

THE OCCURRENCE DURING ACUTE INFECTIONS OF A PROTEIN NOT NORMALLY PRESENT IN THE BLOOD

II. ISOLATION AND PROPERTIES OF THE REACTIVE PROTEIN

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The studies of Abernethy and Avery¹ concerning the reactive substance precipitated by the C polysaccharide of Pneumococcus from serum obtained from human beings and monkeys during certain acute infections, have indicated that the reactive material is of protein nature. In order to gain further knowledge of this "reactive protein," attempts have been made to obtain an active fraction as free as possible from other serum constituents by methods which do not involve the use of the C polysaccharide as precipitant. The present report deals with the isolation of the reactive protein from acute phase sera and with certain of the properties which distinguish it from normal serum proteins. Throughout this paper, for the sake of convenience, the term "acute phase" serum is used when referring to serum obtained from patients acutely ill with an infectious disease. The material precipitated from acute phase serum upon the addition of the C polysaccharide is referred to as reactive protein.

EXPERIMENTAL

In the preceding paper of this series it was shown that the reactive protein in acute phase serum is present in the albumin fraction, and that the serum globulins removed by half saturation with ammonium sulfate do not react with the C polysaccharide. The following experiments were performed to determine whether the reactive protein is distributed uniformly throughout the total albumin or whether it is limited to a particular fraction of the albumin precipitated between half and full saturation with ammonium sulfate.

Fractionation of Serum Albumin with Ammonium Sulfate.—

10 cc. of acute phase serum were diluted with an equal volume of physiological saline, and 20 cc. of a saturated solution of ammonium sulfate added. The mixture was allowed to stand at room temperature for 1 hour, and the precipitated globulin removed by

¹ Abernethy, T. J., and Avery, O. T., *J. Exp. Med.*, 1941, 73, 173.

centrifugation. To the solution of albumin, sufficient saturated ammonium sulfate solution was added to bring the salt concentration to 75 per cent saturation. After standing for 1 hour the precipitate (50 to 75 per cent fraction) was collected by filtration. The filtrate was then brought to full saturation and the final precipitate (75 to 100 per cent fraction) collected by filtration. The globulin and the two albumin fractions were resuspended in 5.0 cc. of distilled water and dialyzed in cellophane sacs against physiological saline until free of sulfate. On removal from the dialyzing sacs, the volume was made up to 10.0 cc. in each case by the addition of saline. To 0.5 cc. portions of each serum fraction an equal volume of C 1:20,000 was added to determine the presence or absence of the reactive protein. The results of these tests are shown in Table I.

TABLE I
Distribution of Reactive Protein in Fractions of Acute Phase Serum

Fraction of acute phase serum	Precipitation reaction with C 1:20,000
Whole serum	++++*
Globulin	-
Albumin (fraction precipitated between 50 and 75 per cent saturation)†.....	+++±
Albumin (fraction precipitated between 75 and 100 per cent saturation)‡.....	±

* In this and the following tables the degree of precipitation is expressed by the symbols:

++++ = flocculent precipitate, clear supernatant.

± = slight turbidity.

† Fraction of albumin precipitated between 50 and 75 per cent saturation with ammonium sulfate (active albumin fraction).

‡ Fraction of albumin precipitated by full saturation with ammonium sulfate following removal of albumin precipitated by 75 per cent saturation.

From the data shown in Table I it can be seen that the reactive protein is restricted to that fraction of the serum albumin precipitated between 50 and 75 per cent saturation with ammonium sulfate. The fraction of the albumin precipitated between 75 and 100 per cent saturation is only slightly reactive with the C polysaccharide, and as previously shown, the globulin fraction does not contain reactive protein. By fractional precipitation considerable separation of inert protein can be readily effected, and for further purification only the fraction of the albumin precipitated between 50 and 75 per cent saturation with ammonium sulfate or sodium sulfate was used. This fraction will be referred to as the "active albumin fraction."

