LXXXI. NOTE ON THE ALLEGED REVERSAL OF THE DENATURATION OF SERUM-ALBUMIN.

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THE claim to have effected the reversal of the denaturation of serum-albumin [Anson and Mirsky, 1931] is so contrary to general experience that it is worthy of close examination. The method consists in dissolving the denatured protein in dilute acid and neutralising with dilute sodium hydroxide solution at room temperature. A portion of the albumin treated in this way crystallises like untreated albumin and behaves similarly in the precipitin reaction [Miller, 1933]. At first sight this evidence appears convincing but it is necessary to consider the various steps in the process and the assumptions involved very critically, since the conclusions reached are of some importance in the study of proteins.

EXPERIMENTAL METHODS.

Serum-albumin.

The serum-albumin used was prepared from horse plasma. Globulins were removed by precipitation with 18 % of anhydrous sodium sulphate at 34°, and crystallisation of the albumin was effected by cautious addition of acetic acid to the filtrate in the ordinary way. Crystallisation of albumin is usually carried out in ammonium sulphate filtrates, but the sodium sulphate process is equally effective. The crystalline albumin was filtered off on cloths, lightly pressed and dialysed in cellophane bags. The resulting solution was brought to p_H 7 and filtered through a sterile Seitz filter. The resulting 800 ml. of sterile 10 % solution of albumin was used as a stock preparation in these and other experiments.

Electrometric p_H determinations.

A compact micro-quinhydrone electrode assembly was devised (Fig. 1). The cell is readily made by drawing out a piece of 7.5 mm. bore glass tubing and bending the ends round into the form of a U-tube. The bottom horizontal portion of the tube is filled with 3 % agar saturated with KCl and acts as a salt bridge. In one limb is placed a buffer solution of known p_H and in the other the solution to be investigated. About 0.3 ml. of solution is sufficient for a determination. A little quinhydrone is added to each limb and platinum wire electrodes of the usual form are placed in each limb, being supported by corks resting on the rims of the limbs. A Cambridge portable potentiometer completes the simple apparatus.

Effect of p_H on denaturation.

A neutral 0.4 % solution of serum-albumin in 0.8 % saline was titrated with 0.1 N HCl, and 4.5 ml. samples of different p_H values were withdrawn at various stages of the titration. For the p_H determinations the quinhydrone vessel described above was used. The samples were placed in 5 × 0.5 in. tubes in a rack immersed in a tank of boiling water from which the burner had just

been removed. The tubes were so arranged in the rack that samples of similar p_H were not adjacent. When the tubes had been in the hot water for 3 minutes they were transferred to a bath of cold water. The precipitates, when present, were filtered off and protein determinations on the filtrates were conducted by means of the phenol reagent of Folin and Ciocalteu. In Fig. 2 are plotted the

Fig. 1.

Fig. 2.

Fig. 1. Quinhydrone electrode vessel.

Fig. 2. Effect of p_H on heat-coagulation of albumin. (The vertical line, indicated by arrow, indicates the isoelectric point.)

amounts of precipitation at different levels of p_H . It will be observed that coagulation has been maximum in the region of the isoelectric point (p_H 4.5 to 5.0) and falls away slowly on the alkaline side but very steeply on the acid side. Thus, taking points equidistant from the isoelectric point (say p_H 4.75), at p_H 5.5 over 80 % of the protein is coagulated, whereas at p_H 4.0 only 9 % is precipitated. Heating at reactions more acid than p_H 4.0 does not effect coagulation when the solution is cooled and brought to the isoelectric point.

Effect of salts.

It is well known that coagulation of proteins on heating does not occur in the absence of salts and this is shown in the following experiment. The stock 10 % solution of albumin was diluted with distilled water giving a final concentration of 0.2 % albumin. Twelve samples were taken and their reactions adjusted with 0.02*N* HCl to p_H values ranging from 4.1 to 7.2. The samples were heated in a boiling water-bath for 15 minutes but no coagulation occurred. A small amount of salt was added to each of the cooled tubes without there being any coagulation, but when the tubes were reheated coagulation occurred, being almost complete in the samples of p_H from 4.6 to 5.0. Electrodialysed albumin is said to coagulate without added salt [Adolf, 1926]. The effect of salts on protein coagulation was not taken into account by Anson and Mirsky.

