STABILITY OF SUSPENSIONS OF SOLID PARTICLES OF PROTEINS AND PROTECTIVE ACTION OF COLLOIDS.

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I.

INTRODUCTION.

While it was formerly held that the forces at the boundary between solids and liquids were purely physical, Langmuir and Harkins have come to the conclusion that these forces are primarily chemical. This viewpoint will be utilized in the problem which forms the object of this paper; namely, an investigation of the nature of the forces which keep solid particles of proteins in suspension. The large molecule of proteins does not act as a homogeneous unit, and it is necessary to discriminate for our problem between "aqueous" groups, i.e. groups which have a strong chemical affinity for the molecules of water (e.g. carboxyl, amino, or imino groups), and "oily" groups (e.g. hydrocarbon groups) which have a stronger affinity for each other than for water. A similar view was expressed by Sheppard. The molecules of gelatin in aqueous solution may adhere to each other wherever they touch each other with their "oily" groups; and if the concentration of the gelatin solution is high enough, the whole mass will set to a solid gel. In this case the affinity of the "aqueous" groups of the gelatin molecule for water need not be, and probably is not, lessened, and when the gelatin sets to a gel, the average distance between the molecules of gelatin remains the same as it was in the solution. When, however, gelatin is precipitated by a salt, the affinity of the "aqueous" groups for water is diminished and the mole-

cules attract each other over a larger area. The result is a coagulation in which the average distance between the molecules of gelatin is very much less than it was in the solution. This view of the difference between gel formation and precipitation or salting out is supported by the fact observed by the writer that it requires the same high concentration of a salt to cause precipitation of a gelatin solution when it is near complete gel formation as is required when the same solution has a very low viscosity; the temperature in both cases, of course, being equal.

These facts are mentioned because they show that when a solid particle, e.g. collodion, is coated with a film of a protein like gelatin, it is not only a priori possible but, perhaps, probable that the affinity of the "aqueous" groups of the gelatin molecule for water continues to act. It is intended to show in this paper that the flocculation or precipitation of suspensions of gelatin-coated particles of collodion by salts is the same process of "salting out" by which solutions of gelatin in water are precipitated by salts; and it is, moreover, intended to show that the suspensions of gelatin-coated particles of collodion are as unstable at the pH of the isoelectric point of gelatin, namely 4.7, as are the solutions of gelatin, and that suspensions of gelatin-coated collodion particles as well as solutions of gelatin are stabilized at pH 4.7 by the addition of neutral salts. It follows from this that the forces which determine the stability of suspensions of gelatin-coated particles of collodion in water are the forces of chemical affinity between the "aqueous" groups of the gelatin molecule and water.

This chemical viewpoint is in strong contrast with the purely physical viewpoint, whereby suspended particles are protected against coalescence merely by their electrical double layers, which have their origin primarily in forces inherent in the water itself. On this assumption, the stability of a suspension depends only on the potential difference between the two strata of the electrical double layer surrounding the particle in water, and this P.D. is reduced to zero by comparatively low concentrations of salts, that ion of the salt being effective which has a sign of charge opposite to that of the particle. It will be shown that when collodion particles are coated with genuine crystalline egg albumin, they behave as if the film formation destroyed the affinity of the protein for water in the same way as does high
temperature ("boiling"), and in this case the chemical forces of attraction between the "aqueous" groups of albumin and water play no part in the stability of the suspensions. Such particles can be kept in suspension only by the electrical double layers surrounding the particles, as the physical theory demands.

II. The Nature of the Forces Which Determine the Stability of Suspensions of Gelatin-Coated Particles of Collodion.

Suspensions of collodion particles were prepared as described in a previous publication. A small quantity of such particles was put over night into 100 cc. of an aqueous solution of 0.1 per cent isoelectric gelatin at a pH of 4.7. The writer has previously shown that under such conditions a solid film of protein is formed on the surface of the collodion. (It was found important not to use a higher concentration of gelatin than 0.1 per cent, since when a stronger gelatin solution is used, larger gelatin aggregates are formed to which several particles of collodion may stick. Such larger masses will settle rapidly without salt, thus rendering the test futile.) The next morning the (now gelatin-coated) collodion particles were centrifuged from the 0.1 per cent gelatin solution and then enough of the particles of a certain size were put into water of the desired pH to form a creamy suspension. 3 drops of such a stock suspension were then put at room temperature over night into test-tubes containing 10 cc. of a salt solution of a definite pH, to find out which concentration of salt was required for precipitation. The P.D. of the double electrical layer of the gelatin-coated particles is known from the cataphoretic experiments published in a preceding paper.

In Table I are given the molar concentrations of different salts required to cause precipitation of the suspension of gelatin-coated collodion particles over night at room temperature.

The concentration of salts required for precipitation of the gelatin suspension bears no relation to the cataphoretic P.D., first, since the

concentrations are far in excess of those required to depress the P.D. to any low value or even zero; and second, since there is no indication of the valency effect which is so strong in the depression of the P.D. The concentrations required to precipitate the suspension of negatively charged collodion particles free from protein were for NaCl, Na$_2$SO$_4$, CaCl$_2$, and LaCl$_3$, $m/2$, $m/4$, $m/32$, and $m/2,048$ respectively. At pH 5.8 and 11.0, the gelatin-coated particles of collodion are also negatively charged; yet the concentrations of LaCl$_3$ required for their precipitation are greater than 1 M, and for CaCl$_2$ greater than 2 M; as a matter of fact, the writer is not absolutely certain that these salts precipitate the suspension of the negatively charged gelatin-coated particles at any concentration, though this may be the case. Moreover, Na$_2$SO$_4$ and Na$_4$Fe(CN)$_6$ are more powerful precipitants for the negatively charged gelatin-coated particles than LaCl$_3$ or CaCl$_2$. These results admit only one explanation; namely, that the forces which determine the stability of suspensions of gelatin-coated collodion particles are not the electrical charges of the particles.