Separation of Reactive Protein by Dialysis of Active Albumin Fraction against Tap Water.—In the previous experiment the albumin fractions were dialyzed against physiological saline to remove the ammonium sulfate. When tap water instead of saline was used for dialysis, it was found that the

reactive protein precipitated whereas the normal albumin remained in solution. Dialysis against tap water was continued until the solution was free of sulfate, and the flocculent precipitate which formed was recovered by centrifugation. On the addition of physiological saline most of the precipitate again became soluble, although a small amount of material generally remained which was insoluble in saline. This appeared to be protein which had become denatured during the course of dialysis at the pH of ammonium sulfate (pH 5.5). Consequently, in subsequent fractionations sodium sulfate was used in place of ammonium sulfate, all procedures up to the point of dialysis being carried out at 37°C.

Fractionation of Serum Albumin with Sodium Sulfate.—

100 cc. of serum were obtained at autopsy from a fatal case of Type III Pneumococcus pneumonia. After filtration through a Berkefeld V candle the serum was diluted with 100 cc. of distilled water. The subsequent steps in fractionation were carried out at 37°C. To the diluted serum 200 cc. of a saturated solution of sodium sulfate were added. After standing for 1 hour the precipitated globulin was collected on a filter paper. 355 cc. of the clear filtrate containing the albumin were brought to 75 per cent saturation by the addition of 177 cc. of a saturated solution of sodium sulfate, and allowed to stand for 40 minutes after which the albumin precipitate was collected on a filter paper. This precipitate was washed off the filter paper with tap water, and the solution was refiltered to remove paper fibers. The final volume was 80 cc. The clear solution was transferred to a cellophane sac and dialyzed against running tap water for 3 hours. At the end of this time the solution in the sac had become turbid and a fine flocculent precipitate was beginning to settle out. The dialyzing sac was then transferred to a 10 liter jar of tap water and dialysis continued in the cold room (approximately + 2°C.) for 16 hours. The copious flocculent precipitate was separated by centrifugation for 1 hour in the cold. The packed precipitate was light yellow in color; the supernatant albumin solution remained slightly turbid but cleared on the addition of sodium chloride in a concentration of 0.85 per cent. The pH of the albumin solution was 6.97. The precipitate was completely soluble in physiological saline, and when redissolved, reacted strongly with the C polysaccharide. The albumin fraction which precipitated between 75 and 100 per cent saturation was dissolved and dialyzed against tap water. No precipitation occurred during dialysis. The salt concentration of the various fractions was adjusted with NaCl to 0.85 per cent.

Each fraction was tested for reactivity with the C polysaccharide. The results of these tests are shown in Table II.

The results shown in Table II indicate that practically all of the reactive protein is precipitated when the active albumin fraction is dialyzed against tap water. If instead of tap water, calcium-free distilled water is used for dialysis, little or no precipitation occurs. This difference in solubility is attributable to the presence of traces of calcium in tap water:

Further Purification of Reactive Protein.—The precipitate of reactive protein obtained by the method described above was further purified by dissolving it in alkaline saline and reprecipitating by dialysis. The protein was dissolved in saline buffered at pH 7.9 with $m/50$ borate buffer and re-dialyzed against tap water. Distilled water is unsatisfactory, since as pointed out above, the reactive protein is soluble in water unless a trace of calcium is present.

90.0 cc. of pooled acute phase serum were fractionated by means of sodium sulfate according to the method described. The yellowish precipitate of reactive protein obtained by dialyzing the active albumin fraction against tap water was dissolved in 25 cc. of saline buffered at pH 7.9 with $m/50$ borate buffer. The somewhat opalescent solution was dialyzed against tap water for 48 hours in the cold room, and the precipitate collected

TABLE II

Precipitation of Reactive Protein on Dialysis of the Albumin Fraction against Tap Water

Serum fraction	Precipitation reaction with C 1:20,000
1. Whole serum.....	++++
2. Active albumin fraction*.....	+++±
3. Protein precipitated from fraction 2 on dialysis against tap water.....	+++
4. Supernatant of fraction 3.....	+

* Fraction of albumin precipitated between 50 and 75 per cent saturation with sodium sulfate.

by centrifugation in the cold. A small portion of the precipitate was dissolved in saline and found to be reactive with the C polysaccharide. The bulk of the precipitate was dissolved in 10.0 cc. of distilled water to which was added 0.4 cc. of $N/100$ NaOH. The lipids were removed by shaking at room temperature in 10 volumes of 50 per cent alcohol-ether. The precipitate was washed once with ether and dried to constant weight. 21.0 mg. of dried material were recovered. Chemical analysis of the dried precipitate gave the following results: N, 13.97 per cent; P, 0.05 per cent.

