THE ULTIMATE UNITS IN PROTEIN SOLUTIONS AND THE CHANGES WHICH ACCOMPANY THE PROCESS OF SOLUTION OF PROTEINS.

BY JACQUES LOEB AND M. KUNITZ.

(From the Laboratories of The Rockefeller Institute for Medical Research.)

(Received for publication, January 20, 1924.)

1.

A number of facts suggest that the genuine proteins are kept in aqueous solution by the forces of attraction between the molecules of proteins or their polar groups and the molecules of water, or, in other words, that they form true solutions. Such facts are the high concentrations of salts required for precipitation of genuine proteins (regardless of valency of ion), the increase in solubility of isoelectric gelatin by salts, increasing with the valency of either ion of the salt,\(^1\) the applicability of the principle of constancy of solubility product,\(^2\) and others. All this, however, does not give us any insight into the nature of the ultimate units in these solutions; namely, whether the ultimate unit is the individual protein molecule or a small or a large aggregate of molecules; since it is quite conceivable that molecules of proteins, though dragged into the water by their polar groups form aggregates through the forces of cohesion between their non-polar groups. Such a possibility is strongly suggested in the case of proteins which set to a gel, e.g. gelatin.

It is possible to arrive at some conclusion concerning the molecular nature of the ultimate particles in protein solutions through measurements of viscosity. When finely powdered particles of solid isoelectric gelatin are put into a solution of an acid, the particles swell. The cause of this swelling is revealed when the concentration of the H ions and


the anions inside the particles and in the supernatant water are compared. It is found that the molar concentration of the two oppositely charged ions is different inside the gelatin granules from what it is outside, and the ratio of the two kinds of oppositely charged monovalent ions of the acid is expressed by the following equation.

\[
\frac{\text{concentration of hydrogen ions inside}}{\text{concentration of hydrogen ions outside}} = \frac{\text{concentration of anions outside}}{\text{concentration of anions inside}}
\]

or

\[
\log \frac{[H^+]}{[H^+]}_{\text{inside}} = \log \frac{[Cl^-]}{[Cl^-]}_{\text{outside}}
\]

or

\[
\text{pH inside minus pH outside} = \text{pCl outside minus pCl inside}.
\]

This was demonstrated in the following way. 1.0 gm. of finely powdered dry isoelectric gelatin granules was put into 100 cc. \(H_2O\) containing different quantities of 0.1 \(n\) HCl; namely, 8, 10, 12, 16, and 20 cc. The suspension was allowed to stand for 1 hour at 20°C. The outside solution was then filtered off and its pH was determined electrometrically. The granules of gelatin were melted and the pH of the liquid was determined in the same way. The pCl outside was, of course, equal to the pH outside. Knowing the volume of the filtrate the quantity of Cl in the filtrate could be calculated and by deducting this value from the value of HCl originally added, it was possible to calculate the Cl held by the gelatin. (It had been shown already that this Cl exists in the form of Cl ions.) In this way the concentration of chlorine inside was obtained. The log \(\frac{[Cl^-]}{[Cl^-]}_{\text{outside}}\) could now be calculated (Table I).

Considering the limits of accuracy the agreement in Table I between the values of pH inside — pH outside and pCl outside — pCl inside is satisfactory.

This distribution of the H and Cl ions between the micelles of gelatin and the supernatant water is characteristic for membrane equilibria and finds its explanation by Donnan's theory of membrane equilibria, which predicts this striking distribution if one ion cannot
diffuse through a membrane or a gel which is readily permeable to the ordinary ions of electrolytes. The non-permeable ion is in this case the protein ion which in the powdered particle or micelle is held by the force of cohesion between the molecules (or rather the non-polar “oily” groups of the molecule) of gelatin.

As was first shown by Procter and Wilson, the unequal distribution of diffusible ions inside and outside the gel leads to an excess of total molar concentration of the diffusible ions inside the gel over that outside, and this excess increases the osmotic pressure inside over that in the outside solution. The consequence is the diffusion of more water into the gel and an increase in the volume of the solid particles.4

It had been shown that the swelling of the solid particles of gelatin and casein in acid leads to an increase in the viscosity of suspensions of these particles and that the influence of acid on the viscosity of suspensions of powdered particles of originally isoelectric gelatin in water runs parallel to the influence of acid on swelling.4 This parallelism is easily understood on the basis of Einstein's formula connecting the volume of particles dissolved or suspended in water with the viscosity of the solution or suspension.

