PROTEIN COAGULATION AND ITS REVERSAL

SERUM ALBUMIN

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The reversal of the denaturation and coagulation of hemoglobin and globin has been described in previous papers (cf. review and literature in: Anson and Mirsky, 1931). This paper describes the preparation of crystalline, soluble, native protein from coagulated serum albumin and discusses the significance of the fact that this preparation offers so little difficulty.

Relation of the Ease of the Reversal of Denaturation to the Solubility of the Denatured Protein

All denatured proteins are insoluble at their isoelectric points but soluble in acid or alkali. There are, however, considerable differences in the solubilities of different denatured proteins just as there are in the solubilities of different native proteins. Denatured hemoglobin is much less soluble than denatured serum albumin. In the absence of salts it is precipitated over a much wider range of hydrogen ion concentration around the isoelectric point than is denatured serum albumin and it is precipitated from acid solution by a concentration of salt which does not precipitate denatured serum albumin. Denatured egg albumin is even less soluble than denatured hemoglobin. Not only is it insoluble over a wide range of hydrogen ion concentration, but even when the denatured egg albumin appears to be dissolved by small amounts of acid or alkali there is aggregation or invisible precipitation of the protein (unpublished viscosity experiments).
Corresponding to these differences in solubility are differences in the ease of the reversal of denaturation. The denaturation of the relatively soluble serum albumin can be reversed with great ease; the denaturation of the less soluble hemoglobin can be reversed only by taking special precautions; while so far it has not been possible to reverse the denaturation of the very insoluble egg albumin at all. Some 75 per cent of denatured serum albumin is converted into soluble native serum albumin on neutralization of an acid solution of the denatured protein. It makes no difference in the yield whether the protein is rapidly brought to the isoelectric point in one step, or first allowed to stand for half an hour in a solution just acid or alkaline enough to prevent precipitation. The same high yield is also obtained if the acid solution is one-tenth saturated with ammonium sulfate. In contrast, if an acid solution of denatured horse hemoglobin is rapidly brought to the isoelectric point, practically all the protein is precipitated. Reversal of denaturation is obtained, however, if the protein is first allowed to stand in a solution just acid or alkaline enough to prevent denaturation. Even with this procedure, no reversal is obtained and all the protein is precipitated, if the acid solution is one-tenth saturated with ammonium sulfate (Anson and Mirsky, 1925, 1929, 1930; Mirsky and Anson, 1929, 1930a). Finally, in the case of the very insoluble egg albumin, there is no reversal of denaturation or neutralization no matter how the neutralization is carried out.

Experimentally, then, there are two different results of neutralization of an acid solution of denatured protein: precipitation or aggregation of the insoluble denatured protein, and conversion of the insoluble protein into soluble native protein by the reversal of denaturation. In the three cases studied the more soluble the denatured protein is under the neutralization conditions, the easier and more complete is the reversal of denaturation.

The fact that in the cases of serum albumin, hemoglobin, and egg albumin, reversal of denaturation is more difficult the more insoluble the denatured protein does not mean that solubility is the only factor which determines to what extent reversal takes place or whether it can...

1Recent experiments, which remain to be confirmed, indicate that the denaturation of egg albumin can under certain conditions be reversed.
take place at all. Unfortunately the theory of the mechanism of the
reversal of denaturation (like the theory of the mechanism of denatura-
tion itself) is as yet in an unsatisfactory state. It is not clear why
reversal should result at all on neutralization, since native proteins
are unstable at their isoelectric points. For instance, isoelectric
methemoglobin or egg albumin coagulates slowly on standing, and
rapidly on being shaken with air or toluol globules. Nor is it clear
why it should not be possible to reverse denaturation completely, at
least on repetition of the reversal procedure. If the reversal procedure
is carried out with denatured hemoglobin, 65 per cent of the protein
is converted into a soluble form while 35 per cent remains insoluble.
If the reversal procedure is repeated with the part that remained
insoluble, this time much less than 65 per cent of the protein is made
soluble. The solution of the non-reversed fraction does not behave the
same as the solution of the original denatured hemoglobin.

A possible factor in causing this incompleteness of reversal is secun-
dary irreversible change in the protein caused by the denaturation pro-
cedure but distinct from denaturation. In the case of hemoglobin there
is not as yet any experimental evidence of such secondary change. It
is known, however, that when egg albumin is heated there is, entirely
apart from the formation of insoluble protein, a splitting off of ammo-
nia (Sørensen and Sørensen, 1925). Particularly interesting experi-
ments have been done with pepsin. Kühne (1877) discovered that
pepsin is inactivated by alkali. Goulding, Borsook, and Wasteney
(1926) showed that there are two different kinds of inactivation by
alkali, a rapid kind which does not go to completion except in strong
alkali, but the extent of which depends on the pH; and a slow kind
which always goes to completion, but the rate of which is propor-
tional to the hydroxyl ion concentration. Northrop (1930; 1931), working
for the first time with the pure pepsin protein, showed that both kinds
of inactivation are accompanied by a formation of protein insoluble
at the isoelectric point. The rapid inactivation and formation of
insoluble protein, however, can be partially reversed by a procedure
identical with that used for the reversal of denaturation, while by the

\*It must be remembered in this connection that surface coagulation is most
rapid at the isoelectric point. Under the conditions for reversal, it is much slower,
indeed may not take place at all.
same procedure the slow kind of inactivation cannot be reversed at all. The simplest explanation of these results is that the rapid inactivation is simply denaturation of the protein, while the slow inactivation is a secondary irreversible change, perhaps a hydrolysis.

