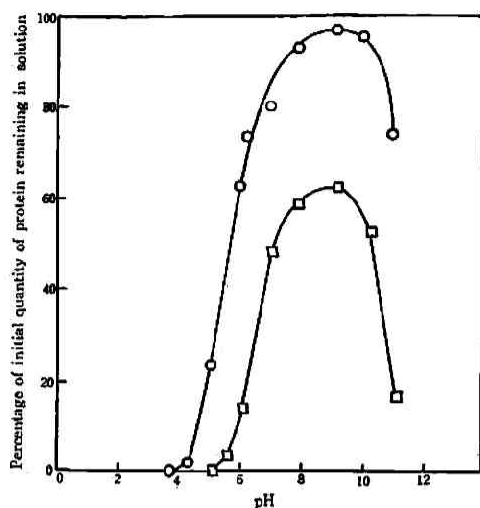


Fig. 4 Effect of pH on pressure denaturation. Samples were compressed for 5 minutes at 6,500 kg/cm²: -○- and 7,500 kg/cm²: -□- and 30°C.

kg/cm², the solution was very slightly opaque after releasing the pressure but did not precipitate at all. Fig. 5 shows the effects of the salt concentration on the solubility of the compressed HSA in water. The compression of the pH 6.0 and salt-free HSA solution gave rise to the precipitate without bringing the pH to the isoelectric point and the precipitate did not resolve, leaving alone at atmospheric pressure. The amount of the precipitate was obtained much more by the compression of the pH 6.0 HSA solution containing only protein molecules and water than by the compression of the pH 4.8 or 6.8 HSA solutions adjusted with hydrochloric acid or sodium hydroxide. When the buffer concentrations in the pH 4.8 and 6.8 HSA solutions were above 0.01M, the compressed HSA did not precipitate at all by bringing the pH to the isoelectric point. And also, when the pH 6.0 HSA solution in the high concentration of sodium chloride above 0.01M was compressed, the compressed HSA did not precipitate at the isoelectric point.

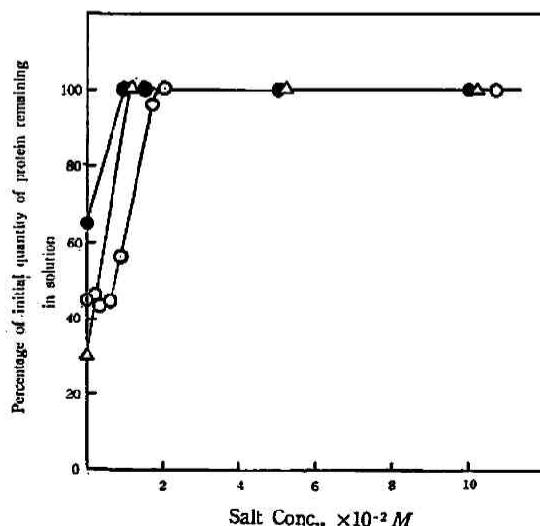


Fig. 5 The effect of salt concentration on the solubility of compressed HSA solution in water at the isoelectric point

- : pH 6.0 solutions (protein conc. 1.0%) containing NaCl at various concentration. They were compressed for 20 min. at 30°C and at 7,000 kg/cm².
- : pH 4.8 solutions (protein conc. 2.0%) containing pH 4.8 acetate buffer at various concentrations. They were compressed for an hour at 30°C and at 10,000 kg/cm².
- △-: pH 6.8 solutions (protein conc. 2.0%) containing pH 6.8 phosphate buffer at various concentrations. They were compressed under same conditions as to the compression of pH 4.8 solutions.

