THE PROPERDIN SYSTEM AND IMMUNITY

V. The Bactericidal Activity of the Properdin System

By ALASTAIR C. WARDLAW,* PhD., and LOUIS PILLEMER, Ph.D.

(From the Institute of Pathology, Western Reserve University, Cleveland)

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Properdin, complement, and magnesium ions constitute the recognized components of the properdin system (1, 2). This system, which is present in the normal serum of man and other mammals, has the ability *in vitro* to lyse certain cells, destroy bacteria, and inactivate viruses, and appears to be involved in the resistance or susceptibility of experimental animals to infection, irradiation, and shock (3–9). Preliminary reports have described briefly the action of the properdin system in bactericidal phenomena (1, 2, 5). The purpose of this paper is to report in detail experimental evidence which shows that the properdin system is the mechanism by which certain bacteria are destroyed by fresh, normal human serum. The factors required for the bactericidal action; and the variety of bacteria affected by the system are also considered.

Properdin was originally recognized by its ability to combine with zymosan, the insoluble cell wall residue of yeast, to form a complex that inactivated the third component of complement (C'3). Two distinct reaction stages were demonstrated: First, the combination of zymosan with properdin, which required the presence of complement and magnesium and second, the destruction of C'3 by the properdin-zymosan complex (PZ) (1, 2). These interactions had a resemblance to the heat-labile viral neutralizing activities of serum described by Ginsberg and Horsfall (10). When it was evident, as a result of preliminary studies (1, 6), that the properdin system had viral inactivating properties, the analogy between zymosan and the bacterial cell wall suggested that the properdin system might also be bactericidal. The experiments, that confirmed this hypothesis, are described below.

Nomenclature

Nomenclature is for the main part the same as employed in previous papers (1, 19). The term *RP* (reagent for properdin) is used in a generic sense to denote a serum rendered deficient in properdin alone; the term RP_b refers specifically to serum rendered deficient in properdin by the technique described below.

* Special Research Fellow in Biochemistry in Pathology. Present address: Wright-Fleming Institute of Microbiology, St. Mary's Hospital Medical School, London.

Materials and Methods

AB Buffer.—Michaelis' buffer, pH 7.5, ionic strength 0.16, containing calcium and magnesium at the concentrations present in normal serum, was prepared as follows: 9.71 gm. sodium acetate $3H_2O$, 14.71 gm. sodium 5,5-diethylbarbiturate, and 17 gm. sodium chloride were dissolved in distilled water; 20 ml. of 0.1 M $MgCl_2$ and 6 ml. of M $CaCl_2$ were added and the volume made up to 2455 ml. The pH was adjusted to 7.5 by adding approximately 45 ml. N HCl, and the buffer then sterilized by autoclaving.

ABA Buffer.—A 2 per cent solution of human albumin was made in AB buffer and sterilized by filtration through an ultrafine sintered glass filter (Virtis, Yonkers, New York). ABA buffer was made by diluting 1 part of 2 per cent albumin with 9 parts of AB buffer to give a final albumin concentration of 0.2 per cent.

Barbital Buffer.—Barbital buffer was prepared as previously described (19) and was sterilized by autoclaving.

Zymosan.—This insoluble cell wall residue of yeast was prepared as previously described (13). Various lots of zymosan were obtained from the Fleischmann Laboratories, Standard Brands Incorporated, Stamford, Connecticut; Lederle Laboratories Division, American Cyanamid Company, Pearl River, New York; or were prepared in our own laboratory.

Zymosan was suspended as evenly as possible in barbital buffer to give a concentration of 10 mg. per ml. The suspension was placed in a boiling water bath for 1 hour. The mixture was then centrifuged for 30 minutes at 4000 R.P.M. and the supernatant discarded. The residue was suspended evenly in barbital buffer to a final concentration of 10 mg. per ml.

Normal Human Serum.—Blood from healthy human donors was drawn under sterile conditions during the later afternoon and allowed to stand at room temperature for 2 hours after which it was stored at 2° overnight. The serum was separated by centrifugation at 1° for 30 minutes at 4000 R.P.M. and recentrifuged under identical conditions to remove residual red cells. The clear serum was then stored at 1°, if used within an 8 hour period, or stored at −70° for further use.

Serum Deficient in Properdin (RP).—As noted elsewhere (2, 11, 19), not all serums, or serum pools, can be satisfactorily depleted of properdin by a single adsorption with zymosan. In addition, different lots of zymosan differ in their activities; for each lot, and for the best results for each serum, or serum pool, the optimal amount of zymosan needed to render a serum properdin-deficient has to be determined empirically on aliquots before any large stock of reagent (RP) is prepared.

A single adsorption of serum with zymosan at 17° for 75 minutes may leave a properdin concentration which, while insufficient for the inactivation of C'3 by zymosan, is sufficient to demonstrate definite bactericidal activity. For this reason it became necessary to employ a second adsorption with zymosan to remove such traces of properdin in the preparation of a properdin-deficient reagent satisfactory for bactericidal studies. A reagent prepared in this manner is termed RP_b.

