XXI. DILATOMETRIC INVESTIGATIONS ON THE HEAT-DENATURATION OF PROTEINS. I.

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It has been commonly assumed that heat-denaturation of proteins is accompanied by dehydration of the protein molecules [Robertson, 1912; Pauli, 1912; 1932; Sørensen, 1925; Spiegel-Adolf, 1926; 1927; 1929; Weber and Versmold, 1931]. The last-mentioned authors showed that the "non-dissolving space" (nichtlösender Raum), *i.e.* such part of the system as does not dissolve an added substance of low molecular weight (sugar), decreases during denaturation. Since the "non-dissolving space" is composed of the volume of the particles and of the hydration layer, in which the water molecules are oriented under the influence of the particles, this result supports the assumption that hydration decreases during heat-denaturation.

Kruyt and de Yong [1934] tried to detect a decrease in hydration by measurements of viscosity. Their experiments however showed that the viscosity increased when protein solutions were heated to a temperature just below the coagulation point, whereas in the case of a decrease in hydration one would have expected a decrease in viscosity. It had already been shown by Loughlin and Lewis [1932] that solutions of completely denatured protein had higher viscosities than those of the natural ones. Kruyt and de Yong rightly remarked however that this increase in viscosity might be due to an increase in particle size and thus need involve no contradiction of the assumption of a simultaneous decrease of hydration. If there is a decrease in hydration during heat-denaturation it is likely that the total volume of the system will increase, since the water of the hydration layer, oriented and compressed as a result of attraction between colloidal particles and water molecules, possesses a smaller specific volume than free water. This view is supported by the observation that, if a protein swells or is dissolved in water, the total volume of the system decreases by about 6–7 ml./100 g. protein [Chick and Martin, 1913].

It is likely that the volume change during heat-denaturation will be only a small fraction of the volume decrease during the solution of the protein, just as the change of the non-dissolving space during the denaturation is only 7 % of the total non-dissolving space (0.09 g. as against 1.25 g./g. protein, Weber and Versmold [1931]). Consequently for a 5 % protein solution an accuracy of at least 0.002 % of the total volume is necessary for qualitative detection of denaturation whilst an accuracy at least ten times as great is required for quantitative measurements.

Investigations on the volume change during heat-denaturation have been carried out by Loughlin and Lewis [1932] and by Haurowitz [1935]. They conclude that there is no volume change. Loughlin and Lewis however used comparatively dilute solutions and rather insensitive dilatometers. Haurowitz's dilatometer, although more sensitive, was of the ordinary pyknometer type. Such instruments are not reliable enough for these measurements, for some portion of their contents might easily be left adhering to the inside walls of the

top section on cooling in the thermostat after heat treatment; this would lead to false capillary readings. Moreover, Haurowitz had to introduce corrections for losses due to evaporation. Experimental errors such as these can be overcome by the employment of a mercury dilatometer as used by Heymann [1935] in studies on sol-gel transformations. The sensitivity of this dilatometer is 0.0002 % of the total volume, *i.e.* 50 times that of Haurowitz's dilatometer. Measurements on the heat-denaturation of egg albumin, serum albumin, serum globulin and zein have therefore been made with this more sensitive instrument.

EXPERIMENTAL.

The apparatus used is shown in Fig. 1. The left arm contained the protein solution and the right arm mercury. The height of the meniscus in the capillary *b* could be varied by means of tap *a*. Leakage at tap *c* was avoided by coating it with vacuum grease and by securing the cock with rubber bands. The protein solution was run in by way of *c* (2 mm. bore) using a very fine funnel. The trap *d* is designed to prevent the possible passage of any sol into the capillary, whilst bulb *e* above the capillary is a reservoir, since considerable expansion of the sol and the mercury occurs on heating.

After filling with protein sol, the dilatometer was left in an electrically regulated thermostat at 23° (constant within 0.003°) for several hours, until the mercury meniscus remained constant. Then the whole apparatus was transferred to a bath at 80° and left there for 30 min. to denature the proteins. The dilatometer was then replaced in the thermostat for about 2 hours. Prolonged temperature equilibration is necessary, because glass vessels heated to higher temperatures do not assume their original volume at once. The difference in mercury level before and after heating gives the volume change during denaturation. Usually the dilatometer was heated again to 80° for 30 min. to make sure that no further volume change occurred, *i.e.* that denaturation was complete.

In making a determination, displacements of the mercury level of the order of 60 mm. occurred; since the capillary radius was 0.23 mm. and the total volume about 80 ml. this represents an expansion of some 0.01 % (see Table I). Displacements of less than 1 mm. are not considered significant, so that the accuracy is about 2 % of the volume change.

Egg albumin I (Table I) was prepared from dried egg albumin (Merck) by electro dialysis with increasing potential (5 days, max. 220 v.) to precipitate the globulins. The final solution of p_H 4.9 coagulated completely on heating. As found by Pauli and Kölbl [1935], solutions at p_H 3.9 or 8.3 did not coagulate directly on heating but coagulated at once on adjustment to p_H 4.8 after heating. Egg albumin II, serum globulin and zein were very kindly supplied by Dr A. Neuberger (University College Hospital Medical School, London). The former was crystalline egg albumin prepared from egg white by accepted methods (but using Na_2SO_4 solution according to an unpublished method of Cannan) and electro dialysed for 5 days before use. The serum globulin was precipitated from ox serum by $(NH_4)_2SO_4$, and the zein was prepared essentially as described by Neuberger [1934]. The serum albumin (for which I am indebted to Dr H. Neurath) was prepared by electro dialysis of horse serum after precipitation of globulin by $(NH_4)_2SO_4$. All solutions were centrifuged from undissolved material

Fig. 1.

and used generally within a day of completing the preparation. During preparation bacterial contamination was minimised by keeping at a low temperature under a toluene-paraffin layer. p_H was adjusted with HCl, alkali or buffer solutions and measured with nitrophenol indicators. Protein concentrations were determined by dry weight of the salt-free solutions or in other cases by dry weight of heat-coagulable proteins. All dilatometric measurements showed satisfactory reproducibility.