By the way, when the isoelectric HSA solution in the presence of 0.1M acetate buffer was immersed in boiling water for 10 min. all protein molecules precipitated, and the resolution did not take place even after cooling. On the other hand, when the HSA solution adjusted to pH 8.0 with 0.1N sodium hydroxide was heated under the same condition as above, and the pH was brought to the isoelectric point instantly after heating, all protein molecules precipitated soon. But when the pH was brought to the isoelectric point after cooling, protein molecules did not soon precipitate, though the heated solution became turbid with the lapse of time and all the protein molecules precipitated in one day. However, if the sodium sulfate (final conc., 10%) besides the pH 4.8 acetate buffer was added to the heated HSA and the mixture was kept at 40°C, the heated HSA completely precipitated within about 20min., even if the pH 4.8 acetate buffer and sodium sulfate were added to the solution after cooling.

And then, the pressure effect on the precipitations of the isoelectric ovalbumin and HSA solutions by heating were observed. The results are shown in Fig. 6. The precipitation of the isoelectric HSA caused by heating was depressed by the compression at pressures up to 8,000 kg/cm² and the effect was larger with increasing pressure. However, being different from HSA, the depression effect of pressure on the precipitation of ovalbumin by heating was maximum at 3,000 kg/cm², and canceled with the effect of the pressure denaturation at pressures above 5,000 kg/cm² (The pressure denaturation of ovalbumin solution takes place at pressures above about 4,000 kg/cm²). Thus, HSA precipitates by heating and not by compression in the presence of salt. Why does HSA not precipitate in the presence of salt by compression unlike by heating? In order to solve this question, the following experiments were performed.

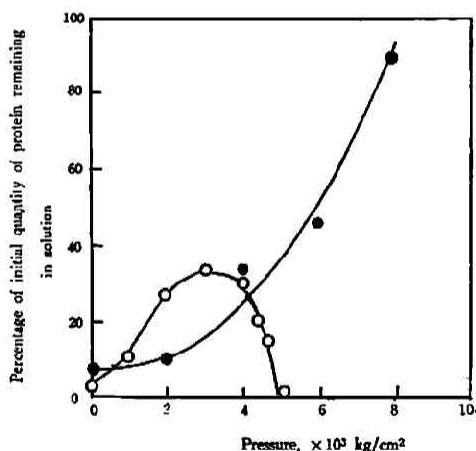


Fig. 6 Pressure effects on the precipitations of isoelectric HSA and ovalbumin solutions by heating

- : pH 4.8 ovalbumin solutions (protein conc. 0.5%) were compressed for 20 min. at 70°C and at various pressures.
- : pH 4.8 HSA solutions (protein conc. 0.7%) were compressed for 10 min. at 30°C and at various pressures.

Oxidation of reactive sulfhydryl groups In order to see whether the denaturation of HSA takes place under high pressure or not, the oxidation of the reactive sulfhydryl groups of protein molecules by PFC was first examined. The denaturation should have taken place under high pressure, if some amount of PFC was consumed in compression of a mixture of protein and PFC, and even if the precipitation did not take place after releasing the pressure. For the sulfhydryl groups of ovalbumin

and serum albumin are reactive only after denaturation⁹⁾. Table 1 shows the results of the oxidation of the reactive sulfhydryl groups of HSA in two cases A and B. Sulfhydryl groups of HSA were not oxidized by PFC after releasing the pressure, but they were oxidized under high pressure and remained in the oxidized state after releasing pressure. It is seen that the pressure denaturation surely occurs under high pressure, but it reverses to the native state by the release of the pressure. In both cases of A and B, the compressed HSA solution were completely transparent. On the other hand, the sulfhydryl groups of ovalbumin could be oxidized with PFC in both cases A and B, though the amount of the consumption of PFC in case A was a little less than in case B*.

Table 1 Consumed quantities of PFC by HSA

	absorbance at 420m μ	decrease of absorbance
Native	0.192	—
Pressed in Case A	0.200	-0.008
Pressed in Case B	0.096	0.096

The mixture of HSA and PFC (pH 6.8) was compressed for 10 min. at 25°C and 8,000 kg/cm² in case A, and PFC was added to the pressure-treated HSA compressed for 10 min. at 25°C and 8,000 kg/cm² in case B.

