PROTEIN COAGULATION AND ITS REVERSAL

THE PREPARATION OF INSOLUBLE GLOBIN, SOLUBLE GLOBIN AND HEME

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The Reversibility of Coagulation

It has already been shown that the coagulation of hemoglobin appears to be reversible. One can prepare from completely coagulated hemoglobin soluble, crystalline hemoglobin which by the tests so far tried is indistinguishable from ordinary native hemoglobin (Mirsky and Anson (13)). Since hemoglobin is a typical coagulable protein (Anson and Mirsky (3)) the conclusion was drawn that protein coagulation in general is probably reversible. In support of this view there is now the fact that one can prepare from globin coagulated by heat or by acid acetone a soluble globin which can be coagulated again by heat and which can combine with heme to form crystallizable hemoglobin. Whether or not this soluble globin is identical with native globin as it exists in hemoglobin is not yet decided. The present paper describes empirically the technique for preparing globin. A later paper will deal with the crystallization of synthetic hemoglobin, the preparation of soluble globin from heat coagulated globin, the factors determining the apparent reversibility of protein denaturation, the temperature of coagulation and the thiol groups of globin and the precipitation of insoluble globin in the presence of soluble globin.

The Preparation of Globin

The Present Procedure.—The method for preparing soluble globin now to be described involves two separate steps. In the *first step*

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acid acetone is added to an acid solution of carbon monoxide hemoglobin whereupon the hemoglobin is separated into a solution of heme and a practically pigment-free precipitate of denatured globin combined with hydrochloric acid. After removal of the acetone the globin powder readily dissolves in water to give an acid solution. The globin may be completely precipitated from this acid solution by third saturation with ammonium sulphate and almost completely precipitated by rapid complete neutralization. In the *second step* of the preparation, the acid solution of the globin powder is gradually neutralized by one of several special procedures which avoid rapid complete neutralization. Thereupon about 65 per cent of the horse globin and about 80 per cent of ox globin are converted into a form which is soluble in a neutral solution 0.4 saturated with ammonium sulfate and which can combine with heme to form hemoglobin.

Historical.—Before describing in detail the preparation which has just been outlined, we shall review the older methods for obtaining globin and heme.

In 1898 Schulz made the first study of the properties of a fairly pure globin prepared by adding acid to hemoglobin and extracting the pigment with alcohol and ether. Schulz's globin was insoluble around pH 8 but readily soluble in acid or alkali. Due to the peculiar properties discovered by Schulz, globin was classified with the histones.

In 1925 it was pointed out (1) that the classical globin of Schulz is not the native globin of hemoglobin but a denatured protein, like other denatured proteins insoluble at its isoelectric point, and (2) that while hemoglobin itself is a compound of native globin and heme, the hemochromogen prepared from hemoglobin is a compound of denatured globin and heme (Anson and Mirsky (2)).

These views were confirmed by Hill and Holden (9) who then made the new and important contribution of preparing for the first time soluble, apparently native globin which can combine with heme to form hemoglobin. It will be shown in another paper that Hill and Holden's theory of the preparation of native globin has no experimental basis and that the preparation based on their theory gives almost the lowest possible yield, only by accident any appreciable yield at all.

The synthesis of hemoglobin, in itself, was not new. Bertin-Sans and de Moitessier had already synthesized hemoglobin from pigment-free globin in 1893 (4). The writers (1) likewise had prepared hemoglobin from Schulz's globin before they realized that denaturation of the protein is involved in Schulz's procedure. Holden and Freeman (10) who repeated and confirmed our experiments have suggested, perhaps correctly, that Schulz's globin although mainly denatured

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and insoluble, nevertheless contains some soluble globin and that it was this soluble fraction which was the source of our synthetic hemoglobin.

Holden and Freeman (10) have also improved the technique for the preparation of soluble globin introduced by Hill and Holden. They stated that they paid especial attention to the yield. They did not state what the yield was.

Wu (17) found that Hill and Holden's method gave a yield of only a few per cent. He prepared soluble globin by simply carrying out Schulz's procedure in the cold.

It remains to describe the old acid acetone preparation of heme (Merunowicz and Zaleski (12), Hamsik (7)) which forms the basis for the present preparation of insoluble globin. The procedure consists simply in a slow extraction of coagulated hemoglobin with acid acetone. It yields a protein-free solution of heme without the use of the high temperature or strong acid required by the classical glacial acetic acid technique. It did not in its previous form yield a globin reasonably free of pigment and so was not used for the preparation of globin. The present modification gives an immediate and almost complete separation of heme and globin.

