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

## V. Physical-Chemical Properties of the C-Reactive Protein Crystallized by a Modified Technique

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Crystallization of the C-reactive protein isolated from human serous fluids was first described in 1947 (1). In these experiments the crystalline protein was obtained from two pathological specimens, one a pleural fluid from a patient with streptococcal pneumonia and the other an abdominal fluid from a cirrhotic patient suffering from an intercurrent infection. Upon fractionation of these fluids by the procedure described by MacLeod and Avery for isolation of C-protein from blood serum, an important difference in the behavior of the material was noted (2, 3). In contrast to the findings with serum, it was found that when the "albumin" fraction obtained by ammonium sulfate fractionation of these fluids was dialyzed against tap water or 0.01 per cent calcium chloride, precipitation of the C-reactive protein did not result. Since the calcium precipitability of the serum C-protein had been attributed to the presence of phospholipid, it was tentatively assumed that the protein in the serous fluids employed differed from that of serum in that it was not associated with lipid and that this property was of importance in the success of the crystallization procedure. Support for this interpretation was provided by the fact that chemical analysis of the crystalline protein revealed the absence of detectable phosphorus, while C-reactive protein isolated from serum by the calcium precipitation method contained 0.45 to 0.7 per cent of this element, most of which could be readily removed by extraction of lipid with alcohol and ether. Furthermore, the property of calcium precipitability disappeared if the "albumin" fraction of serum was submitted to lipid extraction prior to dialysis.

The importance of the nature of the starting material in crystallization of C-reactive protein is emphasized by the fact that following the initial success with the two fluids described above, more than twenty specimens were subjected to fractionation in this laboratory without the isolation of significant amounts of crystalline material. In addition, it was found that the most readily available source of fluids containing C-reactive protein, that of serous fluids

from patients with various neoplastic diseases, usually provided only material in combination with lipid. Thus, examination of 125 pleural and abdominal fluids from this source showed that in the great majority of cases C-reactive protein, if present in the fluid, was calcium-precipitable and often gave grossly milky solutions when redissolved. Because of the importance of obtaining larger amounts of crystalline material for studies on the further characterization of the protein as well as to provide antigen for the preparation of specific antisera employed in the clinical test for C-reactive protein, attempts were made to improve the preparative procedures so that crystallization of the protein from this type of material could be regularly accomplished.

The present paper describes a simple modification of the previously published method for crystallization of C-reactive protein which is suitable for preparation of crystalline material from fluids containing lipid in association with the active fraction. In addition, the improved yields of crystalline protein provided by this procedure have made possible studies on the physical-chemical properties of the protein. The protein has been examined by the techniques of free electrophoresis, zone electrophoresis, and ultracentrifugation.

## *Materials and Methods*

*Isolation of the Lipid-Bound Protein.*—C-reactive protein was isolated, purified, and crystallized from nine separate lots of pooled pathological serous fluids. The majority of these fluids were obtained by thoracenteses from patients with Hodgkin's disease and lymphomas.[1] The volumes of the pools ranged between 3 and 6 liters. Before fractionation of the fluids with ammonium sulfate, one-half volume of tap water was added to each pool. The fluids were brought to 0.5 saturation with ammonium sulfate by the addition of solid ammonium sulfate (313 gm. per liter). The precipitate was removed by suction filtration using 1 per cent filter cel and was then discarded. The 0.5 saturated filtrate was brought to 0.75 saturation by the addition of 175 gm. solid ammonium sulfate per liter of filtrate. One per cent each of filter cel and standard super cel was stirred in and the precipitate recovered by suction filtration. The precipitate was redissolved in as small a volume of distilled water as possible and the filter aid was then removed by centrifugation. The protein fractions obtained in this way were clear amber solutions and contained practically all the C-reactive protein of the original pools. Dialysis against several changes of 4 liters of 0.01 per cent calcium chloride was then carried out. Although dialysis against tap water has been used for this purpose in the past, it is not a reliable procedure for general use since in some areas the tap water may contain insufficient ionized calcium to precipitate the lipid-bound C-reactive protein. The C-reactive protein in these serous fluids precipitated on dialysis against dilute calcium chloride and was found to be in combination with lipid.

