

IONIZING INFLUENCE OF SALTS WITH TRIVALENT
AND TETRAVALENT IONS ON CRYSTALLINE EGG
ALBUMIN AT THE ISOELECTRIC POINT.

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I.

INTRODUCTION.

Measurements of the membrane potentials between aqueous protein solutions or gels and surrounding water at equilibrium have yielded the result that salts with trivalent cations give isoelectric protein a positive charge while salts with tetravalent anions give it a negative charge.¹ On the basis of Donnan's theory of membrane potentials it was assumed that salts with trivalent cations, *e.g.* LaCl_3 , form with isoelectric protein ionizable salts which result in the formation of positive protein-La ions and negative Cl ions; and that salts like $\text{Na}_4\text{Fe}(\text{CN})_6$ form with isoelectric protein salts which result in the formation of negative protein-Fe $(\text{CN})_6$ ions and positive Na ions.² In other words, salts with trivalent cations react with isoelectric protein like acids, and salts with tetravalent anions react with isoelectric protein like alkalies; with this difference, however, that the compounds of isoelectric gelatin with acids and bases are much more stable than those with the salts of trivalent cations or tetravalent anions. Salts with divalent ions like Na_2SO_4 , CaCl_2 , or salts with monovalent ions like NaCl, did not produce any measurable charge on isoelectric gelatin in aqueous solutions. Experiments on anomalous osmosis through gelatin-collodion membranes were in harmony with these results.³

¹ Loeb, J., *J. Gen. Physiol.*, 1921-22, iv, 741.

² Loeb, J., *Proteins and the theory of colloidal behavior*, New York and London, 1922, 165.

³ Loeb, J., *J. Gen. Physiol.*, 1921-22, iv, 463.

It seemed of interest to find out whether experiments on the stability of aqueous solutions and suspensions of proteins at the isoelectric point are also in harmony with the results of the direct measurements of the membrane potentials. The reason why experiments on stability and flocculation were selected was that it is often stated that the flocculation of colloids is influenced in an opposite sense by the two oppositely charged ions of a salt, the ion with the same sign of charge as the colloid increasing the stability, the salt ion with the opposite sign of charge to that of the colloidal particle diminishing the stability of the suspension.

If we wish to use observations on the influence of salts on the stability of protein solutions at the isoelectric point for conclusions concerning the influence of ions on the electrical charges of particles, we are confronted with the difficulty that the electrical charges of particles are not the only forces which keep proteins in solution. There are two different kinds of forces determining the stability of solutions or suspensions of proteins, namely; first, the attraction between the molecules of the protein and the solvent, and second, forces of electrostatic repulsion between micellæ. When the forces of attraction between molecules of the solvent and molecules of the solute (which may be secondary valency forces) are greater than the forces of attraction between the molecules of the solute for each other, the solution will be stable. This type of forces acts in the general case of solutions of crystalloids.

When the forces of attraction between the molecules of solute and solvent are weak, the molecules of the solute upon colliding may adhere to each other and aggregates will be formed. This aggregate formation will lead to a flocculation or coagulation of the whole mass unless new forces originate in the small nascent aggregates (or micellæ) which prevent their coalescence into larger aggregates. These forces may be electrical charges whereby the nascent micellæ repel each other. The writer has investigated the origin of these charges in the case of protein micellæ and has found that they are due to the establishment of a Donnan equilibrium between particles and solution.⁴ A membrane equilibrium between particles and

⁴Loeb, J., *Proteins and the theory of colloidal behavior*, New York and London, 1922, 120; *J. Gen. Physiol.*, 1920-21, iii, 667; 1921-22, iv, 351.

solution can, however, only be established when the particles contain protein ions.

There exists a criterion which seems to permit us to decide which one of the two types of forces is responsible for the stability of a solution or suspension. When the stability depends upon the repulsive forces due to a potential difference between micellæ and solution, comparatively low concentrations of solutions will be required for the precipitation of the protein, since comparatively low concentrations of salts (*e.g.* concentrations of $M/8$ or less) suffice for the annihilation of the P.D. When, however, the stability of a solution is not determined by the P.D. between micellæ and solution but by forces of residual valency between molecules of solute and solvent, much higher concentrations of salts are, as a rule, required for precipitation than are sufficient for the annihilation of a P.D. caused by the Donnan equilibrium. Moreover, the efficiency of a salt in annihilating the P.D. between a micella and a solution is the lower the higher the valency of that ion of the salt which has the opposite sign of charge to that of the micella.

