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THE DENATURATION OF PROTEIN UNDER HIGH PRESSURE

II. The Gelations of Ovalbumin Solution by Pressure and by Heat

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The gelations of aqueous solution of ovalbumin by heating and by compression have been investigated and compared with the gelation of urea solution of ovalbumin, which had been studied by Huggins *et al.*[1] and Frensdorff *et al.*[2] The following results are obtained in both the gelation by heating and that by compression: the optimum pH of gelation is around the isoelectric point, and the lowest protein concentration for gelation rapidly increases with progressing upward or downward from a limited pH range (pH 4–7). The gels near the isoelectric point are highly opaque and synerizing, and easily dissolve when immersed in 0.1*N* sodium hydroxide. On the other hand, the gels at pH far from the isoelectric point are less opaque and non-synerizing, and do not easily dissolve when immersed in 0.1*N* sodium hydroxide. This tendency is more remarkable in thermal gel than in pressure gel. These results indicate that the coarse network by the secondary bonds holds the gel formed from the solution near the isoelectric point, and the fine network by chemical bonds holds the gel formed from the solution near neutral pH. The formation of the inhomogeneous and coarse network by the secondary bonds is most favorable in the pressure gelation, and next in the thermal gelation, but it is not favorable in the gelation of urea solution of ovalbumin.

Introduction

When a globular protein solution is denatured under highly specified conditions of protein concentration, pH and salt content, the globular protein solution forms a gel. The gelation of gelatin has been studied by many workers, but there have been only a few studies on the globular protein solution. Huggins *et al.*[1] and Frensdorff *et al.*[2] found that in the gelation of urea solution of ovalbumin, the presence of the sulfhydryl and disulfid reagents retarded the gelation, and pieces of the stiff gels failed to dissolve when immersed in dilute alkali or sodium sulfite, and they did not melt even when heated to 100°C. Weaker gels, however, melted on heating and dissolved with dilute alkali. Therefore, they concluded that in the gelation of urea solution of ovalbumin, the intermolecular disulfid bonds are formed by the exchange reaction,

$$\text{SS (intramolecular)} + \text{SH} = \text{SH} + \text{SS (intermolecular)}$$

and they play an important role in the formation of the network holding the strong gel together. However, the network of the weaker gel is also held by the secondary bonds. The gelation of ovalbumin solution by heating had been studied by Jensen *et al.*[3], but the results are not described ex-

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1) C. Huggins and E. V. Jensen, *Science*, 113, 477 (1951)
2) H. K. Frensdorff, M. T. Watson and W. Kauzmann, *J. Am. Chem. Soc.*, 76, 5157 (1953)
3) E. V. Jensen, V. D. Hospelhorn, D. F. Tapley and C. Huggins, *J. Biol. Chem.*, 185, 411 (1950)

plicitly. The gelation of ovalbumin solution by compressing had been reported by the author and co-worker[4]. This communication will report the results of the experiments of the gelation of ovalbumin solution by heating and by compressing, and discuss them in comparison with the gelation of urea solution of ovalbumin.

Experimentals

Materials Ovalbumin was prepared from hen's egg white by the method of Sørensen and Høyrup[5] and recystallized three times. The solution was used after dialysis with tap water. Salts and other reagents in special grade were used without further purification. If the pH was above 5.6, phosphate buffer solution was employed. If the pH was lower than 5.6, acetate buffer solution was employed.

Methods In the thermal gelation, a sample solution of 2.0 m*l* charged in a test tube (dims., 10mm × 100mm) with a plug, was immersed for 15 minutes in boiling water, and was cooled with tap water. In the pressure gelation, a sample solution of 1.5 m*l* was charged in a polyvinylchloride tube (dims., 9mm × 30mm) with rubber plugs at both ends and compressed hydrostatically for 5 minutes at 8,000 kg/cm^2 and 20°C. The pressure apparatus has been described in the previous paper[6]. In both gelations, gelation was considered to have taken place when the solution failed to flow on taking off the one plug and inverting gently the tube. The abbreviation LPCG will be used for the lowest concentration of protein capable of forming a gel in each condition. The values of the pH of solutions given in this paper refer to the room temperature and atmospheric pressure. The values of LPCG were reproducible within 0.1% protein concentration.

