CLXXXV. THE DENATURATION OF PROTEINS.

IX. THE EFFECT OF THE ADDITION OF ELECTRO- LYTES ON THE VISCOSITY OF SOLUTIONS OF CRYSTALLINE EGG-ALBUMIN.

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(Received August 9th, 1932.)

IN a recent communication, Loughlin and Lewis [1932] examined the effect of denaturation on the viscosity of solutions of crystalline egg-albumin and oxyhaemoglobin, and found that in each case the process of denaturation is accompanied by an increase in viscosity. Since dilatometer measurements show that there is no change in the total volume of the protein solutions on denaturation, the above observation was interpreted to mean that the process of denaturation involves a physical distension of the protein unit accompanied by an increase in the amount of water associated with each protein particle, this conclusion being in accordance with the well-known fact that denaturation will not occur in the absence of water.

It was found that for a given concentration of undenatured protein solution the viscosity was a constant independent of p_H , as was also the viscosity of a given concentration of denatured protein solution. This was unexpected in view of the theory of Smoluchowski [1916] to the effect that when the particles in a solution are electrically charged the viscosity of the solution should be increased as a result of the charges. Alteration of the p_H from the isoelectric point necessarily involves a considerable alteration in the electric charge of the protein units, and thus appears to create conditions in which an electro-viscous effect would be anticipated. Furthermore this electro-viscous effect would be expected to increase as we recede from the isoelectric point.

Consequently in view of the uncertainty which necessarily existed as to the interpretation of the viscosity results at p_H values removed from the isoelectric point, it was deemed advisable, in the paper referred to, to interpret the results in terms of particle size at the isoelectric point alone, pending a further series of determinations of viscosity and conductivity which would settle whether or not an electro-viscous effect had to be taken into account. The results of these further determinations are given in the present communication, and from them a general conclusion is drawn concerning the mechanism of the process of denaturation at all p_H values.

EXPERIMENTAL.

The protein used was crystalline egg-albumin prepared by the method of Sørensen [1917], and then freed from salt by prolonged dialysis. The concentration of protein in the stock solution was estimated by the method of Devoto as modified by Hopkins [1900].

The viscosities were measured by the times of outflow through the capillary tube of an Ostwald viscosimeter, the "relative viscosity" given being the ratio of the time of outflow of a certain volume of protein solution to the time of outflow of the same volume of pure water through the same viscosimeter at the same temperature. The instrument was tested as described [1932] to ensure that the viscosities measured were true viscosities, and in all cases the mean value of at least five readings was taken. Other experimental details are as before.

The p_H determinations were made at a temperature of 18° by the method of Harrison [1930] using the glass electrode.

The conductivities were measured using the ordinary type of conductivity cell, the resistance of the cell in each case being obtained by an adaptation of the Wheatstone bridge method. The cell constant was obtained by measuring the resistance of the cell when it contained $N/50$ KCl. All conductivity measurements were made in a thermostat at a temperature of 25° . In all cases the resistance of the cell was measured against a series of about ten resistances in the resistance box of the Wheatstone bridge, and two samples of each solution were used. Thus each conductivity measurement given in this paper is a mean value of about twenty readings.

The viscosity and conductivity of 3 % salt-free solutions of undenatured crystalline egg-albumin at 25° .

The 3 % solutions over the required p_H range were prepared from the stock solution as described in the previous paper. The viscosity was then measured at 25° exactly as before. The electrodes in the conductivity cell were well washed with the albumin solution of which 10 cc. were measured into the conductivity vessel. The specific conductivity was then determined as described above. The cell was emptied, and a new 10 cc. sample of the albumin solution was introduced. Once again the specific conductivity was measured and the mean value of all the readings was finally recorded.

In this way the viscosity and conductivity of a series of solutions, ranging from the isoelectric point down to p_H 3.0 were measured, the readings being given below.

