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The Influence of Electrolytes on the Solution and Precipitation of Casein and Gelatin

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THE INFLUENCE OF ELECTROLYTES ON THE SOLUTION AND PRECIPITATION OF CASEIN AND GELATIN.

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I. INTRODUCTION.

The modern concepts of colloid chemistry originated largely from a study of the precipitation of colloids by electrolytes, and this accounts, perhaps, for the fact that the tendency to form aggregates was considered the chief characteristic of colloids in solution. As a consequence it is assumed in the text-books of colloid chemistry that the ultimate units of a colloid in solution are not isolated molecules or ions but larger aggregates of molecules or ions, the so called micella (small crumbs) of Naegeli, which are supposed to be kept in a stable solution or suspension through forces of repulsion due to the fact that they are electrically charged. The precipitation of the particles by electrolytes is ascribed to a diminution of the charge of the micella through the adsorption of the ions of the electrolyte. It was noticed by Hardy that the active or precipitating ion of the electrolyte was always that ion which had the opposite sign of charge as the colloidal particle, and Picton and Linder had noticed that the precipitating efficiency of ions increased rapidly with their valency.[1]

The experiments published by one of us have led to results which are compatible with some but not all of the assumptions just enumerated. In the first place, experiments on the osmotic pressure and the viscosity of protein solutions have made it probable that the ultimate units in certain protein solutions, such as crystalline egg albumin, are essentially isolated protein ions or molecules, though such solutions may, in a secondary way, also contain aggregates of ions or molecules.[2]

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[1] Zsigmondy, R., Kolloidchemie, Leipsic, 2nd ed., 1918.

[2] Loeb, J., *J. Gen. Physiol.*, 1920–21, iii, 827; 1921–22, iv, 73, 97.

It was, secondly, found that proteins combine with acids and bases according to the stoichiometrical laws of classical chemistry, and that there is neither any need nor, in fact, any room for the assumption that the ions of electrolytes are adsorbed by the protein particles in solution.[3] Thirdly, the electrical charges of protein particles which occur in such solutions are due chiefly, if not exclusively, to either the electrolytic dissociation of protein salts or to membrane potentials caused by the Donnan equilibrium.[4] Fourthly, the influence of electrolytes on the P.D., the osmotic pressure, and the viscosity of protein solutions[5] and the swelling of gels[6] can be explained quantitatively on the basis of the Donnan equilibrium. Under these circumstances it became necessary to find out whether or not the influence of electrolytes on the precipitation of proteins can be harmonized with these recent results.

All the workers who have studied the influence of electrolytes on the precipitation of colloids have come to the conclusion that there are two distinct groups of phenomena. In the one group the precipitation requires high concentrations of an electrolyte, while in the second, a low concentration of electrolyte suffices for precipitation. The difference between these two cases is so striking that it has been used for a classification of colloids. Thus, according to Zsigmondy, Noyes[7] discriminates between "colloidal solutions" and "colloidal suspensions;" the "colloidal solutions" being characterized by a high degree of viscosity, by a tendency to gelatinize, and by the fact that they are not easily precipitable by electrolytes; while the "colloidal suspensions" are, according to Noyes, characterized by a low order of viscosity, by a lack of tendency to gelatinize, and by the fact that they are easily precipitated by electrolytes.[8]

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[3] Loeb, J., *Science*, 1920, lii, 449.

[4] Loeb, J., *J. Gen. Physiol.*, 1920–21, iii, 667.

[5] Loeb, J., *J. Gen. Physiol.*, 1920–21, iii, 557, 667, 691, 827; 1921–22, iv, 73, 97.

[6] Procter, H. R., *J. Chem. Soc.*, 1914, cv, 313. Procter, H. R., and Wilson, J. A., *J. Chem. Soc.*, 1916, cix, 307.

[7] Noyes, A. A., quoted in Zsigmondy, R., Kolloidchemie, Leipsic, 2nd ed., 1918, 29.

[8] Different authors have introduced different terms for these two groups of colloids, Perrin speaks of hydrophilic and hydrophobic colloids; Freundlich of lyophilic and lyophobic, and Wo. Ostwald of emulsoids and suspensoids.

Of all the characteristics for the two groups mentioned by Noyes, only one is essential, namely, the difference in the ease with which the two groups are precipitated by electrolytes, while the two other differences mentioned by him are only accidental. Thus a solution of crystalline egg albumin requires at ordinary temperature a high concentration of electrolyte for precipitation, although the viscosity of such a solution is low and although the solution has no tendency to gelatinize; while at a sufficiently high temperature, low concentrations of electrolytes will precipitate crystalline egg albumin though the viscosity of the solution is now high and though the solution has now a tendency to gelatinize. Hence only one of the differences mentioned in Noyes' definition remains; namely, the difference in the relative concentration required to precipitate colloids from their solution or their suspension.

The discrimination between the two types of precipitation according to whether high or low concentrations of electrolytes are required, is, however, essential and it becomes our first problem to account for this difference. We shall see that it is accompanied by another difference. When high concentrations of electrolytes are required for the precipitation of proteins the sign of charge of the protein particles is of little significance. Sulfates are much more effective for the salting out of gelatin or crystalline egg albumin from their watery solution than chlorides, regardless of whether the solution of the protein is at the isoelectric point or on the acid or alkaline side of it.

When low concentrations suffice for precipitation, the sign of charge of the protein ions becomes of paramount importance. On the acid side of the isoelectric point the active ion of the precipitating salt is the anion, while on the alkaline side it is the cation. It happens that this is also true for the Donnan effect and that in the Donnan effect also, low concentrations of electrolytes suffice for the depressing effect of a salt. This suggests the possibility that precipitation of the second group of colloids, *i.e.*, where low concentrations are required, is in some way connected with the Donnan equilibrium; and that Hardy's rule is only the consequence of this fact; while where high concentrations of electrolytes are required for precipitation the forces determining the process have no connection with the Donnan equilibrium.

In order to gain more definite information concerning the nature of the forces involved in the two cases it seemed advisable to supplement these investigations by a study of the mechanism of the solution of proteins. We have studied the mechanism of solution of granules of isoelectric casein in acid and in alkali and find that this mechanism is entirely different in the two cases. The solution of casein chloride is controlled by forces connected with the Donnan equilibrium; and it also happens that casein chloride when in solution can be precipitated by low concentrations of electrolytes; *e.g.*, M/8 NaCl. On the other hand, the solution of sodium caseinate is apparently controlled by the forces of chemical attraction between water and certain groups of the casein molecule; and it happens that very high concentrations of electrolytes, *e.g.*, $2\frac{1}{2}$ M NaCl or $3\frac{1}{4}$ M LiCl, may not be sufficient for precipitation.

II. The Precipitation and Solution of Casein Chloride.

1 per cent solutions of casein chloride of pH 2.2 were prepared in different concentrations of salts in water of about the same pH. That concentration was determined which causes an almost instantaneous complete precipitation of the protein from the originally milky solution so that the supernatant liquid became as clear as water. These concentrations were as follows:

NaCl	about M/8
$NaNO_3$	about M/8
$CaCl_2$	about M/8
Na trichloracetate	about M/16
Na_2SO_4	about M/32

Though the results are only semiquantitative, the validity of Hardy's rule and the valency effect are easily recognizable. Beside these two effects, some constitutional effects of the anion (such as the trichloracetate) may exist. It is also obvious that the concentrations of electrolytes required for instantaneous, complete precipitation of casein chloride are considerably lower than those required for the precipitation of Na caseinate from their watery solution, so that we can be sure that in the case of casein chloride we are dealing with a representative of the "suspension" group of colloids, in the sense of Noyes.