Evidence That the Protein Has Been Denatured

Insolubility.—To prove that denaturation has been reversed it must be proved that the protein has been denatured in the first place. The serum albumin used in the neutralization experiments is prepared by the addition to native serum albumin of acid acetone which is, in general, an effective denaturing agent. Usually one can demonstrate that protein treated with acid acetone is denatured by showing that the protein is completely precipitated when brought to the isoelectric point. The relatively soluble denatured serum albumin, however, seems to be converted into native protein by just this procedure. This difficulty may be avoided by neutralizing at a temperature at which reversal does not take place. If native serum albumin is heated in acid solution, the protein is denatured but is kept in solution by the acid. If the acid is neutralized while the solution is still hot, the protein is completely precipitated. This precipitate of insoluble and hence denatured serum albumin may be separated, dissolved, and converted, with a yield of 65 per cent, into protein soluble in half saturated ammonium sulfate.

Effect of Heating and Trichloracetic Acid on Yield.—Were 75 per cent of the acid acetone serum albumin undenatured and were there no reversal on neutralization, then heating the acid acetone protein or precipitating it with trichloracetic acid ought to result in further denaturation and hence in a lowering of the yield of soluble protein on neutralization. Actually heating and trichloracetic acid have no effect on the yield, indicating that denaturation by acid acetone has been complete and that soluble protein is obtained by the reversal of denaturation.

SH and S-S Groups.—The high cystine content of serum albumin facilitates an entirely different and independent test for the completeness of denaturation and the reality of reversal. In general native proteins have few, if any, free sulphhydryl and disulfide groups, while denatured proteins have a number of such groups corresponding to
the total number of cystine and cysteine groups in the protein (Mirsky and Anson, 1930b; and unpublished experiments). Serum albumin treated with acid acetone or trichloracetic acid has the number of free sulfhydryl and disulfide groups characteristic of a denatured protein, and when soluble serum albumin is produced by the reversal of denaturation there is a corresponding disappearance of these groups (unpublished experiments).

**Historical**

The reversal of the denaturation of serum albumin was probably observed by Michaelis and Rona (1910). The experiments were not conclusive and there was some confusion in their interpretation, and so this investigation unfortunately never received the attention it deserved. Spiegel-Adolf (1926), however, showed definitely that if heat coagulated serum albumin is dissolved in alkali or acid and then electrodialyzed, soluble, heat coagulable protein is again obtained. No attempt was made to crystallize the protein. Spiegel-Adolf believed, in addition, that certain compounds of serum albumin with acid and alkali can be heated without the protein being denatured at all. Actually, one cannot tell from the type of experiment given in support of this conclusion whether serum albumin has been denatured or not.

**Experimental**

*Preparation of Native Serum Albumin.*—Defibrinated horse blood is left in the cold until the corpuscles have settled. The serum is siphoned off and half saturated with ammonium sulfate which precipitates the globulins and the few corpuscles which remain suspended in the serum. To each liter of filtrate is added 20 gm. solid ammonium sulfate which results in an immediate and complete precipitation of the serum albumin in the amorphous form. Crystallization which is much slower and less complete than amorphous precipitation has the disadvantage that the protein may be fractionated and may in any case be changed on standing. The amorphous precipitate is filtered off and dialyzed in the cold against distilled water.

*Preparation of the Acid Acetone Powder.*—The preparation is the same as that of the acid acetone powder of globin (Anson and Mirsky, 1930). To a 5 per cent solution of serum albumin in 0.05 N HCl is added ten times its volume of acetone containing 2 cc. of 5 N HCl per liter. The resulting precipitate is filtered, washed with acetone, pressed as dry as possible, and then dried in the air. The dry albu-
min hydrochloride readily dissolves in water to give a clear solution. Most of the pigment present with the native serum albumin remains in the acid acetone when the protein is precipitated. The small amount of pigment which remains with the protein is more green and less yellow than the original pigment.

Neutralization Experiments.—If 0.1 N NaOH is gradually added to a 2 percent solution of the acetone powder a point is reached at which a small precipitate is produced which increases with time. If 10 per cent more alkali is added the precipitate is redissolved. If 15 per cent less alkali is added no precipitate at all is formed.

The addition of an equal volume of ammonium sulfate to a solution which has been neutralized to the precipitation point causes further precipitation. Some 70 to 75 per cent of the protein, however, remains in the solution and has the properties of native serum albumin. The same result is obtained when the protein is allowed to stand half an hour in more acid or alkaline solution before being brought to the precipitation point and half saturated with ammonium sulfate. The table gives the results of such an experiment.