Mean time of flow for water = 131.8 secs.

p_H	Time of flow (seconds)	Relative viscosity	Spec. conductivity $\times 10^4$ (mhos)
3.01	152.5	1.157	66.1
3.29	152.9	1.160	32.3
3.88	153.0	1.161	10.0
4.34	152.4	1.156	4.4

Mean value of the relative viscosity = 1.158.

It is thus seen that although the sulphuric acid required to reduce the p_H from the isoelectric point down to 3.0 causes a large increase in the conductivity, there is no appreciable effect on the viscosity. The significance of this will be pointed out later.

There was no necessity to perform a similar series of experiments, either with denatured egg-albumin or with oxyhaemoglobin, since previous viscosity measurements have shown that constant values of the viscosity were obtained for any given solution irrespective of its p_H . Since the p_H was adjusted with sulphuric acid a similar increase in conductivity on either side of the isoelectric point would necessarily be experienced. Consequently the one series of quantitative results suffices for our present purpose.

The viscosity and conductivity of 3 % solutions of undenatured egg-albumin containing sodium chloride.

It has been shown that an alteration in the conductivity of a given albumin solution by means of sulphuric acid does not affect the viscosity. In view of this finding, an alteration in conductivity produced in any other way, *e.g.* by the addition of a neutral salt, should not affect the viscosity, but as a greater range of conductivity could be covered by this means, it was thought worth while to carry out a series of experiments in the presence of sodium chloride. Furthermore, assuming for the moment that Smoluchowski's view is the correct one, the greater the change in conductivity produced, the greater should be the deviation in viscosity due to the electro-viscous effect. Similar measurements for denatured egg-albumin could not be made as the addition of salt causes flocculation.

The experimental details were exactly as above, excepting that each 3 % solution was made *N*/100 with respect to sodium chloride by addition of the requisite amount of sodium chloride solution in the process of dilution of the stock albumin solution down to 3 %. As before, the p_H was adjusted with sulphuric acid.

3 % solutions of undenatured egg-albumin containing 0.01 N sodium chloride at 25°.

Mean time of flow for water = 131.8 seconds.

p_H	Time of flow (seconds)	Relative viscosity	Spec. conductivity $\times 10^4$ (mhos)
2.31	153.1	1.161	105.4
3.46	153.3	1.163	79.4
4.18	153.3	1.163	26.4
5.30	152.9	1.160	12.2

Mean value of the relative viscosity = 1.162.

Thus once again the viscosity is found to be a constant independent of p_H , although the conductivity increases rapidly as the p_H is removed from the isoelectric point. The mean value of the viscosity is 1.162 which is slightly greater than the value 1.158 for the salt-free solutions, the difference however,

less than 0.4 %, being within the limits of experimental error. Experiment shows that the viscosity of *N*/100 NaCl is not materially different from that of pure water.

Although examination of the available work shows that a concentration of 10 milli-equiv. of electrolyte is quite sufficient to produce the maximum depression of the viscosity of those sols for which an electro-viscous effect has been observed, it was deemed advisable to make one or two measurements of viscosity and conductivity with a much higher electrolyte concentration, viz. 100 milli-equiv. per litre. It should be noted that albumin solutions are quite stable at this concentration of neutral salt, and that the process of denaturation, although accelerated, has been shown by Lewis [1926] to be still unimolecular, and quite normal in all respects in the presence of even as high concentrations of sodium chloride as 3.4 *N*.

*3 % solutions of undenatured egg-albumin containing 0.1 N
sodium chloride at 25°.*

p_H	Time of flow (seconds)	Relative viscosity	Spec. conductivity $\times 10^4$ (mhos)
2.40	153.8	1.167	121.4
4.00	151.6	1.150	104.1

Mean value of the relative viscosity = 1.159.

From this it is seen that even when the concentration of sodium chloride is increased to 0.1 *N* there is still no appreciable effect on the viscosity.

DISCUSSION.

