PROTEIN COAGULATION AND ITS REVERSAL

GLOBIN

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)

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It has already been shown that the coagulation of hemoglobin is reversible (review and literature in Anson and Mirsky, 1931). Hemoglobin, however, is a conjugated protein consisting of the simple colorless protein, globin, combined with the red iron porphyrin complex, heme. It might be supposed that the result obtained from the study of the reversibility of the coagulation of hemoglobin reflects not a property of proteins in general but a peculiarity which hemoglobin displays because it contains heme. We have accordingly studied the simple protein, globin, itself. If acid acetone under certain conditions is added to hemoglobin, the heme and the globin are separated, the heme remaining in solution and the globin being precipitated. By a suitable neutralization procedure it is possible to obtain from this acid acetone globin, with a yield of about 65 per cent, soluble, heat coagulable globin which can combine with heme to form crystallizable hemoglobin (Anson and Mirsky, 1930). The facts that acid acetone is in general a denaturing agent and that the denaturation of hemogobin can be reversed by exactly the same kind of neutralization procedure used to prepare soluble globin (Mirsky and Anson, 1930) suggest that the preparation of soluble globin from acid acetone globin consists essentially in the reversal of denaturation. In confirmation of this view, the present experiments show that acid acetone globin is denatured globin and that soluble globin can be prepared from globin which has been precipitated with trichloracetic acid or heated. The opinion of Hill and Holden (1927) that the preparation of soluble globin depends solely on the avoidance of denaturation is shown not to be justified by their experiments.

Evidence That Acid Acetone Globin is Denatured Globin.—Native proteins in general are soluble at their isoelectric points. Denatured proteins are insoluble at their isoelectric points but soluble in acid or alkali. The solubility of acid acetone globin is that of a denatured protein. As prepared from hemoglobin acid acetone globin is a compound of globin with hydrochloric acid which dissolves in water to give a strongly acid solution. It is completely precipitated by rapid, complete neutralization of the acid with a buffer salt and almost completely precipitated by rapid neutralization with NaOH. For instance, if an equal volume of 1 M K_2HPO_4 is added to a 5 per cent solution of acid acetone globin, the filtrate gives only a slight haze with trichloracetic acid. It is *a priori* possible that denatured proteins when precipitated carry down any native, soluble protein present and that therefore complete precipitation at the isoelectric point is not proof of complete insolubility and denaturation. Experimentally, however, we have not been able to obtain evidence that any significant amount of carrying down takes place under the conditions of our experiments. When denatured hemoglobin is precipitated in the presence of native hemoglobin, the concentration of native hemoglobin in solution is not changed (Anson and Mirsky, 1929). Similarly, if one adds ammonium sulfate at 0°C. to a mixture of heat-denatured globin (heated in a solution acid enough to prevent precipitation) and unheated globin in acid, all the heated globin is precipitated and all the unheated globin remains in solution. Northrop (1930) has done essentially the same experiment and obtained the same result with known mixtures of native and denatured pepsin globulin, and he has shown further that when a given fraction of pepsin is denatured by alkali the fraction denatured as estimated by loss of enzyme activity without any precipitation whatsoever, is the same as the fraction denatured as estimated by the amount of protein nitrogen remaining in solution after the denatured pepsin has been precipitated at the isoelectric point. These experiments do not mean that coagulated protein cannot adsorb native protein at all. Such adsorption does take place. But it is significant in amount only when the concentration of coagulated protein is very great in comparison with that of the native protein.

In general when proteins are denatured there becomes free a number of SH and S-S groups which is equivalent to the total number of cysteine

and cystine groups in the protein.* The acid acetone globin has the number of free SH and S-S groups characteristic of denatured proteins (unpublished experiments). Meldrum and Dixon (1930) state that denatured globin has no sulfhydryl groups. A simple qualitative test with nitroprusside shows that such groups are in fact present.

Finally, if the native globin obtained from acid acetone globin were simply native globin which had escaped denaturation, then the further treatment of the globin with trichloracetic acid or heat, both well known denaturing agents, ought to result in further denaturation and hence in a much lower yield of soluble globin. In fact, as will now be seen, trichloracetic acid and heat have little effect on the yield.

