AN IMMUNOLOGICAL AND ELECTROPHORETIC COMPARISON OF THE ANTIBODY TO C POLYSACCHARIDE AND THE C-REACTIVE PROTEIN OF ACUTE PHASE SERUM*

By ELY PERLMAN, M.D., JESSE G. M. BULLOWA, M.D., AND RUTH GOODKIND

(*From Harlem Hospital, Department of Hospitals, City of New York*)

(Received for publication, October 9, 1942)

The polysaccharide obtained from the chemical fractionation of rough strains of pneumococci, called C polysaccharide, has been shown to precipitate with the sera of patients suffering from a number of infections (1, 2) as *e.g.* those due to pneumococci, staphylococci, and streptococci. The reactive protein in these sera has been called acute phase protein or C protein. This C protein, discovered by Tillett and Francis (1), was subsequently shown to be peculiar in several respects: (*a*) it was usually present during the acute stage of such a disease as pneumococcic pneumonia, disappearing with recovery, (*b*) the precipitate obtained by the interaction of C polysaccharide and C protein would not develop in the absence of calcium ions, and (*c*) the protein is associated with the albumin fraction of serum (3, 4). C protein is present in the sera of monkeys experimentally infected with pneumococci, whereas it has not been demonstrable in the sera of mice, guinea pigs, and rabbits similarly infected (5). Nevertheless, it is generally known that the sera of horses and rabbits hyperimmunized with pneumococci develop an antibody to C polysaccharide. This antibody will be referred to as C antibody in contradistinction to the reactive protein in acute phase human sera, referred to as C protein. It seemed of interest to compare the immunological behavior of these two reactive proteins and, if possible, by means of electrophoretic analysis to obtain more information concerning the nature of the C protein.

EXPERIMENTAL

C polysaccharide was prepared according to the method outlined by Tillett, Goebel, and Avery (5), from a rough strain of pneumococcus Type 2. Another sample of C polysaccharide of a high degree of purity was kindly supplied us by Dr. W. F. Goebel.

C antibody has been shown to be present in the sera of horses and rabbits hyperimmunized with pneumococci (6, 7). Large numbers of refined and concentrated therapeutic antipneumococcus horse and rabbit sera of all types were available to us[1] and were roughly tested for C antibody.

* This study received additional financial support from the Metropolitan Life Insurance Company, and from Mr. Bernard M. Baruch, Mr. Bernard M. Baruch, Jr., Miss Belle N. Baruch, and Mrs. H. Robert Samstag.

[1] Kindly supplied by Dr. W. G. Malcolm of Lederle Laboratories, Inc., Pearl River, N. Y.

Downloaded from http://rupress.org/jem/article-pdf/77/2/97/1182638/97.pdf by guest on 19 September 2021

Standard precipitin tubes were used and 0.2 ml. of each of the various antisera were mixed with 0.2 ml. of a solution containing 20 gamma/ml. of C. polysaccharide and with 0.2 ml. of diluent. The diluent used throughout was physiological saline containing 50 gamma of calcium chloride per ml. The mixtures were incubated at 37°C. for 2 hours and were read after remaining overnight in the ice box.

About half of the immune sera (both horse and rabbit) gave positive reactions with the C polysaccharide. There was no consistent tendency for any of the types to give positive reactions more frequently than other types. Indeed, different lots of antisera to the same pneumococcus type reacted differently. Thus, for example, rabbit antipneumococcus Type 12 serum 482H28J

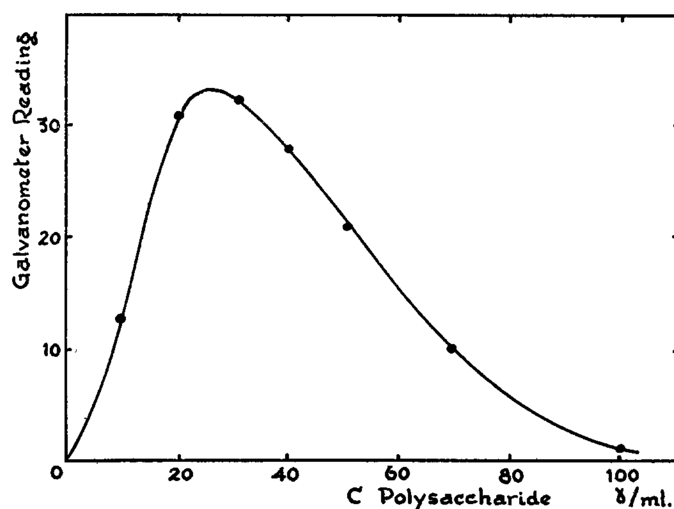

FIG. 1. Turbidity titration of a 1:5 dilution of rabbit serum Type 12 No. 482H28J, 37°C., 25 minutes.

gave a copious precipitate whereas two other Type 12 rabbit sera (482H112H and 482H320H) failed to give any. This same generalization is true of the horse antisera.