II.

The Prevention of Heat Coagulation of Isoelectric Egg Albumin by Trivalent and Tetravalent Ions.

Isoelectric crystalline egg albumin is quite soluble in water as long as the temperature is low. 8 per cent solutions kept at 2°C. remained perfectly clear for more than a year—and they would probably have kept clear indefinitely. Since it requires very high concentrations of salts to cause a precipitation of isoelectric crystalline egg albumin from aqueous solution at ordinary temperature, we may assume that the forces determining the stability of solutions of isoelectric crystalline egg albumin at sufficiently low temperature are not the electrical charges of micellæ but the attraction between molecules of isoelectric albumin and molecules of water. When, however, the temperature of a 1 per cent solution of isoelectric crystalline egg albumin is raised to about 73°C. or above, crystalline egg albumin is flocculated. Through the rise in temperature a change occurs in the molecule of crystalline egg albumin, whereby the attraction of the molecules of

albumin for each other becomes greater than the attraction between the molecules of albumin and water.

If the albumin is practically non-ionized (as is the case at the isoelectric point) no Donnan equilibrium between the nascent micellæ and the surrounding solution can be established and no p.D. between the nascent micellæ and the solution can prevent the coalescence of the micellæ. When, however, part of the albumin is ionized, the molecules of albumin will also unite upon heating to form micellæ, but these micellæ will begin to repel each other as soon as they contain protein ions. For in this case the protein ions in the nascent micellæ will cause the establishment of a Donnan equilibrium between the micellæ and the solution, and the electrical charge produced thereby on the particles will prevent the further coalescence of the nascent micellæ. This charge will increase with the relative concentration of ionized protein contained in the micellæ. It is obvious that the average size of the micellæ will remain the smaller the greater the relative concentration of protein ions in solution; since the greater the relative concentration of ionized protein the smaller will be the average number of protein molecules which can form an aggregate without including protein ions. This argument is supported by the well known fact that when we add some acid or alkali to isoelectric albumin, the solution will become only opalescent on heating but heat precipitation of the albumin will no longer occur. A comparison of the effect of increasing concentrations of acid shows that the relative size of the micellæ will become the smaller the greater the relative mass of ionized protein. To demonstrate this, 10 cc. of an aqueous 0.2 per cent solution of almost isoelectric crystalline egg albumin and containing varying amounts of 0.1 N HCl were put into test-tubes, and these test-tubes were put into boiling water until the temperature of the albumin solution rose to 90°C. Then the test-tubes were allowed to cool at room temperature and the appearance of the solution was noticed. Table I gives the result.

When the 10 cc. contained 0.01 cc. of 0.1 N HCl the protein remained practically isoelectric (pH 4.8), practically no ionization was produced, and hence flocculation occurred upon heating.

TABLE I.
Influence of HCl on Heat Coagulation of Crystalline Egg Albumin in Aqueous Solution. 10 cc. of 0.2 Per Cent Albumin (Nearly Isoelectric), Containing Various Concentrations of 0.1 N HCl, Heated to 90°C.

cc. 0.1 N HCl in 10 cc. of 0.2 per cent albumin.....	0	0.01	0.02	0.03	0.04	0.05	0.1	0.2	0.4	0.8	1.6	3.2
Appearance of solution.....	Coagulated.		Very opaque.	Very opalescent.	Clear but slightly opalescent.	Very clear, like water.						

The addition of 0.02 cc. of 0.1 N HCl prevented coagulation but the solution was opaque showing that only when the micellæ were comparatively large did they assume electrical charges; owing to the fact that the concentration of albumin ions was small compared with that of non-ionized protein. These charges sufficed, however, to prevent the further coalescence of the large micellæ. When the solutions contained 0.03 cc. of 0.1 N HCl the relative concentration of ionized protein was increased and hence the micellæ remained smaller; the solution was no longer opaque but opalescent. With the addition of 0.04 cc. of 0.1 N HCl the solution became very transparent, showing only slight opalescence. With the increasing concentration of ionized protein the average number of molecules in a micella was considerably diminished, and this small size of the average micellæ manifested itself in the greater transparency of the solution. With a still greater concentration of HCl the average size of the micellæ diminished still further and the solution became as clear as water.

