THE OCCURRENCE IN THE RABBIT OF AN ACUTE PHASE PROTEIN ANALOGOUS TO HUMAN C-REACTIVE PROTEIN

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C-reactive protein is a substance which appears in human blood during the acute phase of a variety of infectious diseases as well as during the course of certain non-infectious diseases. The presence of C-reactive protein in serum was first recognized as the result of its property of reacting to form a precipitate with the somatic C polysaccharide of pneumococcus (1). This reaction is responsible for the name that has been applied to the substance and provides a specific technique for its detection and isolation.

As the result of a series of investigations, many of the properties of the C-reactive protein have been defined (2–7). On salt fractionation of serum it is found in the albumin fraction (3), although electrophoretic studies indicate that it is probably an α globulin (6). Calcium ion is required for the reaction between the polysaccharide and the protein, and the precipitation can be prevented by the use of calcium-binding agents such as oxalate or citrate (3). A further relationship to calcium ion is indicated by the fact that the C-protein, as it occurs in the albumin fraction of serum, will precipitate upon dialysis against tap water or dilute calcium chloride solution (4). The precipitability of the protein under these conditions is determined by another component, presumably lipid in character, combined with the protein, and is eliminated by defatting the serum with alcohol and ether. However, defatting does not alter the reactivity of the protein with the C polysaccharide.

The C-reactive protein is immunologically distinct from the proteins of normal serum, and antisera prepared against purified preparations are specific and do not react with normal human sera (5). The protein has been crystallized from human serous fluids, and the serological specificity has been confirmed with this material (7).

Interest has been maintained for several years in the possible significance of the C-reactive protein, and numerous experiments have been undertaken in this laboratory in an effort to determine its function and site of origin. Recently, the clinical value of the determination of C-reactive protein in the serum as a

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measure of the activity of the disease process in acute rheumatic fever has been described (8). The parallelism between the fluctuations in rheumatic activity and the amount of this abnormal protein in the serum is a further stimulus to studies on the nature of the C-reactive protein.

Since investigation of the more basic problems concerning C-protein would obviously be greatly facilitated if they could be carried out in a suitable laboratory animal, our attention was directed to this line of attack. In earlier studies, Abernethy (2) was able to demonstrate the presence of a C-reactive protein in the sera of monkeys infected with pneumococci, but found that the acute phase sera of rabbits did not react with C polysaccharide. However, in the subsequent work of others there were indications that rabbits formed a substance analogous to the C-reactive protein even though it could not be demonstrated by the precipitation reaction used with human sera. For example, Löfström (9, 10) found that acute phase rabbit serum caused non-specific capsular swelling of certain strains of pneumococci comparable to that which he had described as occurring with acute phase human sera. Since Löfström (11) had advanced good evidence that the substance in human acute phase serum responsible for capsular swelling is identical with C-reactive protein, his studies provided presumptive evidence for the presence of a similar protein in the rabbit. Furthermore, Hotchkiss and MacLeod (12), in unpublished experiments, found that on fractionation of acute phase rabbit serum, a substance is present in the albumin fraction which is precipitable by calcium under the same conditions as human C-protein.

The present investigation was undertaken to obtain further information concerning the acute phase protein produced by the rabbit, and to determine its suitability as a model for the study of C-reactive protein. The possibility was considered that some polysaccharide other than the somatic C polysaccharide of pneumococcus might serve as a comparable test reagent in this species, and consequently a search for a suitable polysaccharide was undertaken. The present paper deals with the description of a polysaccharide, closely related to the classical C polysaccharide, which precipitates with the acute phase protein of the rabbit, and with the properties of the protein and its relationship to human C-reactive protein.

EXPERIMENTAL

Bacterial Polysaccharides.—Samples of the following polysaccharides were obtained through the courtesy of Dr. Walther F. Goebel: Type-specific pneumococcal capsular polysaccharides of Types I, II, III, and XIV; the pneumococcal C polysaccharide, the pneumococcal heterophile antigen (F polysaccharide), and the type-specific polysaccharides of Friedländer's bacillus Types A, B, and C.

