THE EFFECT OF DENATURATION ON THE VISCOSITY OF PROTEIN SYSTEMS

By M. L. ANSON and A. E. MIRSKY

(From the Laboratories of The Rockefeller Institute for Medical Research, Princeton, N. J., and the Hospital of The Rockefeller Institute for Medical Research, New York)

(Accepted for publication, December 2, 1931)

Concentrated solutions of proteins in acid or alkali may become very viscous on denaturation of the protein and under suitable conditions a moderately viscous solution can be converted by heating into a clear gel. These striking changes in viscosity, although they have been known for a long time, have not been studied in detail in recent years and in general the study of denaturation has been neglected. The main reason for this neglect has been the belief that denaturation is an irreversible reaction, probably an irreversible splitting up of the protein, and that therefore denaturation is of little physiological interest. The experiments described in previous papers (reviewed in Anson and Mirsky, 1931a) led us to the conclusion that the denaturation and coagulation of proteins, contrary to the usual notion, are reversible; and that therefore denaturation is a biologically possible process. We accordingly became interested in resuming the older investigations of the gross change in viscosity accompanying denaturation. The present experiments make more precise than was possible a generation ago the conditions for obtaining very viscous solutions and gels, and they show that one can convert not only a concentrated protein solution but even a solution containing only one per cent of protein into a clear gel. Although, as will be seen, there is an increase in viscosity due to denaturation itself apart from any aggregation of the molecules, in the very viscous solutions containing little protein the denatured protein seems to be in the form of aggregates, invisible precipitates, which occlude water.

Some of the biological processes, such as muscular contraction, which there is some reason to believe are accompanied by denaturation are also accompanied by a gross increase in viscosity. It might be supposed that the viscosity changes are a result of denaturation. If, however, the viscosity change due to denaturation is, in general, due to aggregation, then since aggregation is not peculiar to denatured proteins, an increase in viscosity cannot by itself be taken as proof of denaturation. The proof that denaturation occurs biologically must come from tests more specific for denaturation.

The experiments on the viscosity of denatured egg albumin solutions provide a possible explanation of the difficulties encountered in the reversal of the denaturation of egg albumin. The essential step in the preparation of native protein from an acid solution of denatured protein is neutralization. But neutralization also causes precipitation of a denatured protein and if the protein is precipitated reversal of denaturation is prevented. With the relatively soluble denatured serum albumin one can get reversal without any particular precaution to avoid precipitation (Anson and Mirsky, 1931c). With the less soluble denatured hemoglobin and globin, rapid complete neutralization causes complete precipitation and no reversal takes place. To obtain reversal the neutralization is stopped before enough alkali has been added to cause visible precipitation (Mirsky and Anson, 1929, 1930; Anson and Mirsky, 1930, 1931b). With the very insoluble denatured egg albumin it is still more difficult to reach the reversal conditions without the protein becoming aggregated. The viscosity experiments show that denatured egg albumin becomes aggregated under the very conditions under which denatured hemoglobin is converted into native hemoglobin. In harmony with this result, only recently and under favorable conditions have we been able to reverse the denaturation of egg albumin at all and then only to a small extent (unpublished experiments).

EXPERIMENTAL

The egg albumin used in these experiments was thrice crystallized and finally dialyzed in a shaking dialyzer (Kunitz and Simms, 1928) against distilled water at 5°C. A 10.3 per cent solution had a conductivity of 5.1×10^{-5} reciprocal ohms, a value which shows the needlessness of electrodialysis.

To prepare the ox hemoglobin used, laked red blood corpuscles were diluted

with an equal volume of water, shaken with a fifth the total volume of toluol and allowed to stand in the cold. The next morning the tuluol and swollen stromata were removed by centrifugation.[1]

Relative viscosity of centrifuged solutions was measured with an Ostwald viscosimeter. A few measurements made with the du Noüy coaxial cylinder viscosimeter gave essentially the same results.

Effect of Acid.—In the first series of experiments (see Table I) solutions of 4 per cent egg albumin containing various concentrations of HCl are heated for 4 minutes in large test tubes kept in boiling water and then cooled to 25°C. If not enough acid is added the protein is precipitated. If just enough acid is added to prevent precipitation the

TABLE I

Effect of pH on Viscosity of Heated 4 Per Cent Albumin

HCl	pH	Appearance	Relative viscosity
mols per liter			
0.004	4.29	Precipitate-gel	
0.005	3.96	Slightly opalescent—almost a gel	
0.008		Clear	8.08
0.010	3.70	Clear	3.92
0.012		Clear	2.09
0.040		Clear	1.52
0.065		Slightly opalescent	About 40

solution is very viscous. The more acid the solutions the less viscous they are after being heated and then cooled, until finally further addition of acid makes them more viscous again and slightly opalescent. As in other protein phenomena, after a certain point the addition of acid has the same effect as the addition of salt. It may be seen in these as in other experiments, that small differences in hydrogen ion concentration may be accompanied by great differences in viscosity, especially

[1] All the experiments described in the present paper, except the one on the hydration of hemoglobin in urea solution, were done in the winter of 1927–28. Since then we have found it desirable to use alumina cream to facilitate the removal of toluol and stromata. The remark is made to avoid giving the impression that the use of alumina cream described in other papers has been abandoned. It may be that the commercially available Filter-cel which is very useful in the filtration of some protein solutions can be substituted for alumina cream.

in the range near the precipitation zone where the solutions are very viscous.