The influence of salts on the stability of suspensions of gelatin-coated particles of collodion is especially interesting when the pH of the solution is 4.7; i.e., that of the isoelectric point of gelatin. The cataphoretic measurements show that at the isoelectric point, the particles are entirely uncharged. It happens that at pH 4.7 suspensions of gelatin-coated particles in water free from salt are unstable. At first sight, this would seem to suggest that electrical charges of the particles are required to make suspensions of gelatin-coated particles stable at the isoelectric point of gelatin. This assumption

<table>
<thead>
<tr>
<th>pH</th>
<th>NaCl</th>
<th>CaCl$_2$</th>
<th>LaCl$_3$</th>
<th>Na$_2$SO$_4$</th>
<th>Na$_4$Fe(CN)$_6$</th>
</tr>
</thead>
<tbody>
<tr>
<td>3.0</td>
<td>2</td>
<td>&gt;2</td>
<td>&gt;1</td>
<td>1</td>
<td>1/2</td>
</tr>
<tr>
<td>4.0</td>
<td>&gt;2</td>
<td>&gt;2</td>
<td>&gt;1</td>
<td>1</td>
<td>1/2</td>
</tr>
<tr>
<td>4.7</td>
<td>&gt;2</td>
<td>&gt;2</td>
<td>&gt;1</td>
<td>1</td>
<td>1/2</td>
</tr>
<tr>
<td>5.8</td>
<td>&gt;2</td>
<td>&gt;2</td>
<td>&gt;1/2</td>
<td>1</td>
<td>1/2</td>
</tr>
<tr>
<td>11.0</td>
<td>&gt;2</td>
<td>2</td>
<td>1/2</td>
<td>1</td>
<td>1/2</td>
</tr>
</tbody>
</table>
is, however, refuted by the fact that comparatively low concentrations of salts, which leave the particles uncharged, make the suspensions stable. Thus M/16,000 CaCl₂ or M/16,000 Na₂SO₄ will suffice to make the suspension of gelatin-coated collodion particles stable at the isoelectric point; NaCl stabilizes the suspension in a concentration of M/512. The measurements of the cataphoretic p.d. of gelatin-coated collodion particles at the isoelectric point of gelatin show that neither NaCl nor Na₂SO₄ nor CaCl₂ causes the particles to be charged at pH 4.7, not only in concentrations as low as M/16,000 CaCl₂ or Na₂SO₄, but even in much higher concentrations. Table II gives the concentrations of salts required for stabilization at the isoelectric point of gelatin.

**TABLE II.**

Concentrations of Salts at Which Suspensions of Gelatin-Coated Particles of Collodion Are Stable at pH 4.7 (Isoelectric Point of Gelatin).

<table>
<thead>
<tr>
<th>Salt</th>
<th>Stable</th>
<th>Precipitated</th>
</tr>
</thead>
<tbody>
<tr>
<td>NaCl</td>
<td>M/512 to 2 M (or higher).</td>
<td>0 to M/1,024.</td>
</tr>
<tr>
<td>CaCl₂</td>
<td>M/16,000 to 2 M.</td>
<td>0</td>
</tr>
<tr>
<td>LaCl₃</td>
<td>M/130,000 to 1 M.</td>
<td>0</td>
</tr>
<tr>
<td>Na₂SO₄</td>
<td>M/16,000 to M/2.</td>
<td>0 to M/32,000 and also above M/2.</td>
</tr>
<tr>
<td>Na₄Fe(CN)₆</td>
<td>M/1,000,000 to M/4.</td>
<td>0 also above M/4.</td>
</tr>
</tbody>
</table>

If then the forces which determine the stability of suspensions of gelatin-coated collodion particles are not the potential differences of the electrical double layer surrounding the particles, the question arises, What other forces determine this high degree of stability of these suspensions? The answer is that these forces are the same as those which keep gelatin in solution. It can be shown that the concentrations of salts required for the precipitation of suspensions of gelatin-coated particles are practically identical with those required for the salting out of gelatin from true aqueous solutions. Table III gives the minimal concentration of NaCl, CaCl₂, LaCl₃, Na₂SO₄, and Na₄Fe(CN)₆ required to “salt out” 1 per cent solutions of gelatin at pH 3.0, 4.0, 4.7 (isoelectric point), 5.8, and 11.0. The solutions were allowed to stand over night at room temperature, and were prepared in the following way.
1 gm. of originally isoelectric gelatin was dissolved in 100 cc. of water containing enough HCl or NaOH respectively to bring the solution to the desired pH and also the required concentration of salt.

The values in Table III are almost identical with the values in Table I.

It is obvious that the concentrations of salts, e.g. CaCl₂ or LaCl₃, required for precipitation of solutions of gelatin in water are many times greater than would be required if gelatin were in suspension; i.e., if the gelatin particles were prevented from coalescing by the electrical double layers around each particle. Moreover, Tables I and III show that Na₂SO₄ is a better precipitant of gelatin solutions than CaCl₂ even if the gelatin particles are negatively charged; i.e., at pH 5.8 or at pH 11.0. If the gelatin particles were kept in solution by electrostatic charges due to electrical double layers, CaCl₂ should be a better precipitant than Na₂SO₄ when the particles of gelatin are negatively charged, which is, however, not the case. Since the concentrations of salts required for the salting out of gelatin from its aqueous solution are almost identical with the concentrations required to precipitate suspensions of gelatin-coated collodion particles, the forces determining the stability of the suspensions must be identical with those determining the stability of true solutions of gelatin, and these latter forces are forces of affinity between solute and solvent.