Pneumococcal Cx Polysaccharide.—The substance designated herein as pneumococcal Cx polysaccharide was first encountered in the course of experiments on the purification of the pneumococcal transforming substance (13). It was concluded on the basis of the available

evidence that this substance probably represented a more highly polymerized form of the somatic C polysaccharide. However, in view of the additional differences between the C and Cx polysaccharides described in the present paper it appears possible that a chemical difference other than degree of polymerization may be involved. The primary difference in the techniques employed in the isolation of the two forms of the polysaccharide lies in the manner in which the material is released from the cells. In the original method of Tillett, Goebel, and Avery (14), the cells were disrupted by repeated freezing and thawing; and in the later method of Goebel *et al.* (15), the cells were allowed to autolyze under toluene for 3 days. In the preparations from which the Cx polysaccharide was isolated, on the other hand, the bacterial cells were suspended in sodium citrate solution and rapidly lysed (5 to 15 minutes) by the addition of sodium desoxycholate, followed by immediate deproteinization. The rapid lysis and deproteinization, rather than the presence of citrate, appear to be responsible for the different character of the end-product. It is inferred that enzymatic degradation occurring during lysis of the cells is responsible for breaking down the polysaccharide to the form in which it is usually isolated.

Although the precise nature of the chemical difference between the two polysaccharides has not been determined up to the present time, the evidence for the close relationship between them has been extended. The Cx polysaccharide contains both nitrogen and phosphorus in essentially the same amount as does the C polysaccharide. In general, the Cx polysaccharide is more reactive with antipneumococcal horse and rabbit sera than is the C polysaccharide, and in some instances sera which give little or no precipitin reaction with the latter give good reactions with Cx. The best evidence that this is not merely the result of an additional antigenic substance contaminating the Cx preparations is obtained from precipitin inhibition tests. In the case of sera such as those mentioned which react only with Cx, or sera that have been adsorbed with C polysaccharide, the reaction with Cx polysaccharide is specifically inhibited by the addition of C polysaccharide. These facts are compatible with the interpretation that C is a somewhat more degraded form of Cx.

In contrast to these differences between the C and Cx polysaccharides in precipitin reactions with pneumococcal antisera, the two substances appear to be identical in their reactivity with human C-reactive protein. Thus, they have the same titer when tested in the precipitation reaction with human acute phase serum, and either substance is capable of removing all of the C-protein from solution.

Preparation of Cx Polysaccharide.—The procedure for the isolation of Cx polysaccharide used in the early part of this work was a modification of that used in the preparation of the desoxyribonucleic acid fraction of pneumococcus (13). It proved to be unnecessarily long and complex, however, and a simpler, more rapid procedure has been devised. Since the serological reactivity of the polysaccharide is not affected by exposure to 100°C., it is possible to use heat to achieve prompt destruction of enzymes and at the same time cause coagulation of much of the inactive material as in the original procedure of Tillett, Goebel, and Avery (14).

The cells from 1 to 50 liters of broth culture of a strain of rough pneumococcus derived from Type II are recovered by centrifugation and washed with physiological saline. The washed cells are suspended in saline at one-fiftieth the volume of the original culture and sodium desoxycholate is added in a final concentration of 0.1 per cent. Lysis occurs rapidly at room temperature, and after 5 minutes the lysate is acidified with acetic acid (1.0 ml. of 2 N acetic acid to each 100 ml. of lysate) and immediately placed in a boiling water bath. After 10 minutes, the lysate is cooled, centrifuged, and the clear supernate removed. The active material is contained in the supernate and is precipitated by the addition of four volumes of ethyl alcohol, recovered by centrifugation and redissolved in a volume of saline equal to one-fifth that of the lysate. The bulk of the nucleic acid remaining in the preparation is removed by precipitation with alcohol in the presence of calcium ion. One part of 10 per cent

calcium chloride is added to nine parts of the solution, and upon the addition of 0.2 volume of ethyl alcohol a flocculent precipitate is formed which is removed by centrifugation. The Cx polysaccharide remains in the supernate and is recovered by precipitation with four volumes of alcohol. The precipitate is washed with absolute alcohol and ether and dried *in vacuo*. Alternatively, drying can be carried out from the frozen state after dialysis of the material against distilled water.