When, however, the concentration of HCl was increased beyond a certain limit, the P.D. between the micellæ and solution was diminished again on account of the depressing effect of the Cl ions demanded by Donnan's theory. When 100 cc. of 1 per cent solution of originally isoelectric albumin contained 30 cc. of N HCl, the protein coagulated at a temperature of 66°C. In this case all the protein was practically ionized but the P.D. between the micellæ and the liquid was nevertheless depressed to zero on account of the high concentration of Cl ions.

By measuring the concentration of salt required to precipitate crystalline egg albumin from a 1 per cent solution in water of pH 3.0 at a temperature of 70°C. we can show that the forces preventing heat coagulation in this case are the electrical charges of the micellæ, since the concentration of salt required to cause precipitation is of the order of M/8 or below, and since sulfates are more efficient than chlorides.

The fact that ionization of protein prevents heat coagulation of albumin can be used to find out whether other electrolytes than acids or alkalis are able to produce ionization of isoelectric egg

albumin. If other ions, like La, Ca, Na, SO_4 , have such an effect on aqueous solutions of isoelectric albumin, it should show itself in the prevention of heat coagulation and in the optical appearance of the albumin solution after heating.

The experimental procedure was as follows: 7 cc. of water of pH 4.8 (this pH being the isoelectric point of crystalline egg albumin) were added to 2 cc. of 1 per cent solution of isoelectric crystalline egg albumin (of course, also of pH 4.8) and then 1 cc. of a salt solution containing different salts of different concentration, but always of pH 4.8, was added. The test-tubes containing the 10 cc. of the mixtures were put into boiling water until the liquid in the test-tubes reached a temperature of 90°C . and then the test-tubes were taken out of the water bath and allowed to cool at room temperature. Table II gives the appearance of the various mixtures after standing over night.

These experiments show first that the heat coagulation of isoelectric solutions of crystalline egg albumin is prevented by the addition of low concentrations of LaCl_3 or $\text{Na}_4\text{Fe}(\text{CN})_6$ of pH 4.8. The concentration of LaCl_3 sufficient for this purpose was $\text{m}/5,000$ and that of $\text{Na}_4\text{Fe}(\text{CN})_6$ about the same. Hence these two salts acted on the heat coagulation of isoelectric egg albumin like acids or alkali respectively. Moreover, it is obvious from Table II that at first the size of the micellæ formed diminishes with increasing concentration of LaCl_3 ions until the molecular concentration of LaCl_3 is about $\text{m}/160$. With a further increase of concentration of salt the size of the micellæ increases again (at $3\text{ m}/80$) owing to the fact that the p.d. is depressed by the Cl ions; and at $\text{m}/20$ LaCl_3 this depressing action of the Cl ions on the p.d. is sufficient to permit again the heat coagulation of the albumin. In the case of $\text{Na}_4\text{Fe}(\text{CN})_6$ the solution ceases to be clear when the concentration becomes $6\text{m}/80$; in this case the depressing action of the Na ions on the p.d. of the negatively charged micellæ is so great that the micellæ begin to coalesce again.

None of the other salts tried, CaCl_2 , BaCl_2 , NaCl , or Na_2SO_4 , is able to prevent heat coagulation of isoelectric egg albumin in an aqueous solution of pH 4.8. It is, of course, possible that certain

TABLE II.
Influence of Different Salts on Heat Coagulation of Crystalline Egg Albumin in Aqueous Solution at pH of Isoelectric Point.