Turbidity measurement under high pressure In the point of view of the reactivity of sulfhydryl groups, it is seen that the pressure denaturation of HSA solution surely occurs under high pressure, but it could not be recognized after the release of pressure, that is, the pressure denaturation of HSA is reversible. Does HSA precipitate in the presence of salt under high pressure, then? And does the precipitate resolve into a solution at the release of pressure? If it should be true, the precipitate once produced under high pressure should resolve into the solution at the instant of releasing the pressure. Such a fact may not be easily believed, because the resolution of the precipitate is assumed to take a considerable time. In order to ascertain the precipitation and the resolution, the turbidity changes of HSA and ovalbumin solution at the isoelectric point were directly measured under high pressure by means of the high pressure apparatus II with optical windows. The changes were followed by the intensities of the light passing through those solutions.

Fig. 7 shows the results on the isoelectric ovalbumin solutions, one of which was adjusted with pH 4.8 acetate buffer (final conc., 0.1M) and the other was adjusted with 0.1N hydrochloric acid. On both solutions, the intensities of the light sharply decreased when those ovalbumin solutions were compressed over 4,000 kg/cm², indicating that the denatured protein aggregated to make a turbid solution. And the ovalbumin solutions remained turbid after releasing the pressure. Therefore, the turbidity change of the isoelectric ovalbumin solution was a little dependent on the salt content of the extent of 0.1M in the solution**. However, the turbidity change of the pH 6.8 ovalbumin solution was considerably

* The exact values can not be reported since the compressed ovalbumin containing the pH 6.8 phosphate buffer solution was slightly opaque.

** However, the precipitation of the isoelectric ovalbumin considerably depends upon the salt concentration over about 0.5M and the kind of salt¹⁰⁾.

9) R. Cecil and J. R. McPhee, "Advances in Protein Chemistry" Academic Press, New York, Vol. 14, 256 (1959)

The Denaturation of Protein under High Pressure

93

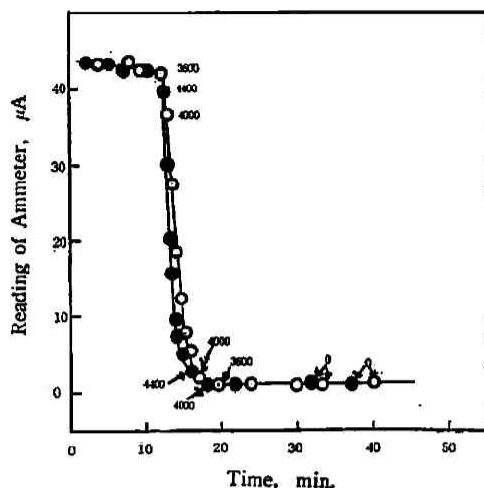


Fig. 7 Changes of light intensities passing through the pH 4.8 ovalbumin solutions (protein conc. 0.5%) on compressions
 —○—: Containing 0.1M acetate buffer solution.
 —●—: Salt-free solution adjusted with 0.1N hydrochloric acid.
 Numerals in figure show pressure magnitude in the unit of kg/cm^2 .

dependent even on the small salt concentration. Fig. 8 shows the results on the pH 6.8 ovalbumin solutions, one of which was adjusted with pH 6.8 phosphate buffer solution (buffer conc., $1/40M$) and the other was adjusted with 0.1N sodium hydroxide. Ovalbumin solution containing buffer solution became considerably turbid at the pressures over $6,000 \text{ kg}/\text{cm}^2$ and remained turbid after releasing the pressure. But in the salt-free solution, the solution became only a little turbid by compression. Thus, ovalbumin solutions were turbid more or less under high pressure depending on the pH and salt concentration of the solution, and the turbidities remained unchanged after the release of the pressure in any case.