<table>
<thead>
<tr>
<th>ML of 0.1 N NaOH added to 10 ml of 2 per cent acid albumin</th>
<th>Per cent of protein not precipitated by half saturation with ammonium sulfate</th>
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</thead>
<tbody>
<tr>
<td>2.6 — precipitation point</td>
<td>74</td>
</tr>
<tr>
<td>0.8 × 2.6</td>
<td>77</td>
</tr>
<tr>
<td>0.9 × 2.6</td>
<td>73</td>
</tr>
<tr>
<td>1.1 × 2.6</td>
<td>68</td>
</tr>
<tr>
<td>1.2 × 2.6</td>
<td>78</td>
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Estimation of Yield with the Phenol Reagent.—To 1 ml of filtrate from the solution half saturated with ammonium sulfate (in a Pyrex test tube, not a pointed centrifuge tube) is added 9 ml of water, and after mixing, 2 ml of 20 per cent trichloracetic acid. The suspension is centrifuged and the supernatant solution poured off. The precipitate is dissolved in 2 ml of 0.1 N NaOH and transferred with 23 ml of water to a 50 ml Erlenmeyer flask. 1 ml of the phenol reagent (Folin and Ciocalteau, 1927) is added and then 1 ml of 3 N NaOH. After 10 minutes the blue color developed is compared with the blue color developed from a known amount of acid acetone albumin similarly precipitated with trichloracetic acid.

The estimation of proteins with the original phenol reagent of Folin and Denis (1912) as introduced by Wu (1922) does not yield colors proportional to the protein concentrations. Proportionality is obtained by the use of a higher concentration of the more soluble form of the phenol reagent described by Folin and Ciocalteau. Greenberg (1929) has made this same modification of Wu's procedure. The quantities he uses are somewhat different from ours and he adds the
phenol reagent after the alkali. In our experience, the procedure of Greenberg does not always yield perfectly clear solutions.

**Effect of Salt.**—If 1 ml. of saturated ammonium sulfate is added to the 10 ml. of the 2 per cent acid albumin, then on neutralization with 2.6 ml. of 0.1 N NaOH and half saturation with ammonium sulfate, 68 per cent of the protein remains in solution.

**Effect of Heating.**—The acid acetone solution is heated to 100°C for 3 minutes. On neutralization a 70 per cent yield of soluble protein is obtained.

**Effect of Trichloracetic Acid.**—To 10 ml. of 2 per cent acid albumin is added 20 ml. of water and 4.5 ml. of 20 per cent trichloracetic acid. The precipitate is centrifuged, dissolved by the addition of a minimum amount of 0.1 N NaOH, and the solution after being made up to 12.6 ml. with water is half saturated with ammonium sulfate. The yield of soluble protein is 76 per cent.

In a similar experiment, native serum albumin is precipitated with 5 per cent trichloracetic acid and the precipitate dissolved in an amount of alkali exactly equivalent to the trichloracetic acid which remains with the protein. The yield of soluble protein is 74 per cent.

**Reversal of Heat Denaturation.**—A mixture of 10 ml. of 2 per cent dialyzed native serum albumin and 3 ml. of 0.1 N HCl is heated for 3 minutes at 100°C. and then while still hot neutralized with 2.5 ml. of 0.1 N NaOH. The resulting suspension is centrifuged. The clear supernatant liquid (which gives only a haze with trichloracetic acid) is rejected and the precipitate is readily dissolved in 3 ml. of 0.1 N HCl plus enough water to give a final volume of 15 ml. 1 ml. is removed to provide a standard in estimating the yield. The 14 ml. are neutralized with 2.9 ml. of 0.1 N NaOH and half saturated with ammonium sulfate. 65 per cent of the protein is left in the solution.

**Crystallization of “Reversed” Serum Albumin.**—The serum albumin may be crystallized from the neutralized solution of 2 per cent albumin by adding to each 10 ml. of the solution half saturated with ammonium sulfate 2 to 2.5 ml. of saturated ammonium sulfate. It is better, however, to start with a more concentrated solution such as can be obtained by neutralizing a 5 per cent solution of acid albumin with 0.2 N NaOH. In this case 1.5 to 2 ml. of saturated ammonium sulfate per 10 ml. of half saturated suffices. The soluble serum albumin can be crystallized completely which indicates that half saturation with ammonium sulfate results in complete precipitation of denatured serum albumin even in the presence of a great excess of native serum albumin. The crystals of “reversed” serum albumin appear to have the same form as those of normal, native serum albumin.

**CONCLUSIONS**

1. It is possible to prepare crystalline, soluble, heat-coagulable serum albumin from coagulated serum albumin.

2. In the cases so far studied, the more soluble a denatured protein, the more easily its denaturation can be reversed.
REFERENCES

Folin O., and Denis, W., 1912, *J. Biol. Chem.*, 12, 239.