Results

Effect of pH The effects of pH in the thermal gelation and the pressure gelation were examined in the presence of 0.1 *M* acetate buffer or phosphate buffer. Figs. 1 and 2 show the LPCG or ovalbumin against the pH of system in the thermal gelation and in the pressure gelation, respectively. In either case, gelation can occur only in a limited pH range, that is, on progressing the pH of solution upward from about 7.0 or downward from about 4.0, the LPCG rapidly increases. On the other hand, over the pH range between 4.0 and 7.0, the LPCG is low and nearly constant, though there are some differences in the LPCG at 5.6 where phosphate buffer was substituted for acetate buffer. The absence of gelation at the lowest or highest pH is undoubtedly caused by mutual repulsion between the highly charged molecules. The thermal gels are less opaque, the further from the isoelectric point the pH's of the solutions are, and the lower the protein concentrations are. Photograph 1 illustrates the comparison of the opacities of the thermal gels formed from the solution at each pH indicated and at a

4) C. Suzuki and K. Suzuki, *Arch. Biochem. Biophys.* in press.
5) S. P. Sørensen and M. Høyrup, *Compt. rend. trav. lab. Carlsberg*, **12**, 12 (1915)
6) C. Suzuki, *This Journal*, 33, 85 (1963)

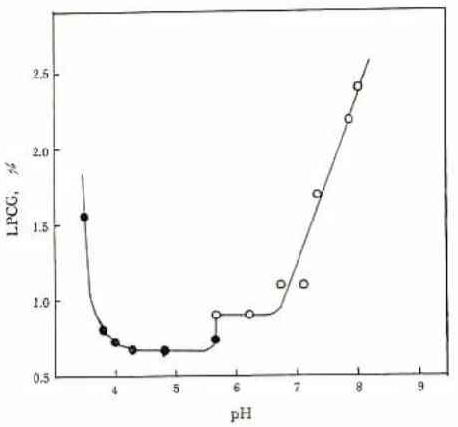


Fig. 1 Effect of pH on LPCG of thermal gelation
The samples were heated for 15 min. at temperature 97 to 99°C. Each sample contains acetate buffer 0.1*M* (●) or phosphate buffer 0.1*M* (○).

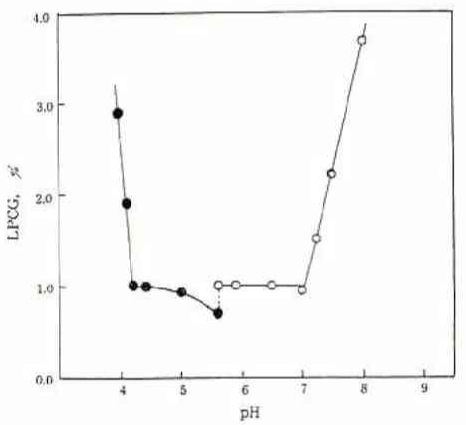


Fig. 2 Effect of pH on LPCG of pressure gelation
The samples were compressed for 5 min. at 8,000 kg/cm² and 20°C. Each sample contains acetate buffer 0.1*M* (●) or phosphate buffer 0.1*M* (○).

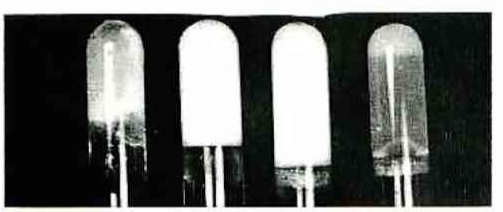
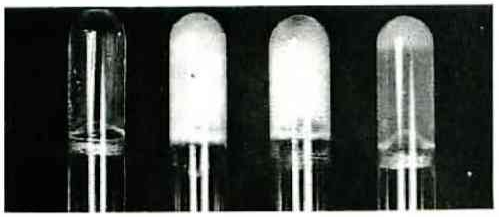


Photo. 1.

