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

I. Distribution of the Reactive Protein in Patients' Sera and the Effect of Calcium on the Flocculation Reaction with C Polysaccharide of Pneumococcus

By THEODORE J. ABERNETHY, M.D., and OSWALD T. AVERY, M.D.

(*From the Hospital of The Rockefeller Institute for Medical Research*)

In 1930 Tillett and Francis (1) found that in certain infectious diseases, notably lobar pneumonia, the serum obtained from patients during the acute stage of the illness yields a precipitate in the presence of dilute solutions of the C polysaccharide of Pneumococcus. In pneumonia the precipitation test is positive during the height of the disease and becomes negative shortly after the onset of recovery. Thus the precipitating action of the serum closely parallels the clinical course of the infection. Although the particular carbohydrate used as test reagent is derived from Pneumococcus, the presence of the active substance in serum is not limited to pneumococcal diseases but occurs in a number of other infections such as acute rheumatic fever, bacterial endocarditis, and staphylococcal osteomyelitis. Ash (2) confirmed and extended these findings and showed that the "C-reactive" substance is demonstrable in sera obtained from children during the acute stage of infections due to Gram-negative bacilli of the colon-typhoid group. In view of the observations cited above it is evident that the phenomenon is associated with a variety of acute pathological conditions and is not specific with respect to the inciting agent.

In 1934 Francis and Abernethy (3) published a preliminary report on the occurrence in patients during the acute stage of pneumonia of a characteristic skin reaction following the intracutaneous injection of 0.1 mg. of the C carbohydrate. They showed that the skin reaction is correlated with the precipitating action of the serum *in vitro*. These results were later confirmed by Finland and Dowling (4). Recently Abernethy and Francis (5) using a more highly purified preparation of the C polysaccharide corroborated the earlier work and emphasized the striking agreement between the results of the serological and cutaneous tests. In all patients in whom the disease terminated in uneventful recovery they found that both the serum and skin tests were positive during the acute febrile period and became negative shortly after crisis. With the development of complications, the capacity of the skin and serum to react may persist or reappear. Only in fatal cases have the results of the serological and cutaneous tests failed to agree. In these instances it has been observed that the skin may fail to react although the active substance can be demonstrated in the blood at the time of death.

In a subsequent paper (6) Abernethy reported the results of a study of the precipitation reaction with sera obtained from experimentally infected animals. He found that the serum of monkeys (*Macacus cynomolgos*) acutely ill with experimental Type III Pneumo-

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coccus pneumonia precipitated with the C carbohydrate in dilutions as high as 1:500,000. The reactive substance was present in serum obtained within the first 24 hours of the experimental disease, but was not demonstrable in the blood of recovered monkeys. The capacity of the serum to react is not dependent upon the site of the lesion, since the sera of monkeys inoculated by the intraperitoneal or intradermal route were found to be equally reactive. The occurrence of the phenomenon during the course of experimental infection in monkeys is the same as that noted in the spontaneous disease in man. Similar tests carried out with sera from infected mice and rabbits, however, were invariably negative throughout the infection irrespective of the site or severity of the lesion. The seemingly discordant results of the precipitation test in different species of animals infected with the same microorganisms appear at first glance to be somewhat paradoxical. However, the presence during infection of a similarly reacting substance in the blood of allied species such as man and monkeys, and the failure to demonstrate the substance in more distant species such as rabbits and mice are probably to be explained on the chemical dissimilarities of the proteins of the unrelated hosts. Differences in the degree of zoological relationship between the species tested would then account for the observed differences in results. The assumption that during infection an analogous but not identical substance exists in the blood of unrelated species would require for its demonstration the use in each instance of a selective test reagent.

The present paper deals with (a) the effect of heat on the C-reactive substance, (b) its distribution in the serum of infected man and monkeys, and (c) the rôle of calcium in the flocculation reaction with the C polysaccharide.

EXPERIMENTAL

The Effect of Heat on the C-Precipitable Substance.—In order to determine the effect of heating on the reactivity of the precipitable substance in human serum the following experiment was carried out.