It would appear justifiable in the light of this and other experiments to refer to the C-precipitable material in acute phase serum as reactive protein. The nitrogen content of various purified preparations has been of the same order as that found in the above experiment, namely between 13 and 14 per cent. The phosphorus content of material not extracted with alcohol and ether has been found to lie between 0.45 and 0.7 per cent, but upon extraction of the lipids the phosphorus content fell to a very low value as in the above preparation where it was found to be 0.05 per cent.

Absorption of Acute Phase Serum with C Polysaccharide and Subsequent

Fractionation.—It was of interest to determine whether or not absorption of acute phase serum with the C polysaccharide will remove the reactive protein precipitable from the active albumin fraction upon dialysis against tap water.

20 cc. of acute phase serum were absorbed with the C polysaccharide so that no further precipitation occurred on the addition of an excess of C. The C-absorbed serum was fractionated by means of sodium sulfate as outlined above. The albumin fraction precipitated between 50 and 75 per cent saturation was dialyzed against tap water for 72 hours. No precipitation of reactive protein occurred under these circumstances.

It would appear, therefore, that the material precipitated from acute phase serum on the addition of the C polysaccharide, and the material precipitated from serum of the same origin by dialysis of the albumin fraction against tap water, are identical.

Relation of Lipids to Reactive Protein.—In order to determine the relation of lipids to the reactive protein, acute phase serum was treated with alcohol and ether in the cold, and an attempt made to separate the reactive protein from the "defatted" serum.

Removal of Lipids from Acute Phase Serum.—100 cc. of acute phase serum were chilled to 0°C. and added slowly, with constant stirring, to a mixture of 750 cc. absolute alcohol and 250 cc. of absolute ether chilled to -12°C. in a salt-ice bath. The suspension was kept in the salt-ice bath for 1 hour with frequent shaking, after which it was centrifuged at -1°C. to collect the precipitated protein. The precipitate was washed three times with absolute ether at -12°C., then distributed in a thin layer and dried *in vacuo*.

The defatted and dried serum was taken up in the original volume of normal saline, in which it was completely soluble. On fractionation with sodium sulfate, the albumin which precipitated between 50 and 75 per cent saturation appeared definitely less in amount than in the case of serum from which the lipids had not been removed. After dialysis against tap water for 36 hours only slight turbidity of the solution occurred. The small amount of water-insoluble material was collected by centrifugation, and when dissolved was found to be reactive with the C polysaccharide. The supernatant albumin which still showed marked reactivity with the C polysaccharide, was dialyzed for an additional period of 48 hours, but no further precipitation occurred.

The various fractions of the defatted albumin were tested for reactivity with the C polysaccharide as shown in Table III.

The results shown in Table III indicate that the serum lipids are of importance in determining certain of the properties of the reactive protein, particularly its sensitivity to calcium as indicated by increased solubility in tap water. The reactivity of the defatted albumin with the C polysaccharide remains unchanged. After removal of the serum lipids, although the reactive protein is still almost quantitatively precipitated by 75 per

cent saturation with sodium sulfate, its solubility in tap water is greatly increased. For this reason only a small amount of the reactive protein is precipitated upon dialysis against tap water, and the supernatant albumin retains practically all of its original reactivity with the C polysaccharide. Moreover, it has been found that the reactive protein remains soluble in the presence of a concentration of calcium sufficient to precipitate it from undefatted albumin.

TABLE III

Effect of Extraction of Lipids on the Solubility of the Reactive Protein in Acute Phase Serum

Serum fraction	Precipitation reaction with C 1:20,000	
	Before removal of lipids	After removal of lipids
Whole serum.....	++++	++++
Globulin.....	—	—
Active albumin fraction*.....	+++±	+++±
Supernate of active albumin fraction after dialysis against tap water.....	+	+++
75 to 100 per cent albumin fraction†.....	±	±

* Fraction of albumin precipitated by 75 per cent saturation with sodium sulfate.

† Fraction of albumin precipitated by full saturation with sodium sulfate following removal of albumin precipitated by 75 per cent saturation.