Effect of Salt Added before Heating.—In the previous section there was described a heated solution of 4 per cent albumin in 0.012 N HCl which had a relative viscosity of 2.09. If NaCl is added before the heating so that its concentration is 0.005 M then after the heating the solution is more than three times as viscous as without the salt (see Table II). The same effect is produced by 0.0002 M K_2SO_4 and by even smaller concentrations of $K_4Fe(CN)_6$. Similar experiments can be done in great variety with the general result that whenever a moderate increase in viscosity can be caused by heating in the absence of salts and the presence of a small concentration of salt results in visible

TABLE II

Effect of Salt on Viscosity of Heated 4 Per Cent Albumin

Salt	Concentration	Time after heating	Relative viscosity
		hrs.	
NaCl	0.005 M	0	7.3
		2	11.8
		4	13.4
		22	17.4
K_2SO_4	0.002 M	0	7.3
		2	10.3
		4	11.5

precipitation of the protein, then a still smaller concentration of salt can be found which can cause a great increase in viscosity on heating.

It is characteristic in these experiments that the viscosity of a heated self-containing solution gradually increases with time. Thus the solution containing 0.005 M NaCl in 22 hours reaches a viscosity almost nine times as great as that obtained if no salt is added.

Effect of Salt Added after Heating.—If salt is added to a suitable solution of albumin which has been heated and then cooled the viscosity rises and continues to rise for a long time. The gradual rise in viscosity can be speeded up and made more extreme by raising the temperature. For instance, a solution whose relative viscosity is only 1.55 is obtained by adding an equal volume of water to a 3 per cent solution of

egg albumin in 0.006 N HCl which has been heated 4 minutes and cooled to 25°C. When an equal volume of 0.002 M K_2SO_4 is added instead of the water the viscosity is 2. After 22 minutes it is 2.76 and after 2 days the solution has become a slightly opalescent hard gel. The hard gel may be obtained in 30 seconds by placing a test tube of the solution containing 0.001 M Na_2SO_4 in boiling water.

A similar experiment illustrates the facts (1) that one can obtain very viscous solutions whose protein content is only 1 per cent and whose salt concentration is only 0.0008 M, and (2) that whether or not albumin is precipitated when its solution is heated depends not only on the composition of the solution but also on its history. An equal volume of 0.0016 M K_2SO_4 is added to a heated 2 per cent solution of albumin in 0.004 N HCl. The viscosity is 1.28 and increases gradually. Warming to 37°C. causes a gross increase in viscosity and heating in boiling water for 3 minutes makes the solution slightly opalescent and almost a gel. If an equal volume of 0.0016 M K_2SO_4 is added to an unheated 2 per cent solution of albumin in 0.004 N HCl, then on being heated the protein is definitely precipitated.

Effect of Alkali Added after Heating in Acid.—If a solution of albumin in acid is heated and then neutralized the protein is precipitated. If, however, the amount of alkali added is just not enough to cause precipitation then the resulting solution in time becomes very viscous even if the concentration of protein is low. For example, to a heated 3 per cent solution of albumin in 0.006 N HCl is added an equal volume of 0.00268 N NaOH. The viscosity at first is 1.67. After 19 hours it is 6.82.

Effect of Addition of Native Protein.—The addition of native, salt-free egg albumin to albumin heated in acid has the same sort of effect as the addition of NaOH. The solutions become more viscous and the viscosity continues to rise with time. Very characteristic in these solutions is the great increase in viscosity produced by warming the solutions only a few degrees. The native albumin probably acts not only by combining with the acid to produce a less acid solution in which the denatured albumin is less soluble, but also by combining with denatured albumin itself, since, at the pH produced, denatured egg albumin would precipitate in the absence of native protein. In general, in many so-called mixtures of soluble and insoluble proteins of

all sorts when the conditions are such that the insoluble protein does not precipitate, although it would precipitate in the absence of the soluble protein, then the solution of the two proteins may have a variety of physical properties not possessed by solutions of either of the two proteins taken alone.

The experiment consists in adding to a 3 per cent solution of albumin heated in 0.006 N HCl a 10.3 per cent solution of dialyzed native albumin. When an equal volume of native albumin is added, the viscosity is at first 1.73 and the next morning 7.50. When a third more native albumin is added the viscosity is at first 1.75 and the next morning too great to be measured.