According to Smoluchowski [1916], when the particles in a solution are electrically charged, the viscosity of the solution is increased as a result of the charges, the value of the viscosity being given by the equation

$$\eta = \eta_0 \left[1 + 2.5 \phi_E \left\{ 1 + \frac{\sigma}{a^2 \eta_0} \left(\frac{\kappa \zeta}{2\pi} \right)^2 \right\} \right],$$

where σ is the specific resistance of the solution, a is the particle radius, ζ is the electrokinetic potential difference at the interface particle-medium, κ is the dielectric constant of the medium, and η_0 its viscosity. It will be noticed that at the isoelectric point, i.e. when ζ is zero, the formula becomes the simple Einstein formula. Thus the ϕ value is that which appears in the Einstein equation, and it is accordingly represented as ϕ_E .

On Smoluchowski's formulation, therefore, the electro-viscous term is expressed as a correction factor of the form $(1 + x)$ multiplied on the term ϕ which stands for the fraction of the total volume of the system occupied by the disperse phase. The factor x is given the value

$$\frac{\sigma}{a^2 \eta_0} \left(\frac{\kappa \zeta}{2\pi} \right)^2 \quad \text{or} \quad \frac{4\sigma \eta_0 v_0^2}{a^2},$$

where v_0 is the electrophoretic velocity of the particles under unit electrostatic potential gradient, and the other symbols have the same significance as before.

It follows from the above expression that an increase in ζ (due to increase in surface charge) should increase the viscosity, whilst on the other hand, any reduction in ζ (say by the addition of salt) should decrease the viscosity.

Clearly Smoluchowski had in mind the case of a lyophobic system in which the radius a likewise determines the value of ϕ . In the case of proteins we are dealing in general with essentially hydrated structures. In this case the term a must stand for the radius of the ionisable protein kernel upon the surface of which are located electric charges whose lines of force pass outwards through the surrounding shell of water of hydration. On the other hand, the volume occupied by the disperse phase will depend not only on a but also upon the degree of hydration, and upon whether this varies with p_H or not. In view of this, let us consider very briefly the attempts that have been made from time to time to verify Smoluchowski's equation for various systems.

By far the most work has been performed on sols of agar, gum arabic, and starch, and to a less extent on colloidal hydroxides, sulphides and oxides, and on certain dyes. Very little attention has been paid to proteins, and none at all to the two proteins with which we are dealing in the present paper.

Kruyt and de Jong [1928] state that all electrolytes diminish the viscosity of agar sols even in small concentrations. Salts containing univalent cations in equivalent concentrations cause practically the same decrease. Salts containing bivalent cations in equivalent concentrations also cause equal decreases in the viscosity, the magnitude of the decrease being greater than in the previous case. The decrease in viscosity in fact is found to depend chiefly on the valency of the cation of the added electrolyte. It may be added that cataphoretic velocity measurements showed that the agar sols are negatively charged. The conclusion drawn from these experimental results is that the Smoluchowski formula cannot be applied to agar sols quantitatively, but it can be applied qualitatively.

Sols of soluble starch were observed by de Jong [1924] to act towards electrolytes exactly as did the agar sols above. Kruyt and Tendeloo [1929, 1] observed the same effects with gum arabic.

Dhar and Ghosh [1929] examined more than thirty sols of the colloidal metallic hydroxide, sulphide, *etc.* types, and found that addition of electrolyte lowered the viscosity.

In the case of proteins, Pauli and Handovsky [1908; 1910] state that the viscosity of ox-serum is diminished by the addition of neutral salts. Kruyt and Tendeloo [1929, 2] state that gelatin and caseinogen act in a manner similar to starch and gum arabic. No observations appear to have been made on egg-albumin or oxyhaemoglobin.

In addition to the investigations referred to above, wherein it is concluded that the Smoluchowski equation gives a qualitative idea of the behaviour to be expected when salts are added to the sols mentioned, a few attempts at a quantitative application have likewise been made.