According to Einstein, the viscosity of aqueous solutions is a linear function of the relative volume occupied by the solute in the solution,

$$\eta = \eta_0 (1 + 2.5 \varphi)$$

in which $\eta_0$ is the viscosity of the water at the temperature of the experiment, $\eta$ the viscosity of the solution, and $\varphi$ the fraction of the volume of the solution occupied by the solute. This formula can be

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used only when $\phi$ is very small and when the particles of the solute are spherical and large in comparison with the molecules of the solvent.

When powdered particles of gelatin swell in acid, water enters the particles and the value of $\phi$ in Einstein's equation increases. The main fact was that under the influence of acid the value of $\phi$ varies in the case of suspensions of powdered particles of gelatin or casein in acid according to Donnan's equation.

It was further shown that the viscosity of solutions of gelatin varies with the $pH$ of the gelatin solution in exactly the same way as does the viscosity of suspensions of powdered particles of gelatin, and the only difference was that the viscosity of freshly prepared solutions of gelatin was always smaller than that of the suspension of powdered particles for the same concentration of originally isoelectric gelatin, the same $pH$ of the gelatin, and the same temperature. The inference was that the influence of acid on the viscosity of gelatin solutions was also due to the swelling of micelles which in the solution of gelatin were, however, not visible; but from the fact that the freshly prepared solution of gelatin had a lower viscosity than the powdered gelatin and that the influence of acid on viscosity was also smaller in the solution than in the case of the powdered gelatin, it was to be inferred that when a solid particle of gelatin goes into solution, the result is a formation of units of different size, some units being possibly individual molecules or aggregates of so few molecules that the aggregates were too small to give rise to a Donnan equilibrium, while others were units consisting of enough molecules to give rise to a Donnan equilibrium and to undergo acid swelling.4

This inference was supported by the fact that solutions of genuine crystalline egg albumin which do not set to a gel (unless too much acid is added or unless the temperature is too high) have a very low viscosity. The addition of acid to solutions of genuine crystalline egg albumin causes only a slight increase in the viscosity of the solutions, while the same change in pH causes a considerable increase in the viscosity of solutions of gelatin.4

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Hedestrand\(^5\) has shown that acid causes a slight increase in the viscosity of solutions of amino acids of a high concentration. This increase may have been due to the possible attachment of some water molecules to the ions of the amino acids (hydration). The amino acids form true solutions and it is not probable that they form aggregates capable of giving rise to a Donnan equilibrium. The low order of magnitude of the influence of acid on the viscosity of solutions of genuine crystalline egg albumin suggests that the influence of acid is either not due at all to swelling of micelles, but, perhaps, only or chiefly to a hydration of the individual molecules; or, if it is due to swelling, that there are only few aggregates capable of swelling in albumin solutions, since it can be shown that wherever there are particles capable of acid swelling present in a solution, the influence of acid on the viscosity is of a very high order.

It seems, therefore, as if the changes in viscosity which accompany the process of solution of solid particles of protein in acid might give us some information concerning the ultimate units of protein which are formed in the process of solution.

II.

*The Viscosity of Solutions of Genuine and of Suspensions of Denatured Crystalline Egg Albumin at 24°C.*

1 gm. of dry, finely powdered isoelectric egg albumin, denatured by heating, going through a sieve with mesh 200, was stirred up in 100 cc. H\(_2\)O containing 10 cc. of 0.1 N HCl and the time of outflow was measured at constant temperature of 24°C. at stated intervals (Table II) through a straight viscometer.

Table II gives also the relative viscosity of a 1 per cent solution of originally isoelectric genuine crystalline egg albumin, also in 100 cc. 0.01 N HCl. The viscosity was in both cases of a very low order of magnitude and did not change.

The influence of different concentrations of acid was almost negligible for both forms of albumin, denatured and genuine. 1 gm. of originally isoelectric albumin was put into 100 cc. water containing

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varying quantities of 0.1 N HCl. The viscosity was measured after the albumin had been in the acid solution for 1 hour at 24°C. (Table III).