It can also be shown that the stabilizing effect of salts on suspensions of collodion particles coated with gelatin at the isoelectric point is due to an influence on the affinity of the protein film for water.
The writer had already shown in his book and in a preceding paper that neutral salts increase the solubility of isoelectric gelatin, and the more the higher the valency of either ion of the salt.\(^6\) That we are dealing in this case with ordinary solubility follows from two facts; namely, first, that the time required to dissolve a given mass of solid isoelectric gelatin is diminished upon the addition of salts; and second, that the amount of isoelectric gelatin dissolved in water increases with the addition of salt. Both effects increase with the valency of one of the ions of the salt.

From the practical identity of the influence of salts on the stability of solutions of gelatin and of suspensions of gelatin-coated collodion particles, it follows that the forces which prevent the coalescence of gelatin-coated collodion particles in water are the forces determining the ordinary solubility of gelatin in water; \textit{i.e.}, the chemical affinity between the "aqueous" groups (carboxyl, amino, or imino groups) and the molecules of water. The forces of cohesion between the "oily" groups of the gelatin molecules are no longer noticeable in the case of gelatin-coated particles of collodion.

\section*{III.}

\textit{Solutions of Genuine Crystalline Egg Albumin.}

Egg albumin exists in two modifications, "genuine" and "denatured" egg albumin. While genuine crystalline egg albumin is highly soluble in water, denatured egg albumin is practically insoluble. The simplest (but not the only) way to transform genuine egg albumin into its water-insoluble modification is by bringing an aqueous solution of the substance to a sufficiently high temperature. It is possible to show that the forces which keep \textit{genuine} egg albumin in solution are those determining ordinary solubility, while the forces which keep \textit{denatured} egg albumin in solution, or rather suspension, are the electrostatic forces of repulsion due to the electrical double layer around the particles. We will first discuss the solubility of genuine egg albumin in water.

It is understood that in all the following experiments with genuine egg albumin the temperature is ordinary room temperature not

\(^6\)Loeb, J., \textit{Arch. néerl. physiol. homme et animaux}, 1922, vii, 510.
STABILITY OF PROTEIN SUSPENSIONS

exceeding 24°C., since at temperatures of 60°C. or above, crystalline egg albumin may be transformed into its insoluble modification.

Since the particles of genuine isoelectric egg albumin do not migrate at the pH of the isoelectric point, they are not kept in solution by electrical charges; moreover, the viscosity of such solutions is of so low an order of magnitude that they can contain comparatively few (if any) aggregates. The idea that crystalline egg albumin forms a true solution is also held by Sørensen and is contradicted by no fact.

The concentrations of salts required to precipitate crystalline egg albumin, in the neighborhood of the isoelectric point, i.e. at 4.0, 4.8, and 5.8, are very high, and show no relation between the sign of charge of the protein particle and the precipitating ion. At higher hydrogen ion concentrations, e.g. pH 2.0, lower concentrations of salts are sufficient for precipitation. Salts like LaCl₃ have a greater precipitating power for egg albumin at any pH than salts like CaCl₂ (Table IV).

These facts leave no doubt that the solubility of genuine crystalline egg albumin is not determined by double electrical layers surrounding the molecules or particles but is determined by the forces responsible for true crystalloidal solution, and that the precipitation of genuine egg albumin from its solution is a true "salting out" but is not caused by a diminution of the P.D. of a double layer.

IV.

*The Stability of Suspensions of Collodion Particles Coated with Crystalline Egg Albumin.*

Collodion particles coated with genuine crystalline egg albumin do not behave like solutions of genuine egg albumin, but like suspensions of particles of denatured egg albumin; or, in other words, when genuine crystalline egg albumin forms a film on a collodion surface, it behaves as if its "aqueous" groups had ceased to react with water. Suspensions of albumin-coated collodion particles are stable only as long as the cataphoretic P.D. of the particles is above about 12 or 13 millivolts.

Collodion particles were kept over night in 1 per cent solutions of crystalline egg albumin of pH 4.8, centrifuged off from the solution, and then a milky stock suspension was prepared in water of pH 4.8. 3 drops of that suspension were added to 50 cc. of various concentrations of salt at pH 11.0, 5.8, 4.5, 4.0, and 3.0, and the velocity of migration in an electric field was measured under the microscope.

The results of these measurements are represented graphically in Figs. 1 to 5. The ordinates of the curves representing the influence of salts on the cataphoretic P.D. of the albumin-coated collodion particles are the millivolts calculated from the mobility measurements as described previously; the values are given as negative when the particle bears a negative charge. The curves are practically identical with those for the cataphoretic P.D. of particles of denatured egg albumin published in the preceding article, except at pH 5.8 and 11.0, where the collodion particles coated with egg albumin behave almost like collodion particles free from albumin.

The influence of salts on the stability of suspensions of albumin-coated particles of collodion was tested in the following way. 3 drops of the stock suspension of the particles were shaken up in 10 cc. of various concentrations of salts at different pH and allowed to settle overnight at room temperature. The results are given by the horizontal line "critical P.D." in Figs. 1 to 5. In all salt solutions between that line and the zero line the particles were generally precipitated over night. We shall see later that the critical P.D. for the stability of collodion particles coated with albumin is almost the same as the
critical P.D. for suspensions of particles of denatured egg albumin; namely, above 10 to 11 millivolts.