	pH	buffer	protein conc.
1-1	4.1	(acetate buf. 0.1*M*)	2.5%
1-2	4.8	(〃)	2.5%
1-3	6.0	(phosphate buf. 0.1*M*)	2.5%
1-4	8.0	(〃)	2.5%

protein concentration. Photograph 2 illustrates the comparison of the opacities of the thermal gels at the LPCG's at various pH's. However, the relation between the opacity of the gel and the pH is more ambiguous in the pressure gels than the thermal gel. Photographs 3, 4 and 5 illustrate the pressure gels formed from the ovalbumin solutions at the concentrations around LPCG at some pH's. All pressure gels are white opaque, but the gel at the isoelectric point is especially dense white and not glossy. When the isoelectric solutions at a little lower concentration than LPCG is compressed, it turns to many films and the solvent as illustrated in Photo. 4-3. On the other hand, the gels at pH 8.0 and 3.9 as illustrated in Photos. 3 and 5 are glossy and elastic.



2-1 2-2 2-3 2-4

Photo. 2.

	pH	buffer	protein conc.
2-1	4.1	(acetate buf. 0.1*M*)	0.8%
2-2	4.8	(〃)	0.7%
2-3	6.0	(phosphate buf. 0.1*M*)	0.9%
2-4	8.0	(〃)	2.5%

3-1

3-2

3-3

Photo. 3 pH 3.9 acetate buffer 0.1*M*
protein conc.: 3-1 3.6%
3-2 3.1% (LPCG)
3-3 2.9%

4-1

4-2

4-3

Photo. 4 pH 4.8 acetate buffer 0.1*M*
protein conc.: 4-1 3.6%
4-2 0.9% (LPCG)
4-3 0.4%

Effect of electrolytes In both gelations, the LPCG's are dependent on the concentrations of the added salts and of the buffer solution. Fig. 3 and Table 1 show the relations between the LPCG and the buffer concentration in the pressure gelation and in the thermal gelation, respectively. In both cases, the LPCG's tend to be less with the increased of buffer concentration at any pH except in the thermal gelation at pH below 3.8, but the effects are little at pH near 5.0. Fig. 4 shows the effects of salts on the LPCG's by heating and by compression at pH 7.0 (0.02*M* phophate buffer), The presence of salts can make the LPCG decrease in both gelations. Moreover, the opacity of thermal gel increases

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5-1 5-2 5-3

Photo. 5 pH 8.0 phosphate buffer 0.1*M*
protein conc.: 5-1 6.2%
5-2 3.6% (LPCG)
5-3 3.3%

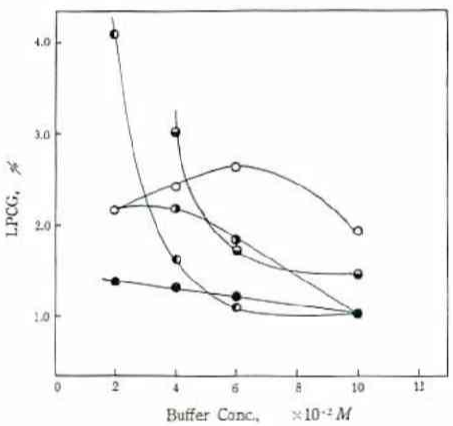


Fig. 3 Effect of buffer concentration on the LPCG of pressure gelation
Samples were compressed for 5 min. at 8,000 kg/cm², 20°C and pHs. pH 4.1 (○), pH 4.3 (◑) and pH 5.0 (●) were obtained with asetate buffer, and pH 7.0 (◐) and pH 7.2 (◒) were obtained with phosphate buffer.

Table 1 Effect of buffer conc. on LPCG in thermal gelation

pH	kind of buffer soln.	buffer conc. (*M*)	LPCG (%)
3.5	acetate buffer	0.10	1.6
3.5	〃	0.05	1.2
3.8	〃	0.10	0.8
3.8	〃	0.10	0.7
4.8	〃	0.10	0.7
4.8	〃	0.05	0.7
6.2	phosphate buffer	0.10	1.4
6.2	〃	0.05	1.4
7.0	〃	0.10	1.1
7.0	〃	0.05	1.3
7.0	〃	0.02	3.0