**TABLE II.**

*Viscosity at 24°C. of 1 Gm. Albumin in 100 Cc. of 0.01 N HCl.*

<table>
<thead>
<tr>
<th>Time after mixing (min.)</th>
<th>Relative viscosity</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1 per cent suspension of denatured egg albumin</td>
<td>1 per cent solution of genuine egg albumin</td>
</tr>
<tr>
<td>5</td>
<td>1.02</td>
<td>1.04</td>
</tr>
<tr>
<td>10</td>
<td>1.02</td>
<td>1.05</td>
</tr>
<tr>
<td>15</td>
<td>1.02</td>
<td>1.04</td>
</tr>
<tr>
<td>25</td>
<td>1.02</td>
<td>1.04</td>
</tr>
<tr>
<td>35</td>
<td>1.02</td>
<td>1.04</td>
</tr>
<tr>
<td>45</td>
<td>1.04</td>
<td>1.04</td>
</tr>
<tr>
<td>55</td>
<td>1.03</td>
<td>1.04</td>
</tr>
<tr>
<td>65</td>
<td>1.04</td>
<td>1.05</td>
</tr>
<tr>
<td>75</td>
<td>1.03</td>
<td>1.04</td>
</tr>
</tbody>
</table>

**TABLE III.**

*Relative Viscosity of 1 Gm. Albumin in 100 Cc. HCl Solutions after 1 Hour.*

<table>
<thead>
<tr>
<th>Concentration of 0.1 N HCl in 100 cc. H2O</th>
<th>1 per cent suspension of denatured egg albumin</th>
<th>1 per cent solution of genuine egg albumin</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>1.03</td>
<td>1.03</td>
</tr>
<tr>
<td>2</td>
<td>1.01</td>
<td>1.04</td>
</tr>
<tr>
<td>4</td>
<td>1.01</td>
<td>1.04</td>
</tr>
<tr>
<td>8</td>
<td>1.01</td>
<td>1.05</td>
</tr>
<tr>
<td>12</td>
<td>1.01</td>
<td>1.05</td>
</tr>
<tr>
<td>16</td>
<td>1.00</td>
<td>1.05</td>
</tr>
<tr>
<td>20</td>
<td>1.00</td>
<td>1.04</td>
</tr>
<tr>
<td>30</td>
<td>1.00</td>
<td>1.04</td>
</tr>
<tr>
<td>40</td>
<td>1.01</td>
<td>1.04</td>
</tr>
<tr>
<td>50</td>
<td>1.01</td>
<td>1.03</td>
</tr>
</tbody>
</table>

It is obvious that there is no measurable effect of the acid on the viscosity of suspensions of finely powdered particles of denatured albumin; there is a slight but unmistakable effect on genuine egg albumin, the viscosity rising from 1.03 to 1.05 when acid is added and falling again to 1.03 when more acid is added.
Similar results are obtained with suspensions of insoluble casein particles when 1 gm. of very finely divided isoelectric casein (going through mesh 200) is put into 100 cc. of water containing acids which form insoluble casein salts—such as trichloroacetic, sulfuric, and sulfosalicylic. The viscosity of the casein remains very low since no swelling can occur. This is obvious from Table IV.

Similar results were obtained with sulfuric and sulfosalicylic acid. All these experiments show that where there are no particles capable of giving rise to a Donnan equilibrium and to swelling in acid, the order of magnitude of the viscosity is low and the effect of acid on the viscosity is zero or very small.

TABLE IV.

<table>
<thead>
<tr>
<th>0.1 N trichloroacetic acid in 100 cc. H₂O.</th>
<th>Relative viscosity of casein.</th>
</tr>
</thead>
<tbody>
<tr>
<td>cc.</td>
<td>Relative viscosity of casein.</td>
</tr>
<tr>
<td>0</td>
<td>1.02</td>
</tr>
<tr>
<td>2</td>
<td>1.02</td>
</tr>
<tr>
<td>4</td>
<td>1.04</td>
</tr>
<tr>
<td>8</td>
<td>1.08 (?)</td>
</tr>
<tr>
<td>12</td>
<td>1.02</td>
</tr>
<tr>
<td>16</td>
<td>1.04</td>
</tr>
<tr>
<td>20</td>
<td>1.03</td>
</tr>
<tr>
<td>30</td>
<td>1.02</td>
</tr>
<tr>
<td>40</td>
<td>1.02</td>
</tr>
<tr>
<td>50</td>
<td>1.02</td>
</tr>
</tbody>
</table>

Hence we come to the conclusion that a low order of viscosity in a protein solution in the presence of acid indicates that the solution contains few or no micelles capable of swelling.