Since the Type 12 rabbit serum 482H28J gave the most copious precipitate of any of the sera tested, careful precipitin titrations were done by means of the photoelectric turbidometer.

The method is described in detail elsewhere (8). 1 ml. of a 1:5 dilution of this serum was mixed with 1 ml. of increasing concentrations of C polysaccharide. The mixtures were incubated by one-third submersion in a water bath at 37°C. for 25 minutes. The resulting turbidities were read in the photoelectric turbidometer and are indicated in terms of arbitrary galvanometer divisions. The results are shown graphically in Fig. 1.

Downloaded from http://rupress.org/jem/article-pdf/77/2/97/1182638/97.pdf by guest on 19 September 2021

This curve shown in Fig. 1 is similar to those obtained when antipneumococcus sera are titrated with their homologous type specific polysaccharides (8) in every respect, *i.e.*, the curve is asymmetrical showing a rapid rise as the concentration of C polysaccharide is increased, reaching a maximum, and gradually tapering off into a zone of inhibition as the concentration of polysaccharide is increased beyond equivalence.

A titration with the same antiserum was performed in the same manner, this time using a fixed concentration of 10 gamma/ml. of C polysaccharide and increasing concentrations of serum (beta procedure). The results are shown in Fig. 2. The concentrations of antiserum are recorded in terms of the volume of the original serum contained in the 1 ml. of each dilution used. We find an S-shaped curve similar to that obtained with other systems.

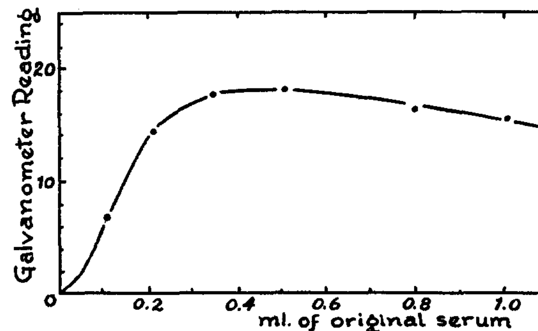

FIG. 2. Turbidity titration of rabbit serum Type 12 No. 482H28J using the beta or Ramon procedure.

In order to determine whether the technique of Heidelberger and Kendall would yield values comparable to those obtained with other precipitating systems, the following experiments were done:—

Refined and concentrated antipneumococcus Type 1 and 2 horse serum 239H1671R was "aged" by repeated incubation and refrigeration followed by centrifugation in order to remove the turbidity in the original serum. A 1:10 dilution of this serum was prepared and 1 ml. portions were mixed with various concentrations of C polysaccharide. The mixtures were incubated for 2 hours at 37°C. and then placed at 1°C. for 24 hours. They were then centrifuged in the cold for ½ hour. The precipitates obtained formed discs similar to those obtained with pneumococcal specific polysaccharides and their homologous antibodies. These precipitates were washed three times with chilled saline containing 50γ of calcium chloride per ml., and transferred to Kjeldahl digestion tubes for nitrogen determinations in the usual manner.

The results of this test are shown in Table I. The rather high blank obtained was in part due to the incomplete clearing of the serum initially and in part to calcium chloride added to the diluent.

Downloaded from http://rupress.org/jem/article-pdf/77/2/97/1182638/97.pdf by guest on 19 September 2021

To determine whether the initial concentrations of calcium chloride would affect the amount of protein precipitated, the same horse antiserum was mixed with 75 gamma of C polysaccharide but in one instance the final concentration of calcium chloride was 2 mg. per cent and in the other 10 mg. per cent. Table II shows the nitrogen precipitated. It can be seen that the differences obtained were within experimental error, although both concentrations of calcium chloride may have been beyond the amount necessary.