*Removal of Lipid.*—The precipitated C-reactive protein was recovered by centrifugation, the supernatant decanted off, and the precipitate redissolved in 50 to 100 cc. of 0.1 M sodium citrate made up in 0.1 M sodium chloride. There was great variation in the appearance of these solutions. Some were clear, others were slightly opalescent, and still others were of a heavy, creamy consistency. The solution of C-reactive protein was layered over an equal

[1] All pathological fluids were obtained through the courtesy and cooperation of Memorial Hospital, New York City.

volume of chloroform and stirred gently for 1 hour with a mechanical stirrer in the cold. Avoidance of violent agitation of the preparation is important to prevent denaturation of the protein. The preparation was then centrifuged at 4°C. and the citrate-saline phase pipetted off. In all instances three phases were present in the centrifuge tube, the chloroform on the bottom, a solid gel of material in the middle, and on the top the citrate phase containing the serologically active C-reactive protein. The procedure of stirring the citrate solution over chloroform was repeated a second time, and after centrifugation the solution containing the C-reactive protein was again layered over chloroform in the cold and left for 12 to 18 hours. Following another centrifugation the protein solution was dialyzed for 18 hours against physiological saline, 0.01 per cent with respect to calcium chloride, to remove citrate and to assess the completeness of the defatting procedure.

Following dialysis, pneumococcal C polysaccharide was added to the solution to precipitate out the C-reactive protein. The final concentration of C polysaccharide was approximately 0.1 mg./cc. After incubation at 37°C. for 2 hours and refrigeration overnight, the precipitated protein-carbohydrate complex was recovered by centrifugation and washed three times with physiological saline containing 0.01 per cent calcium chloride. The washed protein-carbohydrate complex was redissolved in a small volume, 10 to 20 cc., of the 0.1 M citrate-saline solution. In some instances a small amount of insoluble material was removed by centrifugation and discarded.

*Crystallization.*—The procedure employed for crystallization was that described by McCarty (1). The citrated solutions of the protein were brought to 0.75 saturation with the addition of saturated sodium sulfate (prepared at 37°C.) and were kept in an incubator at 37°C. In each instance an amorphous precipitate formed early and crystallization occurred from the amorphous state. All C-reactive protein used for electrophoresis was recrystallized at least once.

*Free Electrophoresis*[2].—The electrophoretic studies were carried out at 1.0°C. in the semimicro apparatus described by Longsworth (4). Prior to electrophoresis a 1 per cent solution of the protein, or a 1:2 dilution of serum, was dialyzed at 4°C. for 2 days against the buffer used in the analysis. Owing to the desirability of minimizing the concentration of ionized calcium, modified McIlvaine buffers, prepared by mixing 0.1 M disodium citrate and 0.1 M disodium phosphate in the proper proportions to give the required pH, were used.

*Zone Electrophoresis.*—Further electrophoretic studies were carried out in a starch supporting medium as described by Kunkel and Slater (5). After the sample of serum, or solution of purified protein, was applied to the starch block, electrophoretic separation was allowed to proceed for 36 hours at 400 volts at 4°C. The block was then cut into 1 cm. segments. Each segment was suspended in 3.0 cc. of saline and after the starch had settled, aliquots of supernatants were removed for analysis. Analysis for total protein was carried out by the modified Folin-Ciocalteu tyrosine method. A sample of each aliquot was tested for the presence of C-reactive protein using specific rabbit antiserum. In all but one experiment, in which veronal buffer of pH 8.0 was used, the experiments were carried out in modified McIlvaine buffer of pH 7.7.

*Ultracentrifugal Analysis.*—Studies were made in a Spinco model E analytical ultracentrifuge. The C-reactive protein dissolved in 0.1 M sodium citrate was centrifuged at concentrations of 1.0, 2.0, and 3.0 mg. protein per ml. With the aid of the factors given by Svedberg (6), the sedimentation constants were corrected to water at 20°C. and then extrapolated to zero protein concentrations to give a value for $s_{20,w}$.

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[2] The studies employing free electrophoresis were kindly carried out by Dr. Lewis K. Longsworth.

## RESULTS

*Behavior on Crystallization.*—All nine preparations of the C-reactive protein crystallized from the amorphous state in 0.75 saturated sodium sulfate. The period of time required for first crystallization of the protein ranged from 4 days to 2 weeks. Recrystallization occurred more rapidly and was complete in 2 to 4 days. In none of the nine preparations did the crystals have the form of tiny needles seen in the initial crystallization of the C-reactive protein (1). In each case they appeared in the form of rhomboid plates which appeared to form from amorphous aggregations of the protein which had a roughly rhomboid shape.