III.

Changes in Viscosity Accompanying the Solution of the Sparingly Soluble Type of Casein.

Entirely different results are observed when finely powdered isoelectric casein is put into solutions of an acid such as HCl or H₃PO₄, in which the casein is soluble. In this case the solution of the micelles
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is preceded by swelling and the swelling varies with the concentration of acid and with the addition of neutral salts in agreement with the theory of membrane equilibria. It had also been shown in a book and in a preceding paper⁶ that the viscosity of the casein chloride or phosphate solution varies with the relative volume of the casein particle in the solution, and that the comparatively high viscosity of casein solutions in acid is due to the swelling of micelles.

Two processes go on simultaneously in such casein solutions which affect the viscosity of the solution in an opposite sense, first, the swelling of the micelles under the influence of the acid by which the viscosity increases (since the value of $\phi$ in Einstein’s equation increases with the swelling), and second, the solution of the micelles whereby the micelles are divided into units too small to give rise to a Donnan equilibrium.

In the older experiments on viscosity referred to, a casein preparation was used which was sparingly soluble. In this sparingly soluble form of casein, the power of cohesion between the molecules of casein was so great that a considerable hydrostatic pressure of the water forced into the micelles by the excess of osmotic pressure due to the Donnan equilibrium was required to smash the solid particles of casein, as a preliminary to their solution.⁹ As a consequence the solution of the micelles became a function of the swelling inasmuch as solution of the micelles became possible only when the swelling reached a certain limit.

It is, however, possible to prepare a form of casein which is much more readily soluble in solutions of HCl so that the solution either ceases to depend upon the swelling of the micelles, or depends to a much smaller degree upon the swelling. We may assume that in this latter form of casein the molecules are held together with a smaller force of cohesion than in the case of the more difficultly soluble form of casein. The difference between the two forms lies in the preparation of the isoelectric casein from skimmed milk. To produce the less soluble casein, 1 $N$ acid must be added very rapidly to the skimmed milk with moderate stirring. In this case some of the casein is perhaps

"denatured." To produce the more rapidly soluble casein, 0.1 N acid must be added very slowly (running in through a capillary tube) with very energetic stirring. This latter type of casein can dissolve without undergoing any swelling, though as a matter of fact if the proper concentration of acid is selected, it can be shown that the actual solution may be preceded by a swelling. We will call the two types of casein the "less soluble" and the "more soluble," respectively.

It seemed of interest to compare the changes in viscosity which accompany the solution of the two types of casein in solutions of HCl. Since it is also possible to measure the change in size of an individual micelle under the microscope, and furthermore to measure the quantity of non-dissolved material, an exact method exists to test once more the conclusion arrived at in earlier publications that the changes in viscosity of the casein solution under the influence of acid are primarily and chiefly caused by the swelling of the casein micelles.

We will first discuss experiments on the "less soluble" casein. Dry isoelectric casein was sifted through a sieve No. 200 and only the very fine granules which passed through this sieve were used. 1 gm. of such sifted powdered casein was put into 4, 8, 15, and 50 cc. of 0.1 N HCl to which enough water was added to make the volume 100 cc. Measurements of the rate of outflow were then made in stated intervals in which a straight form of a viscometer tube was used as described in previous publications. The temperature was always 24°C.

The curves in Fig. 1 represent the variations in the viscosity of 1 gm. of originally isoelectric casein during the first 3 hours in 100 cc. aqueous solution containing respectively 4, 8, 15, and 50 cc. of 0.1 N HCl. In 4 cc. of 0.1 N HCl the casein micelles swell slowly and the relative viscosity of the solution or suspension increases slowly in 3 hours from 1.02 to 1.55. Later some of the casein goes into solution and the viscosity drops a little.