Serum obtained from a patient during the acute stage of Type VIII Pneumococcus pneumonia was diluted with an equal part of M/20 borate buffer at pH 7.8. 1.5 cc. samples of the diluted serum were heated for 30 minutes in a water bath at temperatures ranging from 55° to 70°C. After cooling, the specimens of serum heated at the different temperatures were tested for the presence of the reactive substance by adding 0.25 cc. of each to an equal volume of 1:20,000 solution of C polysaccharide. The mixtures were incubated at 37°C. for 2 hours and overnight in the refrigerator. The results of precipitation tests are given in Table I.

The data presented in Table I indicate that under the experimental conditions the C-precipitable substance in diluted serum is inactivated after exposure to temperatures above 65°C. The samples of diluted serum heated at the higher temperatures became opalescent, and it seems probable that the loss of activity was associated with denaturation of the serum proteins since in each instance loss of activity occurred following exposure to temperatures at which most proteins are known to undergo denaturation.

Distribution of the C-Precipitable Substance in Human Sera.—In order to determine whether the reactive material in patients' serum is associated with the albumin or the globulin fraction the following experiment was carried out. Specimens of serum known to be reactive with the test carbohydrate together with a control serum from a normal individual were fractionated by salting out the respective proteins with ammonium sulfate. This procedure was carried out with blood serum obtained from patients suffering from acute lobar pneumonia, active rheumatic fever, and bacteremia due to colon bacillus. In the last instance the patient had aplastic anemia complicated by fatal secondary infection and at autopsy abscesses of the kidney and bronchopneumonia were found. The method of fractionation of the serum proteins was as follows:—

5 cc. of serum were diluted with an equal volume of physiological salt solution, and 10.0 cc. of saturated ammonium sulfate were then added. The mixtures were allowed to

TABLE I

Effect of Heat on the C-Precipitable Substance in Human Serum

Blood obtained during acute infection	Precipitation reactions with C 1:20,000				
	Unheated serum	Serum heated for 30 min. at:			
		55°C.	60°C.	65°C.	70°C.
Type VIII Pneumococcus pneumonia....	+++	+++	+++	+++	–

stand for 15 minutes at room temperature and the globulin precipitated at half saturation was collected by centrifugation at 20,000 R.P.M. The supernatant liquid was decanted and the precipitate redissolved in 5.0 cc. of distilled water. The latter was reprecipitated at half saturation with ammonium sulfate and collected as above. The supernatants from the first and second precipitation were then combined and brought to full saturation by the addition of solid ammonium sulfate. The precipitated albumin was collected by filtration through paper on a Büchner funnel. The albumin and globulin precipitates were separately dissolved in 3.0 cc. of M/100 phosphate buffer pH 7.4 and dialyzed in cellophane sacs against the same buffer solution for 48 hours. The dialysates were then removed and made isotonic by the addition of the requisite amount of solid NaCl. The final protein solutions were clear. Most of the serum pigment was noted in the albumin fraction.

For comparative purposes the whole untreated serum and the respective protein fractions were adjusted to the same volume with physiological salt solution. Precipitation tests with the globulin and albumin fractions together with the original serum were carried out by adding to 0.25 cc. of each an equal volume of a 1:20,000 solution of the C polysaccharide. The final readings were made after incubation at 37°C. for 2 hours and overnight in the refrigerator. The results are given in Table II.

From the results shown in Table II it is evident that the active material in patients' serum which combines to form a precipitate with the C poly-

saccharide is associated with the serum albumin. In each instance the precipitating titre of the albumin fraction was comparable to that of the whole serum. The serum globulins on the other hand were wholly inactive. It is interesting to note that in one of the cases of pneumonia (Type VII) cited in Table II, pleural fluid as well as the blood serum was found to be active in the precipitation tests with the polysaccharide. On fractionation with ammonium sulfate the reactive material in both the blood and chest fluid was present in the albumin and not in the globulin fraction precipitated by half saturation with ammonium sulfate.

Distribution of the C-Precipitable Substance in the Serum of Monkeys with Experimental Type III Pneumococcus Pneumonia.—The clinical course and

TABLE II

Distribution of C-Precipitable Substance in the Albumin and Globulin Fractions of Human Serum

Serum of patients during acute infection	Precipitation reactions with C 1:20,000		
	Whole serum	Albumin	Globulin
Type III Pneumococcus pneumonia..............	++++	+++	—
Type VII Pneumococcus pneumonia.............	++++	++++	—
Rheumatic fever................................	+++	+++	—
*Bacteremia (*B. coli*)...........................	+++	+++	—
Control—normal individual.....................	—	—	—

* Patient had aplastic anemia with terminal bacteremia, and at autopsy abscesses of kidney and bronchopneumonia were found.

pathological findings of the experimental disease in these animals have been described in a preceding paper (6).

