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

IV. Crystallization of the C-Reactive Protein

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In 1930 Tillett and Francis showed that a precipitate occurs when sera obtained from patients during certain acute infectious diseases are mixed with a solution of the somatic C polysaccharide of pneumococcus (1). It was found that the phenomenon is not limited to pneumococcal infections but occurs also in some other diseases; *e.g.*, acute rheumatic fever, osteomyelitis, and subacute bacterial endocarditis. In addition to this non-specificity with respect to the etiologic incitant of the disease, the reaction was shown to differ from known antigen-antibody reactions in that the titer of the serum is maximal in the active stage of the disease and rapidly decreases after the onset of convalescence. Subsequent studies by Abernethy and Avery (2) and MacLeod and Avery (3, 4) established the fact that the reaction is due to the appearance in the blood of a protein not normally present, which precipitates selectively with the C polysaccharide. The reaction between C polysaccharide and the protein was further differentiated from classical immune precipitation by the following facts: (*a*) Removal of calcium ion from the serum by the use of oxalate, citrate, or dialysis results in loss of reactivity, and readdition of calcium chloride to sera thus inactivated fully restores the precipitability of the protein. (*b*) On fractionation with ammonium or sodium sulfate the reactive protein is associated with the albumin rather than the globulin fraction of the serum (2). The reactive protein was isolated in relatively purified form and some of its chemical and immunoligical properties described (3, 4).

Isolation of the C-reactive protein was accomplished by dialysis of serum albumin fractions (precipitated between 0.5 and 0.66 saturation with sodium sulfate) against tap water, with resultant precipitation of the C-protein. This effect was shown to be dependent on the presence of traces of calcium ion in the tap water, and no precipitation occurred if the dialysis was carried out against calcium-free distilled water (3). Precipitation by calcium ion appears to be related to the association of lipids with the reactive protein, since preliminary treatment of serum with alcohol and ether to remove lipid abolishes the calcium effect. It should be emphasized, however, that the removal of lipids does not interfere with the reaction between the C polysaccharide and the protein, nor does it alter the calcium requirement of this reaction.

The present paper deals with the crystallization of the C-reactive protein

from human serous fluids and with certain of the properties of the crystalline protein.

EXPERIMENTAL

In the studies here described the source material has been chest or abdominal fluids from patients, rather than blood serum, primarily because larger volumes of fluid containing the reactive protein are more readily available in this form. Experience with a variety of serous fluids of this type has revealed a consistent difference in the behavior of the C-reactive protein from that of the same protein in the blood serum. When the albumin fraction from chest or abdominal fluids is dialyzed against tap water, precipitation of the C-reactive protein does not result. In this respect, therefore, these fluids behave like blood serum after the removal of lipids, and the results suggest that in serous fluids the protein may not be associated with lipid. It is possible that this fact may in part be responsible for the success of the crystallization procedure. Since it was not possible to make use of calcium precipitation for initial isolation of the protein, it was necessary to depend upon precipitation by the pneumococcal C polysaccharide to separate the active material from the bulk of serum proteins. Crystallization occurred in the course of attempts to dissociate the polysaccharide-protein precipitate and to separate the two substances by means of sodium sulfate fractionation.

Crystallization of C-Reactive Protein

Initial Fractionation with Ammonium Sulfate.—1500 cc. of yellow fluid was obtained from the pleural cavity of a patient suffering from acute streptococcal pneumonia. The fluid contained both sulfadiazine and penicillin, and cultures yielded no growth. Tests for C-reactive protein by the standard precipitation method using the pneumococcal C polysaccharide were strongly positive.

After storage in the refrigerator for several days, the fluid was centrifuged to remove the accumulated fibrin. The clear supernatant fluid was diluted to 3 liters with distilled water, and solid ammonium sulfate was added to 0.5 saturation (314 gm. per liter). The inactive precipitate was removed by suction filtration and discarded. The 0.5 saturated filtrate was brought to 0.75 saturation by the addition of 172 gm. of solid ammonium sulfate per liter of filtrate and allowed to stand at room temperature overnight. One per cent each of filter cel and standard super cel were stirred in and the precipitate recovered by suction filtration. The precipitate was suspended in 500 cc. distilled water and filtered to remove the filter aid. The protein fraction so obtained was a clear amber solution and contained all the C-reactive protein of the original fluid. The material was further concentrated by precipitating a second time at 0.75 saturation with ammonium sulfate and redissolving the precipitate in 100 cc. of distilled water. This solution was dialyzed first against running tap water and finally against 0.01 per cent calcium chloride without the formation of any visible precipitate.