Preparation of Hemoglobin

Ox corpuscles washed four times with isotonic salt solution are shaken with an equal volume of alumina cream (Tracy and Welker (15)) and a quarter of the total volume of toluol (Heidelberger (8)) and allowed to stand in the cold. The next morning the clear solution on bottom is siphoned off and filtered. The suspension of stromata is centrifuged and the clear solution on bottom is filtered. In the case of horse blood it is desirable to add 10 per cent more alumina cream after adding water to the corpuscles to the extent of 10 per cent of their volume. This greater dilution is to prevent the crystallization of the less soluble horse hemoglobin. Unfortunately the alumina cream used in these preparations did not have the property described by Tracy and Welker of precipitating quantitatively the serum proteins but it proved useful, nevertheless, in facilitating the rapid and complete separation of stromata and toluol.

Preparation of Insoluble Globin Powder and Heme Solution

For each 10 cc. of hemoglobin solution cooled in ice water are added 10 cc. of cold 0.1 N HCl and 200 cc. of acetone (not cooled) containing 2 cc. of 1 N HCl. The suspension is thoroughly shaken and filtered with gentle suction. The filtrate, which is saved for the preparation of heme, gives no further precipitate on the addition of acetone, dilute sodium acetate or trichloracetic acid. While the globin is still on the filter paper it is washed with acetone and drained with full suction. It is then pressed between filter papers, removed and dried in the air. It loses 5 per cent of its weight on being dried further at 100°. This dry acid globin is practically free of pigment, is readily dissolved in water to give a clear

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solution, and is completely precipitated from such a solution when it is one-third saturated with ammonium sulphate.

If the slightly pigmented globin is dissolved in alkali and the reducer $Na_2S_2O_4$ added, a small amount of hemochromogen is obtained whose α band (in the case of horse globin) is about 10 Å. to the blue of the α band of the globin hemochromogen prepared from hemoglobin, hemin or acid acetone heme. In other words, the small amount of pigment which remains attached to the globin is in part at least a modified heme. If oxalic acid is used instead of HCl in the preparation of the globin then the globin contains still less pigment and the globin hemochromogen this pigment yields has its α band in the normal position. This oxalic acid globin, however, is less useful for the preparation of soluble globin than is the HCl globin. The procedure with oxalic acid is simply to add to a 5 per cent solution of carbon monoxide hemoglobin ten times its volume of acetone containing 2.5 gm. oxalic acid per 100 cc. acetone.

Preparation of Soluble Globin

When an acid solution of the acetone globin is neutralized part of the protein is precipitated and part remains in solution. On 0.4 saturation of the neutral filtrate with ammonium sulfate a small additional precipitate is obtained. The insoluble fraction has the characteristics of denatured protein; it is insoluble at its isoelectric point (about pH 8) and it combines with reduced heme to form hemochromogen. The fraction soluble in 0.4 saturated ammonium sulfate has the properties one would expect native globin to have; it is soluble in distilled water, it can be salted out by seven-tenths saturation with ammonium sulfate and then redissolved by the addition of a little water, it can be coagulated by heat or by shaking, and it can combine with heme to give a substance with the spectrum of methemoglobin. The methemoglobin may be reduced with $Na_2S_2O_4$ to give a pigment with the typical spectrum of reduced hemoglobin. When a solution of this reduced hemoglobin is shaken with air there appears the spectrum of oxyhemoglobin, the α band being in the normal position, within the experimental error of 2 Å. Whether the soluble globin is *identical* with globin as it exists in hemoglobin is not decided by the present experiments.* It is likewise not proven that some insoluble

* In solubility experiments normal hemoglobin and hemoglobin synthesized from soluble globin behave as if they were one and the same component in the sense of Willard Gibbs.

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globin is not kept in solution by soluble globin even in 0.4 saturated ammonium sulfate.

Factors Influencing the Yield.—What fraction of the globin is precipitated on neutralization and 0.4 saturation with ammonium sulfate and what fraction is obtained in the form of soluble, apparently native globin depends primarily on how the neutralization is carried out. If the neutralization is rapid and complete, practically all the globin is precipitated. If the alkali is added in two steps, first just not enough to cause turbidity and after an interval of time, the rest, then most of the globin remains in solution. Similarly a high yield of soluble globin is obtained if most of the acid is first removed gradually by dialysis.

A variety of neutralization experiments together with a discussion of their bearing on the question of what factors influence of the apparent reversal of denaturation will be presented in a following paper. The purpose of the present paper is merely to describe empirically a technique for obtaining soluble globin.

Neutralization Method.—To a 5 per cent solution in water of the acetone globin powder there is gradually added with shaking an amount of 0.1 N NaOH which is 95 per cent of that required to give the first permanent turbidity. Then, after ten minutes, enough more 0.1 N NaOH is added to give the maximum precipitation. The amounts of NaOH required are determined empirically for each batch of globin. In the case of one sample of horse globin to each 100 cc. of 5 per cent solution there were added first 50 cc. 0.1 N NaOH and then 12.5 cc.