We will now go into some details. In Fig. 1 are given the cataphoretic P.D. of the albumin-coated particles of collodion at pH 4.5 at

![Graph](https://example.com/graph.png)

**Fig. 1.** Influence of salts on the cataphoretic P.D. of collodion particles coated with a film of crystalline egg albumin at pH 4.5, where the cataphoretic P.D. without salt was about zero. The two broken lines in Fig. 1 and in the following figures, with the designation “critical P.D.,” give that P.D. below which the suspensions of the albumin-coated particles are no longer stable. It is obvious that only in solutions of Na₄Fe(CN)₆ between concentrations of M/16,000 and M/32 was the suspension stable.

which without salt the charge was zero. The isoelectric point of crystalline egg albumin is 4.8, but the cataphoretic P.D. was not zero at pH 4.8 and it was necessary to add a trace of acid to annihilate the cataphoretic migration.
The addition of NaCl, Na₂SO₄, CaCl₂, or LaCl₃ did not raise the cataphoretic P.D. to the critical value required for stability. Na₄Fe(CN)₆ in concentrations of M/16,000 or higher raised the P.D. to 13 millivolts and above, and the suspension was stable. When the concentration of Na₄Fe(CN)₆ reached or exceeded M/32, the P.D. was depressed below the critical value and flocculation occurred.

At pH 4.0 (Fig. 2) the cataphoretic P.D. was above the critical value without salt, but the addition of LaCl₃, CaCl₂, NaCl, or Na₂SO₄...
depressed the P.D. below that of the critical value and flocculation occurred. Stable suspensions were obtained in solutions of Na₄Fe(CN)₆ in concentrations between M/8,000 and M/32, because in these solutions the cataphoretic P.D. was above 13 millivolts.

At pH 3.0 (Fig. 3) the P.D. was over 30 millivolts without salt and the suspension was stable. The addition of high concentrations of salts was required to depress the P.D. below the critical value of 13 millivolts. 

\[
\text{Concentration} \\
0 0.016 0.032 0.064 0.128 0.256 0.512 1.024 2.048 4.096 8.192 16.384 32.768 \\
\text{Genuine albumin pH 3.0} \\
\text{Critical P.D.} \\
\text{NaCl} \\
\text{CaCl}_2 \\
\text{LaCl}_3 \\
\text{Na}_2\text{SO}_4
\]

Fig. 3. Influence of salts on cataphoretic P.D. and stability of albumin-coated particles of pH 3.0. Without salt the cataphoretic P.D. is about 31 millivolts and the particles are positively charged. The suspension is stable as long as the concentration of the salt is not too high. Flocculation occurs when the values for the P.D. fall between the line for critical P.D. and the zero line.

\[\text{millivolts} \quad \text{M/16 NaCl, M/32 CaCl}_2, \text{about M/64 LaCl}_3, \text{and M/256 Na}_2\text{SO}_4 \text{were required to cause flocculation.} \]

Figs. 4 and 5 give the result of experiments at pH 5.8 and 11.0. The curves are so much like those for the P.D. of colloidion particles free from protein that the suspicion exists that the albumin had been dissolved by the alkali.
Hence, collodion particles coated with genuine egg albumin form stable suspensions only by virtue of their electrical double layers; as soon as the P.D. of the double layer falls below a critical value, the suspension is no longer stable. The particles attract each other in

![Diagram showing the influence of salts on the cataphoretic P.D. and the stability of suspensions of albumin-coated collodion particles at pH 5.8.](image)

**Fig. 4.** Influence of salts on the cataphoretic P.D. and the stability of suspensions of albumin-coated collodion particles at pH 5.8.

the same way as do the particles of denatured egg albumin when the P.D. falls below the critical value of about 12 millivolts.

It is difficult to understand why genuine egg albumin when it forms a solid film on collodion particles should lose its solubility in water
and behave in that respect like boiled egg albumin, yet the fact that albumin is denatured when forming a film is supported by the observations of Ramsden. This author found that proteins have a tendency to form a solid film at the surface of liquids on account of their lowering the surface tension of the water; but he also found that these proteins (or, perhaps, more correctly certain proteins) undergo an irreversible coagulation in this case. Applied to crystalline egg

Fig. 5. Influence of salts on the cataphoretic P.D. and the stability of suspensions of albumin-coated collodion particles at pH 11.0. In both Figs. 4 and 5 the P.D. is so much like that of collodion particles free from albumin that the suspicion is warranted that the film of albumin had been partly or entirely destroyed by the alkalies.

Genuine albumin
pH 11.0

Critical P.D.

Concentration
albumin it would mean that genuine egg albumin is denatured when it forms a film.

Herzfeld and Klinger, as well as Wiechowskl, found that mechanical grinding of a dry powder of soluble blood albumin renders the albumin insoluble.

The mechanism of denaturation is unknown; should it be possible that the albumin molecule of the film is oriented in such a way as to render ineffective the action of the groups with a high affinity for water? The observations of Langmuir leave no doubt that the molecules of surface films are definitely oriented.

Whatever the explanation may be, the fact remains that the influence of electrolytes on the cataphoretic P.D. is practically the same for collodion particles coated with gelatin or with genuine crystalline egg albumin; while the influence of salts on the stability of suspensions of the two types of particles is entirely different. This difference finds its explanation in the fact that the forces determining the stability of suspensions of gelatin-coated particles in water are the chemical forces acting in true solubility; while the forces determining the stability of suspensions of albumin-coated collodion particles are essentially the electrostatic forces of the double electrical layer surrounding the particle.

