THE COMPLEMENT FIXATION REACTION WITH PNEUMOCOCCUS CAPSULAR POLYSACCHARIDE

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The extreme sensitivity of the complement fixation reaction as usually applied in immunology is well recognized. However, certain unique findings have been reported with the use of bacterial polysaccharides as antigens. For example, Zinsser and Parker (1) found that no fixation could be obtained with combinations of pneumococcus capsular polysaccharide and specific immune horse sera, although complement was bound when the same substances were combined with specific immune rabbit sera. Similar results were reported by Pittman and Goodner for the specific polysaccharide of *Hemophilus influenzae*, Type b (2).

The present experiments constitute a critical survey of this subject with especial regard to the general mechanism of the complement fixation reaction. It must be appreciated that the use of purified specific polysaccharides offers some advantage in that the quantity and character of at least one reagent in this most complicated of tests can be carefully controlled.

Materials and Methods

Complement Fixation Test.—The reagents used were: fresh guinea pig serum (complement), a 5 per cent suspension of washed sheep red blood cells, and the serum of a rabbit immunized with sheep red blood cells (amboceptor). Each reagent was used in a volume of 0.5 cc. The amboceptor was titrated with excess of complement and so diluted that 0.5 cc. contained 2 units. The complement was titrated against 2 units of amboceptor. The final volume in each tube of the titrations was 1.5 cc. Further details of the methods used in the various experiments will be presented with the respective protocols.

The majority of these experiments have been carried out with the capsular polysaccharide (acetyl form) of Type I Pneumococous. Type-specific anti-pneumococcus horse and rabbit sera have possessed equivalent agglutinin and precipitin titers.

The Sensitivity of the Complement Fixation Test

In order to establish the essential sensitivity of the reaction, the results of an experiment, which consists of a comparison between precipitin and complement fixation titers of Type I antipneumococcus rabbit serum, are reproduced in Table I. It will be noted that, as the amount of immune serum was decreased, smaller amounts of polysaccharide were required in order to obtain maximum reactions. In our hands the limit of the reaction with complement fixation was reached

TABLE I

Comparison of Precipitin and Complement Fixation Titers of Type I Antipneumococcus Rabbit Serum

Dilution of acetyl polysaccharide	Dilution of immune rabbit serum					
	1:2		1:10		1:50	
	Precipitin reaction	Complement fixation	Precipitin reaction	Complement fixation	Precipitin reaction	Complement fixation
1:2,500	++++	++++	−	+++	−	−
1:12,500	++++	++++	+	++++	−	−
1:62,500	++++	++++	+++	++++	±	+
1:312,500	+++±	++++	++	++++	−	+++±
1:1,562,500	+	+++	+	++++	−	++++
1:7,812,500	−	+	−	+++	−	++++
1:39,062,500	−	−	−	−	−	±

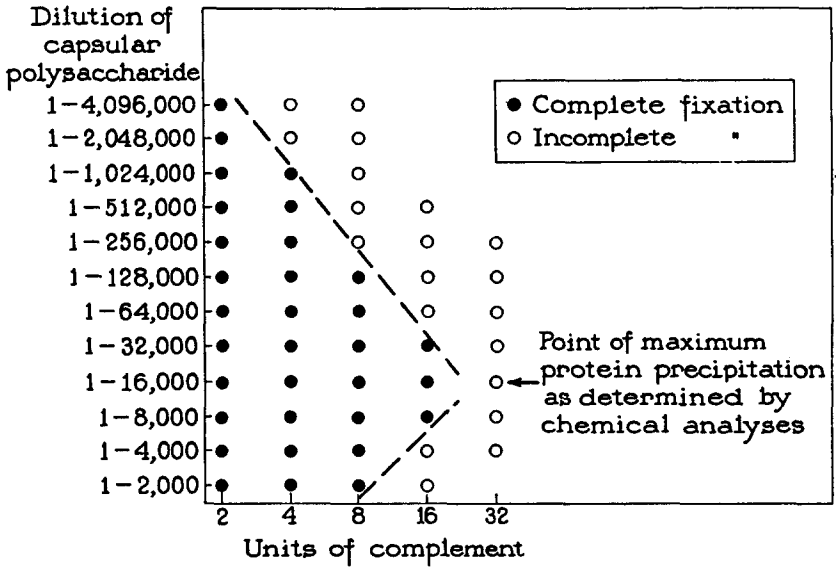
with serum diluted 1:200 and specific polysaccharide diluted 1:20,000,000. It may be pointed out that in many instances combinations gave complete fixation in the absence of any visible precipitation.