The neutralized solution of globin is now 0.4 saturated with ammonium sulfate to precipitate the insoluble globin as completely as possible and is filtered. The globin left in solution is precipitated by the addition of 16 gm. solid ammonium sulfate for each 100 cc. of solution. After filtration the precipitate is put in collodion membranes and dialyzed overnight at 5°C. in a shaking dialyzer (Kunitz and Simms (11)). Thus a concentrated salt-free solution is obtained.

Yield.—The yield is conveniently determined by estimation of the globin concentration in the solution which is 0.4 saturated with ammonium sulfate. To 10 cc. of a diluted solution containing about 3 mg. of protein are added 2 cc. of 20 per cent trichloracetic acid. After centrifugation and rejection of the supernatant fluid, the precipitate is dissolved in 2 cc. 0.1 N NaOH and estimated by means of the color developed by the phenol reagent of Folin and Ciocalteau (5), in essentially the manner first described by Wu (16). The modifica-

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tions of the directions of Wu which have been used in this laboratory for several years for a number of proteins are practically the same as the modifications recently published by Greenberg (6). As a standard the original solution of the acetone globin powder is used. This is justified because both the insoluble and soluble forms of globin after precipitation with trichloracetic acid have the same color value per milligram of nitrogen. The ammonium sulfate which remains with the trichloracetic acid precipitate has no influence on the color.

Yield by the neutralization method: Horse globin, 65 per cent.

Dialysis Method.—A 2.5 per cent solution of the acetone powder is dialyzed overnight at 5°C. against distilled water in a shaking dialyzer. No precipitate is formed because the solution is not completely neutralized by the dialysis. The insoluble globin may be precipitated by the addition of an equal volume of a $K_2HPO_4-(NH_4)_2SO_4$ solution containing one part 1 M K_2HPO_4 to five parts saturated ammonium sulfate. Or the solution may be neutralized by dialysis against M/30 K_2HPO_4. When the precipitate formed is filtered off, 0.4 saturation of the filtrate with ammonium sulfate causes only a faint haze to appear.

Yields: Horse globin, 35 per cent; ox globin, 80 per cent.

Preparation of Heme

There is available a number of ways of precipitating heme from the acid acetone solution, removing the acetone which remains with the precipitate and redissolving the acetone-free heme without the use of strong alkali which may modify the heme.

Water Precipitation.—The heme is precipitated by the addition to the acid acetone solution of twice its volume of water. After filtration the heme precipitate is washed on the filter paper with water and dissolved while still fresh by the addition of 1 M K_2HPO_4.

NaAc Precipitation.—Much more convenient is the precipitation by addition to the acid acetone solution of 1 per cent its volume of 2 N sodium acetate. There is less solution to filter than when water is added, the filtration is faster, and the acetone may readily be recovered from the filtrate by distillation after removal of the water with $CaCl_2$.

The NaAc precipitate may be washed with 1/15 M KH_2PO_4 and then dissolved in a buffer consisting of equal parts of Na_2CO_3 and $NaHCO_3$. Or the precipitate may be dissolved in acetone phosphate solution by adding 0.5 M K_2HPO_4 to a suspension of heme in acetone and then diluting with water. The acetone may be removed by vacuum distillation or if the acetone concentration is not too high, by

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dialysis against any slightly alkaline buffer such as K_2HPO_4. In slightly alkaline solution heme does not pass through collodion membranes made to retain hemoglobin. Apparently under these conditions heme exists not in the form of molecules containing one iron atom and having a molecular weight of 650 but in the form of aggregates. An alternative procedure for removing the acetone from the acetone phosphate solution is to precipitate the heme with HCl, wash with water and redissolve in K_2HPO_4.

If it is desired to store the heme indefinitely, the NaAc precipitate may be washed with acetone and dried in the air. The powder dissolves slowly in 0.1 N NaOH, and not at all in K_2HPO_4. It may be dissolved slowly in the acetone phosphate solution.

The precise directions for the preparation of the acid acetone solution were worked out in order to get the best separation of globin and pigment. If only the heme is desired, it is not essential to use CO or low temperature and the procedure may be modified to require less acetone. Economy of acetone, however, is not important since the acetone may readily be recovered.

The properties of the acid acetone heme have not as yet been investigated. In particular, it has not been determined whether the heme dissolved in K_2HPO_4 is identical with the heme dissolved in NaOH and then neutralized with KH_2PO_4.

SUMMARY

1. By a procedure involving the use of acid acetone hemoglobin may be rapidly separated into a precipitate of denatured globin and an acetone solution of heme.

2. By neutralization procedures the denatured globin may be largely converted into a soluble, apparently native form which can combine with heme to form hemoglobin.

3. The heme may be obtained in acetone-free, slightly alkaline solution without the use of strong alkali which may modify the heme.

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