The Cx polysaccharide so obtained gives negative qualitative tests for protein, usually contains traces of ribonucleic acid as indicated by the orcinol reaction, and gives a pink color with the diphenylamine reagent. The Molisch reaction is positive and is greatly increased in intensity by prior acid hydrolysis. The yield of Cx polysaccharide is 10 to 20 mg. per liter of culture.

TABLE I

Precipitation Reaction of Acute Phase Rabbit Serum with Bacterial Polysaccharides

Polysaccharide, *1 mg./cc.*	Acute phase serum	Reaction
Friedländer Type A	Rabbit	—
" Type B	"	—
" Type C	"	—
Pneumococcus C	"	—
Pneumococcus F (heterophile)	"	—
Pneumococcus Type I	"	—
" Type II	"	—
" Type III	"	+
" Type XIV	"	—
" Cx	"	+
Pneumococcus Type III	Human	+
" Cx	"	+

Reactions between Rabbit Acute Phase Sera and Various Polysaccharides

For preliminary screening of the polysaccharide preparations, acute phase rabbit sera were obtained from animals infected intradermally with 0.2 ml. of a blood broth culture of pneumococcus Type I (SVI). The rabbits were bled 40 hours after injection. After initial experiments employing a capillary precipitation test, the more sensitive ring test was used as a routine. In these tests, saline solutions of the polysaccharides (1 mg./ml.) were carefully layered over a column of the acute phase serum. Saline controls were employed with each serum. The tubes were incubated at 37°C. in a water bath and read for the presence of interfacial precipitates after 2 hours. The results of the tests are given in Table I.

It will be seen that the acute phase rabbit serum reacts only with the Cx polysaccharide and the pneumococcal Type III polysaccharide. In addition, both of these polysaccharides react with acute phase human serum. Since not all preparations of Type III polysaccharide show this type of reactivity, the possibility was suggested that the preparation used contained the Cx type of somatic polysaccharide as contaminant. It was possible to show that this is probably the case and that the reactivity is not inherent in the Type III

polysaccharide itself. This was done by the following experiment employing the bacterial enzyme which hydrolyzes the Type III polysaccharide (16).

Three-tenths ml. of a 1.0 mg./ml. solution of the Type III polysaccharide was incubated with 0.3 ml. of a preparation of the SIII enzyme for 24 hours at 37°C. in the presence of one drop of chloroform. The control tube contained saline in place of the enzyme. The reaction mixtures were then tested for their reactivity with rabbit and human acute phase sera and with Type III antipneumococcal rabbit serum. The results are given in Table II.

It appears that the SIII enzyme almost completely destroys the capsular polysaccharide, as indicated by the loss of reactivity with specific antiserum, but does not alter the reaction with acute phase rabbit and human sera. Therefore, it is most likely that the reaction between the untreated Type III poly-

TABLE II

Effect of SIII Enzyme on the Reactivity of an SIII Polysaccharide Preparation with Acute Phase Sera

Serum	Reaction system	
	SIII polysaccharide + SIII enzyme	SIII polysaccharide + saline
	Serological reaction	
Acute phase rabbit serum—pooled..............	+	+
Acute phase human serum.....................	+±	+±
Type III rabbit antipneumococcal serum.......	? Trace	++++

saccharide solution and rabbit acute phase serum is due to the presence of Cx polysaccharide.