Total concentration of salt in 10 cc. of 0.2 per cent albumin.....	Concentration of salt (M)																		
	10M/80	8M/80	6M/80	4M/80	3M/80	2M/80	M/80	M/160	M/320	M/640	M/1,280	M/2,560	M/5,120	M/10,240	M/20,480	M/40,960	M/81,920	0	
LaCl_3	Coagulated.	Coagulated.	Coagulated.	Coagulated.	Opalescent.	Clear.	Bluish clear.	Opalescent and turbid.	Milky.	Coagulated.	Coagulated.	Coagulated.	Coagulated.	Coagulated.	Coagulated.	Coagulated.	Coagulated.	Coagulated.	Coagulated.
$\text{Na}_2\text{Fe}(\text{CN})_6$	Very opaque.	Slight opalescence.	Clear.	Increasing opalescence.	Coagulated.	Coagulated.	Coagulated.	Coagulated.	Coagulated.	Coagulated.	Coagulated.	Coagulated.	Coagulated.	Coagulated.	Coagulated.	Coagulated.	Coagulated.	Coagulated.	Coagulated.
BaCl_2	Coagulated.	Coagulated.	Coagulated.	Coagulated.	Coagulated.	Coagulated.	Coagulated.	Coagulated.	Coagulated.	Coagulated.	Coagulated.	Coagulated.	Coagulated.	Coagulated.	Coagulated.	Coagulated.	Coagulated.	Coagulated.	Coagulated.
CaCl_2	Coagulated.	Coagulated.	Coagulated.	Coagulated.	Coagulated.	Coagulated.	Coagulated.	Coagulated.	Coagulated.	Coagulated.	Coagulated.	Coagulated.	Coagulated.	Coagulated.	Coagulated.	Coagulated.	Coagulated.	Coagulated.	Coagulated.
Na_2SO_4	Coagulated.	Coagulated.	Coagulated.	Coagulated.	Coagulated.	Coagulated.	Coagulated.	Coagulated.	Coagulated.	Coagulated.	Coagulated.	Coagulated.	Coagulated.	Coagulated.	Coagulated.	Coagulated.	Coagulated.	Coagulated.	Coagulated.
NaCl	Coagulated.	Coagulated.	Coagulated.	Coagulated.	Coagulated.	Coagulated.	Coagulated.	Coagulated.	Coagulated.	Coagulated.	Coagulated.	Coagulated.	Coagulated.	Coagulated.	Coagulated.	Coagulated.	Coagulated.	Coagulated.	Coagulated.

other bivalent and monovalent ions act differently, since the valency is not the only variable determining the combining action.⁵

These results are in entire agreement with the experiments published in a preceding paper¹ showing that only salts with trivalent and tetravalent ions can produce a membrane potential on isoelectric gelatin while Na, Ca, Ba, and SO₄ have no such effect.

When crystalline egg albumin is dissolved in a solution with little water and much alcohol, salts have not the same influence on the stability of the solution that they have in an aqueous solution. This is due to the difference in the nature of the solvent, since the influence of salts on the stability of such alcoholic solutions of crystalline egg albumin is similar to the influence of salts on the stability of solutions of gelatin in a solution with much alcohol and little water. The stability of isoelectric gelatin in a mixture with little water and much alcohol is increased not only by salts with trivalent and tetravalent ions but also by salts with bivalent ions, such as MgCl₂, CaCl₂, SrCl₂, BaCl₂, and Na₂SO₄, while salts like MgSO₄, LiCl, NaCl, or KCl have no such effect. The clearing effect of Ba was considerably greater than that of Mg. We know too little about the p.D. and solubility in alcoholic solutions and for this reason the publication and discussion of these results may be postponed.

SUMMARY AND CONCLUSION.

1. While crystalline egg albumin is highly soluble in water at low temperature at the pH of its isoelectric point, it is coagulated by heating. It has long been known that this coagulation can be prevented by adding either acid or alkali, whereby the protein is ionized.

2. It is shown in this paper that salts with trivalent or tetravalent ions, *e.g.* LaCl₃ or Na₄Fe(CN)₆, are also able to prevent the heat coagulation of albumin at the isoelectric point (*i.e.* pH 4.8), while salts with a divalent ion, *e.g.* CaCl₂, BaCl₂, Na₂SO₄, or salts like NaCl, have no such effect.

⁵ Michaelis, L., and Rona, P., *Biochem. Z.*, 1919, xciv, 225.

3. This is in harmony with the fact shown in a preceding paper that salts with trivalent or tetravalent ions can cause the ionization of proteins at its isoelectric point and thus give rise to a membrane potential between micellæ of isoelectric protein and surrounding aqueous solution, while the above mentioned salts with divalent and monovalent ions have apparently no such effect.