DISCUSSION

One of the most unique properties of the reactive protein is its precipitability by the C polysaccharide of *Pneumococcus*. Under these conditions the reactive protein is almost quantitatively precipitated. The effect of calcium in this reaction has been described.¹ It was shown that flocculation does not occur when the polysaccharide is added to a reactive serum from which the calcium has been removed, but that this capacity is completely restored on the addition of calcium in a concentration much lower than that normally present in blood. The precipitate formed in the presence of the polysaccharide becomes soluble when the calcium is split off at acid or alkaline reactions or by the addition of sodium citrate. However, these procedures have not as yet proved useful in further purification of the reactive protein because of the difficulty in removing the polysaccharide without at the same time denaturing the protein.

Because of the importance of calcium in the precipitation reaction it was thought that this reagent might be used as a direct means of isolating the

reactive protein. It was found that when an excess of calcium is added to acute phase serum the reactive protein is precipitated even in the absence of the C polysaccharide. This procedure however has certain disadvantages since precipitation of normal serum proteins also occurs under these conditions. In addition most acute phase sera contain large amounts of lipid material which flocculates upon the addition of an excess of calcium chloride. The copious fatty precipitate is difficult to separate from the reactive protein without causing denaturation of the latter. A further objection to the use of calcium chloride is that the precipitate formed is not soluble at neutral reaction.

As a consequence of these observations procedures were sought for obtaining the reactive protein in a relatively pure state without making use of either the C polysaccharide or an excess of calcium as precipitants.

The reactive protein has been found to be present in the fraction of albumin precipitated by ammonium or sodium sulfate between 50 and 75 per cent saturation. Sodium sulfate was found to be more satisfactory since the slight acidity of ammonium sulfate usually caused denaturation of part of the reactive protein, particularly if dialysis were prolonged. By the use of sodium sulfate denaturation is avoided.

The reactive protein is only partially precipitated from the active albumin fraction of serum on dialysis against distilled water. However, more complete separation results if tap water is used for dialysis in place of distilled water. This difference is due to the trace of calcium contained in tap water. The reactive protein can then be freed of extraneous material by dissolving the precipitate in physiological saline at alkaline reaction and redialyzing against tap water. The purified material obtained by this means is fully reactive with the C polysaccharide.

The property of insolubility in tap water makes it possible to separate the reactive protein from serum albumin. It has been found that this property is due in part to the association of the protein with certain lipids. If acute phase serum is first extracted with alcohol-ether in the cold to remove the lipids the reactive protein is still almost quantitatively precipitated by sodium sulfate between 50 and 75 per cent saturation. However, it now remains soluble upon dialysis against tap water and is not precipitable by calcium. It is noteworthy that the reactivity of the protein with the C polysaccharide is not altered by removal of the lipids.

It is evident, therefore, that the solubility of the reactive protein is conditioned by the interaction of two factors; namely, the presence of the lipid or lipids with which it is intimately associated, and the marked sensi-

tivity of this lipo-protein complex to calcium ions. The effect of both these factors on solubility is evidenced by the fact that following removal of the serum lipids, the active protein is far less sensitive to calcium, as shown by its solubility in tap water. Although important in determining the solubility of the protein, the lipids are not essential in the C-reaction, since they can be extracted either before or after fractionation of the serum without impairing the capacity of the protein to react with the polysaccharide.

Studies of the lipid constituents of the reactive protein have not as yet been made. However, it is probable that a phosphorus-containing lipid is associated with the protein since phosphorus determinations on preparations of the reactive protein after extraction with lipid solvents show a much lower phosphorus content than similar preparations from which the lipids have not been removed.

SUMMARY

Methods are described for isolating a protein commonly present in the blood of patients during the acute phase of various infections which, unlike the normal serum proteins, is precipitable by the C polysaccharide of *Pneumococcus*.

The reactive protein is present in the fraction of serum albumin precipitated by either ammonium or sodium sulfate between 50 and 75 per cent saturation. From this fraction the reactive protein separates out on dialysis against tap water.

Following removal of the alcohol-ether-soluble lipids from acute phase serum the reactive protein becomes soluble in tap water, and is no longer precipitable by traces of calcium but still retains its precipitability with the C polysaccharide.