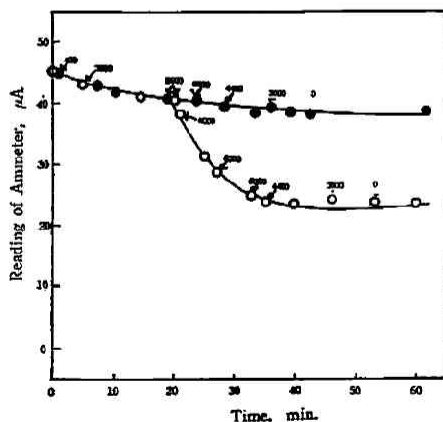


Fig. 8 Changes of light intensities passing through the pH 6.8 ovalbumin solutions (protein conc. 0.5%) on compressions
 —○—: Containing $1/40M$ phosphate buffer solution.
 —●—: Salt-free solution adjusted with 0.1N sodium hydroxide.
 Numerals in figure show pressure magnitude in the unit of kg/cm^2 .

It was described above that the solubility in water of the pressure-treated HSA solution remarkably depended upon the salt content. Fig. 9 shows the turbidity changes of the isoelectric HSA solu-

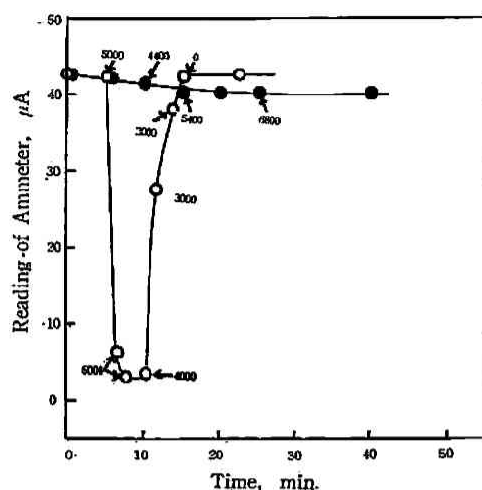


Fig. 9 Changes of light intensities passing through the pH 4.8 HSA solutions (protein conc. 0.6%) on compressions
 -○-: Containing 0.1 *M* acetate buffer solution.
 -●-: Salt-free solution adjusted with 0.1 *N* hydrochloric acid.

tions on compressing, one of which was adjusted with pH 4.8 acetate buffer solution, and the other was adjusted with 0.1 *N* hydrochloric acid. The turbidity of the isoelectric HSA solution in the presence of 0.1 *M* acetate buffer rapidly increased by the compression over 5,000 kg/cm², but it reversed completely by releasing the pressure. On the other hand, the pH 4.8 and salt-free HSA solution was hardly turbid under the high pressure up to 6,800 kg/cm². If crystallized HSA was dissolved in water without any other material and the salt in the solution was dialyzed off thoroughly, the pH of the solution became 6.0. Fig. 10 shows the turbidity change of pH 6.0 HSA solutions on compression, one of which contained 1/20 *M* phosphate buffer solution and the other was salt-free containing only water and protein molecules. The turbidity changes of both HSA solutions increased by compression over a pressure of 3,000 or 4,000 kg/cm², and then partly reversed at the release of the pressure. The salt-free solution was easier to be turbid but more difficult to reverse than the HSA solution containing phosphate buffer solution. Fig. 11 shows the turbidity changes of the pH 6.8 HSA solutions on compression, one of

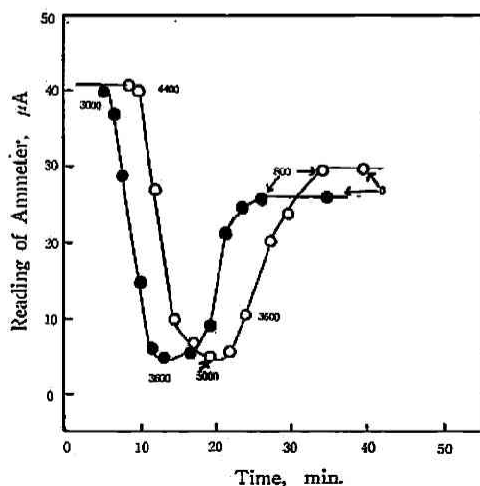


Fig. 10 Changes of light intensities passing through the pH 6.0 HSA solutions (protein conc. 0.6%) on compressions
 -○-: Containing 1/20 *M* phosphate buffer solution.
 -●-: Salt-free solution containing only water and protein molecules.