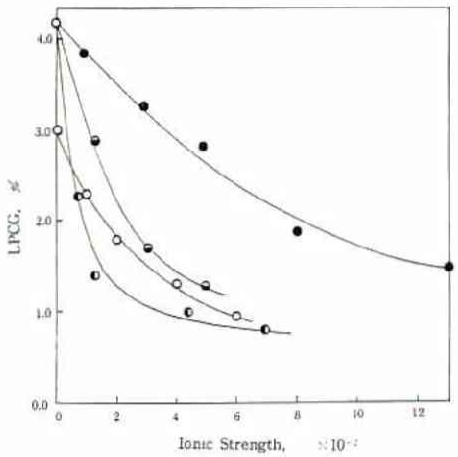
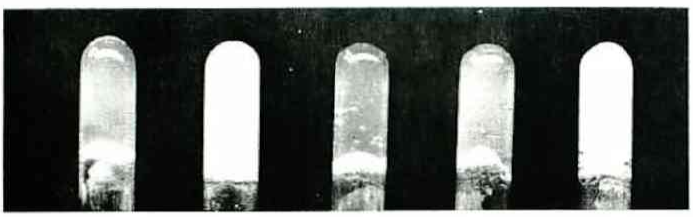


Fig. 4 Effects of salts on LPCG's of thermal gelation and of pressure gelation
The samples were heated for 15 min. at 97–99°C in the presence of NaCl (○), or compressed for 5 min. at 8,000 kg/cm² and 20°C in the presence of NaCl (●), $NaSO_4$ (◒), and $CaCl_2$(◐). The pH was adjusted to 7.0 with phosphate buffer, and the concentration was 0.02*M*.



6-1 6-2 6-3 6-4 6-5

Photo. 6.

	buffer conc.	NaCl conc.	protein conc.
6-1	0.02*M*	0 *M*	3.2%
6-2	0.10*M*	0 *M*	1.3%
6-3	0.02*M*	0.02*M*	2.0%
6-4	0.02*M*	0.06*M*	1.1%
6-5	0.02*M*	0.06*M*	3.2%

by the addition of salts as shown in Photo. 6, but the effects of salts on the opacity of the pressure gel are little.

Effects of various sulfhydryl reagents Figs. 5 and 6 show the influences of various sul-

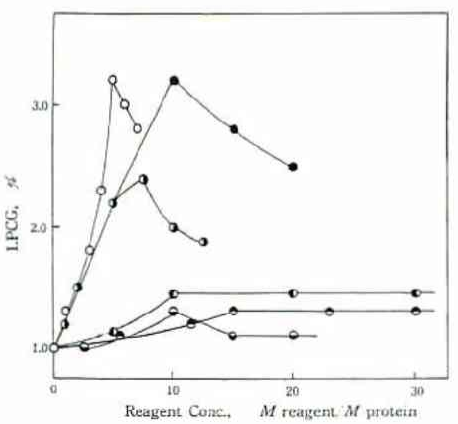


Fig. 5 Effects of sulfhydryl reagents on the LPCG of pressure gelation
The samples were compressed for 5 min. at 8,000 kg/cm² and 20°C, with additions of $AgNO_3$ (○), $HgCl_2$ (◑), PCMB (●), iodoacetate (◐), potassium ferricyanide (◓) and iodoacetamide (◒), respectively. The pH was adjusted to 7.0 with phosphate buffer (0.1*M*).

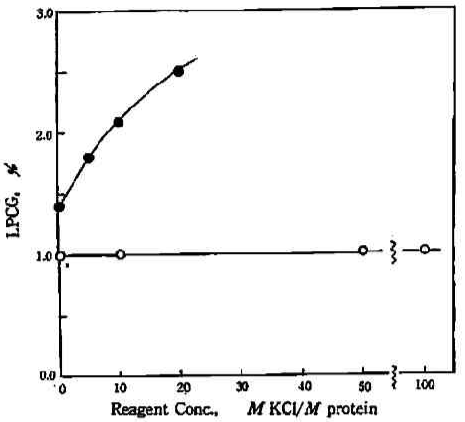


Fig. 6 Effect of KCN of the pressuer gelation
The samples were compressed for 5min. at 8,000 kg/cm² and 20°C. The pH was adjusted to 7.0 (○) and 7.5 (●) with phosphate buffers (0.1*M*).

fhydryl and disulfid reagents on the pressure gelation. The pressure gelation is inhibited by every reagent examined. Especially, the effects of mercuptide forming reagents: silver nitrate, mercuric chloride, and *p*-chloromercuribenzoate are marked. On the other hand, the effects of sulfhydryl and disulfid reagents on the LPCG of the thermal gelation do not show a definite trend as illustrated in Figs. 7 and 8.