TABLE I

Milligrams of Specifically Precipitable C Antibody Nitrogen

Horse serum 239H1671R 1:10 dilution	C polysaccharide	N	C protein N precipitated
ml.	*gamma*	*mg.*	*mg.*
1	10	0.169	0.049
1	25	0.190	0.070
1	50	0.203	0.083
1	75	0.241	0.120
1	100	0.236	0.117
1	200	0.256	0.135
1	400	0.156	0.035
1	—	0.121	—

TABLE II

The Effect of Different Initial Concentrations of $CaCl_2$ on the Precipitation of C Antibody

Horse serum 239H1671R 1:10 dilution	C polysaccharide	Final concentration of $CaCl_2$	N precipitate	Difference
ml.	*gamma*	*mg. per cent*	*mg.*	*mg.*
1	75	2	0.242	0.138
1	None	2	0.104	
1	75	10	0.255	0.149
1	None	10	0.106	

To determine the solubility of the precipitate in normal saline as compared with its solubility in saline containing 10 mg. per cent calcium chloride, precipitates were obtained as above with 75 gamma of C polysaccharide, but one was washed twice with chilled normal saline, the other twice with saline containing the calcium chloride. Table III shows the results and indicates that under the conditions employed, the precipitate obtained from this horse serum was no more soluble in the one than in the other.

The quantity of C antibody in a rabbit serum was also determined. Refined and concentrated antipneumococcus Type I rabbit serum 471H167H contain-

Downloaded from http://rupress.org/jem/article-pdf/77/2/97/1182638/97.pdf by guest on 19 September 2021

ing 3,500 Type I mouse protection units per ml. was mixed undiluted in 1 ml. portions with various concentrations of C polysaccharide, incubated for 2 hours at 37°C., and placed at 1°C. for 24 hours. The precipitates were centrifuged in the cold and were washed twice with normal saline containing 50 gamma of $CaCl_2$ per ml. The results are recorded in Table IV and the curve obtained is similar to that shown in Fig. 4.

TABLE III

The Solubility of the Precipitate

Horse serum 239H1671R 1:10 dilution	C polysaccharide	Washed 2 times with	N
ml.	*gamma*		*mg.*
1	75	N saline	0.224
1	75	10 mg. per cent $CaCl_2$ in N saline	0.225

TABLE IV

Milligrams of C Antibody Nitrogen Precipitable from Rabbit Antipneumococcus Serum Type I

Rabbit serum 471H167H	C polysaccharide	N precipitate	C protein N
ml.	*gamma*	*mg.*	*mg.*
1	25	0.204	0.159
1	50	0.218	0.173
1	75	0.256	0.211
1	100	0.239	0.194
1	200	0.242	0.197
1	400	0.241	0.196
1	None	0.045	—

In order to determine if the C antibody in rabbit serum differed from the antibody to specific polysaccharide in the same serum, the following experiment was done.

The Type 1 antibody in rabbit serum 471H167H was completely absorbed with a Type 1 specific polysaccharide. This specific polysaccharide was negative for C polysaccharide when tested with a high-titered C antibody. The supernatant was then tested for C antibody with various concentrations of C polysaccharide and was found to be negative after 48 hours. The precipitate from the original absorption was washed three times with chilled physiological saline and then a solution of 10 per cent sodium chloride was added to it. The mixture was incubated at 37°C. for 2 hours in order to dissociate the antibody (9). The mixture was centrifuged and the supernatant was dialyzed against physiological saline in the cold for 3 days. This preparation was then tested with C polysaccharide and gave a faint positive reaction.

Downloaded from http://rupress.org/jem/article-pdf/77/2/97/1182638/97.pdf by guest on 19 September 2021

The result of the test suggests that at least a portion of the C antibody is contained in the same molecules as the type specific antibody.

Rabbit serum 482H28J employed above was tested for its ability to precipitate C polysaccharide in the absence of calcium ions.

0.1 ml. of a 20 per cent solution of calcium oxalate was added to 5 ml. of rabbit serum 482H28J. After standing 6 hours the precipitate was removed by centrifugation. The clear supernatant was dialyzed against frequent changes of physiological saline for 4 days. The serum was then tested for activity by mixing 0.2 ml. of the serum with 0.2 ml. of a 1:50,000 dilution of the C polysaccharide. After 2 hours at 37°C., the reaction was 3 plus. To 1 ml. of this serum was added 0.04 ml. of a 30 per cent solution of sodium citrate and the resultant mixture again tested for C protein. The reaction was again 3 plus.