Precipitation with Pneumococcal C Polysaccharide.—25 mg. of pneumococcal C polysaccharide dissolved in 2.0 cc. of water was added to the dialyzed solution of the albumin fraction. The mixture was incubated at 37°C. for 2 hours and refrigerated overnight. The polysaccharide-protein precipitate was recovered by centrifugation, and washed three times with physiological saline containing 0.01 per cent calcium chloride. The washed precipitate was

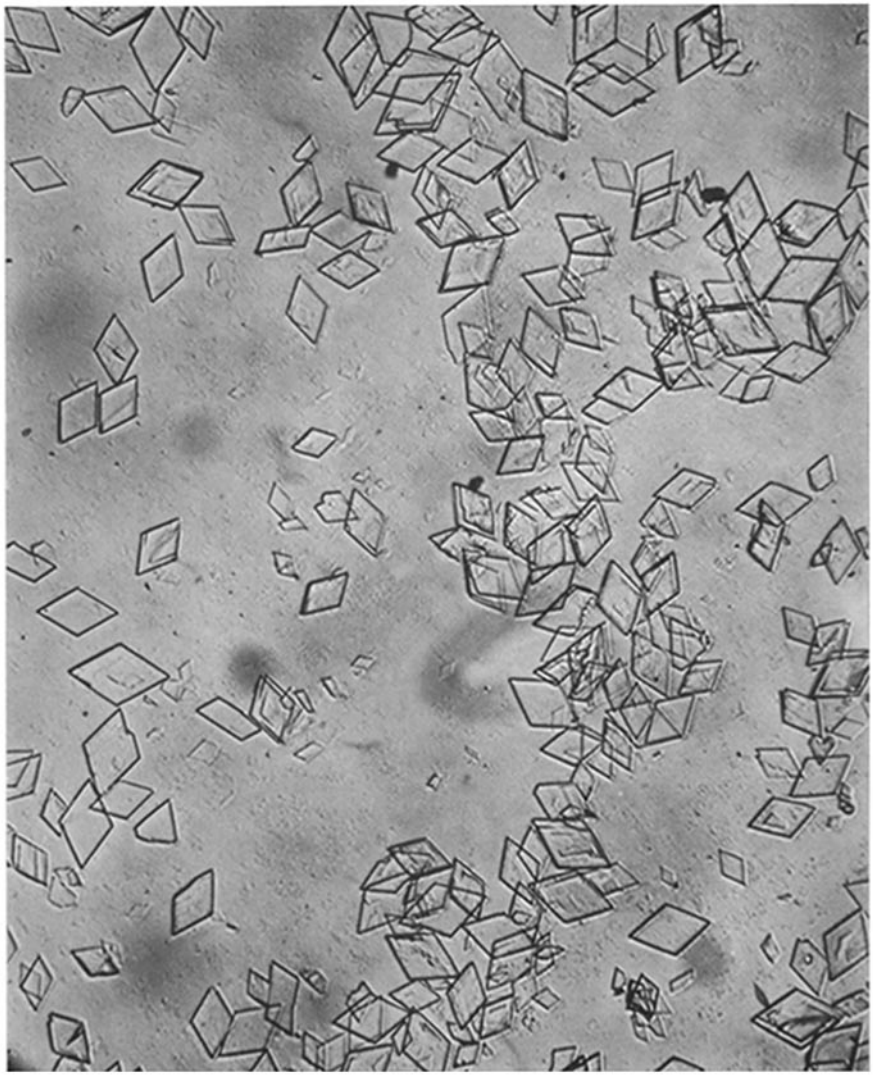

FIG. 1. Crystals of C-protein. × 100.

suspended in 35 cc. of physiological saline and brought into solution by the dropwise addition of saturated sodium citrate. A small amount of insoluble material was removed by centrifugation and discarded.

Crystallization.—The citrated solution (36 cc.) was mixed with an equal volume of saturated sodium sulfate solution (prepared at 37°C.) and held at 37°C. This step was included for its possible effect in causing further dissociation of the polysaccharide-protein complex by the action of the high salt concentration. The half-saturated solution remained entirely clear. After 2 hours an additional 72 cc. of saturated sodium sulfate was added, bringing the final concentration to 0.75 saturation. A light amorphous precipitate formed which

did not increase and did not settle on standing. After several days at 37°C. the suspension was found to have a crystalline sheen, and microscopic examination revealed the presence of very small, irregular needles. There was very little evidence of amorphous material.