V.

The Stability of Suspensions of Particles of Denatured Egg Albumin.

The conditions for the stability of suspensions of particles of denatured egg albumin are practically identical with those for the stability of suspensions of particles of collodion coated with genuine egg albumin. This supports the idea that genuine egg albumin, when forming a film on collodion, undergoes a change whereby its affinity for water no longer seems to exist.

Careful experiments on the flocculation of denatured egg albumin have been made before, especially by Chick and Martin, but the cataphoretic P.D. of the particles was not measured, and we are here

10 Wiechowski, W., Biochem. Z., 1917, lxxvi, 278.
concerned with the relation between that P.D. and the stability of suspensions.

Suspensions of denatured egg albumin were prepared in the following way. A 1 per cent solution of isoelectric crystalline egg albumin (pH 4.8) was heated to 90°C. and the coagulated mass was allowed to settle. It was then ground up in a mortar with a small amount of water to a milky suspension. 1 drop of a sufficiently concentrated stock suspension was put into 10 cc. of a solution of different salts at different pH, and the solution was allowed to stand over night at room temperature.

In order to bring the cataphoretic P.D. of the particles of (boiled) denatured egg albumin to zero, the surrounding solution had to have a pH of about 5.0. Flocculation occurred in all concentrations of NaCl, Na₂SO₄, and CaCl₂. Only in certain concentrations of Na₄Fe(CN)₆ and LaCl₃ was the suspension stable and these concentrations were in the case of Na₄Fe(CN)₆ between M/65,000 and M/16. Inside these concentrations the cataphoretic P.D. was above 10 millivolts. The suspension of particles of denatured egg albumin was stable in concentrations of LaCl₃ between M/2,000 and M/16, and in this case the P.D. was also above the critical level of about 12 millivolts.

At pH 4.0 the cataphoretic P.D. of the particles was about 20 millivolts without salt, and since this is above the critical value the suspension was stable. The addition of a trace of Na₄Fe(CN)₆, M/8,000 or less, brought the P.D. to about zero and flocculation occurred. The addition of more Na₄Fe(CN)₆ reversed the sign of charge and the P.D. increased. At M/2,048 the P.D. was about 12 millivolts and the suspension was stable. The other salts depressed the P.D. below the critical value in the following concentrations, NaCl M/16, CaCl₂ M/32, LaCl₃ below M/16, and Na₂SO₄ M/1,024; and in these and higher concentrations caused flocculation.

From these and other experiments no doubt was left that the concentration where flocculation occurs can be predicted from the cataphoretic P.D. since the suspension is stable only when the P.D. is above 10 to 12 millivolts. The maximal concentration where the suspension was stable and the minimal concentration where flocculation occurred are given in Table V.
The figures in Table V show that the suspension of particles of denatured crystalline egg albumin no longer remains stable when the P.D. falls below about 9 millivolts, while it is always stable when it is above 10 to 12 millivolts. It must also be remembered that the

\[\begin{array}{c|c|c|c|c}
\text{pH} & \text{Stable} & \text{Complete precipitation} \\
\hline
11.0 & \text{CaCl}_2 & \text{m/256} & 10 & \text{m/16} \\
 & \text{NaCl} & \text{m/4 to 0.} & 24 to 9. & \text{m/128} \\
 & \text{Na}_2\text{SO}_4 & \text{m/4 to 0.} & 31 to 10. & \text{m/2,048} \\
5.8 & \text{NaCl} & \text{m/256} & 8 & \text{m/128} \\
 & \text{CaCl}_2 & \text{m/4,096} & 8 & \text{m/32,000} \\
 & \text{LaCl}_3 & \text{m/2,048} & 13 & \text{m/32,000} \\
 & \text{Na}_2\text{SO}_4 & \text{m/4,096} & 12 & \text{m/512} \\
 & \text{Na}_4\text{Fe(CN)}_6 & & & \text{m/4} \\
5.0 & \text{NaCl} & & & 0 to \text{m/4.} \\
 & \text{CaCl}_2 & & & 0 to \text{m/8.} \\
 & \text{Na}_2\text{SO}_4 & & & 0 to \text{m/2.} \\
 & \text{LaCl}_3 & \text{m/2,048 to m/16.} & 13 to 10. & \text{m/32,000 to 0.} \\
 & \text{Na}_4\text{Fe(CN)}_6 & \text{m/65,000 to m/16.} & 19 to 10.5. & 8 to 6. \\
4.0 & \text{NaCl} & \text{m/32} & 9 & \text{m/16} \\
 & \text{CaCl}_2 & \text{m/128} & 13 & \text{m/32} \\
 & \text{LaCl}_3 & \text{m/65,000 to m/16.} & 22 to 9. & \text{m/1,024} \\
 & \text{Na}_2\text{SO}_4 & \text{m/16,000} & 14 & \text{m/8,192} \\
 & \text{Na}_4\text{Fe(CN)}_6 & \text{m/4,096 to m/2,048.} & 12 to 8. & 3 \\
3.0 & \text{NaCl} & \text{m/16} & 10.5 & \text{m/4} \\
 & \text{CaCl}_2 & \text{m/16} & 11 & \text{m/4} \\
 & \text{LaCl}_3 & \text{m/16} & 9.5 & \text{m/4} \\
 & \text{Na}_2\text{SO}_4 & \text{m/256} & 9.5 & \text{m/128} \\
\end{array}\]

measurements of the cataphoretic P.D. are accurate only within ± 2 millivolts.