Antipneumococcus Type 1 horse serum 77-835 was also tested for its dependence upon calcium ions by adding 0.04 ml. of a 30 per cent solution of sodium citrate to 1 ml. of a 1:5 dilution of this serum. The mixture was tested as above and found to give a 3 to 4 plus reaction with C polysaccharide, and the serum remained reactive to the same extent when 0.1 ml. of the citrate solution was added to 1 ml. of the serum dilution.

The turbidometric and quantitative precipitin tests of C antibody were repeated, this time using C protein.

Sera obtained from several patients suffering from pneumonia and giving a copious precipitate with C polysaccharide were pooled. The pooled material was then diluted with four parts of diluent and titrated with various concentrations of C polysaccharide. Readings were taken in the photoelectric turbidometer after incubation at 37°C. for 270 minutes.

The results of these tests are shown in Fig. 3. The pattern is similar to that shown in Fig. 1 with the exception that the peak was considerably broader. This serum gave a very copious precipitate when tested with C polysaccharide. Kjeldahl determinations of the precipitable C protein nitrogen content of this serum were done as above. Parallel determinations were done on this serum after inactivation at 56°C. for 1 hour. The results are given in Table V and are plotted in Fig. 4. The slightly lower results obtained with the inactivated serum, although fairly consistent, are almost within experimental error. The difference is more likely due to some destruction of the C protein rather than the fixation of complement. The results are similar to other such determinations with specific polysaccharide and homologous type specific antibody except for a difference in the antibody to antigen ratios.

Serum obtained from patient C.P. gave a 2 plus reaction when tested with C polysaccharide. 0.1 ml. of a 30 per cent solution of sodium citrate was added to 1 ml. of this serum and the reaction became negative and remained so after 2 hours at 37°C.

Downloaded from http://rupress.org/jem/article-pdf/77/2/97/1182638/97.pdf by guest on 19 September 2021

and 18 hours in the ice box. The serum from another patient N.P. when treated and tested in the same fashion was found to give a faint positive reaction after 18 hours in the ice box, whereas the initial reaction with the untreated serum was 2 plus.

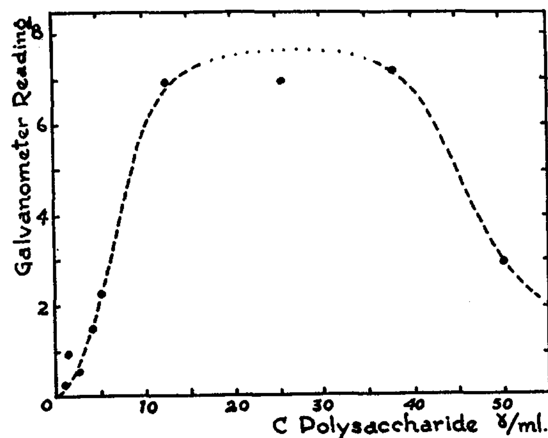

FIG. 3. Turbidity titration of pooled, acute phase human serum, 37°C., 270 minutes.

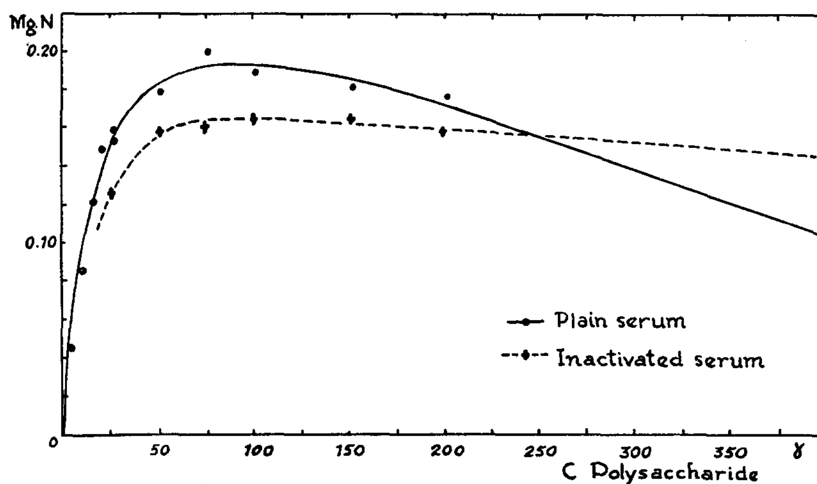

FIG. 4. Milligrams of precipitable C protein nitrogen in serum before and after inactivation at 56°C. for 1 hour.