Quantitative Complement Fixation

In an experiment the results of which are plotted in Text-fig. 1, the maximum number of units of complement fixed by given combinations of polysaccharide and immune rabbit serum was determined. It will be noted that, with certain amounts of the polysaccharide, the amount of complement which was fixed was large, that is, at least 16 units. This point of maximum fixation is also the point of maximum protein precipitation as determined in a parallel experiment carried out by the method of Heidelberger, Sia, and Kendall (3).

Thus the results suggest that, within certain limits, the property to fix complement is proportional to the quantity of immune precipitate formed in the same reaction.

As a means of testing this hypothesis, quantitative fixation tests were carried out with three forms of Type I pneumococcus capsular polysaccharide: (*a*) the viscous product described by Heidelberger and Kendall (4), (*b*) the acetyl polysaccharide of Avery and Goebel (5), a product which may be derived from the first mentioned form by



TEXT-FIG 1. The number of units of complement fixed by varying amounts of pneumococcus capsular polysaccharide in combination with specific immune rabbit serum diluted 1:4.

means of heat, and finally, (*c*) the deacetylated product which results when the acetyl form is treated with alkali (5). As shown by Heidelberger and Kendall (4), the capacities of these three forms of the polysaccharide to precipitate immune globulin from specific antipneumococcus rabbit serum are in this same order.

The results of the quantitative complement fixation test are shown in Table II. It will be noted that with the deacetylated form only 2 units of complement were fixed, with the acetyl form 16 units were fixed, while the viscous polysaccharide

completely fixed 32 units of complement. The maximum amounts of protein specifically precipitable by these three forms of polysaccharide are also shown in Table II.

These results are a convincing demonstration that under these conditions the amount of complement which can be fixed is proportional to the amount of immune precipitate formed.

If one conceives of the precipitin reaction as at least diphasic, that is, consisting of a stage of specific union of antigen and antibody fol-

TABLE II

Amount of Complement Fixed by Combinations of Immune Rabbit Serum and Various Forms of Type I Capsular Polysaccharide

Type I polysaccharide	Specifically precipitable protein per cc. of immune serum	Units of complement					
		2	4	8	16	32	64
	mg.						
Deacetylated polysaccharide	1.595	++++	++±	+	−	−	−
Acetyl polysaccharide	4.139	++++	++++	++++	++++	+++	−
Viscous acetyl polysaccharide of Heidelberger and Kendall	4.640	++++	++++	++++	++++	++++	++±

The complement fixation reactions were carried out with immune rabbit serum diluted 1:10 and the various polysaccharides diluted 1:100,000. Complement was added immediately after admixture of antiserum and polysaccharide. Precipitable protein determinations were carried out after the method of Heidelberger, Sia, and Kendall (3).

lowed by a second stage of flocculation, there arises the question of the temporal position of complement fixation in this series of reactions.

As an approach to this problem, quantitative fixation tests were carried out with mixtures which differed only in the time which elapsed before the addition of complement. The results of one experiment are shown in Table III. It will be noted that, in instances in which the addition of complement was made an hour or more after preliminary incubation of polysaccharide and specific antibody, only 2 units were completely fixed. When, however, the preliminary incubation had

occupied only 10 minutes, 8 units were completely fixed. When complement was present at the time of admixture of antigen and antibody, 32 units were fixed.

With preliminary incubations of 1 hour or longer, flocculation had occurred; in the instance of the 10 minute period no flocculation was apparent when complement was added. It is obvious that under these circumstances the total surface exposure of immune aggregates had diminished with the time of incubation, for the surface presented by large flocs is much less than that which would be presented by all of

TABLE III

Amount of Complement Fixed with Reference to the Time of Addition of this Reagent

Conditions	Units of complement				
	2	4	8	16	32
A. Polysaccharide + serum 4 hrs. 37° then complement added	++++	+++±	+	−	−
B. Polysaccharide + serum 1 hr. 37° then complement added	++++	+++±	++	−	−
C. Polysaccharide + serum 10 min. 37° then complement added	++++	++++	++++	+++	−
D. Complement added before admixture of polysaccharide and immune serum	++++	++++	++++	++++	++++

In this experiment all tubes contained equal amounts of Type I antipneumococcus rabbit serum diluted 1:10 and the acetyl polysaccharide diluted 1:100,000.

their component particles, were these evenly suspended. It would appear, therefore, that the amount of complement which can be fixed is a function of the total surface exposure of the immune aggregates.