Samples of the sera of monkeys (1-37 and 1-44) were obtained 24 and 48 hours after intrabronchial inoculation of these animals with a virulent culture of Pneumococcus Type III. The pooled specimens from each were found to be highly reactive with the test carbohydrate. The serum of each animal was fractionated with ammonium sulfate by the method described in the preceding experiment. The albumin and globulin fractions were tested with the polysaccharide and the results compared with those of the corresponding original serum as shown in Table III.

It is again evident that the reactive material present in the blood of experimentally infected monkeys is intimately associated with the albumin fraction as was previously shown to be the case with serum of patients acutely ill with the spontaneous disease. The serum globulins separated by half saturation with ammonium sulfate failed in each instance to react. The striking parallelism between these findings and those with human serum

clearly demonstrates that during the spontaneous disease in man and the experimental infection in monkeys the active component is found in the albumin and not with the globulin fraction of the serum.

The Effect of Calcium on the Precipitation Reaction.—In comparative tests on the blood plasma and serum of patients with pneumonia it was found that whereas the serum from coagulated blood gave a marked precipitate with C, the plasma derived from an oxalated sample of the same blood failed to react when similarly tested. This difference in activity between serum and plasma suggested that calcium might be involved in the reaction. In order to determine whether the presence of calcium is essential in the precipitation reaction with C, the following experiments were carried out.

0.1 cc. of a 10 per cent solution of potassium oxalate was added to 5.0 cc. of reactive serum. After standing at room temperature for 6 hours the mixture was centrifuged

TABLE III

Distribution of C-Precipitable Substance in Serum of Monkeys with Experimental Type III Pneumococcus Pneumonia

Serum obtained within 48 hrs. after intrabronchial inoculation	Precipitation reactions with C 1:20,000		
	Whole serum	Albumin	Globulin
Monkey 1-37	+++	++	—
" 1-44	++++	++++	—
Normal monkey	—	—	—

and the clear supernatant serum pipetted off from the deposit of insoluble calcium oxalate. This procedure was repeated once more and the supernatant serum was then transferred to a sterile cellophane sac and dialyzed to remove the excess of potassium oxalate and residual traces of calcium. Dialysis was carried out against 0.85 per cent NaCl solution adjusted to pH 7.4 by means of sodium bicarbonate and hydrochloric acid according to Loeb (7). The sac was transferred daily to fresh buffer solution and dialysis was continued for 7 days at 4°C. The serum was then tested for reactivity with C both before and after the addition of calcium.

To a series of tubes each containing 0.4 cc. of the serum from which calcium had been removed there was added 0.1 cc. of an appropriate dilution of $CaCl_2$ containing the amounts shown in the protocol. An equal volume (0.5 cc.) of 1:10,000 solution of C was then added to each tube. The reaction mixtures were incubated at 37°C. for 2 hours and left overnight in the refrigerator. The final readings are shown in Table IV.

From the data presented in Table IV it is evident that sera which are highly reactive in the test with C lose their precipitating action after removal of the calcium. The inactivation is reversible since on the addition of minute traces of calcium chloride the serum regains its capacity to pre-

cipitate with C. The amount of calcium required to reactivate the serum is considerably less than that normally present in the blood. This observation together with the fact previously noted that the polysaccharide itself is not precipitated by an excess of calcium alone, suggests that in the reaction between C and the active component of serum, calcium combines to form an insoluble complex which then precipitates from solution. The calcium appears to be loosely bound since on washing with saline the precipitate becomes increasingly more soluble with each successive extraction. If instead of saline a dilute solution of calcium chloride is used the precipitate remains insoluble.