It follows from this that the stability of suspensions of denatured crystalline egg albumin depends on the P.D. surrounding each particle.
There is, however, one statement to be added. At pH 4.0, 5.8, and 5.0, there occurs a suspension in concentrations of CaCl₂ and NaCl of M/2 or above. Since at these high concentrations the P.D. is very low in water, we must conclude that the salt depresses the cohesive forces between the particles, or increases the forces of attraction between water and albumin, so that the particles cannot coalesce even if the P.D. around each particle is zero. Northrop and De Kruif observed a similar phenomenon in their experiments on the influence of salts on bacterial suspensions and they proved by measurements that the cohesive forces between bacteria may be sufficiently diminished by high concentrations of salt solutions, so that no agglutination occurs between the bacteria even if the P.D. between the bacteria is low or zero.¹²

VI.

Influence of Salts on the Heat Coagulation of Denatured Egg Albumin.

When crystalline egg albumin is heated, it undergoes a change whereby it becomes insoluble. Its molecules upon colliding will adhere to each other and form aggregates and these aggregates may further coalesce upon colliding, provided the cataphoretic P.D. is below that of the critical value for coalescence, which was shown to be about 9 millivolts in the preceding paragraph. When the cataphoretic P.D. is above this critical value, no such coalescence will occur and the suspension will be stable. But the average size of the particles will be the smaller the higher the P.D. because the probability of the particles approaching each other with sufficient kinetic energy to break through the barrier of electrostatic repulsion becomes the smaller the higher the cataphoretic P.D. This will show itself in the appearance of the solution. When the P.D. is very high, the solution must remain clear as water, because there may be aggregates of, at the utmost, a few molecules, but no coalescence of such aggregates can occur. When the P.D. is a little less, a small percentage of particles may possess the kinetic energy to break through the barrier of electrostatic repulsion and some coalescence of aggregates may occur. Such suspensions may appear slightly bluish. Upon further dim-

inution of the P.D. the relative percentage of coalescence will increase, the suspension will appear gray, finally milky, and when the P.D. falls below the critical value, coalescence will be so general that the majority of the colliding aggregates will coalesce and flocculation will occur, since the rate of settling of a suspension depends on the relative size of the particles.

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 to room temperature. Table VI gives the appearance of the various mixtures after standing over night.

Without salt the cataphoretic charge of the aggregate is zero and flocculation occurs. When either NaCl, or Na₂SO₄, or CaCl₂ is in the solution, flocculation will always occur, since these salts raise the P.D. little or not at all. When, however, Na₄Fe(CN)₆ or LaCl₃ is added, not only is there a stable suspension, but the suspension becomes almost as clear as water at that concentration of these salts at which the P.D. of denatured egg albumin is high. Thus the suspension of denatured egg albumin remains clear though slightly bluish in appearance in concentrations of LaCl₃ between $\frac{1}{2}$,800 and $\frac{1}{4}$,000 where the cataphoretic P.D. is 15 millivolts or more. At $\frac{1}{2}$,25 and at $\frac{1}{10}$,000 the suspension is still stable but opaque; i.e., the particles are larger but not large enough to settle rapidly. At these concentrations of LaCl₃ the P.D. was about 10 millivolts, i.e. it was just above the critical value of 9 millivolts. In concentrations of $\frac{1}{2}$,000 or less LaCl₃ flocculation occurred, since the P.D. was below the critical value of 9 millivolts.

Acid acts in the same way. When traces of acid are added to isoelectric albumin, heat coagulation is prevented but the appearance of the solution of egg albumin after heating depends on the P.D. of the particles in the way described. 10 cc. of an aqueous 0.2 per cent solution of almost isoelectric crystalline egg albumin and containing
TABLE VI.

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

<table>
<thead>
<tr>
<th>Total concentration of salt in 10 cc. of 0.2 per cent albumin</th>
<th>10cc./80</th>
<th>6cc./80</th>
<th>4cc./80</th>
<th>2cc./80</th>
<th>1cc./80</th>
<th>0.5cc./80</th>
<th>0.2cc./80</th>
<th>0.1cc./80</th>
</tr>
</thead>
<tbody>
<tr>
<td>LaCl₃</td>
<td>Coagulated</td>
<td>Opalescent</td>
<td>Bluish clear</td>
<td>Opalescent and turbid</td>
<td>Milky</td>
<td>Coagulated</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Na₂Fe(CN)₅</td>
<td>Very opaque</td>
<td>Slight opalescence</td>
<td>Clear</td>
<td>Increasing opalescence</td>
<td>Milky</td>
<td>Coagulated</td>
<td></td>
<td></td>
</tr>
<tr>
<td>BaCl₂</td>
<td>Coagulated</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CaCl₂</td>
<td>Coagulated</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Na₂SO₄</td>
<td>Coagulated</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NaCl</td>
<td>Coagulated</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

TABLE VII.

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.

<table>
<thead>
<tr>
<th>cc. 0.1 N HCl in 10 cc. of 0.2 per cent albumin</th>
<th>0</th>
<th>0.01</th>
<th>0.02</th>
<th>0.03</th>
<th>0.04</th>
<th>0.05</th>
<th>0.1</th>
<th>0.2</th>
<th>0.4</th>
<th>0.8</th>
<th>1.6</th>
<th>3.2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Appearance of solution</td>
<td>Coagulated</td>
<td>Very opaque</td>
<td>Very opalescent</td>
<td>Clear but slightly opalescent</td>
<td>Very clear, like water</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
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 to room temperature and the appearance of the solution was noticed. Table VII gives the result.