As a further demonstration of this point, advantage was taken of the fact that, within certain limits, flocculation is more rapid the smaller the diameter of the tube. Quantitative fixation tests were carried out in tubes of three different diameters and the results are shown in Table IV. It will be noted that in tubes having a diameter of 0.81 cm. only 4 units of complement were fixed, while in tubes of 2.40 cm. 16 units were completely fixed. These results can be explained only by the fact that in smaller tubes flocculation is rapid and consequently the time-surface factor is greatly reduced.

A third direct demonstration of this surface phenomenon was obtained by centrifugation. Mixtures of polysaccharide and antibody which had been incubated 1 hour were whirled in the angle centrifuge at high speed for 10 minutes. Complement fixation tests were then carried out on both the clear supernatant fluid and the resuspended precipitates.

TABLE IV

The Amount of Complement Fixed with Reference to the Speed of Flocculation as Conditioned by Tube Diameter

Tube diameter	Mean flocculation time	Units of complement					
		2	4	8	16	32	64
cm.	*min.*						
0.81	21	++++	++++	±	−	−	−
1.26	33	++++	++++	++++	+++	++	−
2.40	43	++++	++++	++++	++++	+++	+±

All tubes contained equal amounts of specific immune rabbit serum diluted 1:10 and acetyl polysaccharide diluted 1:100,000.

TABLE V

Complement Fixation after Centrifugation

Acetyl polysaccharide	Whole mixture not centrifuged	Precipitate	Supernatant
1:8,000	++++	−	++++
1:32,000	++++	−	++++
1:256,000	++++	−	+++±
1:1,024,000	++++	−	+++

Immune rabbit serum diluted 1:4. Incubated with polysaccharide 1 hour at 37°C. One set of tubes then centrifuged 10 minutes and the sediment and supernatant separated. Sediments thoroughly resuspended in saline. 2 units of complement added to each tube.

The results of one of these experiments are shown in Table V. It will be noted that the uncentrifuged mixture gave complete fixation with all dilutions of the polysaccharide. None of the resuspended precipitates gave any fixation. On the other hand, the property of fixation remained in the supernatant fluid, although in somewhat reduced amount. In other experiments in which *quantitative* fixation was carried out, this decreased capacity for fixation was clearly demonstrated. Thus a combination capable of fixing several units of complement lost a

large proportion of this capacity after 10 minute centrifugation, and all of it after 30 minutes at very high speed. In any instance in which centrifuged combinations possessed the capacity of fixation this property resided in the clear supernatant fluid and not in the precipitate.

It may be pointed out that centrifugation speeds up the flocculation or secondary phase of the precipitin reaction, and in this sense greatly reduces the time-surface factor essential to the property of fixation.

Another question which remains to be answered: Is complement fixed by immune combinations in true solution, or rather by aggregates in a finely dispersed colloidal state? As an approach to this problem, experiments were carried out with certain "synthetic" antigens and the corresponding antisera.[1] This system consisted of the following reagents: (*a*) aminobenzyl glucoside, (*b*) the same coupled to chicken serum protein, and (*c*) serum of rabbit immunized to (*a*) coupled with the proteins of horse serum. The azobenzyl-gluco-chicken serum antigen has the property of reacting in precipitin tests with the immune rabbit serum. This serum, however, gives no visible reaction with the uncoupled glucoside. If, however, the glucoside is added to the immune serum, the latter loses the capacity of reacting with the azobenzyl-gluco-chicken serum antigen, that is, the uncombined glucoside has the capacity of combining with the antibody even though no precipitation occurs. (For the details of this phenomenon see Avery and Goebel (6).)

It has been found that complement is fixed only by the combination of immune serum and the azobenzyl-glucose-chicken serum antigen, not with the uncoupled glucoside. The evidence of the nature of this combination of uncoupled glucoside and immune serum is not sufficient to permit an exact interpretation of the findings with complement fixation, but the results suggest that the soluble immune combinations are incapable of fixing complement.

In so far as can now be determined, therefore, complement fixation is a phenomenon associated with particulation. It appears to be a matter of selective adsorption.