Modifications of Anson and Mirsky's experiment.

Unless the experimental conditions, especially p_H , are carefully selected it is not possible to repeat Anson and Mirsky's experiment, and some care is necessary in finding the appropriate conditions, since in their paper no p_H

measurements are recorded. The following experiment is of some interest and will be described in detail.

A 2 % solution of serum-albumin in 0.8 % saline was rendered acid (p_H 2.3) by addition of hydrochloric acid and heated for 4 minutes in a boiling water-bath. The solution whilst still hot was divided into three portions. One portion was brought to the isoelectric point by addition of sodium hydroxide to the hot solution and then immediately cooled (Part I), a second portion was brought to the isoelectric point and allowed to remain hot for 2 minutes before cooling (Part II), whilst the third portion was allowed to remain hot for 2 more minutes, then cooled and finally brought to the isoelectric point when cold (Part III).

Part I, which had been brought to the isoelectric point and immediately cooled was in the form of a granular precipitate; Part II which had been heated for 2 minutes at the isoelectric point was in the form of a gummy coagulum; whilst Part III, which had been heated in acid solution and cooled before being brought to the isoelectric point, was a perfectly clear solution. The method of reversal of denaturation described by Anson and Mirsky was applied to Parts I and II only, since Part III was already totally in solution. Part I dissolved readily in dilute hydrochloric acid giving a clear solution but Part II gave a highly viscous gel with which efforts at reversal were quite unsuccessful. When the acid solution of Part I was again brought to the isoelectric point by addition of dilute sodium hydroxide at room temperature a turbidity was produced, and, on addition of an equal volume of saturated ammonium sulphate, there was a considerable amorphous precipitation. Now when an equal volume of saturated ammonium sulphate solution was added to Part III the protein remained soluble and on cautious addition of dilute acetic acid in the usual way the albumin crystallised out in fine rosettes of needles, no amorphous material being detected microscopically. If the granular precipitate (Part I) be centrifuged down and ground by an agate pestle in an agate mortar some 40 % dissolves up in the supernatant fluid.

Comment on these experiments is made in the following section, but a point raised by Miller [1933] must be referred to here. He mentions the fact that, since the solubility of native albumin in half-saturated ammonium sulphate solution near the isoelectric point is limited, the precipitate obtained is not necessarily all denatured protein. As a matter of observation however the addition of an equal volume of saturated ammonium sulphate to serum-albumin at the isoelectric point (p_H 4.8) results in a considerable change in p_H (to p_H 5.2) and at this reaction undenatured albumin is soluble.

Using the disulphide reaction [Walker, 1925] and observations of optical rotatory power [Hewitt, 1927] evidence of reversal of denaturation was not obtained.

DISCUSSION.

At the outset we are faced by the significant fact that, in order to observe the reversal of denaturation, it is necessary to adhere rigidly, not only to the conditions essential for the reversal, but also to Anson and Mirsky's methods of preparing the denatured albumin. The exact repetition of their experiments is rendered difficult by their neglect to record the p_H values of their solutions.

The first two methods employed by Anson and Mirsky for preparing denatured albumin, namely precipitation with trichloroacetic acid and with acidified acetone, need not be considered for the moment as there is no satisfactory evidence that the albumin has been denatured. The third method consists in warming the albumin in acid solution and adjusting the reaction of

the hot solution to the isoelectric point. If immediately cooled the precipitate may be redissolved in dilute acid preparatory to the reversal of the denaturation. This is effected by the addition of 0.1*N* NaOH to the solution at room temperature until the isoelectric point is reached. It may seem that the partial neutralisation with dilute caustic soda at room temperature is not a process likely to effect a reversal of denaturation. The dissolving of the precipitate in dilute acid would seem more likely and, indeed, Northrop [1931] has reactivated a small portion (up to 1 %) of inactivated pepsin by treatment with acid. Actually however the crux of the matter has appeared at an earlier stage. The fundamental difficulty lies in the exact definition of a denatured protein. Anson and Mirsky's [1931] definition, "All denatured proteins are insoluble at their isoelectric points but soluble in acid or alkali," is not of general application, but it will serve our present purpose if accepted with suitable qualifications.