Recrystallization.—The crystalline precipitate was recovered as completely as possible by centrifugation and redissolved in 10 cc. of distilled water. 30 cc. of saturated sodium sulfate was added with the immediate formation of an amorphous precipitate which gradually assumed a crystalline character after several days at 37°C. On this occasion, only a small portion of the material had the form of tiny needles and the remainder consisted of well formed, flat, rhomboid plates (Fig. 1). Further recrystallization of the material by the same procedure became increasingly rapid (48 hours), and on the 3rd and 4th crystallizations all of the crystals had the rhomboid form illustrated in Fig. 1. In each case the crystals arose from the amorphous precipitate, and attempts to obtain crystals at salt concentrations which did not throw down amorphous material met with no success. This may be due to the fact that the concentration of protein was relatively low. The yield of recrystallized material was less than 100 mg.

Preparation of Crystalline Protein from Abdominal Fluid.—10 liters of abdominal fluid was obtained from a patient suffering from cirrhosis of the liver. The fluid had accumulated during an intercurrent infection and gave a definite, though not heavy, precipitation reaction when tested with pneumococcal C polysaccharide. It is of interest that fluid from the same patient had been tested on previous occasions in the absence of known infectious processes and proved to be devoid of C-reactive protein.

Fractionation of the abdominal fluid was carried out as described above for the chest fluid. As in the previous case, the fraction obtained at 0.75 saturation with ammonium sulfate did not form a visible precipitate upon dialysis against tap water or 0.01 per cent calcium chloride, and isolation of the protein was again achieved by the use of the C polysaccharide. Both the initial and subsequent crystallizations were slower and more difficult in this case, but as would be expected the process could be accelerated by inoculation. The crystals had the same symmetrical rhomboid form as those obtained from the chest fluid.

Properties of the Crystalline Protein

The crystalline protein has a very low solubility in the absence of salts and can be almost quantitatively precipitated by dialysis against distilled water. Apparently the presence of other proteins can exert a solubilizing effect since prolonged dialysis of the albumin fraction of serum containing the protein results in no precipitation. Temperature also appears to have a pronounced effect on solubility, and solutions of relatively low concentration (0.1 to 0.5 per cent) prepared at 37°C. tend to precipitate rapidly on cooling.

Chemical analysis of the crystalline protein reveals a nitrogen content of 14.66 per cent and no detectable phosphorus.[1] The absence of phosphorus is of interest for two reasons. First, it provides conclusive evidence that the protein has been effectively separated from the pneumococcal C polysaccharide employed in its isolation. The preparation of C polysaccharide used in these experiments contains 5.21 per cent phosphorus[1] and since the crystalline protein contains none, it is apparent that the final product cannot represent crystallization of the polysaccharide-protein complex. Secondly, the absence of phosphorus confirms the assumption discussed above that the C-protein as obtained

[1] The chemical analyses were made by Dr. A. Elek of The Roekcfeller Institute.

from serous fluids is not associated with phospholipid. MacLeod and Avery (3) obtained good evidence that the lipid combined with C-protein isolated from serum is phosphorus-containing, since "defatting" with alcohol and ether reduced the phosphorus content from values between 0.45 and 0.7 per cent to 0.05 per cent.

The crystalline protein retains its affinity for the pneumococcal C polysaccharide. The addition of C polysaccharide at a final concentration of 0.05 mg. per cc. to solutions of the protein in the presence of traces of calcium ion results in prompt precipitation.

Immunological Traits.—In their study of the immunological properties of the C-reactive protein, MacLeod and Avery (4) demonstrated that this substance is highly antigenic and serologically unrelated to the normal serum proteins. These investigators prepared antisera by the intravenous injection into rabbits of carefully washed C polysaccharide-protein precipitates. The sera

TABLE I

Precipitin Reactions of Crystalline C-Protein with Specific Rabbit Antisera

Rabbit serum	Final concentration of crystalline C-protein, *mg./cc.*					
	0.1	0.03	0.01	0.003	0.001	0.0003
Rabbit 2-0, normal	−−	−−	−−	−−	−−	−−
Rabbit 2-0, immune	++++	++++	++	+	Trace	−−

++++, flocculent precipitate with clear supernate.
−−, no precipitate.

reacted in good titer with purified preparations of C-protein and with acute phase human sera, but gave only weak or negative reactions with normal human sera. Cross-reacting antibodies were readily removed by adsorption with normal human serum without affecting the titer of the antiserum with C-protein as antigen.

In the present study, rabbit antisera were prepared using crystalline C-protein. The serological studies serve the major purpose of providing a test of the purity of the protein, since it is possible by this means to demonstrate whether or not appreciable quantities of normal serum proteins are present. In addition, the antiserum serves as a superior reagent for detection of C-protein, and provides a test of considerably greater sensitivity than the usual procedure using C polysaccharide (4).