Inhibition of Complement Fixation by Immune Horse Serum

Zinsser and Parker (1) not only showed that immune horse serum gave no fixation of complement with specific bacterial polysaccharides,

[1] Furnished through the courtesy of Dr. Walther F. Goebel.

but they also demonstrated that immune horse serum would completely block the fixation normally obtained with immune rabbit serum.

In order to illustrate the type of inhibition by immune horse serum, a protocol of an experiment essentially similar to those reported by Zinsser and Parker (1) is shown in Table VI. The capsular polysaccharide of Type I Pneumococcus and the specific immune rabbit serum were allowed to react for 30 minutes. Dilutions

TABLE VI

Inhibition of Complement Fixation by Specific Immune Horse Serum

Tube	Type I pneumococcus capsular polysaccharide (acetyl form)	Type I antipneumococcus rabbit serum		Type I antipneumococcus horse serum					Result (fixation)
	0.5 cc.	*0.5 cc.*		*0.5 cc.*	10 minutes 37°C.	2 units complement added to each tube	30 minutes 37°C.	1 cc. sensitized sheep cells	30 minutes 37°C.	
1	1:100,000	1:5		—						++++
2	"	1:25		—						++++
3	"	1:5		1:2						−
4	"	"		1:10						−
5	"	"		1:25						+++
6	"	1:25		1:2						−
7	"	"		1:10						−
8	"	"		1:25						−
9	"	—		1:2						−
10	"	—		1:10						−
11	"	—		1:25						−

Final volume in each tube, 3 cc.

In this, as in all subsequent experiments, suitable controls showed that none of the reagents were anticomplementary in the amounts used.

of Type I antipneumococcus horse serum were then added, followed by a second incubation of 10 minutes; guinea pig complement was then added and a third incubation of 30 minutes was carried out. At the end of this time sensitized sheep cells were added and the tubes were incubated again for 30 minutes.

From the results shown in Table VI it will be seen that the specific immune rabbit serum in various dilutions in combination with the bacterial polysaccharide gave positive fixation (tubes 1 and 2). Immune horse serum, on the other hand, did not exhibit this property (tubes 9, 10, 11). If immune horse serum was subsequently added to potentially active combinations of polysaccharide and immune

rabbit serum, the reaction was blocked in all instances except one in which the amount of immune horse serum was relatively small (tube 5).

It may be pointed out that the immune horse serum was added after the reaction between the polysaccharide and rabbit antibody had occurred. It is entirely conceivable that the antibodies of the immune

TABLE VII

The Reverse of the Inhibition Experiment

Tube	Type I pneumococcus capsular polysaccharide	Type I antipneumococcus horse serum		Type I antipneumococcus rabbit serum						Result (fixation)
	0.5 cc.	*0.5 cc.*		*0.5 cc.*	10 minutes 37°C.	2 units guinea pig complement	30 minutes 37°C.	1 cc. sensitized sheep cells	30 minutes 37°C.	
1	1:100,000	1:5		—						−
2	"	1:25		—						−
3	"	1:5		1:2						+++
4	"	"		1:10						−
5	"	"		1:25						−
6	"	1:25		1:2						++++
7	"	"		1:10						++++
8	"	"		1:25						++++
9	"	—		1:2						++++
10	"	—		1:10						++++
11	"	—		1:25						++++
12	"	—		—						−
13	—	1:5		—						−
14	—	1:25		—						−
15	—	—		1:2						−
16	—	—		1:10						−
17	—	—		1:25						−

horse serum reacted in some way with the already formed rabbit aggregates. Thus it might be assumed that the core of the final aggregates was composed of rabbit antibody-polysaccharide combination with only the surface reoriented by the addition of the horse serum antibodies, in themselves not capable of bringing about fixation and thus transforming potentially active particles of immune precipitate into

inactive ones. If this assumption were true it should be possible to reverse the typical inhibition experiment, that is, to add the immune rabbit serum as the second reagent and thus bring about fixation. The results of an experiment of this type are shown in Table VII.

It will be noted that the capsular polysaccharide in combination with immune horse serum failed to give fixation (tubes 1 and 2) whereas the rabbit immune serum gave positive results (tubes 9, 10, 11). If, however, immune rabbit serum was added to combinations of the polysaccharide and immune horse serum, fixation was obtained in every instance in which the amount of rabbit serum was equal to or greater than the amount of horse serum.