Cx polysaccharide reacted consistently with a large number of different samples of acute phase rabbit sera obtained following infection with pneumococci, and also with acute phase rabbit sera obtained after the injection of other stimuli. The Cx polysaccharide did not react with normal rabbit sera, including control bleedings on the animals used for the production of acute phase sera and a large number of sera prepared for the purpose of typing group A streptococci.

It appears, therefore, that the Cx polysaccharide provides a reagent for the further study of an acute phase protein of rabbit serum. While this polysaccharide is similar in many respects to C polysaccharide obtained by the usual methods, it is sufficiently different to react consistently with the acute phase rabbit protein which gives no visible reaction with C polysaccharide. The reaction between the C and Cx polysaccharides and rabbit acute phase sera is markedly inhibited by C polysaccharide. This inhibition is illustrated by the following experiment.

Aliquots of acute phase rabbit serum were mixed with falling twofold dilutions of C poly-saccharide, prepared according to the procedure of Goebel *et al.* (15). Each of the mixtures was tested by the ring test using an 0.1 mg./ml. solution of Cx polysaccharide as the upper layer. The results are recorded in Table III.

It is apparent that the C polysaccharide completely inhibits the reaction at a final concentration of 0.25 mg./ml. and has a perceptible inhibitory effect at a final concentration of 0.03 mg./ml. In addition to confirming the relationship between the two polysaccharides, this experiment is evidence for the relationship between the human and rabbit acute phase proteins. The similarities between the two proteins are further discussed in the following section.

Comparison of the Acute Phase Rabbit Protein with Human C-Reactive Protein

General Properties.—The properties of the acute phase rabbit protein precipitable by the Cx polysaccharide are analogous in practically all respects to the human C-reactive protein. As in the case of the human C-protein, calcium ion

TABLE III

The Inhibition by C Polysaccharide of the Reaction Between Rabbit Acute Phase Serum and Cx Polysaccharide

	Final concentration of C polysaccharide in rabbit acute phase serum mg./cc.							
	1.0	0.5	0.25	0.125	0.06	0.03	0.015	None
Reaction with Cx Polysaccharide	−	−	−	±	++	++	+++	+++

is required for the reaction between Cx polysaccharide and rabbit acute phase protein. The addition of oxalate or citrate eliminates the reaction, and Cx polysaccharide-protein precipitates can be redissolved in sodium citrate solutions.

On fractionation of acute phase rabbit serum with ammonium sulfate the reactive protein is found in the albumin fraction. Dialysis of the albumin fraction against tap water or 0.01 per cent calcium chloride solution results in quantitative precipitation of the acute phase substance. In addition, as in the case of human C-protein, defatting of the serum results in an alteration so that precipitation no longer occurs under these conditions.

Time of Appearance of Acute Phase Protein.—While acute phase sera drawn very early in the clinical course of pneumonia in human subjects contained C-reactive protein, it was not known exactly when this protein first appeared in the blood after the onset of disease. Experiments on two human volunteers were carried out in this laboratory to determine the appearance time of C-reactive protein. Triple typhoid vaccine, 0.5 ml. subcutaneously, was used as the stimulus. Bleedings were obtained at 2 hour intervals for the first 12 hours, then at 6 hour intervals for the next 18 hours in one subject, and at 0, 12, 18, and 36 hours in the other. In both subjects C-reactive protein was not present in

detectable amounts in the 12 hour bleeding but had appeared in the blood by the 18th hour after the injection of the vaccine. Similar experiments were carried out by Hedlund, Frisk, and Bucht (17) with essentially the same results. They administered to human subjects continuous intravenous infusions of a vaccine made from *Aerobacter aerogenes* and measured the appearance time of C-protein as evidenced by the non-specific pneumococcal capsular swelling test.