**TABLE VII.**

*Heat Coagulation of Crystalline Egg Albumin.*

<table>
<thead>
<tr>
<th>pH</th>
<th>NaCl</th>
<th>Concentration</th>
<th>P.D. in millivolts</th>
</tr>
</thead>
<tbody>
<tr>
<td>11.0</td>
<td>0 to m/2</td>
<td>24 to 9.</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0 to m/512</td>
<td>31 to 12.5</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0 to m/8</td>
<td>31 to 12.</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0 to m/8</td>
<td>29 to 9.5</td>
<td></td>
</tr>
<tr>
<td>5.8</td>
<td>NaCl</td>
<td>m/16 and above</td>
<td>5 and less.</td>
</tr>
<tr>
<td></td>
<td>m/512 to 1 m.</td>
<td>5 and less.</td>
<td></td>
</tr>
<tr>
<td></td>
<td>m/16 to m/2.</td>
<td>7 and less.</td>
<td></td>
</tr>
<tr>
<td>4.0</td>
<td>NaCl</td>
<td>m/1024 to 1 m.</td>
<td>8 and less.</td>
</tr>
<tr>
<td></td>
<td>m/64 to 2 m.</td>
<td>11 and less.</td>
<td></td>
</tr>
<tr>
<td></td>
<td>m/128 to 2 m.</td>
<td>13 and less.</td>
<td></td>
</tr>
<tr>
<td></td>
<td>m/4</td>
<td>11 and less.</td>
<td></td>
</tr>
<tr>
<td>3.0</td>
<td>NaCl</td>
<td>m/32</td>
<td>13.5 m/4</td>
</tr>
<tr>
<td></td>
<td>m/32</td>
<td>14 m/8 to 2 m.</td>
<td>8 and less.</td>
</tr>
<tr>
<td></td>
<td>m/32</td>
<td>13 m/4</td>
<td></td>
</tr>
<tr>
<td></td>
<td>m/512</td>
<td>10 m/128</td>
<td>8 and less.</td>
</tr>
<tr>
<td>4.8</td>
<td>NaCl</td>
<td>m/2,500 to m/26</td>
<td>At all concentra-</td>
</tr>
<tr>
<td></td>
<td>m/2,500 to m/20</td>
<td>13 to 11.</td>
<td>tions. About 3 or</td>
</tr>
<tr>
<td></td>
<td>m/2500 to m/20</td>
<td>30 to 14.</td>
<td>less.</td>
</tr>
</tbody>
</table>

When the 10 cc. contained 0.01 cc. of 0.1 N HCl the protein remained practically isoelectric (pH 4.8), the P.D. remained below that of the critical point, and hence flocculation occurred upon heating. When 0.02 cc. of 0.1 N HCl was added, coagulation no longer occurred,
but enough particles could coalesce because the P.D. was not very high. Only when 0.05 cc. or more acid was added did the cataphoretic P.D. become high enough to keep the solution clear on boiling. When the concentration of acid was sufficiently increased so as to bring the cataphoretic P.D. down again below the critical value, flocculation occurred again.\textsuperscript{12}

Table VIII compares the maximal concentrations of salts at which solutions of genuine crystalline egg albumin no longer flocculate upon heating to 90\textdegree C. and the minimum concentrations required for flocculation. The table also gives the cataphoretic P.D. of denatured particles of white of egg at these concentrations.

VII.

Proteins as Protective Colloids.

In his experiments on anomalous osmosis the writer showed that when collodion membranes are filled with a 1 per cent solution of a protein, such as gelatin, crystalline egg albumin, casein, or oxyhemoglobin, there is formed over night inside the membrane a durable film of solid protein which cannot be washed away even if the interior is rinsed out as often as ten or twenty times with warm water.\textsuperscript{4} This film betrays itself by its color in the case of oxyhemoglobin. The forces which make the film adhere to the collodion must be very strong, but they do not depend upon the ionization of the protein, since the films are formed no matter whether the protein is at the isoelectric point, or whether it is on the alkaline or on the acid side of the isoelectric point. The forces which cause the film formation must be those forces of secondary valency responsible for phenomena of adhesion and cohesion in general.

This film formation is responsible for the so called protective action of certain colloids. Zsigmondy and his collaborators showed that

\textsuperscript{12} Some of these results had been discussed in a preceding paper (\textit{J. Gen. Physiol.}, 1921–22, iv, 759) on the assumption that the double electrical layer is determined by the membrane equilibrium. Since it seems that membrane potentials and cataphoretic potentials are not identical and since the stability of suspensions seems to depend on cataphoretic potentials, it seemed necessary to interpret these phenomena on the basis of cataphoretic potentials.
suspensions of colloidal gold, which were precipitated by low concentrations of salts, were protected against such precipitation when the gold particles were suspended in a gelatin solution; and to make the work quantitative he introduced the term gold number, defining it as that number of milligrams of protective substance which is just sufficient to prevent a definite degree of agglutination of the gold particles caused by the addition of 1 cc. of 10 per cent NaCl to 10 cc. of suspension of gold particles.\textsuperscript{14}

Gelatin was found to be especially active as a protective colloid, while egg albumin—which Zsigmondy did not purify by crystallization—had little protective action. The experiments reported in this paper give an explanation why gelatin is a good protective colloid and why crystalline egg albumin is not. Suspensions of collodion particles not treated with protein are precipitated by low concentrations of salt because the particles are kept in suspension only by virtue of their double electrical layers, the P.D. of which is brought below the critical value by comparatively low concentrations of salts. When collodion particles are put into a solution of gelatin, a gelatin film is formed at the surface, the molecules of which retain the high affinity of gelatin for water, and this affinity is not destroyed by even very high concentrations of salts. Consequently the stability of the suspension of gelatin-coated collodion particles no longer depends on the double electrical layer which determined the stability of the collodion particles before they were coated with protein, and which is reduced below the critical value by relatively low concentrations of salts.