Effect of Water Added before and after Heating.—If an acid solution of albumin is diluted with water before it is heated, the water has a much greater effect in lowering the viscosity than if it is added after the heating of the more concentrated solution. For instance, if a 4 per cent solution of albumin in 0.008 HCl is diluted with an equal volume of water before heating, then after heating the viscosity at 25°C. is 1.48. If the dilution is done after the heating the viscosity is 3.85. Even more striking results can be obtained by using somewhat more viscous solutions. It is easy to obtain two solutions of the same composition whose viscosities differ more than five times.

Acid likewise has a greater effect on the viscosity when it is added before rather than after the heating. As has already been shown, near the precipitation zone the addition of a little more acid before the heating makes the viscosity on heating much lower. The addition of the same amount of acid after heating has little more effect than the addition of the same amount of water.

Without multiplying examples it may be said that in general it is easier to prevent the formation of a viscous solution by adding water, acid or alkali, than to decrease grossly the viscosity of an already viscous solution, just as it is often easier to prevent the precipitation of a substance than to dissolve it once it is precipitated. Heat coagulated and precipitated albumin does not dissolve readily in a concentration of acid which is sufficient to prevent the precipitation on heating.

Effect of Urea.—The addition to saturation of solid urea to a very viscous solution of denatured egg albumin always results in a gross lowering of viscosity. For instance, 4 gm. of neutral urea were added

to 5 cc. of a solution whose viscosity thereupon dropped from 8.18 to 2.14. Furthermore in many cases the viscosity is about the same whether the urea is added before or after the heating. It is impossible to get a very viscous solution of denatured albumin in saturated urea solution with the concentrations of proteins and electrolytes used in the experiments already described.

The experiments with egg albumin can be varied in many ways, always with the result that if only one factor is varied at a time then a range can be found in which the viscosity is very sensitive to that factor. It is in this range, which is always close to the precipitation zone, that the highest viscosities are obtained.

Experiments similar to those with egg albumin and giving similar results can be done with other denatured proteins. A few experiments with hemoglobin are summarized in Table III. The hemoglobin concentration is 5 per cent, the time of heating 4 minutes. The solutions are filtered before the viscosity measurements and are all clear.

TABLE III

Effect of pH on Viscosity of Heated 5 Per Cent Hemoglobin

HCl	Relative viscosity
mols per liter	
0.0021	7.60
0.0024	3.18
0.0063	2.37

DISCUSSION

Viscosity Change Due to Aggregation.—All the theories of viscosity agree that the viscosity of a solution depends in some way on the fraction of the total volume occupied by the solute. Kunitz (1926) has shown empirically that when partial volume is plotted against viscosity the same curve results for a number of different colloids. By the use of this curve it is possible to calculate in a new case the partial volume of the solute from the viscosity of the solution. When the value so obtained is higher than that calculated from the concentration and

density of the solute, then one must assume that the solute contains water. When the amount of water is greater than can reasonably be attributed to ordinary hydration then one must further assume that the solute is not dispersed into molecules of the ordinary size but that it has a structure which consists of aggregates occluding water. Such *a priori* considerations lead to the view that in the more viscous solutions of denatured protein the protein is in part aggregated. This view is in harmony with all the experimental facts.

1. In general the conditions for a high viscosity are close to those for visible precipitation. If an acid solution of heated, denatured protein is brought just a little closer to the isoelectric point or a little salt is added, the solution becomes much more viscous or the protein actually precipitates. If the solution before being heated is slightly further away from the isoelectric point or contains slightly less salt then the viscosity of the heated solution is very much reduced. It is characteristic of colloidal aggregates that in a narrow critical range slight changes in ionic concentrations should cause either further aggregation or dispersion.

2. The great increase in viscosity which follows the heating of a suitable protein solution can readily be prevented by the addition of water or acid *before* the heating. The addition of water or acid *after* the heating has much less effect on the viscosity. It is known that it is easier to influence the formation of aggregates and precipitates than it is to dissolve them once they are formed.

3. Urea which can dissolve denatured proteins always lowers the viscosity of a very viscous solution of denatured protein. Were there no solubility effect, the addition of solid urea ought to increase the viscosity.