It can be shown that the solution of the casein chloride depends on forces regulated by the Donnan equilibrium and that the rule of Hardy is in this case at least only a consequence of this fact. This can be proven by microscopic observation of the mechanism of the solution of solid particles of originally isoelectric casein in solutions of acids of different concentration. It was found that the particles of casein swell in a solution of HCl, becoming more and more transparent the more they swell, and that when the swelling has reached a certain stage, the particles disappear—they are dissolved. When in the swollen stage, slight agitation may make them fall apart. T. B. Robertson had suggested such a mechanism for the solution of Na caseinate,[9] but it was found that the mechanism of solution in this latter case is different. There is no doubt, however, that the swelling of casein particles is a necessary prerequisite for the solution of casein-acid salts, since such particles are only dissolved when their swelling exceeds a definite limit.

The method of procedure was as follows: A small number of granules of isoelectric casein of the same size (going through a sieve with mesh 100 but not through a sieve with mesh 120) were put into 50 cc. of water containing different quantities of different acids and kept at 24°C. In various intervals, *i.e.*, after 15, and 60 minutes, and 6, and 24 hours, the diameter of about 15 grains was measured with a micrometer in a microscope and the average diameter calculated. The particles were not stirred, and care was taken to avoid their breaking into smaller fragments. The averages after 1 hour are plotted in Fig. 1. The abscissæ are the logarithms of the concentrations of acid of the watery solution, the ordinates are the average diameters of the particles. It is obvious that the average diameter of the particles increases at first with the increase of the concentration of the acid, reaching a maximum at about pH 2.0 of the outside solution, and with a further increase in the concentration of the acid the swelling becomes less again.

Fig. 2 gives the measurements of the same particles after 24 hours. At this time all the particles in the region of greatest solubility for HCl and for H_3PO_4, *i.e.*, between pH of the outside solution of 1.8 and 2.9, had completely dissolved and could no longer be measured.

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[9] Robertson, T. B., The physical chemistry of the proteins, London, Bombay, Calcutta, and Madras, 1918, 275 ff.

Figs. 1 and 2 show another fact; namely, that the rate of swelling is not the same in different acids. It is about the same in HCl and H_3PO_4 (for the same pH) but decidedly less in HNO_3 and still less in H_2SO_4 and trichloracetic acid. It was found that the rate of solution of casein in these different acids followed closely the rate of swelling. It took longer to dissolve casein in HNO_3 than it did in HCl (at 20°C.); and the casein was practically insoluble in H_2SO_4 and trichloracetic acid in 24 hours.

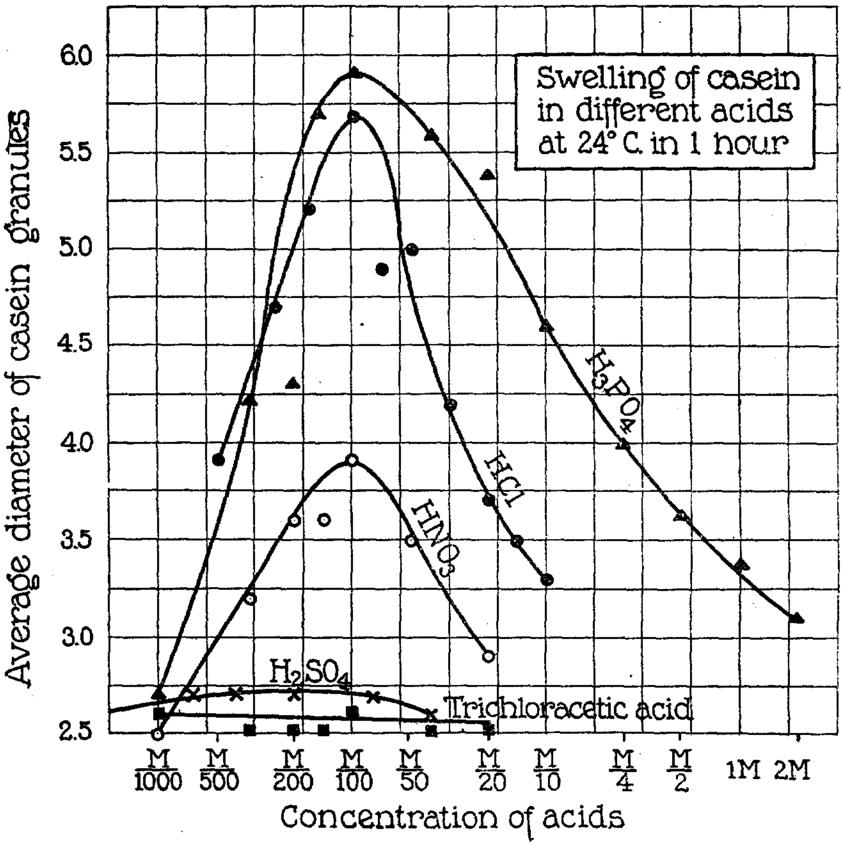

FIG. 1. Relative swelling of isoelectric granules of casein when put into acids of different concentrations, at 24°, after 1 hour. The hydrogen ion concentration of the casein particles is less than that of the outside solution on account of the Donnan equilibrium. The latter concentrations are plotted as abscissæ. The ordinates are the relative average diameters of the granules.

The rate of swelling is a function apparently not only of the Donnan equilibrium, but also of the force of cohesion between the particles. Procter and Wilson[6] have suggested that the rapid increase of swelling of solid gelatin with a rise in temperature is due to a corresponding diminution of cohesion between the molecules of gelatin with rising temperature. The influence of the anion of gelatin-acid salts on the cohesion of the particles of a solid gel is apparently much less than the influence of the anion on the cohesion of casein particles. The