In 8 cc. of 0.1 N HCl the viscosity rises in 20 minutes from about 1.02 to 1.7. Then the process of solution proceeds more rapidly than the process of swelling and the viscosity drops rapidly to about 1.22. On

By relative viscosity is meant the ratio of time of outflow of solution from viscometer over the time of outflow of pure water.
account of the Donnan equilibrium, the maximal swelling reached in 15 cc. of 0.1 \text{n} HCl is less than in 8 cc. HCl and hence the maximal viscosity is lower in 15 than in 8 cc. HCl. The solution proceeds at least as rapidly in 15 cc. as in 8 cc. and the viscosity drops parallel with that in 8 cc.

**Fig. 1.** Changes of relative viscosity of 1 gm. of powdered, nearly isoelectric casein of the less soluble type when put into 100 cc. H\textsubscript{2}O containing 4, 8, 15, and 50 cc. of 0.1 \text{n} HCl. (The pH of these solutions after the casein was added was about 3.15, 2.45, 1.9, and 1.4, respectively.) Abscissae, time after the powdered casein was put into the HCl solution; ordinates, relative viscosity. The viscosity rises at first on account of swelling of the particles and falls later on account of solution.

The Donnan equilibrium demands that the swelling should be considerably less in 50 than in 8 or 15 cc. 0.1 \text{n} HCl, and accordingly the viscosity rises but little in 50 cc. HCl, namely only to 1.1. On account of the small amount of swelling but little of this type of casein goes into solution in this concentration of HCl. Hence the viscosity remains almost unaltered at about 1.1 during the next 3 hours.
The application of the Donnan equilibrium to the explanation of swelling of the micelles demands that the addition of neutral salts to acid should diminish the swelling of the micelles, and hence should diminish also the viscosity; but since, for this difficultly soluble type of casein, solution depends upon the swelling reaching a certain quantity, the addition of salt should not only influence the viscosity (i.e. the ascending branch of the time curve) but also the descending part due to solution of the casein. It is therefore possible to predict the effect of the addition of salt on the viscosity curves and the prediction is fulfilled. Fig. 2 gives the results of one series of experiments. The changes of viscosity with time were measured in the following four solutions.

\[
\begin{align*}
8 \text{ cc. of } 0.1 \text{ N HCl} & + 92 \text{ cc. H}_2\text{O.} \\
8 \text{ " " } + 7 \text{ " m/10 NaCl} & + 85 \text{ cc. H}_2\text{O.} \\
8 \text{ " " } + 22 \text{ " " } & + 70 \text{ " " } \\
8 \text{ " " } + 42 \text{ " " } & + 50 \text{ " " }
\end{align*}
\]
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The theory of the Donnan equilibrium demands that the maximal swelling and hence the maximal viscosity should diminish with increasing concentration of salt, which is the case. While without NaCl the viscosity reaches a maximum of 1.76, the maximal viscosity is 1.46 in 7 cc. M/10 NaCl, 1.12 in 22 cc., and 1.06 in 42 cc. M/10 NaCl.

Furthermore, while the drop of the viscosity in the solution without salt is steep it is very slight when 7 cc. M/10 NaCl are added and there is no drop (and no solution) when 22 or 42 cc. of M/10 NaCl are added, because in these latter solutions the swelling is too low to permit the casein particles to go into solution.

The correlation between swelling and solution of micelles on the one hand and viscosity on the other can be tested by observing directly the changes in volume of individual micelles of the originally isoelectric casein in various solutions of acid. An individual micelle was observed under the microscope and its change in dimensions in an HCl solution recorded in certain intervals. The measurements of the outline were made with the aid of a micrometer ruled in squares. The outline of the granule under observation was drawn on paper also ruled in squares, so that one square of the paper corresponded to one square of the micrometer scale in the eyepiece of the microscope. These drawings were enlarged and the area of each drawing of a micelle measured with a planimeter. This area raised to the $\frac{3}{2}$ power was considered a measure of the volume of the micelle. The first measurement was given the value 1.