Some of the crystalline C-reactive protein thus obtained served as antigen for the preparation of rabbit antisera to be used in clinical testing. Further confirmation of the immunological traits described by MacLeod and Avery, McCarty, and Wood was obtained (3, 1, 7). It was again demonstrated that the crystalline C-reactive protein is antigenic in the rabbit and that the antisera obtained gave no cross-reaction with normal human serum.

*Electrophoretic Properties.*—From free electrophoresis of the crystalline protein in four buffers ranging in pH from 4.91 to 7.46 its isoelectric pH was estimated to be 4.82. A tendency to flocculate was noted at pH values approaching the isoelectric pH.

Comparison of the electrophoretic patterns at pH 7.4 of an acute phase serum and the same serum after removal of the C-reactive protein with C polysaccharide showed no significant difference. This result was not unexpected since it has been shown that sera giving maximal precipitation with antiserum to C-reactive protein contain only 0.165 mg. C-reactive protein per cc. of serum (8). However, comparison of the patterns of either the crystalline C-reactive protein, or this material prior to defatting, with that of normal human serum, obtained under the same conditions of free electrophoresis indicated that the C-reactive protein has a mobility in the same range as $\beta$-globulin. This result was confirmed, as shown in Fig. 1, by obtaining patterns of a normal serum before and after the addition of purified, lipid-bound C-reactive protein. It is evident from inspection of the figure that addition of the C-reactive protein caused a marked increase in the area under the $\beta$-globulin peak.

In none of the free electrophoresis experiments described thus far was the C-reactive protein allowed to migrate more than a few centimeters. When, however, a 1 per cent solution of the crystalline protein was subjected to prolonged electrophoresis at pH 7.33, the patterns of Fig. 2 were obtained and indicate some electrophoretic inhomogeneity. In this respect the behavior of C-reactive protein is not unlike that of crystalline egg albumin and may indicate the presence of molecular species differing only in a relatively small number of amino acid residues.

Zone electrophoresis of an acute phase human serum known to contain a large amount of C-reactive protein was carried out in veronal buffer of pH 8.0

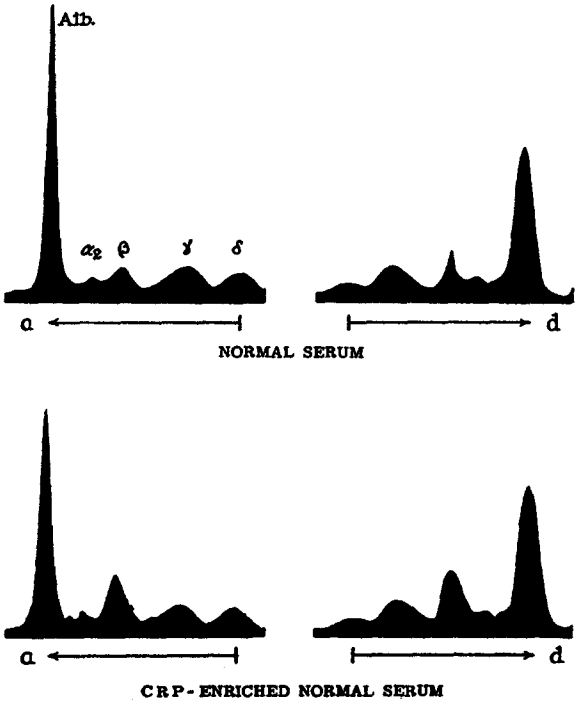


FIG. 1. Electrophoretic patterns of a normal serum with and without added purified lipid-bound C-reactive protein. Electrophoresis carried out in phosphate-citrate buffer of pH 7.45. The two upper figures show the ascending and descending boundaries of the patterns obtained with normal human serum. The lower two figures show patterns obtained with the same serum to which C-reactive protein had been added.