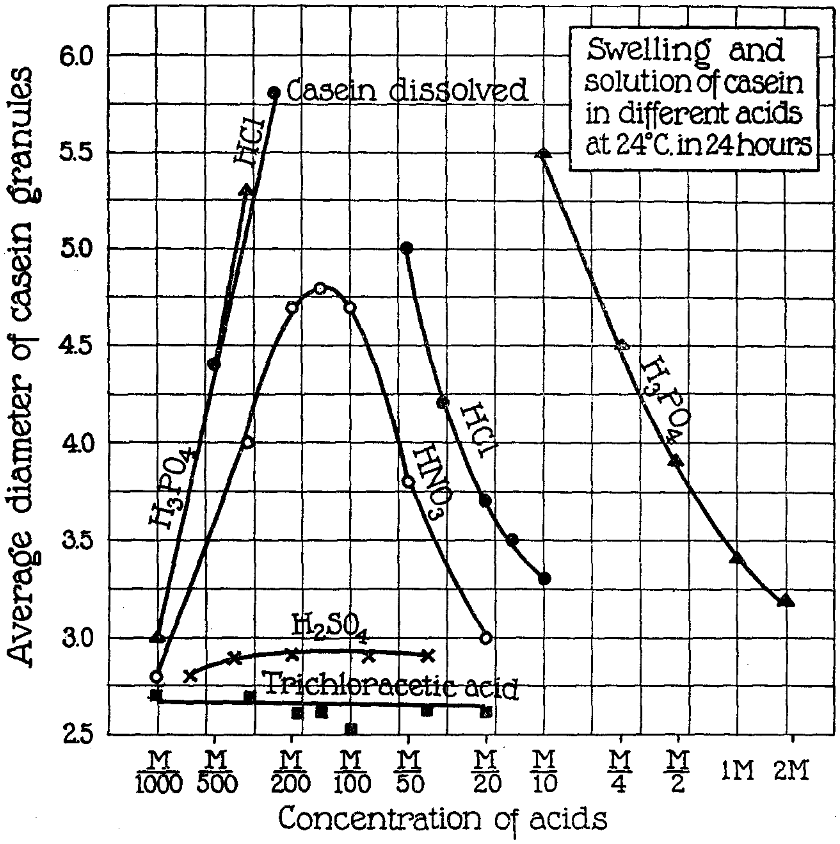

FIG. 2. Swelling of the same granules after 24 hours. Where the swelling exceeds a certain limit, between 5 and 6 in our scale, the particles are dissolved, thus showing that swelling is of importance in the mechanism of solution of casein chloride.

forces of cohesion in the case of casein sulfate and casein trichloracetate seem to be so great that they cannot be overcome by the osmotic pressure due to the Donnan equilibrium; and hence no swelling (and as a consequence no solution) of solid casein is possible in H_2SO_4 or trichloracetic acid.

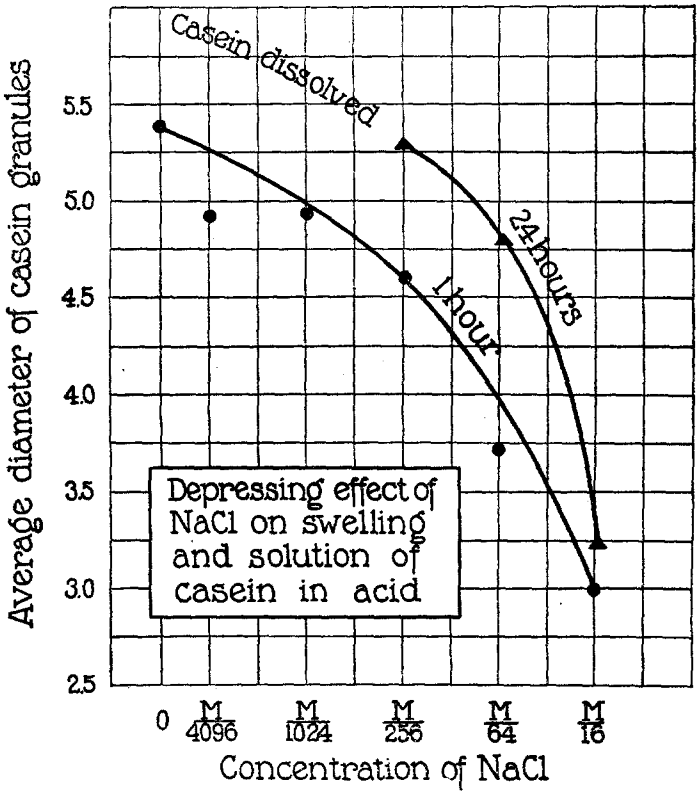

FIG. 3. Depression of swelling of casein particles in M/100 HCl by the addition of NaCl. A low concentration of acid suffices already for a noticeable depression of the swelling. The lower curve represents swelling after 1 hour; the upper curve swelling after 24 hours. Solution of the swollen particles occurs when the average diameter of the particles exceeds the value 5 to 6 in our scale.

Procter and Wilson have shown that the theory of the Donnan equilibrium explains the depressing effect of a salt on the swelling of solid gelatin.[6] Microscopic measurements of the influence of NaCl on the rate of swelling of individual grains of casein particles in M/100 HCl were made at 24°C. and the results plotted in Fig. 3.

The ordinates are the average diameters of the particles after 1 and 24 hours respectively. The abscissæ are the concentrations of NaCl. The depressing effect is similar to that found in the case of the swelling of a jelly of gelatin. After 24 hours the particles had dissolved in the NaCl solutions of a concentration of below M/256, but not in concentrations of NaCl higher than M/256.

That the solution of casein chloride is thus regulated to a large extent by the Donnan effect was ascertained also by measurements of the quantity of casein chloride dissolved at 20°C. at various pH of the solution. 1 gm. of isoelectric powdered casein was put into 100 cc. of solutions of HCl of different concentration and kept in these solutions in one case for 1 hour, in a second case for 22 hours. The mass was then poured into graduated cylinders and the undissolved part was allowed to settle to the bottom for 2 and for 6 hours respectively at 20°C. The supernatant liquid was removed and the sediment dried over night in an oven at about 100°C. Table I gives the result. The dry weight of 1 gm. of isoelectric casein was found to be 0.870 gm. and this weight diminished by the dry weight of the sediment was the amount dissolved. Table I shows that, the rate of solution increases with diminishing pH from 4.4 to 2.18 where the solubility of casein chloride is a maximum; with a further decline in pH the solubility diminishes again. This is in agreement with the Donnan effect.

TABLE I.

Amount of Casein Dissolved at 20°C in HCl of Different pH.

pH	4.36	3.32	3.11	2.97	2.94	2.84	2.75	2.64	2.53	2.36	2.18	2.06	1.87	1.66	1.50	1.40
Mg. dissolved after 1 hour................	42	55	86	249	265			348	408		547	538	401	366	272	219
Mg. dissolved after 22 hours..............	102	133	164	267	342	459	536	634	646	733	788	779	710	528	374	300

In a similar way the depressing effect of NaCl on the rate of solution of casein chloride was ascertained. Solutions of 12.5 cc. of 0.1 N HCl in 100 cc. and containing 1 gm. of powdered, originally isoelectric casein were prepared in 0, M/2048, M/1024, to M/4 NaCl. The pH of a solution of 1 gm. casein in 100 cc. containing 12.5 cc. of 0.1 N HCl was 2.12 and this pH was the same in all solutions made up in NaCl.

The solution was kept at 20° for 16 hours and then allowed to settle for 24 hours at 20° in 100 cc. graduate cylinders. The dry weight of the sediment was determined and this weight when deducted from the dry weight of 1 gm. isoelectric casein, namely, 0.870 gm., was the amount that had gone into solution after a correction was made for the free NaCl held in 2 cc. solution which was arbitrarily assumed not to have been removed. Though this latter correction was somewhat arbitrary, it could have caused a noticeable error only when the concentration of the salt solution exceeded M/64. For the solutions of M/64 and below this error was negligible. Table II gives the number of milligrams of casein which had gone into solution.

TABLE II.

	Concentration of NaCl.					
	M/2048	M/1024	M/512	M/256	M/128	M/64
Mg. dissolved...............	714	685	665	615	449	282

The main fact is that a slight increase in the concentration of NaCl causes already a noticeable drop in the rate of solution. Thus M/1024 NaCl causes already a noticeable diminution in the solubility of a 1 per cent solution of casein chloride of pH 2.12 at 24°C.