Two rabbits were injected intravenously on each of 4 consecutive days with 0.5 cc. of a solution containing 2 mg. per cc. of crystalline C-protein. The animals were bled 7 days after the last injection. Precipitin tests were carried out using serum diluted 1:2 in physiological saline. Equal volumes of diluted serum and serial dilutions of the antigen were mixed, and readings were made after incubation at 37°C. for 2 hours and refrigeration overnight.

The results of a titration of the crystalline C-protein with rabbit antiserum are recorded in Table I. It will be seen that the protein gives a maximal reaction at a final concentration of 0.03 to 0.1 mg. per cc. and forms a visible precipitate when present in amounts as low as 0.001 mg. per cc. It is thus apparent that the rabbit antiserum provides a sensitive reagent for the detection of small amounts of the C-protein.

Reactions of anti-C-protein rabbit serum with human sera are illustrated in Table II. The antiserum gives no trace of precipitation with any of the samples of normal human serum tested, so that one can conclude that the crystalline protein used for immunization contained no appreciable quantity of normal serum proteins. On the other hand, the antiserum reacts strongly with the acute phase serum of patients with certain infections. This is shown

TABLE II

Precipitin Reactions of Human Sera with Specific C-Protein Rabbit Antiserum

Rabbit serum	Test antigen	Final dilution of test antigen			
		1:2	1:10	1:100	1:500
Rabbit 2-0, immune	Normal human serum	−−	−−	−−	−−
	Acute phase human serum (Patient H.E. 12/28/42)	++++	+++	++	±
	Convalescent human serum Patient H.E. 1/21/43)	−−	−−	−−	−−

in Table II by the reactions with the serum of a patient obtained on the 2nd day of an attack of Type XIV pneumococcal pneumonia. It is of interest that serum obtained from the same patient 3 weeks later during convalescence gave no reaction whatever.

DISCUSSION

The preparation of crystalline proteins from pathological human sera is not unique. Crystalline proteins have been isolated from sera of patients suffering from multiple myeloma (5), rheumatoid arthritis (6), and periarteritis nodosa (7). In each instance, the pathological proteins have been present in large amounts and crystallization has occurred spontaneously from whole serum. These various proteins appear to be readily distinguishable from the C-protein on the basis of crystalline form as well as other properties, notably solubility.

It seems relatively certain in the light of the evidence available at present that the reaction between the pneumococcal C polysaccharide and the

C-reactive protein is a phenomenon based on a chance complementary relationship between the molecular configuration of the two substances which, in the presence of calcium ion, results in the formation of an insoluble complex. The characteristics of the C-reactive protein differ from those of immune antibody in the following important respects: (1) the occurrence of C-protein in the serum is non-specific with reference to the inciting agent of the disease; (2) the protein is present early in the acute phase and disappears rapidly with the onset of convalescence; (3) it occurs in the albumin rather than in the globulin fraction of serum; and (4) the presence of calcium ion is required in the precipitation reaction with C polysaccharide.

One of the most interesting implications of the precipitation reaction is that it demonstrates the occurrence in the blood, under the stimulus of certain disease processes, of a new constituent, the presence of which would otherwise not have been suspected. Although nothing is known at present concerning the source or function of the C-protein, its regular appearance during the course of various infectious diseases suggests the possibility that it may be related in some way to the host reaction to infection. It is further suggested that the C-reactive protein may be only one of several new constituents that appear in the blood in small amounts during the course of disease, and that others have not been demonstrated in greater numbers simply because specific reagents for their detection comparable to the C polysaccharide are not available.

As stated above, the source of the C-protein has not been determined, and indeed the full range of pathological processes resulting in the appearance of the protein in the serum is not known. Löfström (8) has described a non-specific capsular swelling reaction of certain strains of pneumococci when mixed with sera obtained in the acute phase of various infections. In a comparative study (9), he has shown that the substance responsible for the capsular swelling is probably identical with the C-reactive protein. In the light of this fact, his studies appear to represent the most extensive investigation of the occurrence of C-protein in disease processes, including some clearly non-infectious in nature. For example, Löfström obtained positive reactions in six patients with myocardial infarction. There is some evidence, then, that the occurrence of C-protein in the blood is not limited solely to diseases of infectious origin.

SUMMARY

A procedure is described for the isolation and crystallization from human serous fluids of the C-reactive protein, a substance which appears in the blood especially in the early phase of certain acute infectious diseases. Immunological studies confirm earlier work in showing that the protein is highly antigenic and serologically specific, and demonstrate that crystallization of the protein effectively separates it from normal serum proteins.

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