C protein was found to be associated with the albumin fraction of serum (4). It resembled a globulin in that it precipitated during water dialysis and it resembled an albumin in that it was obtained between 50 and 66.7 per cent saturation with sodium sulfate. Svensson (10) studying ammonium sulfate fractions of various sera showed that 50 per cent saturation does not remove all

Downloaded from http://rupress.org/jem/article-pdf/77/2/97/1182638/97.pdf by guest on 19 September 2021

of the globulin but leaves some of the alpha and beta globulin in solution. The following electrophoretic analyses were done before and after absorption of C antibody and C protein with C polysaccharide, in order to determine, if possible, which of the electrophoretic components of these sera were involved.

Rabbit antipneumococcus serum Type 12 No. 482H28J was completely absorbed by the addition of the required amount of C polysaccharide. The control was diluted to the same volume as the absorbed sample with normal saline. Both samples were then dialyzed for 5 days against a buffer of pH 7.6 which was 0.15 molar with respect to NaCl and 0.02 molar with respect to phosphate. The photograph was taken after 225 minutes with a current of 32 milliamperes.

TABLE V

Milligrams of Precipitable C Protein Nitrogen in Serum from Patient C.McN. before and after Inactivation by Heat at 56°C. for 1 Hour

Serum patient C.McN.	C polysaccharide	N precipitated	N inactivated serum	C protein N	
				Plain serum	Inactivated serum
ml.	*gamma*	*mg.*	*mg.*	*mg.*	*mg.*
1	5	0.062		0.024	
1	10	0.080		0.042	
1	15	0.099		0.061	
1	20	0.113		0.075	
1	25	0.118		0.080	
1	25	0.115	0.097	0.077	0.062
1	50	0.128	0.115	0.090	0.080
1	75	0.139	0.115	0.101	0.080
1	100	0.129	0.117	0.091	0.082
1	150	0.122	0.120	0.084	0.085
1	200	0.129	0.114	0.091	0.079
1	400	0.093	0.110	0.055	0.075
1	None	0.038	0.035		

Electrophoretic analysis showed that the serum consisted almost entirely of gamma globulin with a very minute amount of beta globulin. Since the amount of C protein precipitated from the absorbed sample was small in comparison to the total protein, the electrophoretic areas show no significant difference between the two samples. The same experiment was done using the serum of patient W. The results of this analysis under the same conditions showed that there was a significant decrease in the gamma globulin fraction of the serum after absorption, with but a slight decrease in the albumin fraction. The change in the albumin fraction is within experimental error. The patterns before and after absorption and the areas of the components are shown in Fig. 5.[2]

[2] Electrophoretic analyses of these samples were kindly done by Dr. R. W. G. Wyckoff at Lederle Laboratories, Inc., Pearl River, N. Y.

Downloaded from http://rupress.org/jem/article-pdf/77/2/97/1182638/97.pdf by guest on 19 September 2021

This experiment was repeated with the serum of patient McN., this time using a buffer which resolves the various electrophoretic components of serum more completely (11). The buffer used consists of 0.12 molar diethylbarbituric acid (barbital) in 0.1 molar sodium hydroxide. This buffer has a pH of 8.5 and an ionic strength of 0.10. Electrophoretic analysis was done on