These observations then indicate that the solution of solid particles of casein chloride is brought about by the ultimate elements being forced apart mechanically through the process of swelling. The force acting in this swelling is the hydrostatic pressure of the water which is forced into the interstices of the solid particles by the osmotic pressure in the interstices between the casein ions. Procter and Wilson have shown that the application of Donnan's theory of membrane equilibrium accounts quanitatively for this swelling on the assumption that swelling is caused by the excess of the osmotic pressure inside the gel over that of the surrounding solution. As soon as the osmotic pressure in the gel exceeds the forces of cohesion between the casein ions of the gel the casein ions constituting the gel are separated.

The question then arises: How can the Donnan effect stabilize the particles of casein chloride in solution, and how can we explain the precipitating effect of low concentrations of neutral salts? Let us assume that the ultimate particles in a solution of casein chloride of pH 2.2 are, (*a*) isolated casein ions, (*b*) isolated casein molecules, and (*c*) small casein aggregates or micella. The Donnan equilibrium furnishes two kinds of forces preventing that degree of coalescence of these particles which is required for precipitation; namely, the osmotic pressure and the membrane potentials. When isolated protein ions collide and remain attached to form a micellum, a Donnan equilibrium is established between the nascent micellum and the surrounding solution. The Donnan equilibrium demands that there be a higher concentration of electrolytes inside than outside and this difference in osmotic pressure leads to water being attracted into the micellum. The increase in hydrostatic pressure will force the protein molecules apart again and thus tends to prevent the formation of the micellum. Moreover, if micella exist in the casein chloride solution (aside from isolated casein ions and molecules) the coalescence of different micella into larger aggregates must be prevented by the potential difference between the micella and the surrounding solution. J. A. Wilson suggested in 1916[10] that the source of the charges might be the Donnan equilibrium, and one of us has recently shown that these potential differences between a gel and the surrounding solution, which Donnan's theory demands and which Wilson postulated, actually exist.[11] As a consequence of this P.D., the micella are charged and must repel each other according to the charge. This charge caused by the Donnan equilibrium is a minimum at the isoelectric point, rises with increasing hydrogen ion concentration, reaching a maximum, and diminishes again with a further increase in hydrogen ion concentration as shown in a preceding paper. The osmotic pressure and charge are also diminished by the addition of salt.

Hardy's rule that only that ion of a neutral salt is active in precipitation which has the opposite sign of charge as the colloidal ion, and that the efficiency of the ion increases with the valency is simply the expression of the Donnan effect, as is also the fact that very low concentrations of electrolytes suffice for precipitation. The reader will notice that it is unnecessary to assume that the ions

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[10] Wilson, J. A., *J. Am. Chem. Soc.*, 1916, xxxviii, 1982.

[11] Loeb, J., *J. Gen. Physiol.*, 1920–21, iii, 557, 667.

are adsorbed by the casein and that this adsorption of ions annihilates the electrical charges on the particles of casein.

Emulsions of oil drops in water are prevented from coalescing by their electrical charges (and not by osmotic forces). The investigations of Beutner[12] have shown that the P.D. at the boundary of water and substances immiscible in water is determined by an unequal distribution of crystalloidal ions between the two phases, and the writer ventures to suggest that this distribution may be regulated by Donnan's theory, owing to the fact that, *e.g.*, the oleic acid anion in oil cannot diffuse into the water. The similarity of the behavior of the P.D. between oil and water and between solid gelatin and water is rather striking. The depression of the charge on the particles through the addition of salt can in both cases be explained without the adsorption hypothesis.

III. The Precipitation and Solution of Na Caseinate.

When we prepare solutions of Na caseinate of pH 11.0 containing 1 gm. of originally isoelectric casein in 100 cc., we notice that it requires enormous concentrations of NaCl or LiCl to precipitate the casein. NaCl as concentrated as $2\frac{1}{2}$ M and LiCl as concentrated as $3\frac{1}{4}$ M were not able to precipitate Na caseinate from its solution, while M/8 NaCl was sufficient for instantaneous and complete precipitation of casein chloride from its solution. Hence Na caseinate belongs to the other group of colloidal solutions; namely, those which require high concentrations of electrolytes.

When a grain of isoelectric casein is put into a solution of NaOH Na caseinate is formed at the surface of the solid granule. This dissolves in water, as any crystalloidal substance does (*i.e.*, by the forces of residual valency), except for the accidental fact that the solution of Na caseinate seems to lower the surface tension of the watery solution at the interface, as is suggested by the rather violent spreading of the Na caseinate solution at the interface, which can be observed under the microscope. As a result projecting particles at the surface of the granule are torn away from the surface of the solid casein, collect-

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[12] Beutner, R., Die Entstehung elektrischer Ströme in lebenden Geweben, Stuttgart, 1920.

ing at a short distance, while the originally rough surface of the grain of casein becomes smooth. These phenomena of solution accompanied by spreading occur soon all over the surface. The small particles of casein carried away in the spreading motion are gradually completely dissolved and the result is a solution of Na caseinate of a high osmotic pressure, indicating that the solution consists to a large extent of isolated protein ions.

The mechanism of the solution of casein in NaOH is essentially that of the solution of a crystal except for the purely accidental and secondary phenomenon that the surface tension of the Na caseinate solution is considerably lower than that of a watery NaOH solution, and that thus the orderly process of solution noticeable in the case of crystals is disturbed by phenomena of spreading. The mechanism of solution of grains of solid casein in NaOH resembles the solution of sodium oleate in water, which is also accompanied by a lowering of the surface tension at the interface. This interference of the phenomena of spreading in the process of solution of Na caseinate may account for the fact that Robertson[9] was unable to confirm in this case the solution theory of Noyes and Whitney,[13] according to which there exists at the boundary of crystals and water a film of solution which is always saturated, the velocity of solution being determined by the rate of diffusion of the dissolved crystalloid out of this film into the body of the fluid. It is natural that this theory should be masked whenever the process of solution is accompanied by phenomena of spreading due to a lowering of surface tension at the interface. The forces dragging the particles of Na caseinate into solution are to all appearances those forces of residual valency between certain groups of the molecule of Na caseinate and water which also cause the solution of crystalloids in water, while the forces dragging the particles of casein chloride into water are forces which have their origin in the Donnan equilibrium. A quotation from Langmuir will illustrate the rôle which active groups of a complex molecule may play in the process of true solution.

> "Acetic acid is readily soluble in water because the COOH group has a strong secondary valency by which it combines with water. Oleic acid is not soluble because the affinity of the hydrocarbon chains for water is less than their affinity

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[13] Noyes, A. A., and Whitney, W. R., *Z. physik. Chem.*, 1897, xxiii, 689.

> for each other. When oleic acid is placed on water the acid spreads upon the water because by so doing *the COOH can dissolve in the water* without separating the hydrocarbon chains from each other.
>
> "When the surface on which the acid spreads is sufficiently large the double bond in the hydrocarbon chain is also drawn onto the water surface, so that the area occupied is much greater than in the case of the saturated fatty acids.
>
> "Oils which do not contain active groups, as for example pure paraffin oil, do not spread upon the surface of water."[14]

That the solution of isoelectric casein in NaOH has no connection with the Donnan equilibrium is also evidenced by the fact that there is no point in the concentration of NaOH where further increase in the concentration of NaOH lowers the rate of solution, as would be the case if the Donnan equilibrium influenced this phenomenon.