If our theory is correct that the increase in viscosity of the casein in HCl during solution is due chiefly to the swelling of the micelles and that the decline of the viscosity curves with time (Figs. 1 and 2) is due to solution, i.e. the transformation of parts of the micelles into units too small to give rise to a Donnan equilibrium, it must be possible to show that if we plot the values for the volume of the micelles as ordinates over the time, we must get a system of curves rising in time in a way similar to the rise in the curves in Fig. 1 or 2. The difference to be expected was in the following points. The solutions in which the viscosities were measured were frequently stirred, while the micelles used for measurements were not stirred since the same micelle had to be observed continuously. Hence solution commenced
earlier in the stirred solutions than in the non-stirred individual micelles. Furthermore, only a few individual micelles were observed in the direct volume measurements under the microscope and hence these values are afflicted with the error of individual variation. A third difference is the pH. If we make allowance for these facts and in the measurements of the area of the drawings of the micelles, we find that the changes in volume of the micelles actually observed in solutions of HCl have the same drift as the changes in viscosity. Fig. 3 gives the time as abscissa and the change in volume of an individual micelle in each of the four solutions of HCl.

The reader will notice that the rising branches of the curves for viscosity (Fig. 1) agree with the curves for volume in Fig. 3. The volume rises most sharply in 8 cc. HCl (in Fig. 3) corresponding to an equally sharp rise of the viscosity in Fig. 1. After 30 minutes, the volume curve in 8 cc. HCl had to be discontinued since the surface of the micelle broke into fragments. The volume curve in 16 cc. HCl (Fig. 3) is lower than the volume curve for 8 cc. HCl (Fig. 3), and this also corresponds to the viscosity curve in 16 cc. HCl (Fig. 1). In 4 cc. HCl the volume curve (Fig. 3) rises slowly and no solution occurs and the viscosity curve in 4 cc. HCl (Fig. 1) has the same character. The volume curve in 50 cc. HCl (Fig. 3) is low and runs parallel to the axis of abscisse, the viscosity curve for 50 cc. HCl (Fig. 1) having the same character.

Fig. 4 gives the influence of the addition of NaCl on the increase in volume of individual micelles in solutions containing 8 cc. 0.1 N HCl per 100 cc. solution, the volume curve in 8 cc. HCl (without salt) rises more rapidly and reaches a higher value than the volume curve for 7 or 22 cc. NaCl (Fig. 4) corresponding to the viscosity curves for the same salt values in Fig. 2. The character of the volume curve for 22 cc. NaCl in Fig. 4 agrees also with the character of the viscosity for 22 cc. NaCl in Fig. 2.

We can therefore say that the increase in viscosity which accompanies the first stage of the solution of powdered particles of the less soluble type of casein is due to the increase in the volume of the individual micelles of this type of casein.

Experiments were also made to ascertain the changes in viscosity of this type of casein in alkali where it is more readily soluble. Former
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Fig. 3. Showing that the rise in viscosity in Fig. 1 was due to swelling of the casein particles. Abscissae, time; ordinates, volume of individual granules. The curves for changes in volume of individual particles in Fig. 3 are similar to the curves for changes in viscosity, Fig. 1.

Fig. 4. Showing that the rise in viscosity in Fig. 2 was due to swelling of casein particles.
observations have shown that when powdered particles of isoelectric casein are put into alkali no swelling occurs but that the solution occurs in a different way. It was found that the viscosity of 1 gm. of originally isoelectric casein of this type rises in one minute to 1.11, reaching a maximum in 20 minutes of 1.16, and dropping again in 4 hours to 1.13. This indicates that the process of solution of this type of casein in alkali is accompanied by only an insignificant increase of viscosity and the low order of magnitude of viscosity indicates that the majority of units into which the casein dissolves in alkali are too small to undergo swelling.

IV.

Changes in Viscosity Accompanying the Solution of the More Soluble Type of Casein.

The swelling of the micelles of the more soluble type of casein in solutions of HCl should depend in a similar way upon the concentration of HCl and of salts as does that of the less soluble type discussed in the preceding chapter. The two types differ in regard to the solubility which shows itself in the fact that the micelles of the more soluble type of casein can go into solution even in the presence of higher concentration of acids or salts where the swelling is diminished in agreement with the Donnan equilibrium. In other words, the smashing of the micelle by the hydrostatic pressure of the water due to excess osmotic pressure inside the micelle is not a necessary prerequisite for the solution of this type of casein. Since this powder is not so readily wetted by the solution, the dry powder was ground up in a mortar with a trace of the acid solution, so as to insure its wetting and was then put into the solution of acid. Fig. 5 represents the changes in viscosity of 1 gm. of the more soluble type of casein in different concentrations of HCl. The size of the particles was the same as that used in the case of the less soluble casein; namely, particles of powdered isoelectric casein which went through the sieve No. 200. There is a sharp rise in viscosity in the first few minutes due to swelling of the micelles and this is followed by a rapid drop in viscosity due to the solution. In 4 cc. the drop in the curve is less steep because the casein is less rapidly soluble.
FIG. 5. Changes of viscosity accompanying solution of 1 gm. of powdered casein of the more soluble type in HCl.