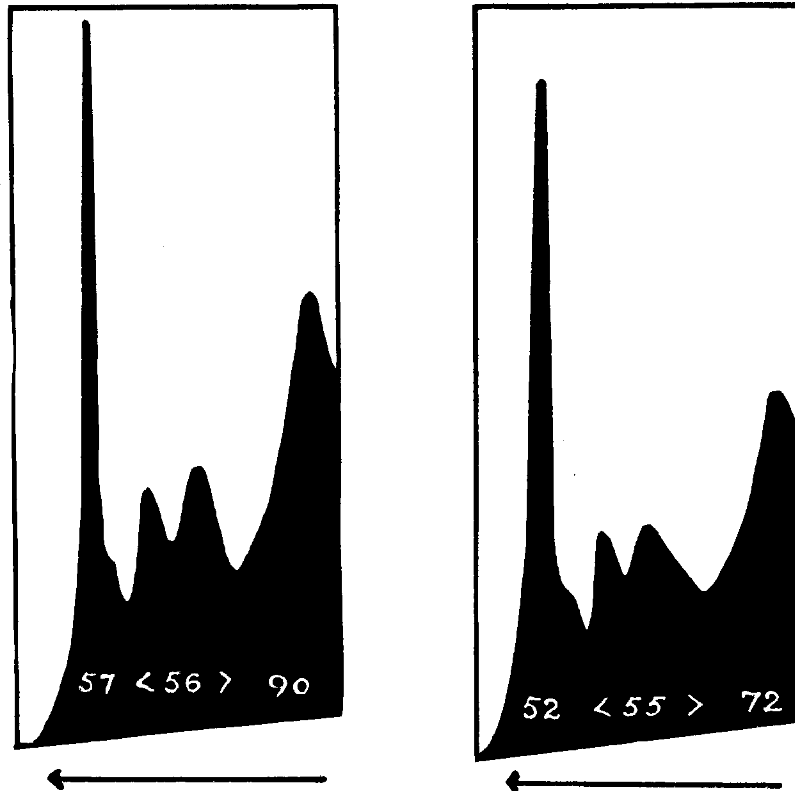

FIG. 5. Left pattern shows serum before absorption with C polysaccharide, right pattern, after absorption.

the serum before and after complete absorption with C polysaccharide. The photographs were taken after 3 hours using a current of 13.5 milliamperes. Fig. 6 shows the photographs obtained before and after absorption with C polysaccharide. The areas of each of the components were obtained by planimetric measurement of enlarged tracings of the patterns. Table VI records the values obtained for each of the components and gives the ratio of each component to the albumin fraction. However since the albumin fraction may be implicated, the ratios of each to the total area and to the $alpha_2$ component are recorded. As can be seen from these figures, the two components which are reduced after absorption are the gamma and the $alpha_1$ globulins.

Downloaded from http://rupress.org/jem/article-pdf/77/2/97/1182638/97.pdf by guest on 19 September 2021