The stability of solutions of this kind is guaranteed by the forces of chemical attraction between certain groups of the molecule of Na caseinate and water; and the precipitation by electrolytes is due to a diminution of these forces.

IV. Precipitation and Solution of Gelatin in Water.

Solutions of gelatin in water require enormous concentrations of salts for precipitation and it is very probable that the forces causing solution are the forces of residual valency discussed in the case of the solution of Na caseinate. These cases have no connection with the Donnan equilibrium, and this is borne out by the well known fact that solutions of gelatin are always more readily salted out by sulfates than by chlorides, regardless of the pH of the gelatin solution. This is illustrated by Table III.

0.8 per cent solutions of gelatin were prepared at three different pH; namely, 4.7 (isoelectric gelatin), 3.8 (gelatin chloride), and 6.4 to 7.0 (Na gelatinate). The purpose was to find the molecular concentration of different salts, namely, $(NH_4)_2SO_4$, Na_2SO_4, $MgSO_4$, KCl, and $MgCl_2$, required for precipitation. Table III shows that regardless of the pH the sulfates are better precipitants than the chlorides. There is, therefore, no definite relation between the sign of charge of the colloidal particles and of the precipitating ion.

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[14] Langmuir, I., *J. Am. Chem. Soc.*, 1917, xxxix, 1850.

Precipitation experiments give no insight into the reason why sulfates are superior to chlorides in salting out; and an attempt was made to get an answer by experiments on the solution of gelatin. Such experiments gave the result that salts increase the solubility of isoelectric gelatin, and the more, the higher their concentration until finally a concentration is reached where the reverse effect is noticed. This reversal occurs at a comparatively low concentration in the case of the sulfates, while in the case of salts like $CaCl_2$ or $MgCl_2$ it does not seem to occur at all in the limit of the concentrations tried. The experiments were carried on at a temperature of 35°C.

Powdered gelatin of not too small a size of grain (going through sieve 30 but not through sieve 60) was rendered isoelectric in the way described in previous articles, and 0.8 gm. was put into 100 cc. of each of a series of solutions of NaCl, $CaCl_2$, or Na_2SO_4 varying in concentration from M/4096 to 2 M. The suspensions of the powdered gelatin were frequently stirred and the time required to practically completely dissolve all the grains of powdered gelatin in suspension at 35°C. was measured. We consider this time the reciprocal of the solubility. The ordinates in Fig. 4 are the solution times (*i.e.*, the reciprocal of the solubility) of isoelectric gelatin, and the abscissæ are the molecular concentrations of the salt used. It is obvious that NaCl and still more $CaCl_2$ increase the solubility (or diminish the solution time) of isoelectric gelatin in water, and the more, the higher the concentration of the salts. There exists a striking discontinuity in the Na_2SO_4 curve. As long as the concentration of Na_2SO_4 is below M/32 it increases the solubility of gelatin, and the more so, the higher the concentration. When, however, the concentration

TABLE III.

Minimal Molar Concentrations Required to Precipitate 0.8 Per Cent Solutions of Gelatin.

pH of gelatin solution.	Approximate molecular concentration of salt required for precipitation.				
	$(NH_4)_2SO_4$	Na_2SO_4	$MgSO_4$	KCl	$MgCl_2$
4.7	15/16 M	6/8 M	10/8 M	>3 M	>3 M
3.8 (gelatin chloride)	13/16 M	5/8 M	7/8 M	3 M	>3 M
6.4 to 7.0 (Na gelatinate)	16/16 M	7/8 M	9/8 M	>3 M	>3 M

of Na_2SO_4 is above M/32, a further increase in the concentration of Na_2SO_4 diminishes the solubility of gelatin, and the more so, the higher the concentration of Na_2SO_4. $(NH_4)_2SO_4$ acts in the same way. We now understand why it is that we cannot precipitate solutions of isoelectric gelatin with KCl or $MgCl_2$ in concentrations up to 3 M (see Table III) while we can precipitate such solutions with sulfates but only at concentrations above M/2; since our curve shows that at such high concentrations of sulfates the solubility of isoelectric gelatin in Na_2SO_4 becomes less than in pure water.

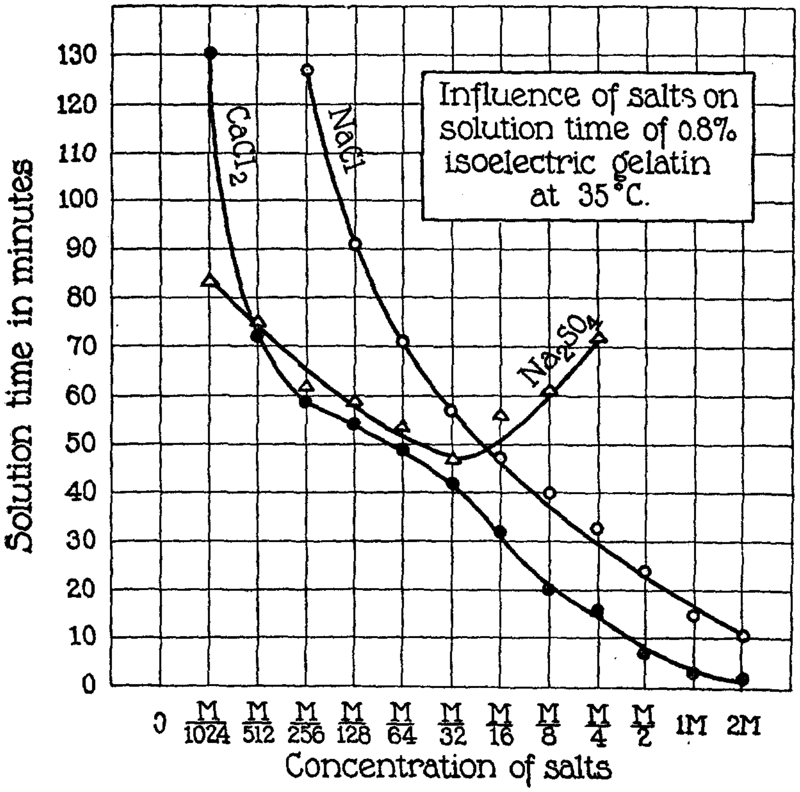

FIG. 4. Influence of salts on the time required to completely dissolve 0.8 gm. of powdered isoelectric gelatin in 100 cc. of salt solution of different concentrations at 35°C. Abscissæ are the concentrations of the salt; ordinates, the time required for complete solution.

While isoelectric gelatin is only sparingly soluble, gelatin salts are highly soluble. 0.8 gm. of powdered gelatin of pH of about 3.3 dissolves very rapidly in 100 cc. HCl of the same pH at 35°C. The addition of NaCl or $CaCl_2$ no longer increases the solubility, except when the concentration of $CaCl_2$ becomes greater than M/16. Na_2SO_4 abruptly diminishes the solubility at a concentration above M/4 and NaCl above a concentration of 1 M (Fig. 5).