which was adjusted with pH 6.8 phosphate buffer (buffer conc., $1/20M$) and the other was adjusted with $0.1N$ sodium hydroxide. The turbidity of the HSA solution containing phosphate buffer solution rapidly increased by the compression over $5,000 \text{ kg/cm}^2$ and completely reversed at the release of the pressure as well as the pH 4.8 HSA solution containing $0.1M$ acetate buffer solution. Whereas, the salt-free solution at pH 6.8 did not become turbid under the pressure of $6,000 \text{ kg/cm}^2$.

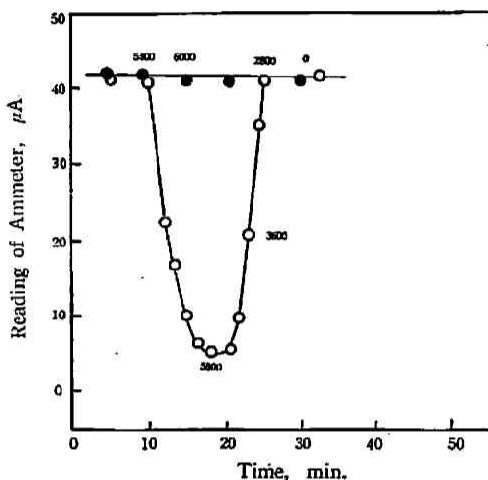


Fig. 11 Changes of light intensities passing through the pH 6.8 HSA solutions (protein conc. 0.5%) on compressions
 -○-: Containing $1/20M$ phosphate buffer solution.
 -●-: Salt-free solution adjusted with $0.1N$ sodium hydroxide.

Other properties of HSA solution reversed in turbidity The HSA solutions containing $0.1M$, pH 4.8 acetate buffer solution and $1/20M$, pH 6.8 phosphate buffer solution, respectively, were compressed for 10 min. at $8,000 \text{ kg/cm}^2$ and 30°C . After the compression, these solutions were transparent and did not precipitate in water at the isoelectric point. Some other properties of the compressed HSA solution which completely reversed to the native state in the view-point of the turbidity were examined. The optical rotations $[\alpha]_D$ of the pH 4.8 and 6.8 compressed solutions were respectively -61 and -62 , and they did not differ from those of the native HSA solutions. And also there was no difference between the ultraviolet absorption spectrum of their compressed HSA and the native one. However, the susceptibilities to proteolysis of their compressed HSA solutions were found out to differ from the native protein, and the amount of the digested products was nearly a half of the heated HSA immersed in the boiling water for 10 min. The compressed solution containing salt at the concentration above $0.01M$ did not precipitate at all in water, but they precipitate in the 10% sodium sulfate solution. 1.5% HSA solutions containing pH 6.0 phosphate buffer or pH 4.8 acetate buffer solution at various concentrations were compressed for 60 min. at $8,000 \text{ kg/cm}^2$ and 30°C , and then the amount of the precipitation in the 10% sodium sulfate solution was measured. Those results are shown in Fig. 12. It was seen that the precipitation depended upon the buffer concentration to decrease with the increase of the buffer concentration, though the effect was not so much in pH 4.8. Thus, the compressed HSA solution containing the amount of salt which did not give rise to any precipitate in water at the isoelectric point, precipitated in the 10% sodium sulfate solution.