Thus Kruyt and Tendeloo [1924] using 0.5 % sols of starch containing

various concentrations of different electrolytes measured in each case the viscosity, the specific conductivity, and the cataphoretic velocity. By substituting the values obtained in the Smoluchowski equation a number of equations involving the two unknowns ϕ and a were thus obtained. These equations were solved in pairs and values of ϕ and a obtained. The values of ϕ varied from 4.8 to 5.8×10^{-2} and a varied from 5.4 to 9.7×10^{-7} cm. Such calculations imply, however, that ϕ and a do not themselves vary with the charge of the sol, which is continually modified by the addition of electrolyte. The authors conclude that constant values of ϕ and a are actually obtained, thus confirming the supposition which was made in the first place.

Tendeloo [1927] measured the viscosities, specific conductivities and cataphoretic velocities of arsenious sulphide sols containing various amounts of electrolytes, and obtaining the value of ϕ from the weight of arsenious sulphide in the sol by assuming its density to be unaltered in solution, he was able to obtain, with the aid of the equation of Smoluchowski, a number of values of a . These ranged from 5 $m\mu$ to 7.4 $m\mu$ giving a mean value of 6 $m\mu$. Tendeloo considers that the constancy of the value for a may be regarded as an indication that the Smoluchowski equation is applicable to the arsenious sulphide sols.

Finally Prideaux and Howitt [1932] found that the viscosities of agar sols between 2 and 7 % are much greater than the values calculated from the equation of Smoluchowski when electrolyte is present. They mention that, strictly speaking, this formula applies only to suspensoids, the solution volume ϕ and radius a of which are known, but that it can also be used in a semi-quantitative manner for lyophilic colloids.

Considering the above work on the electro-viscous effect, it is evident that the Smoluchowski equation has not been verified as yet in an entirely satisfactory manner, although it leads in the cases of starch and arsenious sulphide sols to values of a which are well within the bounds of possibility.

It is significant, however, that practically all the sols upon which work has been performed are of such a nature that their charge is due to the adsorption of ions, and not to structural ionisation of the colloidal unit itself. Consequently the effects to be observed in such cases are of a totally different nature from those which one would expect for egg-albumin and oxyhaemoglobin, and the above work can be of little help in connection with the question as to whether the Smoluchowski equation applies to protein solutions or not.

As we have seen, the striking experimental fact is that the viscosities of egg-albumin and oxyhaemoglobin solutions are constant and independent of p_H , although alteration in p_H from the isoelectric point necessarily involves a considerable alteration in the electric charge of the protein units. How then can this be reconciled with the view of Smoluchowski?

One explanation which immediately suggests itself is that the Smoluchowski correction does in fact exist, but is so small that its effect on the viscosity is insufficient to be observed experimentally. This possibility is immediately disposed of, however, if we take some definite p_H removed from the isoelectric

point, and calculate the value of the correction. Let us take for example 3 % salt-free egg-albumin solution at p_H 3.0. As mentioned above, the correction factor x is given by the expression

$$x = \frac{4\eta_0 v_0^2 \sigma}{a^2} = \frac{4\eta_0 v_0^2}{\kappa a^2} \text{ where } \sigma = \frac{1}{\kappa}.$$

The value of η_0 given in the International Critical Tables is 0.00894 poises, v_0 from the cataphoretic velocity measurements of Scott and Svedberg [1924] is $21 \times 10^{-5} \times 300$ cm. per sec., κ from the writer's experiments is 66×10^{-4} mhos or 6×10^9 electrostatic units, and a is of the order 2×10^{-7} cm., therefore

$$x = \frac{4 \times 0.00894 \times (21 \times 10^{-5} \times 300)^2}{6 \times 10^9 \times (2 \times 10^{-7})^2} = 0.6,$$

which is by no means negligible compared with unity. This means that the equation of Smoluchowski would predict an increase of 60 % in the ratio $\frac{\eta - \eta_0}{\eta_0}$ for a 3 % albumin solution when the p_H is altered from the isoelectric point down to p_H 3.0. Such a large increase in viscosity, needless to say, would readily be observed experimentally. Thus the suggestion that the electroviscous effect is negligible is untenable. At the same time the magnitude of the effect which the application of the formula would lead us to expect is unbelievably large. Such a marked increase in viscosity on the addition of a small amount of sulphuric acid indeed seems improbable. The constancy of viscosity with p_H is therefore in direct conflict with the theory of Smoluchowski.