Advantage was taken of this observation in a preliminary attempt to determine quantitatively, according to the method of Heidelberger and

TABLE IV

Effect of Calcium on Precipitation Reaction between the C Polysaccharide and Reactive Substance in Human Serum

Serum of patient during acute stage of lobar pneumonia	Amount of CaCl₂ added	Precipitation reaction with C 1:10,000
	mg.	
Original serum...............................	0	++++
Calcium removed...............................	0	–
Calcium restored...............................	0.02	++++
" "	0.0012	++++
" "	0.0006	+++
" "	0.0003	++

Kendall (8), the amount of precipitable nitrogen removed from reactive serum by a known amount of polysaccharide. The result of an experiment in which the conditions mentioned above were adequately controlled indicates on the basis of the nitrogen content of the precipitate that the reactive substance in serum is protein in nature. However, the chemical identity of the active component is not as yet definitely known, nor is sufficient evidence available at present to warrant any conclusions concerning its origin or specific relationship to other proteins of the blood.

Potassium oxalate precipitates the blood calcium in an insoluble form, whereas sodium citrate converts it into a compound which remains in solution in equilibrium with a lowered concentration of calcium ions. Because of the difference in the effect of citrate on the solubility, ionization, and diffusibility of calcium in protein-containing fluids (9) its use in the present test involves a number of variables which influence the state and form of calcium in the reacting system. However, despite these variables the

results of experiments in which citrate was used to immobilize the calcium have all been in the same direction as those in the experiments with oxalate, that is, active sera showed progressive and eventually complete loss of precipitating action as the concentration of calcium ions decreased.

In the preceding experiments dialysis was employed primarily to rid the serum of the potassium oxalate and sodium citrate remaining in solution in excess of the theoretical amount required to combine with the calcium. Although the removal of the calcium by dialysis alone is slow and less complete, an experiment was carried out in which an aliquot of the original serum was dialyzed directly against saline buffered at pH 7.4; the same technique as that employed in the case of the oxalated and citrated sera was used. After dialysis had been continued for 7 days it was found that the serum had lost almost completely its capacity to precipitate with the polysaccharide. The faint trace of activity which remained was probably attributable to minute amounts of residual calcium not removed by dialysis. The precipitating action which was lost on dialysis was completely restored when calcium chloride was added to the serum in final concentration of 0.001 mg. per cc.

In view of the action of oxalate and citrate on the precipitation reaction it was of interest to compare the effect of another type of anticoagulant such as heparin which does not remove or alter the state of the calcium in body fluids. It was found that heparin in concentrations effective in preventing blood coagulation does not inhibit the precipitation reaction of active plasma, serum, or joint fluid with the C polysaccharide.

The results of the experiments on the influence of oxalate, citrate, dialysis, and heparin demonstrate that the precipitating action of the serum is conditioned by the presence of calcium in the reacting system. The effect of salts of divalent metals, notably calcium, on the hemagglutinating and precipitating action of concanavalin A, a crystallizable globulin from jack bean, has been reported by Sumner and Howell (10). They showed that when salts of calcium, manganese, or magnesium are added to concanavalin A which has been inactivated by treatment with acid followed by dialysis, the protein completely regains its capacity to agglutinate erythrocytes and starch granules and to precipitate glycogen.

DISCUSSION

The reaction under consideration depends upon the presence in the blood, during certain acute pathological conditions, of a reactive substance presumably protein in nature which combines with the test carbohydrate to form a precipitate in the presence of calcium ions. It will be recalled that

the particular carbohydrate used as test reagent is the so called C polysaccharide which is present in the cell bodies of all types and variants of pneumococci irrespective of their specific derivation (11). It differs from the better known and chemically well defined capsular polysaccharides in containing organically bound phosphorus, an acetylated amino sugar, but no uronic acid as constituent parts of the molecule. Among a limited but fairly representative group of other complex sugars including several of bacterial origin none has been found thus far to substitute for the C polysaccharide in the reaction.

Although the test carbohydrate is derived from pneumococci it must be emphasized that the reaction itself is not restricted to pneumococcal diseases but occurs in a number of other infections of widely diverse origin. It is apparent, therefore, that the phenomenon is not specific with respect to the inciting agent. Since the reactive substance is found in the blood in a number of unrelated infections it is evidently not derived from the bacteria themselves, but seemingly arises in the host as a result of pathological changes induced by or associated with acute infection.