When genuine crystalline egg albumin forms a film around a collodion particle, the albumin loses its high affinity for water and behaves like denatured egg albumin, inasmuch as its affinity for water molecules is considerably diminished. Collodion particles coated with egg albumin depend therefore chiefly on the electrical double layer surrounding each particle and hence will be precipitated by low concentrations of salts.

If we take the effects of the hydrogen ion concentration into consideration, we can, however, single out certain cases where even a

\textsuperscript{14} Zsigmondy, R., Kolloidchemie, Leipsic, 1918, 173, 358.
film of crystalline egg albumin has some protective action on suspensions of collodion particles. Thus, a low concentration of LaCl₃ (about \( \frac{m}{4,000} \)) suffices to flocculate a suspension of collodion particles free from protein at pH 4.0 or 3.0. When, however, the particles are coated with egg albumin or casein, the concentration of LaCl₃ required for that purpose is much higher, about \( \frac{m}{32} \) or even higher; since at the pH mentioned, the particles are positively charged and the P.D. is diminished by the Cl ion instead of by the La ion. A second protective effect is noticed at pH 4.0 or pH 4.7 in high concentrations of CaCl₂, \( \frac{m}{2} \) or somewhat higher, when the particles are coated with casein or albumin.

Casein has generally little or no protective action, since it will be shown in a subsequent paper that the stability of casein-coated collodion particles depends on the cataphoretic P.D. which is depressed below the critical value by low concentrations of salts. In certain cases, however, salts, especially CaCl₂ in high concentrations, can keep the particles in suspension even if the P.D. is zero.

Experiments with edestin showed that it is of very little use as a protective colloid.

These experiments permit us to define the conditions for a general protective action of colloids, such as that by gelatin. Protective colloids must first be capable of forming durable films on the surface of the particles to be protected, and, second, the molecules constituting the film must have a higher attraction for the molecules of the solvent (e.g., water) than for each other; in other words, they must possess true crystalloidal solubility. Those who refuse to believe that proteins may form true solutions will find it difficult to explain the mechanism of the protective action of such colloids as gelatin.

We have no idea of the mechanism whereby a protein can act as an antigen; but it is, perhaps, worth while to point out that gelatin which forms films with a high affinity for water is no antigen, while crystalline egg albumin, casein, and edestin, which form films with practically no affinity for water, are good antigens. It is, however, quite possible that the coincidence is merely accidental.
VIII.

SUMMARY.

1. It is shown that the concentrations of different salts required to precipitate suspensions of gelatin-coated collodion particles in water are practically identical with the concentrations of the same salts required for the "salting out" of gelatin from aqueous solutions. Neither effect shows any relation to the electrical double layers surrounding the particles.

2. It is shown that at the isoelectric point of gelatin, suspensions of gelatin-coated collodion particles are not stable and it had been shown previously that gelatin is least soluble at the isoelectric point. The addition of salt increases both the solubility of gelatin in water as well as the stability of suspensions of gelatin-coated collodion particles in water, and both effects increase with the valency of one of the ions of the salt.

3. This latter effect is not due to any charges conferred on the gelatin particles by the salts, since the cataphoretic experiments show that salts like NaCl, Na₂SO₄, or CaCl₂, which at the isoelectric point of gelatin increase the solubility of gelatin as well as the stability of suspensions of gelatin-coated collodion particles, leave the particles practically uncharged in the concentrations in which the salts are efficient.

4. It follows from all these facts that the stability of suspensions of gelatin-coated particles in water depends on the solubility of gelatin in water; e.g., on the chemical affinity of certain groups of the gelatin molecule for water.

5. Though crystalline egg albumin is highly soluble in water, the stability of collodion particles coated with crystalline egg albumin does not depend upon the affinity of the albumin molecule for water, but depends practically alone on the electrical double layer surrounding each particle. As soon as the p.d. of this double layer falls below 13 millivolts, the suspension is no longer stable.

6. The critical potential for the stability of suspensions of collodion particles coated with genuine egg albumin is the same as that for particles of boiled (denatured) white of egg. Since through the process of heating, egg albumin loses its solubility in water, it is inferred that
egg albumin undergoes the same change when it forms a film around a solid particle like collodion.

7. The influence of electrolytes on the stability of suspensions of collodion particles coated with casein or edestin was similar to that of collodion particles coated with egg albumin. The experiments are, however, complicated by the fact that near the isoelectric point CaCl₂ and even NaCl cause a suspension again at concentrations of about \( \frac{M}{2} \) or 1 M, while still higher concentrations may cause a precipitation again. These latter effects have no connection with double layers, but belong probably in the category of solubility phenomena.

8. These experiments permit us to define more definitely the conditions for a general protective action of colloids. Protective colloids must be capable of forming a durable film on the surface of the suspended particles and the molecules constituting the film must have a higher attraction for the molecules of the solvent than for each other; in other words, they must possess true solubility. Only in this case can they prevent the precipitating action of low concentrations of electrolytes on particles which are kept in suspension solely by the high potentials of an electrical double layer. Thus gelatin films, in which the attraction of the molecules for water is preserved, have a general protective action, while crystalline egg albumin, casein, and edestin, which seem to lose their attraction for water when forming a film, have a protective action only under limited conditions stated in the paper.