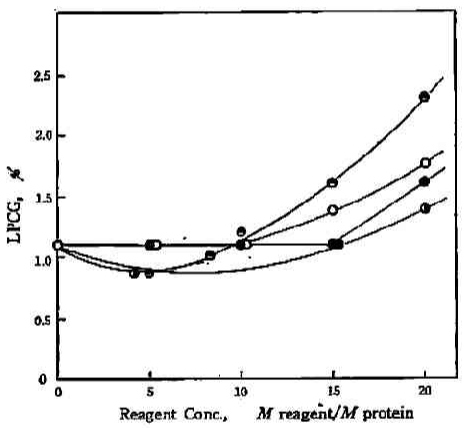


Fig. 7 Effects of alkilating and mercuptide reagents on LPCG of thermal gelation by heating for 15 min. at 97–99°C, in the presence of iodoacetate (◓), iodoacetamide (◉), mercuric chloride (◑), and p-chloromercuribenzoate (○).

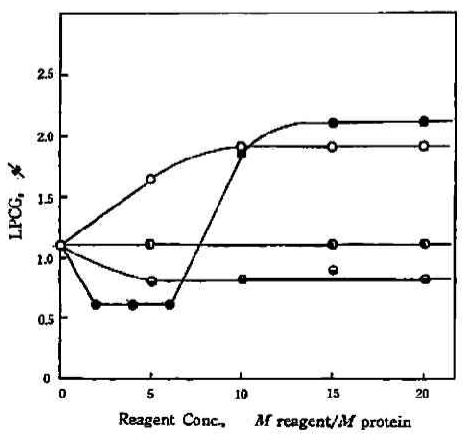


Fig. 8 Effects of oxdizing and reducing reagents on LPCG of thermal gelation
The sample solution were heated for 15 min. at 97-99°C, in presence of potassium ferricyanide (●), potassium iodide (◐), and iodine in potassium iodide (○), and cysteine (◒). The pH was adjusted to 7.0 with phosyhate buffer (0.1*M*).

A definite trend had not been obtained similarly by Jensen *et al.*[3] for the effects of sulfhydryl and disulfid reagents on the thermal gelation of serum albumin solution. These reagents probably combine with the other groups than sulfhydryl and disulfid groups in the protein molecules, since these reagents are present in the protein solution on heating process in the thermal gelation. Therefore, the effects of sulfhydryl and disulfid groups are covered with the effects of the other groups, and each reagent produces the different effect on the gelation from each other according to the kind of the reagent. Therefore, in order to study the roles of the sulfhydryl and disulfid groups on the thermal gelation, the protein solution should be heated after the protein is modified with those reagents under specified conditions for the reaction of their groups and the remainder of the reagents are removed from the solution by dialysis.

The effects of the sulfhydryl and disulfid reagents on the opacity of pressure gels were hardly recognized, except that the addition of ferricyanide makes the pressure gel a little less opaque. And the clear gel can not be obtained by the addition of any reagent. On the other hand, the thermal gel formed from the solution at pH 7.0 containing no reagent is not so opaque. However, if iodoacetate, *p*-chloromercuribenzoate, mercuric chloride, and cysteine are present in the solution, the gels formed by heating are composed of the two parts : the upper part is clear but the lower part is white, and the relative quantities are dependent on the kind and the concentration of the added reagent. The presence of potassium ferricyanide and potassium cyanide causes no change in the opacity of the thermal gel. The presence of iodine with potassium iodine makes the sample solution color before heating according to the amount of iodine (color by I_3^-). but the larger the amount of iodine is, the more transparent and more colorless the gels formed from these solutions by heating become.