The chemical relationships that determine the interaction between the serum component and the test carbohydrate may be quite accidental though perhaps not fundamentally different in principle from those which characterize true antigen-antibody reactions. However, the reaction in question presents certain distinctive features which serve to distinguish it from immunity reactions in general. For example, in pneumonia the C-reactive substance first appears in the blood early in the course of the inflammatory process; it persists during the activity of the lesion and disappears from the circulation shortly after crisis. This sequence of events with respect to the time of appearance and disappearance of the active substance is the reverse of that usually noted in the development and persistence of the specific antibodies. Furthermore, as previously pointed out, although widely different animal species may produce type specific antibodies to pneumococcus, the C-reactive substance has been found in the blood during acute pneumococcal infection only in the related species, man and monkey. Moreover the C-reactive substance is not specifically related to the etiological agent as is the case with most antibodies that arise in response to infection. In addition the results of the present study show that unlike most antibodies the reactive substance in patient's serum is present in the albumin and not in the globulin fraction precipitated from serum by half saturation with ammonium sulfate.

Another striking peculiarity of the precipitation reaction which seemingly distinguishes it from known antigen-antibody reactions is the fact that the

presence of calcium ions in the reacting mixture is essential in the formation of the precipitate, for as the present study shows no precipitation results when the polysaccharide is added to a reactive serum from which the calcium has been removed even though an optimal concentration of sodium chloride is present. However, the precipitating action of the calcium-free serum is completely restored on the addition of calcium in a concentration much lower than that normally present in blood.

In the usual antibody reactions on the other hand, the primary union of precipitins with a soluble antigen is followed by flocculation, provided an appropriate concentration of a salt such as sodium chloride alone is present in the reacting mixture. For example, in a parallel series of experiments in which antipneumococcus serum was used, the removal of calcium from the immune serum by oxalate and subsequent dialysis against physiological saline did not prevent the calcium-free serum from reacting in precipitin tests with the corresponding specific polysaccharide. Although salt is required for the secondary manifestation of flocculation, the precipitating action of immune sera is not selectively determined by the presence of calcium ions as is the case in the reaction under discussion.

SUMMARY

The serum obtained from human beings and monkeys during the acute phase of diverse infections contains a protein which is precipitable by the C polysaccharide of pneumococcus. The distribution of this protein in acute phase serum has been studied, and the effect of calcium on the precipitation reaction with the C polysaccharide is described. Other distinctive features of this reaction are discussed.

1. When heated above 65°C., serum obtained from patients during certain acute infections loses the property of reacting in precipitation tests with the C polysaccharide of pneumococcus. The loss of activity under these conditions occurs at temperatures known to denature many proteins.

2. The reactive component in "acute phase" serum which precipitates with the C polysaccharide is tentatively regarded as a protein.

3. The reactive substance is associated with the albumin fraction of serum.

4. In the reaction between patients' serum and C polysaccharide, flocculation is conditioned by the presence of calcium ions.

5. The following distinctive features of the C-reaction are discussed with reference to known characteristics of antigen-antibody phenomena: (a) the occurrence of the reactive component in blood only during the acute stage of the infection; (b) the lack of specificity of the reaction with respect

to the inciting cause of the disease; (c) the presence of the active substance in the albumin fraction of the serum; (d) the action of calcium in producing flocculation.

BIBLIOGRAPHY

1. Tillett, W. S., and Francis, T., Jr., *J. Exp. Med.*, 1930, **52**, 561.
2. Ash, R., *J. Infect. Dis.*, 1933, **53**, 89.
3. Francis, T., Jr., and Abernethy, T. J., *J. Clin. Inv.*, 1934, **13**, 692.
4. Finland, M., and Dowling, H. F., *J. Immunol.*, 1935, **29**, 285.
5. Abernethy, T. J., and Francis, T., Jr., *J. Exp. Med.*, 1937, **65**, 59.
6. Abernethy, T. J., *J. Exp. Med.*, 1937, **65**, 75.
7. Loeb, R. F., *J. Gen. Physiol.*, 1924, **6**, 453.
8. Heidelberger, M., and Kendall, F. E., *J. Exp. Med.*, 1935, **61**, 563.
9. McLean, F. C., and Hastings, A. B., *J. Biol. Chem.*, 1935, **108**, 285.
10. Sumner, J. B., and Howell, S. F., *J. Biol. Chem.*, 1936, **115**, 583.
11. Tillett, W. S., Goebel, W. F., and Avery, O. T., *J. Exp. Med.*, 1930, **52**, 895. Heidelberger, M., and Kendall, F. E., *J. Exp. Med.*, 1931, **53**, 625.