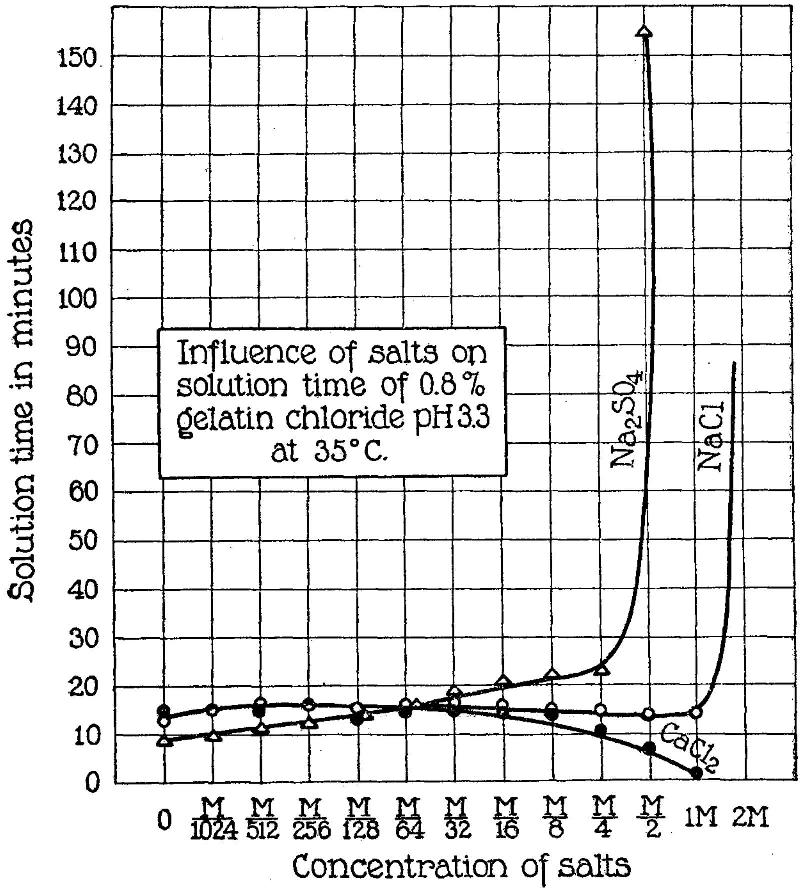

FIG. 5. Influence of salts on time required to dissolve 0.8 gm. of powdered gelatin chloride of pH 3.3 at 35°C.

Fig. 6 shows the influence of the three salts on the solution time of Na gelatinate of pH 10.5. Na_2SO_4 diminishes the solubility abruptly at a concentration above M/8, while both NaCl and $CaCl_2$ increase the solubility of Na gelatinate, NaCl in concentrations above M/2, and $CaCl_2$ in concentrations above M/16.

In all three cases, therefore, is the solubility of gelatin diminished by sulfates, but only exceptionally by chlorides. This explains the results contained in Table III.

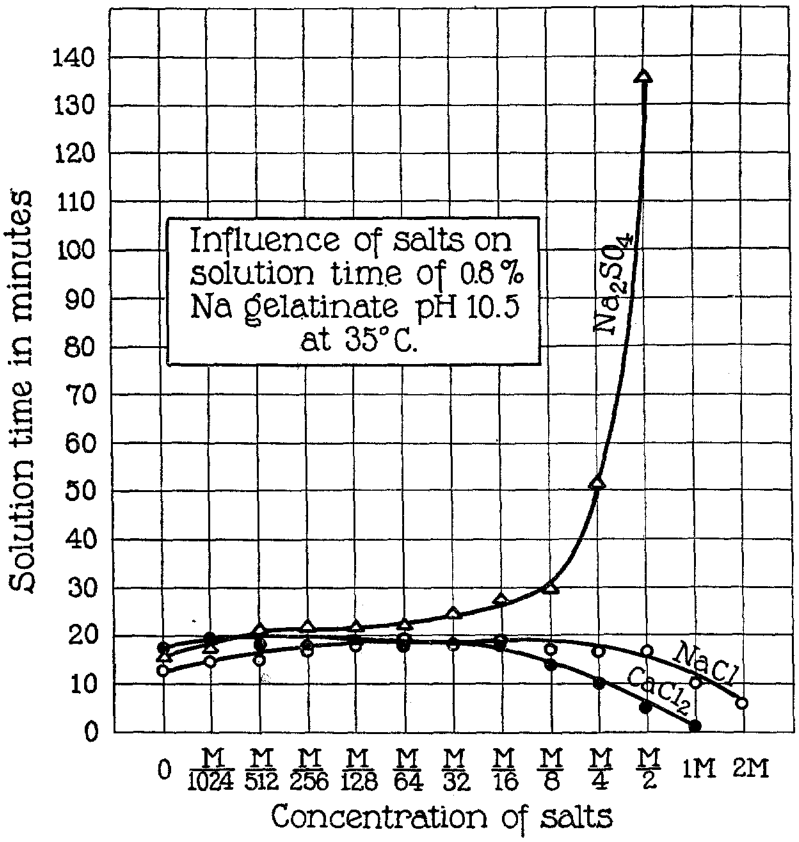

FIG. 6. Influence of salts on the time required to dissolve 0.8 gm. of powdered Na gelatinate of pH 10.5 at 35°C.

We have seen that addition of little acid to isoelectric gelatin increases the osmotic pressure, viscosity, P.D., and swelling with increasing concentration, while beyond a certain pH the addition of more acid has a depressing effect. This is characteristic of the

Donnan effect. It was of interest to find out whether such a maximum followed by a drop existed in the influence of acid on the solubility of gelatin, but this is not the case at least between pH 4.7 and 1.0. Measurements of the dry weight of gelatin dissolved in a certain time at different pH, showed that the amount of gelatin dissolved increases with the hydrogen ion concentration. This corroborates the conclusion that the solution (and precipitation) of gelatin in water is not influenced by forces governed by the Donnan equilibrium and does therefore not show the characteristics of colloidal behavior.

These experiments also contradict the suggestion that the solution of solid gelatin is necessarily preceded by swelling and that swelling and solution are continuous processes. The contradiction lies in the fact that swelling in acid reaches a maximum at pH of about 3.0 and then diminishes upon further increase in hydrogen ion concentration, while the rate of solution of solid gelatin granules continues to increase steadily when the hydrogen ion concentration increases beyond pH of 3.0. The mechanism of swelling and the mechanism of solution of solid gelatin in water are determined by forces of an entirely different character; the swelling by osmotic pressure, and the solution by the secondary valency forces responsible for the solution of crystalloids.

V. Solubility and Viscosity of Gelatin Solutions.

We assumed in a preceding paper that the increase in the viscosity of gelatin solutions on standing is due to the gradual formation of larger aggregates from originally isolated gelatin molecules or gelatin ions. When we melt a solid gel of gelatin by rapidly heating it to 45°C. and cooling it rapidly to 20° there is produced a true solution containing isolated gelatin molecules or gelatin ions side by side with submicroscopic pieces of solid jelly. On standing two opposite processes are constantly going on in such a mixture; *i.e.*, the solution of these aggregates into isolated molecules or ions and the reverse process; namely, that of the formation of new aggregates by the union of formerly isolated gelatin molecules or gelatin ions. When the velocity of aggregate formation prevails over the velocity of the solution of aggregates, the viscosity of the gelatin solution will increase on standing; when the velocity of solution of aggregates prevails over the velocity of formation, the viscosity of the gelatin solution will diminish on standing. All agencies which accelerate the rate of solution of solid gelatin should counteract the aggregate formation and the rise of viscosity of a gelatin solution on standing; while all agencies which diminish the rate of solution of solid gelatin should increase the rise of viscosity of the gelatin solution on standing.