It is obvious that in order to prove the possibility of reversing the denaturation process it is essential to the argument to prove satisfactorily that the protein has in fact been denatured. Now Anson and Mirsky assume, without presenting any evidence to support the assumption, that when heated at 100° in acid solution for 4 minutes albumin is quantitatively denatured. It is a fact however that serum-albumin, after being treated in this way, then cooled down to room temperature and the reaction adjusted to the isoelectric point, gives no evidence of being denatured. The albumin is still soluble in water and dilute salt solutions at the isoelectric point, it is still soluble in 50 % ammonium sulphate and may be crystallised in the usual way. These facts would be explained by Anson and Mirsky as being due to the reversal of the denaturation during the neutralisation of the cold acid albumin solution. In order to avoid this reversal Anson and Mirsky neutralise the acid solution whilst it is still hot, and under these conditions protein is precipitated at the isoelectric point. They assume that reversal of denaturation is avoided by neutralising the hot instead of the cold solution. Reversal of the denaturation is now effected by dissolving the precipitate in dilute acid and then neutralising the cold solution. In this way some two-thirds of the original amount of protein may be recovered in the undenatured form. Anson and Mirsky's explanation however fails to explain why there should be quantitatively complete recovery of undenatured protein in the experiment mentioned above where the albumin heated in acid solution is cooled before neutralisation. Since the reversal process, namely the neutralisation of the cold acid solution, has been identical in each case the different yields of undenatured protein must be due to differences in the denaturation process. Anson and Mirsky suggest that the simplest explanation of their partial recovery of undenatured protein is that there are two effects of heating protein solutions, a reversible and an irreversible denaturation. There is however no necessity to introduce this complication. Let us consider for a moment a further modification of the experiment. If the hot albumin solution when brought to the isoelectric point, instead of being at once cooled, is kept hot for a minute or two longer, the character of the precipitate changes, and the denaturation cannot be reversed. (It should be emphasised that a longer period of heating the acid solution does not have this effect.) The simplest explanation of all the facts and observations is that the heating of the albumin in acid solution has little, if any, denaturing effect. Heating at the isoelectric point however results in very rapid denaturation, so that when the hot acid solution of albumin is brought to the isoelectric point, there is an immediate precipitation of granular floccules. These floccules consist of a shell or skin of denatured protein surrounding a core of undenatured albumin and when ground up in an agate mortar some 40 %

of the precipitate is dissolved. If the granular precipitate be immediately cooled, it dissolves readily in dilute acid, and when again neutralised the undenatured protein remains dissolved, whereas the denatured portion retains its characteristic properties, for example it is insoluble in half-saturated ammonium sulphate solution and is amorphous. If the experiment be varied by keeping the isoelectric solution hot for a minute or two before cooling, the denatured skin of the granular floccules can no longer protect the core and the protein in the interior of the floccules becomes denatured as well. This completion of denaturation is accompanied by a change in the appearance of the precipitate which now becomes more gummy. It is mentioned specifically by Anson and Mirsky that the portion of the protein (about one-third) which has not been reversed in the first instance, cannot be reversed by subsequent treatment. This portion corresponds to the denatured skin mentioned above and in the present experiments it was found that the longer the interval between the adjustment of the reaction to the isoelectric point and the cooling, the less protein could be recovered in the reversed condition. So far therefore there is no evidence that conflicts with the view that protein denaturation is an irreversible process and there is no evidence of two kinds of denaturation, reversible and irreversible.

At this point it is necessary to consider critically the evidence presented by Miller [1933] in support of the possibility of reversible denaturation. Using the precipitin reaction he concluded that native and "reversed" serum-albumin were similar but were different from denatured albumin. This putative evidence is however rendered void by the fact that the denatured protein he used was not a comparable control to the "reversed" albumin. The "reversed" albumin was prepared by Anson and Mirsky's method and the reversed portion had not therefore been denatured, whilst the "denatured" protein had been subjected to further heating processes at the isoelectric point, and was thus truly and irreversibly denatured.

It is clear therefore that the claim to have reversed the heat-denaturation of serum albumin has not been confirmed.

SUMMARY.

1. No evidence for the possibility of reversing the denaturation of serum-albumin was found.
2. Serum-albumin may be heated in acid solution without being appreciably denatured.
3. In experiments purporting to demonstrate partial reversal of denaturation evidence is lacking that the albumin recovered had been denatured.

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