The time of appearance of rabbit Cx protein was investigated in a similar way. At the same time, the effect of three different stimuli on the production of the protein was tested. The following were employed: 0.2 ml. of an 8 hour culture of a group A Type I streptococcus, and 0.2 ml. of an 8 hour culture of a Type I pneumococcus injected intradermally, and 2.0 cc. of a triple typhoid vaccine injected subcutaneously. The results are presented in Table IV. It will be seen that in the rabbit, as in man, the time of appearance of the acute

TABLE IV

Time of Appearance of Cx-Protein in Rabbits after Various Stimuli

Substance injected		Triple typhoid vaccine, 2.0 cc.		Culture of pneumococcus Type I, 0.2 cc.		Culture of group A, Type I streptococcus, 0.2 cc.	
Rabbit		1	2	3	4	5	6
Ring tests employing Cx polysaccharide 1:10,000	Control bleeding	—	—	—	—	—	—
	12 hours after injection	+	—	—	—	—	—
	18 hours after injection	+	+	+	+	+	±
	36 hours after injection	+	+	++	++	++	++

phase protein in most instances is between 12 and 18 hours. Furthermore, the protein appears in response to three of the same stimuli that are known to produce C-protein in human beings: namely, the hemolytic streptococcus, pneumococcus, and typhoid vaccine.

Antigenicity.—Like C-reactive protein, rabbit Cx-reactive protein is antigenic, and specific antisera have been produced in the rooster. A purified, but not crystalline, preparation of rabbit Cx-protein was used as antigen. The reactions of one rooster antiserum in precipitin tests with normal and acute phase rabbit sera are shown in Table V. The data show that the unadsorbed antiserum reacts well with rabbit serum containing Cx-protein and possesses only a slight degree of cross-reactivity with normal rabbit serum proteins. It is apparent that rooster antisera of this type can serve as a sensitive reagent for the detection of rabbit Cx-protein.

Crystallization of Rabbit Cx-Protein

Human C-reactive protein has been isolated in a crystalline form from serous pleural and ascitic fluids but not from blood serum (7). In serum the protein

appears to be combined with a lipid, while in certain pleural and ascitic fluids the same type of combination with lipid does not occur. This fact may be related to the success of the crystallization procedure with human serous fluids. In the case of rabbit Cx-protein, numerous attempts were made to crystallize the protein from serum without success. Since large accumulations of serous fluid containing Cx-protein are not readily produced in the rabbit, an attempt was made to defat rabbit serum as the first step in crystallization. Following the defatting of acute phase rabbit serum with alcohol-ether, it proved possible to obtain a crystalline preparation of Cx-protein by a procedure analogous to that used in the case of human C-protein. The procedure followed is given in detail in the following paragraphs.

Four hundred and sixty ml. of acute phase serum was obtained from 12 rabbits bled 40 hours after the intradermal injection of 0.2 ml. of a blood broth culture of pneumococcus Type I. The serum was chilled and added slowly with stirring to 5 liters of a 3:1 mixture of ethyl

TABLE V

Precipitin Reactions with Serum of Rooster Immunized against Rabbit Cx-Protein

Antigen	Dilution of antigen									
	Undil.	1:2	1:4	1:8	1:16	1:32	1:64	1:128	1:256	1:512
Normal rabbit serum	—	—	—	?	±	±	±	—	—	—
Acute phase rabbit serum	++++	++	++	+±	+	±	±	±	±	?

alcohol and ethyl ether maintained at −12°C. in an ice-salt bath. After 1 hour at a mean temperature of −7°C., the precipitate was collected by centrifugation in a refrigerated centrifuge at −5°C. The precipitate was washed three times with cold ether and finally emulsified with one liter of cold ether and filtered with suction on hard paper in a cold room at 2°C. The filter cake was placed in a desiccator and dried *in vacuo*.