On the other hand, if the pH 7.0 ovalbumin solution adjusted with $0.1N$ sodium hydroxide was

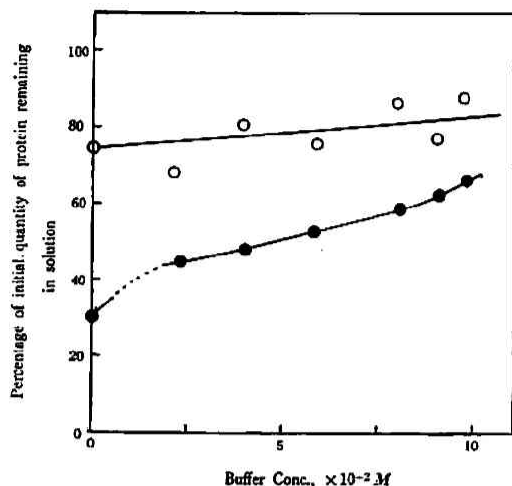


Fig. 12 The amounts of precipitation of HSA compressed at various buffer concentrations in sodium sulfate

—●—: Solutions containing pH 6.0 phosphate buffer solution.

—○—: Solutions containing pH 4.8 acetate buffer solution.

Each 1.5% HSA solution was compressed for 60 min. at $8,000 \text{ kg/cm}^2$ and 30°C .

compressed for 10 min. at $8,000 \text{ kg/cm}^2$ and 30°C , the compressed solution was transparent, but nearly all the molecules precipitated by bringing the pH to the isoelectric point. Its susceptibility to the proteolysis differed from the native one but the same to the heat-treated ovalbumin solution (boiled for 10 min.). However, the optical rotation and the ultraviolet absorption spectra of the above compressed ovalbumin solution differed from the native and the heat-treated ovalbumin solution. The optical rotations, $[\alpha]_D$ of the native, the compressed and the heated solutions were -30 , -50 , and -64 , respectively. The difference spectra of ultraviolet absorption of the pressure-treated ovalbumin and HSA solution in reference of their native protein solutions are shown in Fig. 13.

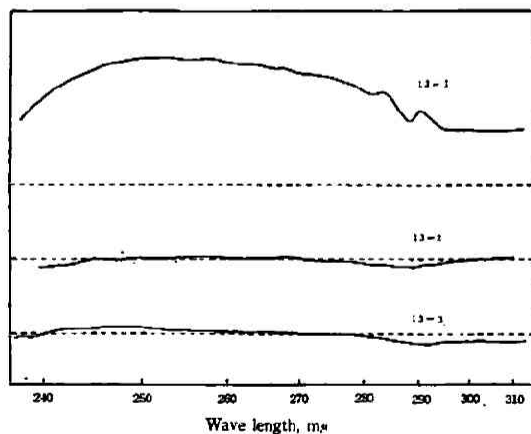


Fig. 13 Difference spectra of ultraviolet absorption of the compressed ovalbumin and HSA solutions in reference to their native solutions

13-1: pH 7.0 salt-free ovalbumin solution.

13-2: pH 6.8 HSA solution containing $1/20M$ phosphate buffer solution.

13-3: pH 4.8 HSA solution containing $1/10M$ acetate buffer solution.

Discussion

The precipitation of the pressure-treated ovalbumin at the isoelectric point and the turbidity under high pressure of ovalbumin solution depend a little, though not seriously, on the presence of salt. And