Properties of gels In the thermal gelation and the pressure gelation, the gels at a pH usually become more stiff and more opaque with increasing protein concentration, and the gels formed from the solution near the isoelectric point are dense white and cause easily syneresis, but the gels at the LPCG tend to be more stiff and less opaque with progressing up from pH 7.0 and down from pH 4.0. If pieces of opaque gels formed by compressing are immersed in 0.1*N* sodium hydroxide solution, the gels formed from the isoelectric solution completely dissolved in a few minutes, but the greater part of the gel formed from the neutral solution dissolved after 10 min. into a clear solution remaining a small quantity of a clear gel which dissolved in a few hours. On the other hand, if thermal gels were immersed in 0.1*N* sodium hydroxide solution, the thermal gel formed from the isoelectric solution at LPCG completely dissolved after a few hrs., but the gels formed from the pH 7.0 and 3.5 solutions at each LPCG did not easily dissolve into the clear solution remaining some quantity of clear gels after one day. Moreover, the gel formed from the pH 8.0 solution at the LPCG hardly dissolved after a few days, yet. The thermal gels and the pressure gels are more difficult to dissolve in dilute alkaline solution with increases of the protein concentration than the LPCG. Neither the thermal gels nor the pressure gels formed from any pH solution dissolved when immersed in 0.1*N* hydrochloric acid.

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Discussion

It is well known[7] that gelations of globular protein solutions can not be interpreted by the solvation theory: the shells of the immobilized solvent surrounding the protein molecules touch or overlap with each other and immobilized the entire system. Since the globuler protein solution of very low concentration is capable of forming a gel by a denaturation. Gels are believed[7] to consist of three-dimensional network formed by some kinds of cross linkages between the polypeptide chains. These cross-linkages may consist of either chemical bonds, secondary forces localized at a few points in the molecules of non-localized secondary forces. The stiffness increases when a greater number of the chains become bound, since the network becomes more closely and knit together. If chemical bonds provide adequate cross-linkages of the network holding a gel, the network should not be ruptured easily by some undrastic treatments. On the other hand, if secondary bonds provide cross-linkages of the network, the network is easily damaged and the gel dissolves by even undrastic treatments. The more coarse the network is, the more easily syneresis takes place. The larger inhomogenities in the coarse network are considered responsible for the much higher opacity, since there is a parallelism between the easiness of syneresis and the opacity of the gel. Either gels formed by heating and by compression from the isoelectric solution doubtlessly is cross-linked by some kinds of secondary bonds, for example, coulombic, dipole and hydrogen bonds and non-polar attractive forces. Moreover, the gels formed from the isoelectric solution are presumed to be held with coarse networks having large pools of solvent, since the gels easily causes syneresis. The formation of such coarse network is favorable at the isoelectric point, because the protein molecules can easily approach each other, and the solution at a very low concentration is capable of forming a gel. The fact that when the gels formed from neutral solutions are imersed in 0.1*N* sodium hydroxide solution, the gels hardly dissolve or they contain some quantities of a clear gel which can not easily dissolve, suggests that the major part of the network of a part of the clear gel consists of chemical bonds, for example, intermolecular disulfid bonds. And this is assured by the inhibitory effects of sulfhydryl and disulfid reagents on the pressure gelation.

Frensdorff et al.[2] had shown that the gels formed from the urea solutions of ovalbumin are quite clear except in the cases of the low urea concentration or of the presence of the large quantity of salts, and the optimum pH of the gelation is not near the isoelectric point but at pH 9. The polypeptide chain may be probably surrounded by urea molecules and can not easily close each other. Therefore, most of the network of the gels formed from urea solution are not formed by the non-localized secondary attractive forces but formed by some chemical bonds, such as disulfid bonds. If the urea concentration decreases enough to decrease the number of urea molecules surrounding polypeptide chain, or if the salt concentration is high enough to decrease the intermolecular repulsion force, polypeptide chain can close easily each other, and the network is formed by the weak secondary bonds. This interpretation does not contradict with the fact that the gels formed at low urea concentration dissolve but the gels formed at higher urea concentration not dissolve when they are immersed in dilute alkaline solution.

7) J. D. Ferry, "*Advances in Protein Chemistry*", 4, 1 (1954)

The formation of the inhomogeneous and coarse network by secondary bonds is more favorable in the pressure gelation than in the thermal gelation, but it is not favorable in the gelation of urea solution of ovalbumin.

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