We have seen that powdered isoelectric gelatin is dissolved the more rapidly, the more HCl we add, and that there is no maximum followed by a drop when a certain hydrogen ion concentration is exceeded. Hence we should expect that the lower the pH of a gelatin solution containing the same concentration of originally isoelectric gelatin, the smaller the increase of the viscosity of the solution on standing. In a preceding paper we have already given curves showing that this is the case and that there is no Donnan effect noticeable in this case.[15]

We have seen in Fig. 5 that Na_2SO_4 commences to diminish noticeably the rate of solution of solid gelatin chloride as soon as the concentration of Na_2SO_4 exceeds M/64, while $CaCl_2$ commences to have the opposite effect as soon as the concentration of $CaCl_2$ exceeds M/4.

We prepared gelatin chloride solutions of pH 3.4 containing 1 gm. of originally isoelectric gelatin in 100 cc. solution. The solutions were made up in various concentrations of Na_2SO_4 and $CaCl_2$. The solutions were rapidly heated to 45°, rapidly cooled to 20°C., and kept at this temperature for 1 hour. The time of outflow of the solution through a viscometer was measured immediately and in intervals of 5 or 10 minutes. The time of outflow of water through the viscometer at 20° was 61 seconds.

The viscosity of a gelatin chloride solution of pH 3.4 rises gradually but very slowly (uppermost curve in Fig. 7) and the rate of increase of viscosity on standing is not materially altered in M/512 Na_2SO_4 and only little in M/128 Na_2SO_4. In M/32 Na_2SO_4 the viscosity increases more rapidly on standing, in M/8 Na_2SO_4 still more rapidly, and in M/2 Na_2SO_4 very sharply. This is exactly what we should expect since the Na_2SO_4 causes a diminution of the rate of solution of gelatin chloride as soon as the concentration of

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[15] Loeb, J., *J. Gen. Physiol.*, 1921–22, iv, 97.

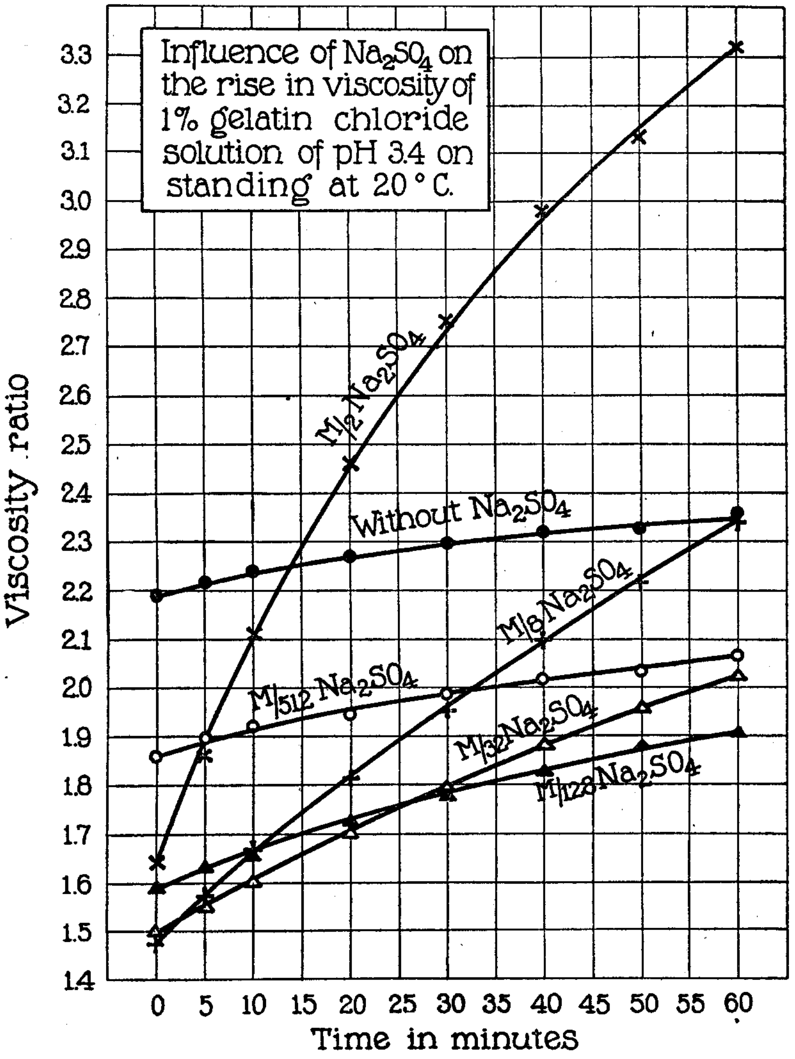

FIG. 7. Showing that concentrations of Na_2SO_4 of M/32 and above cause an increase in the viscosity of gelatin chloride solution of pH 3.4 on standing at 20°C.

Na_2SO_4 is above M/64. In such solutions the rate of solution of micella will be less and less, and since new micella are constantly formed at 20°C., the viscosity will rise more rapidly on standing when the solution contains Na_2SO_4 in concentrations above M/64 than when the solution contains no or less Na_2SO_4.

Fig. 8 shows that $CaCl_2$ in concentrations up to M/8 does not alter the increase in viscosity of gelatin chloride solution on standing but that the viscosity of gelatin chloride of pH 3.4 does no longer increase on standing when the concentration of $CaCl_2$ is M/2 or 1 M. In this concentration $CaCl_2$ causes a slight increase in the rate of solution of gelatin chloride.

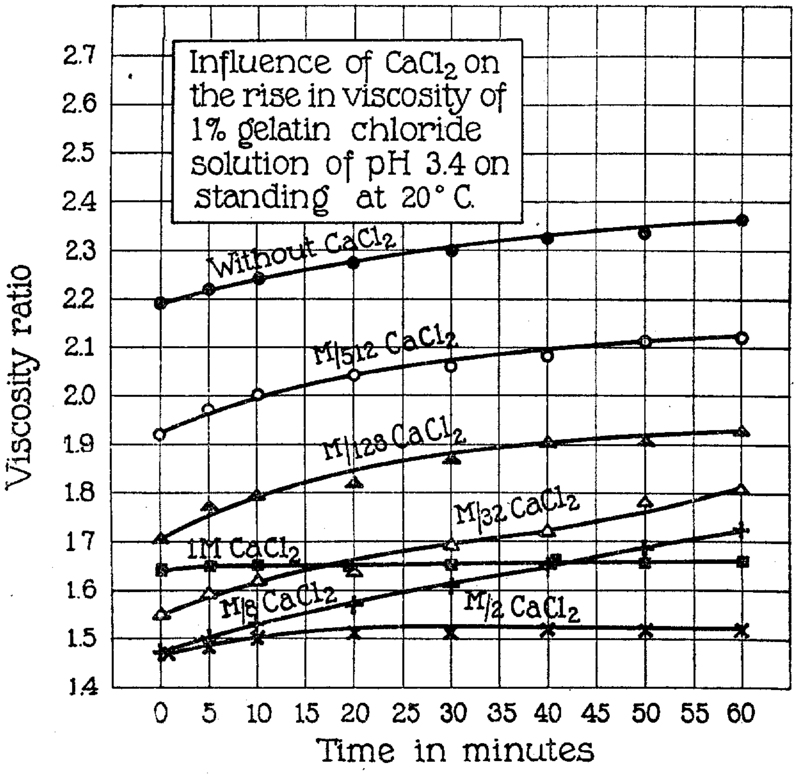

FIG. 8. Showing that concentrations of $CaCl_2$ of M/2 or above prevent the increase in viscosity of gelatin chloride solution of pH 3.4 on standing at 20°C.