Let us next consider whether the experiments on the protein solutions containing neutral salt are also in conflict with the behaviour one would expect on this theory. Once again we will take the case of a 3 % egg-albumin solution at p_H 3.0, but this time in the presence of $N/100$ sodium chloride. The values of η_0 and a are the same as in the previous case, and v_0 is also unaltered, as examination of the cataphoretic velocity measurements of Abramson [1928], Svedberg and Tiselius [1926], and Scott and Svedberg [1924] shows that the cataphoretic velocity is the same at different concentrations of albumin, and also in the presence of different buffer solutions, *i.e.* in the presence of different concentrations of different salts. The value of κ from the table given on p. 1559 is 90×10^{-4} mhos or 8.1×10^9 electrostatic units. Thus

$$x = \frac{4 \times 0.00894 \times (21 \times 10^{-5} \times 300)^2}{8.1 \times 10^9 \times (2 \times 10^{-7})^2} = 0.44.$$

This means that the presence of 0.01*N* sodium chloride at p_H 3.0 should cause the value of the ratio $\frac{\eta - \eta_0}{\eta_0}$ to decrease to $\frac{1.44}{1.60}$ of its original value, *i.e.* a decrease of 10 %. The experiments have shown, however, that the two viscosities are identical within the limits of experimental error, so that once again Smoluchowski's theory is in conflict with the experimental results. The discrepancies are not limited to p_H 3.0 but are general at all p_H values other than the isoelectric point.

In view therefore of these considerations, we can only conclude that the assumption made by Smoluchowski in obtaining his equation, *viz.* that the charge on the particles in the solution causes an increase in the viscosity of the solution, does not apply in the present case. In other words, the viscosity is not affected by a change in charge which has its origin in the ionisation of the individual protein particles. This suggests that the Einstein type of formula is applicable to the protein solutions under consideration, not only at the isoelectric point but also at all other p_H values. In other words, the interpretation of the viscosity results as an indication of the mechanism of the process of denaturation—which was restricted in the previous communication to the isoelectric point—can be extended to include the whole range of p_H covered by the experiments.

It follows that at all p_H values the process of denaturation of egg-albumin and oxyhaemoglobin is accompanied by an increase in the individual protein units to about twice their normal volume, this increase being accompanied by an increase in the amount of water associated with each unit.

SUMMARY.

1. Viscosity and electrical conductivity measurements have been made on a series of solutions of undenatured crystalline egg-albumin in the absence of salts, and likewise in solutions containing 0.01 *N* and 0.1 *N* sodium chloride.

2. The viscosity was found in all cases to be constant and independent of p_H , although the conductivity increased rapidly as the p_H of the solution was removed from the isoelectric point.

3. The Smoluchowski equation for the viscosity of solutions containing charged protein particles has been compared with the results obtained, and is shown to be in conflict with the experimental observations.

4. It is concluded that no electro-viscous term in the sense of Smoluchowski need be taken into account in the case of the proteins under consideration, and the simple Einstein type of equation applies at all values of p_H .

5. The conclusion drawn from the viscosity results (and restricted in a previous communication to the isoelectric point), to the effect that the process of denaturation involves a distension of each individual protein unit accompanied by an increase in the amount of water associated therewith, so that the volume of each is approximately doubled, is now extended to hold at all p_H values.

In conclusion, the writer would like to express his thanks to Prof. W. C. M. Lewis, under whose direction this work was carried out.

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