FIG. 6. Changes of viscosity accompanying solution of 1 gm. of the more soluble type of powdered casein in HCl solutions containing NaCl.
The rapid drop during the first 10 or 40 minutes, respectively, is followed by a very slow drop, the viscosity reaching in all solutions the value of 1.1 or less in the next 24 hours.

The presence of NaCl affects chiefly the degree of swelling and only to a slight extent (if at all) the rate of solution of the micelles of this type of casein. Hence the addition of salt only has the effect of lowering the viscosity (Fig. 6). The low order of viscosity in acid reached at the end of the experiment indicates that there are very few units left in the protein solution large enough to give rise to a Donnan equilibrium.

V.

The Ultimate Units in Casein Solutions in Acid.

The question now arises, what is the nature of the ultimate unit in casein solutions? 1 grm. of finely powdered casein, nearly isoelectric, going through the meshes of a sieve, No. 200, was put into 100 cc. of water containing various numbers of cc. 0.1 N HCl. The solutions were kept at 24°C. for 16 hours and were repeatedly stirred. After 16 hours, the volume occupied by the non-dissolved part of casein was measured in cc. The whole was then stirred up and the viscosity determined.

Fig. 7 contains the results of such an experiment with the less soluble type of casein. The abscissae are the pH of the suspension or solution after 16 hours; for the convenience of the reader the cc. 0.1 N HCl contained in 100 cc. of casein solution at the beginning is written under the pH. The ordinates of one curve are those representing the relative viscosity, the ordinates of the other curve are the volume of the sediment in each solution, i.e. the undissolved part of the casein. Where the volume of the sediment was zero, all the casein was in solution or at least could not settle any more. This happened for the less soluble type of casein between pH of about 2.45 and about 1.85 (or in solutions which contained originally from 9 to 20 cc. 0.1 N HCl in 100 cc.). What interests us is the viscosity in this region of complete solution. It is above 1.1, thus indicating that a certain fraction of the 1 gm. of casein exists still in units large enough to give rise to a Donnan equilibrium and to undergo swelling. Before dis-
cussing this, it will be necessary to compare the curves for volume of sediment and for viscosity where the casein is not all dissolved. It is obvious that there exists a certain parallelism in the two sets of curves. At pH 4.2, the casein is insoluble and incapable of swelling and the viscosity is about 1.02. At pH 3.4 there is a slight amount of swelling, but the casein is still insoluble and both curves for volume of sediment and viscosity rise in a parallel way. At pH 3.0 the swelling increases and the values for viscosity and volume of sediment rise sharply, but the volume curve does not rise as steeply as the viscosity curve. This is due to the fact that some of the casein has gone into solution, thereby diminishing the volume of sediment. Between pH 2.7 and 2.6 there occurs a steep drop of volume of sediment, i.e. the whole gm. of casein goes into solution. There occurs also a steep drop of the viscosity curve but the drop is not so complete as the drop in the volume curve for the reason that some of the units of casein in solution are still in a state of swelling. At pH 1.8,
both curves rise again steeply because now the swelling though still considerable is not enough to permit the casein to go into solution. The reader will remember that unless a critical limit of swelling of the powdered particles of this type of casein is exceeded, the casein cannot be dissolved. At pH 1.8 the swelling is depressed in agreement with the theory of membrane equilibria. The relative viscosity will, however, be high since the relative volume of the particles in solution is high. At pH 1.6 and pH 1.4 the swelling will be more reduced on account of the Donnan equilibrium and the volume of sediment and the viscosity will drop.