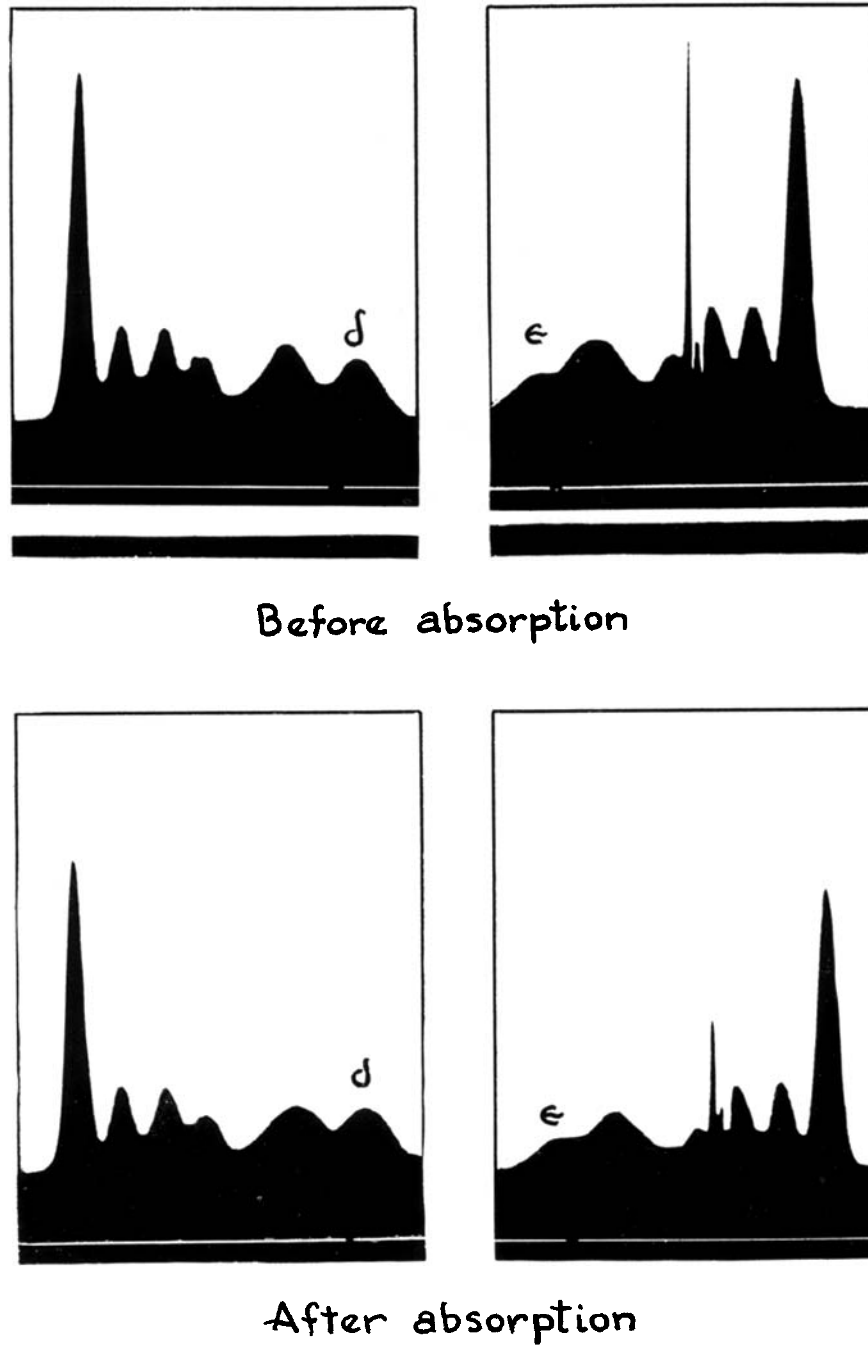

FIG. 6. Acute phase serum before and after absorption with C polysaccharide.

Downloaded from http://rupress.org/jem/article-pdf/77/2/97/1182638/97.pdf by guest on 19 September 2021

A test was next made to determine which of the electrophoretic components of serum were obtained by the fractionation method of MacLeod and Avery (4).

Sera with high C protein content obtained from several patients were pooled. This was diluted with an equal volume (46 ml.) of physiological saline, and filtered

TABLE VI

Electrophoretic component	Areas before absorption*	Areas after absorption
Albumin	170.5	140.0
α_1 globulin	57.0	41.0
α_2 "	46.5	38.0
β "	78.0	64.0
γ "	96.0	69.5
ϵ (boundary)	27.5	28.5
Total	475.5	381.0
Ratio of component to A (albumin)	**Before absorption**	**After absorption**
α_1/A	0.334	0.293
α_2/A	0.272	0.271
β/A	0.457	0.457
γ/A	0.562	0.496
ϵ/A	0.161	0.204
Ratio of component to total area	**Before absorption**	**After absorption**
A/Total	0.358	0.368
α_1/T	0.120	0.108
α_2/T	0.098	0.100
β/T	0.164	0.168
γ/T	0.202	0.182
ϵ/T	0.058	0.075
Ratio of component to α_2	**Before absorption**	**After absorption**
A/α_2	3.66	3.68
α_1/α_2	1.35	1.05
β/α_2	1.68	1.34
γ/α_2	2.06	1.83
ϵ/α_2	0.59	0.75

* Arbitrary planimeter units.

through a Seitz filter. It was warmed to 37°C. and an equal volume of a saturated solution of Na_2SO_4 was added at the same temperature. The precipitate was removed by filtration and to the filtrate was added sufficient saturated Na_2SO_4 to give a mixture which was two-thirds saturated with this salt. After 1 hour the precipitate which formed was removed by filtration and was redissolved in 10 ml. of the barbital buffer. It was then dialyzed for 5 days against $2\frac{1}{2}$ liters of the same buffer. Electrophoretic analysis was done on this fraction. The

Downloaded from http://rupress.org/jem/article-pdf/77/2/97/1182638/97.pdf by guest on 19 September 2021

further fractionation by dialysis against tap water was not done because of the small amount of material available.

Fig. 7 shows the photograph taken after 2 hours at 15 milliamperes. As can be seen, the pattern shows a marked *reduction* of the albumin and of the gamma globulin with some reduction in the beta globulin component. There is a definite preferential increase in $alpha_2$ and a moderate increase in $alpha_1$. Since it has been shown (4) that most of the activity is obtained in this fraction, it is most likely due to one of the alpha globulins and from the evidence of the absorption experiment, probably $alpha_1$. Following the electrophoretic run

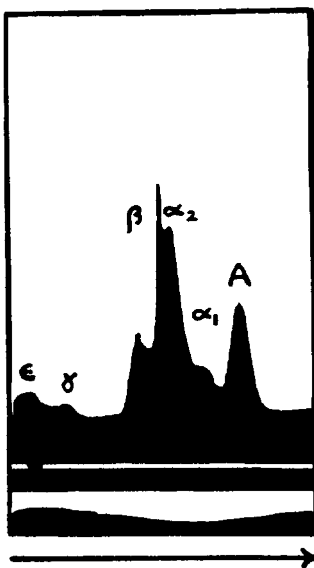

FIG. 7. Electrophoretic analysis of fractionated serum.

the serum fraction was recovered from the cell and was dialyzed against physiological saline for 48 hours. It was then tested for C protein and found to be positive.