Table I.

Protein	Conc. %	Volume of sol ml.	Volume increase			Behaviour on heating
			ml.	%	ml./100 g. protein	
Serum albumin	7.6	81	0.0123	0.0154	0.20	Coagulation
$p_H=5.0$	5.3	80	0.0090	0.0112	0.21	
Serum globulin	9.0	81	0.0120	0.0150	0.17	Coagulation
$p_H=5.6$						
Egg albumin I						
(a) $p_H=4.9$	4.5	80	0.0083	0.0104	0.23	Coagulation
(b) $p_H=3.9$	4.5	80	0.0050	0.0063	0.14	No coagulation
(c) $p_H=8.3$	4.7	79	0.0027	0.0032	0.07	No coagulation
Egg albumin II						
$p_H=5.8$	5.0	81	0.0105	0.0132	0.26	Coagulation
Zein in 90 % alcohol	7.3	80	0	0	0	No coagulation

Table I shows the experimental results. The protein solutions which are denatured on heating all show increase in volume after denaturation. At the same time the dual nature of protein coagulation (firstly denaturation and secondly coagulation of the denatured protein) is illustrated. Only in p_H regions near the isoelectric point is denaturation accompanied by coagulation. Here the volume increase due to denaturation is between 0.20 and 0.23 ml./100 g. protein for serum albumin and egg albumin. This is about 4 g. mols. water per g. mol. egg albumin (mol. wt. 35,000) and about 8 g. mols. water per g. mol. serum albumin (mol. wt. 68,000). It is somewhat smaller for serum globulin. If egg albumin is denatured at p_H values higher or lower than the isoelectric p_H , no coagulation occurs. In this case also the volume changes are smaller, being between 35 and 70 % of the isoelectric values. These lower values cannot be due to incompleteness of denaturation at non-isoelectric p_H , since complete coagulation occurs by adjustment to isoelectric reaction after heating; this is presumptive evidence of previous complete denaturation.

Experiments have been carried out with non-purified egg albumin and "blood albumin". In these cases the volume increase was usually smaller than found with highly purified proteins. Experiments with commercial haemoglobin samples were not easily reproducible. They should be repeated with purified haemoglobin, though dilatometric investigations may present some difficulty since haemoglobin solutions contain considerable quantities of gas.

If we now consider the volume increase during heat-denaturation of isoelectric proteins (0.20–0.23 ml./100 g. protein) and compare it with the volume decrease when albumin is dissolved in water (6–7 ml./100 g. protein) we notice that the former volume change is only about 3–4 % of the latter. We may therefore assume that the decrease in hydration during denaturation is only a small percentage of the total hydration. It should however be borne in mind that there is no simple relation between the volume change and the actual change of the

number of water molecules in the attractional sphere of a protein molecule, since the volume change does not depend only on the number of water molecules influenced by the protein particle, but also on the intensity of the attractive forces.

CONCLUSIONS.

Although many chemical and physical changes during heat denaturation of proteins have been investigated, it is still difficult to give a consistent account of the nature of this important process. Therefore existing theories and opinions will only be briefly examined in this paper. Several authors [Lepeschkin, 1922, 1, 2; 1923; Wu and Wu, 1923] have assumed that heat-denaturation is accompanied by hydrolysis. It is interesting in this connection that sulphhydryl groups appear during denaturation [Hopkins, 1930]. The fact that the volume increases is however not in accordance with the existence of such hydrolytic reactions, since these processes would be connected with an increase in hydration and consequently a decrease in volume [Rona and Fischgold, 1933; Sreenivasaya *et al.*, 1934]. If such processes occurred, we should have to assume that their influence on the volume must be more than compensated by processes which bring about a volume change in the opposite direction.

Such processes have been assumed for a long time [Robertson, 1912; Pauli, 1912; 1932]. According to these authors heat-denaturation is connected with the formation of peptide bonds from —COOH and —NH_2 groups. Cohn *et al.* [1933] assume rather that —COOH groups and —NH_2 groups come into each other's sphere of attraction (*i.e.* that the zwitterionic character of the protein becomes less pronounced [Pauli, 1931]), and in this way acquire a lowered attraction for water dipoles (electrostriction). All these processes are presumably accompanied by decrease in hydration and increase in volume and their existence is thus in good agreement with the experiments of this paper. Away from the isoelectric point, where the zwitterionic character is less marked in the native protein, it is likely that a smaller volume change will occur during denaturation. This is also in agreement with the experimental results.

Several authors have discussed the possibility that denaturation results from a change of intermolecular polarisation consequent upon a change of position of some groups of the protein [Wu *et al.*, 1931; Haurowitz, 1935]. Astbury and Lomax [1935] conclude from X-ray investigations that the polypeptide chains become more regularly arranged in the molecule during denaturation. The number of such possibilities is however so great that it is difficult to foresee their effect on the volume.

SUMMARY.

The heat-denaturation of several proteins has been investigated with a sensitive dilatometer, the accuracy of which was 0.0002 % of the total volume. In all cases heat denaturation was accompanied by a volume increase which amounted to 0.20–0.23 ml./100 g. protein in the case of isoelectric proteins. If non-isoelectric proteins were used the volume increase was smaller. It has been assumed that this volume increase is caused by a decrease in hydration, which is however only a small percentage of the total hydration. Several theories of the heat-denaturation have been discussed on the basis of the experiments of this paper.

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