These experiments demonstrate two facts. The first of these is that immune horse serum is not, as had been deduced by previous workers, of itself inhibitory. The important point is the order in which the two antibodies react with the polysaccharide. It is quite apparent that, in general, the serum last added confers its characteristic properties on the whole aggregate, that is, the second serum reagent masks the reaction expected with the first.

Secondly, there is an obvious quantitative factor. Thus in both experiments the reactive properties of the mixture were those of the second serum reagent in instances in which the concentration of the latter was as great or greater than that of the first serum reagent.

Although there is a possibility that the second antibody may unite with minute traces of uncombined polysaccharide, it seems probable that this reaction is of little quantitative consequence. Furthermore, the experiments in which immune horse serum was added to potentially fixing combinations of polysaccharide and immune rabbit serum yield conclusive evidence to support the assumption that the surface property of the preformed aggregates is reoriented by the second antibody.

The Type Specificity of the Inhibition Phenomenon

It would seem likely that if any actual "inhibitor" were involved it should be present in horse serum in general, and particularly in antipneumococcus horse sera of heterologous types. In order to test the possibilities of this assumption an experiment was arranged to include capsular polysaccharides of two types of pneumococci and their homologous immune horse and rabbit sera. The results of this experiment are shown in Table VIII.

From these results it will be noted that each type polysaccharide gave fixation with its homologous immune rabbit serum (tubes 1 and 6). Controls not reproduced in the protocol showed that these reactions were entirely type-specific. The addition of homologous type antipneumococcus horse sera to potentially fixing mixtures of the above reagents completely blocked the reactions (tubes 2, 3, 9, 10). On the other hand, heterologous type immune horse sera failed to have any effect (tubes 4, 5, 7, 8).

TABLE VIII

Specificity of the Inhibition Phenomenon

Tube	Pneumococcus capsular polysaccharide 1:100,000	Antipneumococcus rabbit serum		30 minutes 37°C.	Antipneumococcus horse serum		30 minutes 37°C.	2 units guinea pig complement	30 minutes 37°C.	1 cc. sensitized sheep cells	30 minutes 37°C.	Result (fixation)
	Type	Type	Dilution		Type	Dilution						
	0.5 cc.	*0.5 cc.*			*0.5 cc.*							
1	I	I	1:5		—	—						++++
2	"	"	"		I	1:5						−
3	"	"	"		"	1:10						−
4	"	"	"		II	1:5						++++
5	"	"	"		"	1:10						++++
6	II	II	"		—	—						++++
7	"	"	"		I	1:5						++++
8	"	"	"		"	1:10						++++
9	"	"	"		II	1:5						−
10	"	"	"		"	1:10						−

Controls showed that none of the reagents were anticomplementary in the amounts used, and that neither type of antipneumococcus horse serum gave fixation with the pneumococcus polysaccharides.

From these results it can be concluded that the inhibition is an entirely type-specific phenomenon. These results also suggest that any hypothetical inhibitor must be intimately associated with the specific antibody, for it is ineffective unless bound by the immune body to the reactive aggregate.

The Time Element in the Inhibition Phenomenon

In an hypothesis previously presented, it was suggested that the striking inhibition by immune horse serum might be related to the fact

that during the first incubation period immune aggregates are increasing in size, so that at the time the immune horse serum is added there is an appreciable diminution in surface, and hence the amount of immune horse serum need not be as large as the original amount of immune rabbit serum, in order to block the reaction. If this hypothesis were correct, the simultaneous admixture of the reagents should give results quantitatively different from those obtained by following the usual procedure.

Table IX shows the results of an experiment in which the two sera were mixed before the addition of the polysaccharide, in comparison with the results of a similar experiment in which the immune horse serum was added 30 minutes after the admixture of polysaccharide and immune rabbit serum.

TABLE IX

Simultaneous Addition of the Two Sera

Immune rabbit serum	Immune horse serum			
	1:10		1:25	
	Added immediately	Added after 30 min.	Added immediately	Added after 30 min.
1:5	++++	+++	++++	++++
1:10	++++	−	++++	+++
1:25	++	−	++++	−
1:50	−	−	++++	−

Acetyl polysaccharide 1:100,000 in all instances.

The results presented of this experiment demonstrate that, with the simultaneous exposure of the polysaccharide to both sera, the presence of immune horse serum did not block fixation except in those instances in which it was present in excessive proportions. On the other hand, in the typical inhibition experiment in which the immune horse serum was added after an interval of 30 minutes, inhibition was obtained when the amount of horse serum was equal to or greater than that of the immune rabbit serum.