The dried, defatted serum was redissolved in 400 ml. distilled water and brought to 0.5 saturation with ammonium sulfate by the addition of 128 gm. of the solid salt. The precipitate was removed by filtration with the aid of filter cel, and the filtrate brought to 0.75 saturation by the addition of 17.5 gm. of ammonium sulfate per each 100 ml. of filtrate. The precipitate at 0.75 saturation was recovered by filtration with the aid of filter cel (1 per cent). This precipitate, containing the Cx-reactive protein, was redissolved in 100 ml. distilled water, and filtered free of filter cel. The filter cel pad was washed with 50 ml. water and the washings combined with the main solution. The combined solution was dialyzed first against tap water and finally against 0.01 per cent calcium chloride to determine whether any material precipitable under these conditions remained. No precipitate formed, and the Cx-protein was recovered by the addition of 5 mg. Cx polysaccharide and sufficient sodium chloride to bring the solution to 0.85 per cent. After 2 hours at 37°C. and refrigeration overnight, the polysaccharide-protein precipitate was centrifuged and washed three times with 0.85 per cent sodium chloride containing 0.01 per cent calicum chloride. The precipitate was redissolved in 5 ml. of a solution containing 0.1 M sodium chloride and 0.1 M sodium citrate and centrifuged free of in-

soluble material. The clear solution was dialyzed against 0.4 saturated sodium sulfate at 37°C. Under these conditions a precipitate formed which showed a distinct silky sheen. The precipitate disappeared at room temperature and reformed on bringing the temperature back to 37°C. Microscopic examination at 37°C. revealed the presence of long needle-like crystals (Fig. 1).
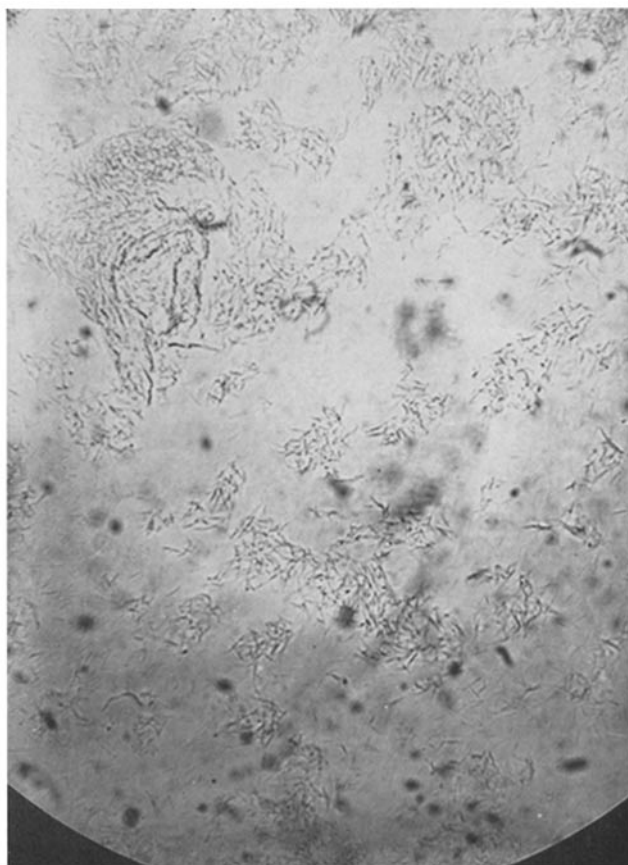

FIG. 1. Crystals of rabbit Cx-protein. × 352.

Recrystallization was accomplished once by dialyzing a solution of the once crystallized material against 0.4 saturated sodium sulfate at 37°C. These crystals were about one-third to one-quarter the size of the original crystals and did not redissolve at room temperature. Further attempts at recrystallization were unsuccessful. Crystallization of Cx-protein from another lot of acute phase rabbit serum was accomplished by the use of final dialysis against 0.5 saturated ammonium sulfate in the cold instead of 0.4 saturated sodium sulfate at 37°C.