NaCl causes no change in the rate of solution of gelatin chloride as long as the concentration of NaCl does not exceed 1 M. Above this concentration it causes coagulation and the viscosity can no longer be measured. Hence NaCl in concentrations up to 1 M should not alter the rate of increase of viscosity of gelatin chloride solutions on standing. Fig. 9 shows that this is correct.

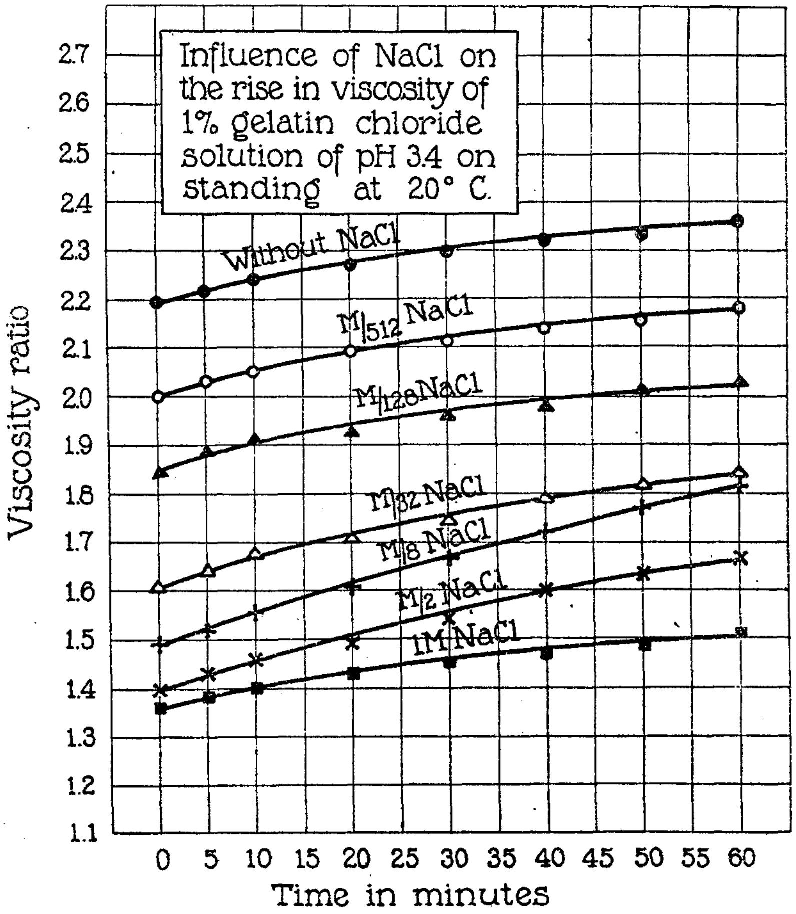

FIG. 9. Showing that NaCl solutions up to a concentration of 1 M have no effect on the increase in viscosity of gelatin chloride solution of pH 3.4 on standing at 20°C.

These experiments corroborate the idea that gelatin solutions are true solutions consisting of isolated gelatin ions and molecules which may contain, in addition, submicroscopic aggregates of gelatin molecules and ions. The formation of new micella and a solution of these already formed occur constantly. When the rate of formation of such micella exceeds the rate of the solution, the viscosity of a gelatin solution rises on standing and the rise is in proportion with the difference in the rate of the formation and solution of these micella. The reader must bear in mind that the micella in the case of gelatin solutions are submicroscopic particles of a reversible jelly of gelatin and not particles of an irreversible precipitate.

SUMMARY.

1. Colloids have been divided into two groups according to the ease with which their solutions or suspensions are precipitated by electrolytes. One group (hydrophilic colloids), *e.g.*, solutions of gelatin or crystalline egg albumin in water, requires high concentrations of electrolytes for this purpose, while the other group (hydrophobic colloids) requires low concentrations. In the latter group the precipitating ion of the salt has the opposite sign of charge as the colloidal particle (Hardy's rule), while no such relation exists in the precipitation of colloids of the first group.

2. The influence of electrolytes on the solubility of solid Na caseinate, which belongs to the first group (hydrophilic colloids), and of solid casein chloride which belongs to the second group (hydrophobic colloids), was investigated and it was found that the forces determining the solution are entirely different in the two cases. The forces which cause the hydrophobic casein chloride to go into solution are forces regulated by the Donnan equilibrium; namely, the swelling of particles. As soon as the swelling of a solid particle of casein chloride exceeds a certain limit it is dissolved. The forces which cause the hydrophilic Na caseinate to go into solution are of a different character and may be those of residual valency. Swelling plays no rôle in this case, and the solubility of Na caseinate is not regulated by the Donnan equilibrium.

3. The stability of solutions of casein chloride (requiring low concentrations of electrolytes for precipitation) is due, first, to the osmotic pressure generated through the Donnan equilibrium between

the casein ions tending to form an aggregate, whereby the protein ions of the nascent micellum are forced apart again; and second, to the potential difference between the surface of a micellum and the surrounding solution (also regulated by the Donnan equilibrium) which prevents the further coalescence of micella already formed. This latter consequence of the Donnan effect had already been suggested by J. A. Wilson.

4. The precipitation of this group of hydrophobic colloids by salts is due to the diminution or annihilation of the osmotic pressure and the P.D. just discussed. Since low concentrations of electrolytes suffice for the depression of the swelling and P.D. of the micella, it is clear why low concentrations of electrolytes suffice for the precipitation of hydrophobic colloids, such as casein chloride.

5. This also explains why only that ion of the precipitating salt is active in the precipitation of hydrophobic colloids which has the opposite sign of charge as the colloidal ion, since this is always the case in the Donnan effect. Hardy's rule is, therefore, at least in the precipitation of casein chloride, only a consequence of the Donnan effect.

6. For the salting out of hydrophilic colloids, like gelatin, from watery solution, sulfates are more efficient than chlorides regardless of the pH of the gelatin solution. Solution experiments lead to the result that while $CaCl_2$ or NaCl increase the solubility of isoelectric gelatin in water, and the more, the higher the concentration of the salt, Na_2SO_4 increases the solubility of isoelectric gelatin in low concentrations, but when the concentration of Na_2SO_4 exceeds M/32 it diminishes the solubility of isoelectric gelatin the more, the higher the concentration. The reason for this difference in the action of the two salts is not yet clear.

7. There is neither any necessity nor any room for the assumption that the precipitation of proteins is due to the adsorption of the ions of the precipitating salt by the colloid.