Perhaps the most striking experiment in the series presented by Zinsser and Parker dealt with the observation that the interfering effect of the horse serum could be eliminated by absorption with pneumococci previously sensitized with immune rabbit serum. The authors state that the reported experiment was only suggestive and that

repeated experiments on this point had in general been inconclusive. A primary objection to this conception is, of course, the already established fact of the marked specificity of the reaction. If an inhibitor exists as such it is undoubtedly an intimate part of the antibody molecule and thus its selective removal should be difficult.

Repeated experiments directed toward the selective absorption of the inhibitor substance have been carried out. These have been uniformly unsuccessful. It is conceivable that absorption of the immune horse serum with pneumococci previously sensitized with immune rabbit serum might under proper conditions result in the partial replacement of the rabbit antibody by the horse antibody with the consequent release of the former. Should the rabbit antibody be released in an amount equivalent to or greater than that of the remaining horse antibody a fixing element might be detected in the supernatant and thus give the impression that the inhibitor had been absorbed from the horse serum. However, on the basis of our present knowledge regarding equilibria in these systems it is doubtful if the concentration of the free rabbit antibody could reach a point greater than that of the free horse antibody. Furthermore, it would seem unlikely that the reaction could continue to a point of complete replacement. Even if the reaction should reach an equivalent point and thus, in theory, render the supernatant fluid potentially active from the point of view of fixation it is extremely doubtful if it could be detected by this technique. In our experience the minimum concentration of the released antiserum would be of necessity greater than the equivalent of a 1:200 dilution of original immune rabbit serum, since with higher dilutions it is extremely difficult to demonstrate complement fixation.

For the purpose of detecting smaller amounts of the rabbit antibody, fowls were immunized with antipneumococcus rabbit serum. The sera of these immunized fowls reacted with rabbit serum in dilutions as great as 1:100,000. With this reagent a large number of experiments have been carried out to demonstrate the release of rabbit antibody both from sensitized pneumococci and from immune precipitates after these had been treated with homologous immune horse serum. These experiments are not reproduced in detail because of their inconclusive character. In about one-third of the total number no rabbit protein was detected in the supernatant fluid. In another third, including all of the experiments with immune precipitates, the controls, untreated with immune horse serum, showed traces of free rabbit protein in the supernatant fluid. In a very few instances minute amounts of rabbit protein appeared to be released after treatment of agglutinates with immune horse serum. These quantities were too small to possess any actual significance.

DISCUSSION

The experiments reported in this paper support the view that the failure to obtain complement fixation with combinations of pneumo-

coccus capsular polysaccharide and specific immune horse serum is not due to some heterologous inhibitor present in immune horse serum but is to be referred rather to some property of the horse antibody itself or some property of the immune aggregate resulting from the union of this antibody and the polysaccharide. It has been somewhat generally assumed that the resultant of an immunological reaction of antigen and antibody has the property of fixing complement. There are, however, instances other than the one cited in which this is not true. Certain information on this subject has been assembled in Table X. Pneumococcus proteins, for example, give fixation in both horse and rabbit immune sera (7). The M or specific protein fraction of *Streptococcus hemolyticus* gives fixation with immune rabbit serum (8). The capsular polysaccharides of Pneumococcus and of *Hemophilus influenzae* give fixation only with immune rabbit sera of homologous type (2). The C or somatic carbohydrate of the Pneumococcus fails to give complement fixation with either antipneumococcus horse or rabbit serum (9). A similar failure is obtained with the C or somatic carbohydrate of *Streptococcus hemolyticus* and its homologous immune rabbit serum (8). It must be emphasized that precipitation occurred in all of these instances. A properly controlled positive complement fixation result, therefore, indicates a specific reaction between antigen and antibody, but a failure in complement fixation does not necessarily imply the lack of specific combination.