also, the turbidity of ovalbumin solution under high pressure does not reverse by the release of pressure, in any case of buffer solution and salt-free solution. The pH 7.0 and salt-free ovalbumin solution was transparent even after the compression for 10 min. at $8,000 \text{ kg/cm}^2$ and 30°C , but the compressed solution was different from the native one in other properties: the solubility in water at the isoelectric point, the optical rotation, the ultraviolet absorption spectra, the reactivity of sulfhydryl groups, and the susceptibility to proteolysis. Therefore, the pressure denaturation of ovalbumin solution can be fairly investigated by the measurements of those properties after the release of pressure*. On the other hand, the compressed HSA at the pH 6.0 and salt-free solution precipitates in the largest quantity. And also, such a solution becomes easily turbid under high pressure, and the turbidity does not reverse completely. Such a pH 6.0 and salt-free HSA solution is at the isoelectric point where the intermolecular repulsion force are smallest, so that the aggregation of molecules takes place easily. And the presence of salt results in the decrease of the turbidity, since the negative ions of salt combine with the positive charges of protein molecules¹¹⁾, and the intermolecular repulsion force increases with the net charge of the protein molecules. In view of the importance of salt content, intermolecular salt linkages are assumed to play an important role in the aggregation of HSA molecules in the pH 6.0 solution under high pressure. Whereas, the compressed HSA molecules in the pH 4.8 and 6.8 salt-free solutions are easy to precipitate at the isoelectric point after the compression in comparison with those of the same pH and buffer-containing HSA solutions and the latter do not precipitate at all if the buffer concentrations are above $0.01M$. The pH 4.8 and 6.8 HSA solutions containing $0.1M$ buffer solutions are easier to become turbid than those of the same pH and salt-free HSA solution under high pressure, their turbidities reverse completely at the release of pressure. It is assumed that salt promotes the decrease of the solubility of HSA under high pressure, and also proceeds the reversal of them by the release of pressure. In the completely reversed HSA solution in the view-point of turbidity, the optical rotation, the ultraviolet absorption spectra, the reactivity of sulfhydryl groups, and the solubility in water at the isoelectric point do not differ from those of the native protein. But the susceptibility to proteolysis and the solubility in 10% solution of sodium sulfate differ from those of the native protein. Barbu et al.¹²⁾ showed from the measurement of the streaming birefringence that the high pressure gave rise to quasispherical aggregates in the solution with a diameter of several hundred Å. Therefore, it is seen that the aggregation does not completely reverse to the native state by the release of pressure, though the solubility in water or the turbidity reverses. Thus, some of the properties of HSA can reverse completely by the release of pressure as well as from the urea denaturation of HSA by dialysis³⁾¹³⁾.

The depression effect of the pressures up to 8000 kg/cm^2 on the precipitation of the pH 4.8 HSA solution containing acetate buffer solution by heating suggests the difference between the aggregation mechanism of the pressure-denatured HSA and of the heat-denatured HSA molecules. This is supported by the following views: (i) Heat-denatured HSA is easier to precipitate than the pressure-

* The properties except the turbidity have not been measured directly under high pressure. Therefore, it is not known whether those properties reverse partially by the release of pressure, or not.

11) G. Scatchard and E. S. Black, *J. Phys. Colloid Chem.*, **53**, 88 (1949)

12) E. Barbu and M. Joly, *Disc. Faraday Soc.*, **13**, 1477 (1953)

13) J. O. Erickson and H. Neurath, *J. Exp. Med.*, **78**, 1 (1943)

treated HSA, and the precipitate once produced by heating is not resolved only by cooling*. (ii) Barbu et al. showed that the heating of HSA solution gave rise to filaments, the length of which could attain several thousand Å by the end-to-end aggregation, while high pressure caused globular-aggregates. (iii) Joly¹⁴⁾ showed that the compression of the pH near 6.0 HSA solution gave rise to the largest quantity of the precipitate in the 10% solution of sodium sulfate, but the compression of pH 4 to 4.5 solutions gave rise to no precipitate. On the other hand, the heating of the pH about 4.5 HSA solution caused the largest quantity of the precipitate in the sodium sulfate solution. Thus, it may be concluded that the pressure denaturation and the heat denaturation of HSA solution differ from each other in the aggregation.

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* The heat-coagulated serum albumin resolves first when immersed in 0.1*N* alkali. Dialysis of this product gives a water soluble protein which is also soluble in salt solution and shows the same conductivity, optical rotation, heat behavior and immunochemical properties as the native protein¹⁵⁾.

14) M. Joly, *Prog. Biophys. Biophysical Chem.*, **5**, 168 (1955)

15) M. Spiegel-Adolf, *Biochem. Z.*, **170**, 126 (1926)