DISCUSSION

C protein was found to be peculiar in several respects soon after its discovery by Tillett and Francis. Unlike the usual antibody, it is found in highest titer in patients during the active stage of an infection, and its titer declines with improvement. It is non-specific, being found in a variety of pathological conditions. Its protective function has never been tested. Its precipitation with C polysaccharide depends upon the presence of calcium ions (3). It is found only in monkeys, during the course of experimental infection, but cannot

Downloaded from http://rupress.org/jem/article-pdf/77/2/97/1182638/97.pdf by guest on 19 September 2021

be demonstrated in rabbits, mice, or guinea pigs. It precipitates with the albumin fraction of serum.

Hyperimmunized horses and rabbits develop an antibody to C polysaccharide superficially resembling C protein in the precipitation reaction with C polysaccharide. Treffers and Heidelberger by tests with specific precipitates showed that C antibody could not be differentiated from other antibody globulins. This, and evidence presented above, place C antibody in the gamma globulin fraction of the serum. MacLeod and Avery (12) have shown that C protein specific precipitates, when injected into rabbits produce an antibody which reacts only very slightly or not at all with normal human serum and is specific for serum containing C protein. The electrophoretic studies in the present paper show that C protein is probably in the alpha globulin component of acute phase serum. Longsworth, Shedlovsky, and MacInnes (13) have shown that the alpha globulin component of serum is quite regularly increased in many pathological conditions. These conditions usually result in detectable C protein. This indirect evidence supports the view that C protein is in the alpha globulin component of serum.

The relationship of antibody gamma globulin to normal gamma globulin is not quite the same as the relation between C protein alpha globulin and normal alpha globulin. Antibody gamma globulin is distinguishable only with difficulty from normal gamma globulin even when the sensitive method of testing with antibodies to specific precipitates is employed (7). Although C protein, at the pH used, seems to migrate with the alpha globulin component, it is readily distinguishable from alpha globulin by the use of antibodies to specific precipitates (of C protein with C polysaccharide) (12). Indeed, antibody so produced is remarkably low in reactivity with normal serum components. Thus it seems that, whereas antibody globulin may be a reoriented normal globulin C protein is probably an entirely new serum protein.

SUMMARY

1. Studies of the precipitation reaction of C polysaccharide with C protein, and of C polysaccharide with C antibody are reported. The similarity between these two systems in this respect is demonstrated.

2. The differences between C protein and C antibody are emphasized. The differences between this protein and antibodies in general have been reported previously by others.

3. Electrophoretic studies show that C antibody is in the gamma globulin fraction of serum whereas C protein migrates with the $alpha_1$ globulin fraction of acute phase protein.

The assistance and guidance of Dr. Theodore Shedlovsky in the electrophoretic studies are gratefully acknowledged.

Downloaded from http://rupress.org/jem/article-pdf/77/2/97/1182638/97.pdf by guest on 19 September 2021

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. Abernethey, T. J., and Avery, O. T., *J. Exp. Med.*, 1941, **73,** 173.
4. MacLeod, C. M., and Avery, O. T., *J. Exp. Med.*, 1941, **73,** 183.
5. Tillet, W. S., Goebel, W. F., and Avery, O. T., *J. Exp. Med.*, 1931, **53,** 625.
6. Heidelberger, M., and Kabat, E., *J. Exp. Med.*, 1938, **67,** 545.
7. Treffers, H., and Heidelberger, M., *J. Exp. Med.*, 1942, **73,** 125.
8. Perlman, E., and Bullowa, J. G. M., to be published.
9. Heidelberger, M., and Kendall, F. E., *J. Exp. Med.*, 1936, **64,** 161.
10. Svensson, H., *J. Biol. Chem.*, 1941, **139,** 805.
11. Shedlovsky, L., personal communication.
12. MacLeod, C. M., and Avery, O. T., *J. Exp. Med.*, 1941, **73,** 191.
13. Longsworth, L. G., Shedlovsky, L., and MacInnes, D. A., *J. Exp. Med.*, 1939, **70,** 399.

Downloaded from http://rupress.org/jem/article-pdf/77/2/97/1182638/97.pdf by guest on 19 September 2021