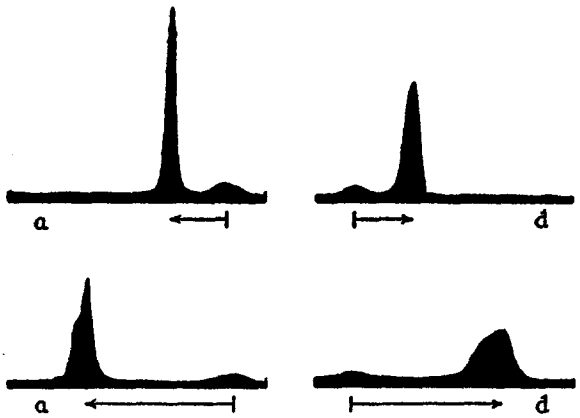


FIG. 2. Electrophoretic patterns obtained with a 1 per cent solution of crystalline C-reactive protein in phosphate-citrate buffer of pH 7.33. The upper two figures show the ascending and descending boundaries of the electrophoretic pattern at 2 hours, the lower two figures show those at 5 hours.

in starch supporting medium. Contrary to the findings in free electrophoresis, the C-reactive protein was located in the fast component of the $\gamma$-globulin. No trace of this protein was found in the $\beta$-globulin. It seemed possible that the apparent discrepancy in this instance was due to the fact that all the free electrophoresis experiments were carried out in the modified McIlvaine buffers. For this reason starch electrophoretic analysis of three separate acute phase sera, all containing concentrations of C-reactive protein in the maximal range, was carried out in modified McIlvaine buffer of pH 7.7. Again in these three experiments as in the experiment carried out in veronal buffer, the C-reactive protein was found to move with the fast components of the $\gamma$-globulin and none

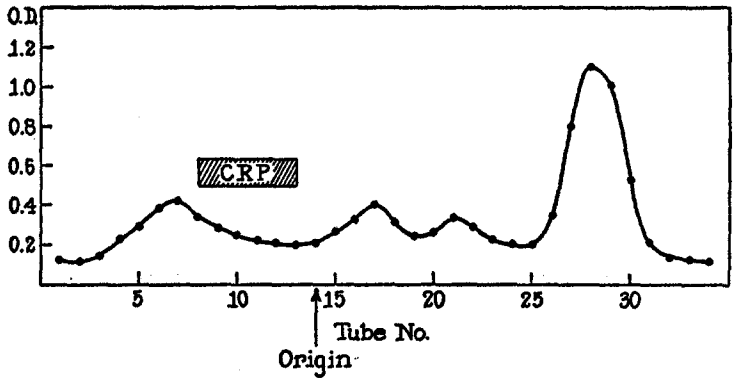


FIG. 3. Starch electrophoretic pattern of an acute phase human serum in phosphate-citrate buffer of pH 7.7. The curve of the protein concentrations in the eluates as determined by the Folin-Ciocalteu tyrosine method is shown. The C-reactive protein was located serologically in eluates 8 through 13.

was present in the $\beta$-globulin. Fig. 3 shows the results obtained in the electrophoresis of one of these three acute phase sera.

In another experiment, 2 mg. of purified lipid-bond C-reactive protein was added to a 3 cc. sample of normal serum. Electrophoresis of this preparation in the pH 7.7 phosphate-citrate buffer was carried out and again the C-reactive protein was found to migrate as $\gamma$-globulin component of high mobility.

A final starch electrophoresis experiment was carried out in which two preparations, one twice crystallized C-reactive protein and the other normal human serum to which crystalline C-reactive protein had been added, were introduced at corresponding points on separate starch blocks. One block was placed on top of the other with an intervening glass plate and both were subjected to electrophoresis simultaneously. In this experiment, which was carried out with the pH 7.7 modified McIlvaine buffer, the crystalline C-reactive protein contained in this normal human serum was found, as in other starch electrophoresis

experiments, in the γ-globulin. The isolated crystalline C-reactive protein was found in the aliquots from the starch segments corresponding exactly to those of the paired blocks which contained the mixed serum and C protein. It is clear, therefore, that the retarded mobility of the protein on starch is not referable to interaction with other serum proteins.