TABLE X

Capacities of Certain Bacterial Derivatives to Fix Complement in Combination with Specific Precipitating Sera

Bacterial derivative	Complement fixation with	
	Immune rabbit serum	Immune horse serum
Pneumococcus protein (7)	+	+
M (specific protein) fraction of *Streptococcus hemolyticus* (8)	+	
Pneumococcus capsular polysaccharide (1)	+	−
Hemophilus influenzae capsular polysaccharide (2)	+	−
C or somatic carbohydrate of Pneumococcus (9)	−	−
C or somatic carbohydrate of hemolytic streptococcus (8)	−	

It would appear from the reports of many workers, particularly Heidelberger and Kendall (10), and Boyd and Hooker (11), that each molecule of pneumococcus capsular polysaccharide is capable of uniting with varying numbers of specific antibody molecules. Similarly it does not seem improbable that a single polysaccharide molecule may unite at one and the same time with antibody molecules of two immune sera derived from two animal species. Whether or not this assumption is true, the experiments have shown that the results of the complement fixation test depend upon which of the two immune sera is present in greater amount, and that, within certain quantitative limits, one serum does not inhibit the action of the other providing both are introduced at the same time. If, on the other hand, one of these sera is added after a definite interval to a system containing the other serum and the antigen, one encounters what has been spoken of as an inhibition phenomenon. It has been shown that under certain quantitative conditions the second serum component predicates the result, and that the reaction of inhibition is type-specific and therefore directly concerned with the capacity of the secondarily added antibody to unite with exposed but unsaturated linkages of the polysaccharide. The results suggest that the effect of the second immune serum is in reorientation of the surface composition of the already formed immune aggregates. This is based on the fact that the amount of the second serum need not be as great as that of the first.

These deductions with reference to the surface phenomena are also supported by the findings as to the amount of complement fixed by combinations of immune rabbit serum and specific polysaccharides. Thus in several types of experiments it was shown that greater amounts of complement were fixed the greater the surface exposure of the immune aggregates. Surface exposure in non-static systems of this order is obviously an expression of the sums of surfaces over progressive time intervals minutely spaced, that is, a true surface-time function, but in its simplest conception it is resolved into total surface.

Moreover, these results support the view that the fixation of complement is a phenomenon of selective adsorption. That one type of aggregate absorbs complement while another fails to do so is curious, but far from unique. A close parallelism is to be found in the fact that horse antibody-polysaccharide aggregates adsorb cephalin, while aggregates containing rabbit antibody selectively adsorb lecithin (12).

It has been clearly shown that complement fixation does not occur in the absence of particulation. When the union of antigen and antibody results in a perfectly soluble combination, complement is not fixed. Since particulation is in itself a secondary phenomenon, complement fixation must be regarded as at least tertiary.

SUMMARY

1. Complement is not fixed by immune aggregates resulting from the interaction of pneumococcus capsular polysaccharide and type-specific immune horse serum, although under proper conditions the substitution of immune rabbit serum gives positive results.

2. The negative results with immune horse serum are due to some poorly understood property of the specific antibodies rather than to some heterologous inhibitor present in the serum.

3. It has been shown that with immune rabbit serum-polysaccharide combinations, complement fixation is an adsorptive phenomenon conditioned upon the surface exposure of the immune aggregates.

4. A close parallelism to the selective adsorption of phosphatides by these immune aggregates has been pointed out.

5. In those instances in which complement is fixed this phenomenon must be regarded as tertiary and conditioned by (*a*) union of antigen and antibody, and (*b*) particulation.

6. The general significance of complement fixation as applied to bacterial polysaccharides has been discussed.

BIBLIOGRAPHY

1. Zinsser, H., and Parker, J. T., *J. Immunol.*, 1923, **8,** 151.
2. Pittman, M., and Goodner, K., *J. Immunol.*, 1935, **29,** 239.
3. Heidelberger, M., Sia, R. H. P., and Kendall, F. E., *J. Exp. Med.*, 1930, **52,** 477.
4. Heidelberger, M., and Kendall, F. E., *Proc. Soc. Exp. Biol. and Med.*, 1935, **33,** 445.
5. Avery, O. T., and Goebel, W. F., *J. Exp. Med.*, 1933, **58,** 731.
6. Avery, O. T., and Goebel, W. F., *J. Exp. Med.*, 1929, **50,** 533.
7. Lancefield, R., and Vollmond, E., cited by Avery, O. T., and Tillett, W. S., *J. Exp. Med.*, 1929, **49,** 262.
8. Lancefield, R. C., unpublished data, personal communication.
9. Goodner, K., unpublished data.
10. Heidelberger, M., and Kendall, F. E., *J. Exp. Med.*, 1929, **50,** 809.
11. Boyd, W. C., and Hooker, S. B., *J. Gen. Physiol.*, 1934, **17,** 341.
12. Horsfall, F. L., Jr., and Goodner, K., to be published.