Reversal Experiments with Globin Precipitated by Trichloracetic Acid.—If the acid acetone globin is rapidly completely neutralized, as has just been seen, it is almost completely precipitated. If it is first only partially neutralized, a little less than enough alkali to cause incipient precipitation being added, then about two-thirds of the globin is found to be soluble native globin when after a time the neutralization is completed (Anson and Mirsky, 1930). If enough alkali is first added to precipitate and just redissolve the globin, then about one-third of the globin is found to be soluble in 0.4 saturated ammonium sulfate. Reversal on the alkaline side of the isoelectric point with globin gives a lower yield than reversal on the acid side. Since trichloracetic acid denatures proteins in acid solution, in the presence of trichloracetic acid only the alkaline reversal procedure is possible. When this is tried with a trichloracetic precipitate the same 33 per cent yield is obtained as from the globin to which no trichloracetic acid has been added. The quantities used are as follows. To 15 ml. of a 3.3 per cent of acid acetone horse globin is added 25 ml. of 2.2 per cent trichloracetic acid. The centrifuged precipitate is suspended in 10 ml. water and dissolved with 13 ml. of 0.1 N NaOH. After a few minutes 0.1 N HCl is added to give a heavy precipitate and the solution is 0.4 saturated with ammonium sulfate. The yield is estimated by the procedure already described (Anson and Mirsky, 1930).

Experiments exactly the same as the one just described can be done with hemoglobin (Mirsky and Anson, 1930) and serum albumin (unpublished experiments).

*See Mirsky, A. E., and Anson, M. L., 1930, *Proc. Soc. Exp. Biol. and Med.*, **28**, 170.

Reversal Experiments with Heated Globin.—A 5 per cent acid solution of the acid acetone ox globin is heated in boiling water for 3 minutes. By the neutralization procedure a 60 per cent yield of soluble globin is obtained. Again the same experiment can be done with hemoglobin and serum albumin.

Soluble globin can likewise be obtained from acid acetone globin prepared from hemoglobin heat coagulated in neutral solution.

Hill and Holden's Theory.—After it was shown (Anson and Mirsky, 1925) that globin prepared by the classical method of Schulz (1898) is denatured globin and that hemoglobin is a compound of native globin and heme while the hemochromogen prepared from hemoglobin is a compound of denatured globin and heme, Hill and Holden (1927) devised a method for preparing native globin. They assumed to begin with that coagulation cannot be reversed and that therefore the mere fact that they obtained native globin was in itself *a priori* proof that their native globin had never been denatured. They did not carry out any experimental tests to see whether the globin in fact had escaped denaturation. Their procedure involved extreme precautions to avoid denaturation, such as the careful maintenance of low temperature during the separation of globin from hemoglobin, the use of an atmosphere free from organic materials, etc. These precautions supposedly were the essential elements of the procedure. Actually these precautions are entirely unnecessary. We have found that by dialyzing globin against cold distilled water one can get the same 60 per cent yield of soluble protein whether one carries out Hill and Holden's procedure for separating heme and globin in the cold or at room temperature. The 60 per cent yield obtained at room temperature is higher than the one Hill and Holden reported for their preparation in the cold. And the yield they reported was higher than the one they obtained since in estimating how much denatured protein remained they did not precipitate the denatured protein completely. The opinion that the denaturation which admittedly takes place in acid at room temperature prevents the obtaining of soluble globin on neutralization is therefore not correct. The differences in yields in the different preparations are due to different conditions of neutralization, not to the greater or less avoidance of denaturation. Since there is no evidence in Hill and Holden's experiments that they avoided dena-

turation and since the present experiments show that one can obtain soluble globin from globin which has been denatured, it is not decided by Hill and Holden's experiments whether or not they were correct in their theory that native globin can be separated directly from hemoglobin. An observation of Holden and Freeman (1928) suggests, however, that Hill and Holden did denature their globin. If Hill and Holden's acid solution of globin is 1/100 saturated with ammonium sulfate then no soluble globin is obtained on neutralization. To explain this fact, Holden and Freeman had to assume that, contrary to experience, this small amount of salt actually brings about a rapid denaturation of the protein. A simpler explanation and one in accord with experience, is that the salt precipitates the already denatured protein, and thereby prevents reversal of denaturization.

CONCLUSIONS

1. The globin prepared from hemoglobin by the acid acetone method is denatured globin.

2. The denaturation and coagulation of globin by acid acetone are reversible.

3. Soluble globin can be obtained from the acid acetone globin even if the globin is first precipitated by trichloracetic acid or heated to 100°C.

4. Hill and Holden's theory that they separated native globin from hemoglobin without any intermediate denaturation is not proven by their experiments.

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