*Ultracentrifugal Analysis.*—In a centrifugal field of 240,000 *g* the crystalline C-reactive protein moved as a single, sharp peak and appeared homogeneous throughout the run. The sedimentation constants which were derived from three dilutions and corrected for temperature and solvent effects are plotted in Fig. 4. The line connecting these three points extrapolated to zero concentration indicates the $s_{20,w}$ of C-reactive protein is 7.5. This value suggests

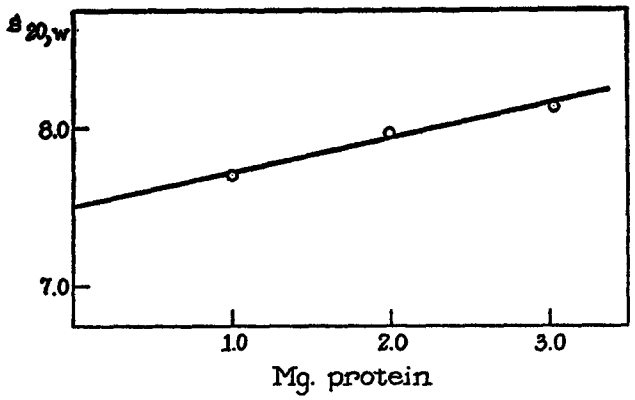


FIG. 4. Ultracentrifugal analysis of C-reactive protein in 0.1 M sodium citrate, pH 7.2 $s_{20,w}$ = 7.5. Plot of the sedimentation constants derived from three concentrations of the protein corrected for temperature and solvent effects.

that the C-reactive protein is a somewhat heavier material than serum γ-globulin for which, under similar conditions, $s_{20,w}$ = 6.3.

## DISCUSSION

A simple method for rendering concentrated solutions of lipid-bound C-reactive protein free of lipid has been described. By the use of this method C-reactive protein obtained from serous fluids can be freed of lipid material and thus made suitable for crystallization or for use as an antigen. Since preparations of lipid-bound C-reactive protein have proved unsatisfactory in the routine production of specific antiserum, this method removed the major obstacle to relatively easy availability of this protein for use as an antigen. Rabbit antisera against lipid-bound C-reactive protein usually cross-react extensively with normal human serum. This fact suggests that a lipoprotein may be removed by the chloroform treatment, a possibility which is supported by the occurrence

of a solid gel phase at the chloroform-water interface. Chemical analysis of the chloroform-soluble fraction or the chloroform gel has not been carried out.

The electrophoretic and ultracentrifugal experiments reveal that the twice crystallized C-reactive protein has been separated from normal serum proteins as a relatively homogeneous material. These findings support the previously described serological specificity of crystalline C-reactive protein and emphasize the desirability of using the crystalline protein as antigen.

The fact that the C-reactive protein has the mobility of a $\beta$-globulin in free electrophoresis and of a $\gamma$-globulin on starch poses an interesting problem which is undergoing further investigation. Present evidence does not suggest that these differences are caused by binding or trailing in the starch medium. It is apparent that the patterns derived by the two methods of electrophoresis will not exactly correspond because of the different methods of measurement employed. The discrepancy between the findings derived by the two methods may be partially explained by the fact that the $\beta$-globulin component of the starch system as measured by the Folin-Ciocalteu technique always reveals a slower mobility than that defined by the optical system of free electrophoresis. The failure of C-reactive protein to precipitate from acute phase serum at half-saturation with ammonium sulfate provides independent evidence against the possibility that this substance is in reality a typical gamma globulin.

The only previous electrophoretic work with the C-reactive protein was done by Perlman, Bullowa, and Goodkind, who studied acute phase sera before and after removal of the C-reactive protein by C polysaccharide and who determined which of the electrophoretic components of serum were obtained by the fractionation method of MacLeod and Avery (9). They were unable to determine conclusively from these studies the fraction of serum with which the C-reactive protein migrated but suggested tentatively on the basis of indirect evidence, that it was the $\alpha_1$-globulin. The results of the present study are not in accord with this view and indicate that C-reactive protein migrates as a $\beta$-globulin in free electrophoresis.

## SUMMARY

A method is described for obtaining crystalline C-reactive protein from serous fluids in which the protein is associated with lipid. Most pathological fluids currently available as a source of this protein appear to fall in this category.

Crystalline C-reactive protein has its isoelectric point at pH 4.82 as determined by free electrophoresis in McIlvaine's buffer. Its mobility in the electrophoresis cell, both alone and after addition to normal serum, coincides with that of the $\beta$-globulin fraction of the serum. In contrast to this finding, by the method of zone electrophoresis on a starch supporting medium the protein migrates with the $\gamma_1$-globulin. The significance of this discrepancy is discussed. Studies in the ultracentrifuge indicate an $s_{20,w}$ of 7.5.

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