The efficacy of the purification achieved by crystallization was indicated by the fact that the material was apparently free of normal rabbit serum protein

as detectable by precipitin tests with specific antisera (Table VI). A solution of the crystalline material gave no reaction with a potent rooster antiserum against normal rabbit serum but reacted well with antiserum against the Cx-protein. The yield of crystalline material was not adequate for detailed studies of its properties or chemical composition.

TABLE VI

Precipitin Tests with Crystalline Cx-Protein

Rooster serum	Dilution of solution of crystals of Cx-protein						
	1:10	1:20	1:40	1:80	1:160	1:320	1:640
Anti-Cx-protein	+	+	++	+	+	±	±
Anti-normal rabbit se-rum	—	—	—	—	—	—	—

DISCUSSION

Since the initial description of human C-reactive protein many of its properties have been defined, but virtually nothing has been determined concerning its function, site of origin, or method of disappearance from the body. The search for the analogue of this protein in the rabbit was carried out as a preliminary to an attempt to obtain information on some of these points. While it is hazardous to reason by analogy in comparing phenomena in two distinct species, the preliminary results indicate that the acute phase proteins studied in human beings and rabbits are remarkably similar. The fact that the Cx-polysaccharide of pneumococcus reacts to form a precipitate with both proteins under the same conditions is evidence for a similar molecular configuration.

The other comparative studies which have been carried out on the two proteins also emphasize their close relationship. Apparently both are combined with lipid in the blood stream and the lipid-protein complex precipitates in the presence of calcium ion if the salt concentration is low. The stimuli which cause the appearance of the abnormal protein in the blood, as well as the time required for its appearance, are the same in the two species. Hedlund (18) has observed a comparable parallelism in the stimuli required for the production of sera in rabbits and human beings giving the non-specific pneumococcal capsular swelling reaction.

The accumulated evidence concerning the relationship between the Cx-reactive protein of the rabbit and human C-reactive protein justifies the use of the rabbit system as an experimental model for further study of the nature and significance of this acute phase protein.

In connection with the fact that a somewhat different polysaccharide is required for the precipitation reaction with the rabbit acute phase protein than for that with the human acute phase protein, it is of interest that Löfström

encountered a similar species difference in his investigations on the capsular swelling phenomenon. He found that in order to obtain capsular swelling with acute phase rabbit sera it was necessary to use strains of pneumococci different from those which react with human sera (9, 10). It is not known whether the difference between the pneumococcal strains which show capsular swelling with acute phase sera of the two species can be explained on the basis of the kind of C polysaccharide occurring in the cells. However, this explanation seems unlikely in view of the present evidence indicating that C polysaccharide is a degradation product of Cx polysaccharide appearing in the course of extraction from the bacteria.

Although not directly related to the studies on the origin and function of the acute phase proteins, the problem of the chemical difference between the C and Cx polysaccharides is of interest. In many respects, the behavior of the two substances is reminiscent of the findings with the capsular polysaccharide of pneumococcus Type I. In this case, it was found that when the method of preparation was varied two substances could be obtained which differed in their reactivity with specific antisera (19). The chemical nature of the difference was defined by the demonstration that one product was essentially the deacetylated form of the other. A comparable slight variation in chemical structure may conceivably be involved in the differentiation of the two forms of the somatic polysaccharide discussed in the present paper.

SUMMARY

The occurrence in the rabbit of an acute phase protein analogous to human C-reactive protein has been confirmed. The acute phase protein of the rabbit reacts with a special form of the pneumococcal somatic polysaccharide, designated Cx polysaccharide, in the same manner that the human C-reactive protein reacts with the classical C polysaccharide. The method of preparation and some of the properties of the Cx polysaccharide are described.

The rabbit Cx-reactive protein has been shown to be remarkably similar to human C-reactive protein in its general properties and in the conditions which govern its appearance in the blood. It has been obtained in crystalline form, and appears to be antigenically distinct from the proteins of normal rabbit serum.

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