4. The increase of viscosity with time in many of the solutions can be accounted for by gradual aggregation.

Viscosity Change Not Due to Aggregation.—Although the gross increase in viscosity, which may under suitable conditions accompany the denaturation of a dilute solution of protein, is probably due to aggregation of the protein, there is evidence that denaturation of itself causes an increase of viscosity apart from any aggregation. Concentrated solutions of urea not only dissolve coagulated proteins (Spiro, 1900; Ramsden, 1902) but also denature native protein (Ramsden,

1902, 1913; Anson and Mirsky, 1929; Hopkins, 1931). Denatured protein precipitates when the urea is removed. The more the protein is denatured as determined by the solubility test the more viscous the solution becomes. When the protein has become completely insoluble at its isoelectric point in the absence of urea, the viscosity ceases to change (Anson and Mirsky, 1929). The final relative viscosity of an 8.5 per cent solution of hemoglobin may be 6.3. Since these experiments were described, Burk and Greenberg (1930) and Huang and Wu (1930) have shown by osmotic pressure measurements that denatured proteins are *not* aggregated in concentrated urea solution. The molecular weight of denatured hemoglobin in urea solution is not greater than the molecular weight of native hemoglobin in water. Burk and Greenberg, however, in order to make the osmotic pressures of solutions of different protein concentrations proportional to the protein concentrations had to assume that hemoglobin is hydrated to the extent of 2.8 gm. of water per gm. of protein. Kunitz (1927) had previously calculated the hydrations of gelatin at different concentrations needed to make the osmotic pressure of gelatin solutions proportional to the protein concentration, and he had shown that the same values for the hydration could be calculated independently from the viscosities of gelatin solutions by the use of his curve which relates viscosity to solute volume. We have now likewise shown that the hydration of denatured hemoglobin in urea solution calculated from the viscosity of the solution is about the same as that calculated by Burk and Greenberg from their osmotic pressure measurements which showed denatured hemoglobin in urea solution not to be aggregated. The increase in the viscosity of a concentrated urea solution of hemoglobin as the protein denatures is thus probably due to ordinary hydration and not to aggregation. This increase in viscosity must not be confused with the *decrease* in viscosity which is obtained when solid urea is added to the very viscous solutions of egg albumin. In such cases the urea is dissolving aggregates of already denatured protein.

A solution containing 0.385 gm. denatured dialyzed horse hemoglobin, 5.5 gm. urea and 10 ml. water has at 30°C. a viscosity of 1.6 relative to the viscosity of a solution containing 5.5 gm. urea per 10 ml. water. From Kunitz's curve one can read off that the solvated hemoglobin occupies a volume of 10 per cent of the total volume or 1.44 ml.

Since 0.385 gm. non-solvated hemoglobin occupies 0.29 ml., the solvation per gm. of hemoglobin is $\frac{1.44 - 0.29}{0.385}$ or 3.0 ml. This calculation involves the assumption that so far as using Kunitz's curve is concerned, a solution of hemoglobin in urea solution can be treated the same as a solution of hemoglobin in water. One can obtain about the same value for the solvation of the hemoglobin without using Kunitz's curve by finding what volume of sugar has to be substituted for the solid hemoglobin in the urea solution to get the same viscosity.

CONCLUSIONS

The viscosity of a protein solution is increased by the denaturation of the protein. This is true both when there is the formation of protein aggregates which occlude water and when there is no aggregation. Under certain conditions, as a result of the aggregation following denaturation, a solution containing only one per cent of protein may be converted into a clear gel. The conditions for obtaining very viscous solutions containing little denatured protein are always close to the conditions for actual precipitation and under these conditions the viscosity is very sensitive to slight changes in the concentration of salts and hydrogen ions.

REFERENCES

Anson, M. L., and Mirsky, A. E., 1929, *J. Gen. Physiol.*, **13**, 121.
Anson, M. L., and Mirsky, A. E., 1930, *J. Gen. Physiol.*, **13**, 469.
Anson, M. L., and Mirsky, A. E., 1931a, *J. Phys. Chem.*, **35**, 185.
Anson, M. L., and Mirsky, A. E., 1931b, *J. Gen. Physiol.*, **14**, 605.
Anson, M. L., and Mirsky, A. E., 1931c, *J. Gen. Physiol.*, **14**, 725.
Burk, N. F., and Greenberg, D. M., 1930, *J. Biol. Chem.*, **87**, 197.
Hopkins, F. G., 1931, *Nature*, **126**, 328, 383.
Huang, T., and Wu, H., 1930, *Chinese J. Physiol.*, **4**, 221.
Kunitz, M., 1926, *J. Gen. Physiol.*, **9**, 715.
Kunitz, M., 1927, *J. Gen. Physiol.*, **10**, 811.
Kunitz, M., and Simms, H. S., 1928, *J. Gen. Physiol.*, **11**, 641.
Mirsky, A. E., and Anson, M. L., 1929, *J. Gen. Physiol.*, **13**, 133.
Mirsky, A. E., and Anson, M. L., 1930, *J. Gen. Physiol.*, **13**, 477.
Ramsden, W., 1902, *J. Physiol.*, **28**, xxiii.
Ramsden, W., 1913, *Tr. Faraday Soc.*, **9**, 93.
Spiro, K., 1900, *Z. physiol. Chem.*, **30**, 182.