The agreement between swelling of particles of casein and viscosity is so good that we have a right to apply this agreement to that part of the curve where the volume of the sediment is zero, i.e. where the whole casein is in solution. Figs. 1 and 3 show that with 8 or 9 cc. HCl in 100 cc. solution, i.e. at pH 2.5 or 2.4, the initial swelling and viscosity are enormous, the relative viscosity reaching a value of about 1.7, but that the viscosity drops rapidly. This can only be due to the fact that part of the casein is dissolved into units too small to give rise to a Donnan equilibrium. The viscosity drops finally to about 1.2. If all the casein in solution consisted of units too small to give rise to a Donnan equilibrium, the viscosity should on the basis of Einstein's theory have dropped to about 1.03 as it does in solutions of crystalline egg albumin, since the molar concentration and density of the 1 per cent casein solution was probably not very different from that of the 1 per cent solution of egg albumin. It is therefore possible to find out what percentage of the 1 gm. of casein is still in solution in the form of particles capable of giving rise to a Donnan equilibrium. These units must undergo the maximal swelling for a pH of 2.4. It was therefore necessary to find out what fraction of 1 gm. of the less soluble casein will give a maximal viscosity of about 1.2 at pH 2.45 at a temperature of 24°C. Fig. 1 shows that the maximal swelling is reached in about 30 minutes, which gives ample time to carry out the experiment. Doses of 0.25, 0.35, and 0.5 gm. of nearly isoelectric finely powdered casein of the less soluble type, going through mesh 200, were put into 100 cc. H₂O containing so much HCl that the pH of the casein mixture with acid was 2.45 in each case and the viscosity of each dose was measured in short intervals during the first 30 minutes at 24°C. The results are given in Table V.
The reader will see that 0.3 gm. of casein in 100 cc. solution of HCl, to give a pH of 2.45, has a viscosity of about 1.14, while the maximal viscosity of 0.5 gm. is above 1.2. We may therefore infer that at pH 2.45 more than one-half of 1 gm. of casein consisted of units too small to give rise to a Donnan equilibrium, while less than one-half of 1 gm. consisted of larger units.

The 1 per cent solutions of this type of casein are always slightly turbid, owing probably to the fact that they contain a number of small particles of insoluble denatured casein which, however, adds little to the viscosity.

Table V shows the curves for viscosity and volume of sediment after 16 hours at 24°C. at varying pH for the more soluble type of casein. 1 gm. of finely powdered casein (not quite isoelectric) of this type, going through mesh 200, was put into 100 cc. H2O containing various numbers of cc. of 0.1 N HCl. At pH 3.0 and below all the casein went into solution. For this type of casein the solubility increases with the hydrogen ion concentration and no longer depends on preliminary swelling of the particles. Between pH 1.4 and 3.3 the viscosity curve
is a straight line, indicating that the viscosity is a linear function of the pH. This straight curve may be chiefly the expression of the Donnan effect on viscosity. The curves for the membrane potentials or osmotic pressure are also practically straight lines for the same pH interval. The viscosity value at pH 2.2 is 1.15 which is the same as that for the less soluble type of casein for the same pH. This would mean that the same relative proportion of casein, i.e. less than 0.5 forms units capable of giving rise to a Donnan equilibrium.

Fig. 8. Influence of pH on viscosity of solutions of the more soluble type of casein at different pH.

SUMMARY AND CONCLUSIONS.

1. The experiments show that suspensions of finely divided particles of insoluble proteins incapable of swelling in acid (denatured egg albumin, casein trichloroacetate, sulfate, etc.) raise the viscosity of the suspension but little and that the influence of acid on the viscosity is negligible.

2. The same is true for solutions of certain genuine proteins such as genuine crystalline egg albumin.

3. In contrast with these are proteins which swell in acid. It can be shown that where acid swelling of particles occurs, the viscosity is of a higher order of magnitude than where the swelling of particles is impossible and that the influence of acid on viscosity runs in these
cases parallel to the influence of acid on swelling. This is shown to be the case for casein in HCl.

4. It is shown that the swelling of the powdered particles which determines the high order of viscosity varies according to the theory of membrane equilibria.

5. These results are used to ascertain with the aid of viscosity measurements whether the ultimate units of genuine protein in solutions are aggregates large enough to give rise to a Donnan equilibrium; or whether they consist of particles below this limit; and in what proportion the two kinds of units are contained in the solution.

6. It is found on the basis of viscosity measurements that when 1 gm. of isoelectric casein is dissolved in HCl so that the solution has a pH of 2.45, more than one-half of 1 gm. of casein must exist as units too small to give rise to a Donnan equilibrium, while the rest must exist in units still capable of undergoing swelling in acid.