To prepare RP_b, 15 ml. of zymosan suspension (10 mg. per ml.) was pipetted into each of two 100 ml. centrifuge tubes and centrifuged at 4000 R.P.M. for 15 minutes. The supernatants were discarded and one tube with the moist zymosan residue placed in a 17° water bath. 50 ml. of serum equilibrated to 17° was poured onto the residue, the zymosan stirred into an even suspension, and the mixture incubated at 17° with periodic stirring for 75 minutes. The mixture was then cooled to below 10° by immersing the tube in a −5° bath and centrifuged at 4000 R.P.M. for 30 minutes at 1°. The supernatant was decanted and warmed to 37°, then poured into the tube containing the second zymosan residue, which also had been warmed to 37°. The zymosan was stirred into even suspension and maintained at 37° for 30 minutes with periodic stirring, then cooled and centrifuged as before. The supernatant RP_b was poured

off, distributed in 5 or 10 ml. volumes in pyrex or lusteroid tubes and stored at −70°. RP_b could be stored at least 2 months at −70° and at least 10 days at −20° without significant deterioration. Sterile glassware was used in the preparation of RP_b, and tubes were covered during incubation and centrifuging. RP_b was tested for sterility by plating 0.1 ml. on agar, and only rarely was a contaminant encountered.

To determine whether satisfactory conversion of serum into RP_b has been achieved, RP_b was tested for bactericidal activity, alone and in the presence of added properdin. Buffer, properdin, and the untreated serum were also tested as controls. RP_b was considered satisfactory for bactericidal studies when it met the following criteria: RP_b, alone, should kill less than 10 per cent of the inoculum; RP_b plus properdin (1 to 5 units per ml.) should kill fully 95 per cent. Buffer and properdin alone should not be bactericidal while the parent serum should be fully bactericidal. Usually one or two of every 10 randomly chosen human serums yielded satisfactory RP_b.

As will be discussed below, RP_b generally contained 30 to 50 per cent of the total hemolytic complement that was present in the corresponding untreated serum. The component most affected by the zymosan treatment was C'3. An ideal RP_b should be completely lacking in properdin but should contain all of the original complement component activities. Such a reagent was not obtained by the procedure described above because not all of the properdin could be removed at the first zymosan treatment at 17°, and the second zymosan treatment at 37° then inactivated C'3 to a varying extent.

Serum Deficient in Complement Activity.—Human serum was made deficient in complement activity by a variety of procedures known to inactivate or remove certain components of complement. R1, R2, R3, and R4, which respectively lack C'1, C'2, C'3, and C'4 but contain the other components of complement, were prepared and characterized by standard methods (12). SK serum (14) was prepared by incubating normal human serum with 1500 units of streptokinase (Lederle) per ml. of serum for 1 hour at 37°. CF serum (15) was prepared by treating normal human serum with antigen-antibody aggregate (SIII-anti SIII, rabbit). Complement was also inactivated by heating serum in a constant temperature bath at 56° for 30 minutes. Partially purified C'1 (16) and C'3 (17) were prepared respectively from R2 and from human plasma fraction III (Cohn). Each was shown to contain only the designated component of complement and small amounts of properdin. The concentrated solutions of C'1 and C'3 were diluted with barbital buffer to levels approximately equal to those in fresh, normal human serum.

Resin-Treated Serum.—Previous investigations (18, 20) showed that the treatment of human serum with the cation exchange resin, amberlite IRC-50 in the sodium cycle, reduced the calcium and magnesium content to concentrations below 1×10^{-6} M without any inactivation of complement or properdin. For the studies reported here the resin was placed in a graduated centrifuge tube, moistened with distilled water; the tube stoppered, and the contents heated to 70° for 30 minutes to destroy bacterial contaminants. The resin was cooled to 1°. A volume of serum also cooled to 1°, equal to or less than the volume of the loosely packed resin, was added and the contents kept mixed by gentle inversion and swirling. The resin was kept in suspension in the serum for 10 minutes, care being taken to insure that the mixture remained cool. The serum was then decanted and centrifuged at 1° to remove residual particles of resin.

Properdin.—Properdin, purified 3000- to 7000-fold on the basis of protein nitrogen with respect to the original serum, was prepared as described previously (1). Human properdin, derived both from serum and from placental extracts, and bovine properdin derived from bovine serum have all been found satisfactory for bactericidal studies. The experiments described below utilized properdin derived from human serum. These properdin preparations contained no detectable amounts of any of the components of complement. The properdin

was dissolved in AB or barbital buffer at concentrations of 100 units per ml. and 2 to 5 units per ml. These dilutions were then stored at −20° until used. For some bacteriological studies the properdin was sterilized by passage through an ultrafine sintered glass filter prior to storage. However, properdin as generally prepared did not contain contaminating organisms sufficient in number to interfere with bactericidal tests.

Testing of Serologic Materials.—Serum, complement reagents, RP, and properdin were tested for the presence of complement components by standard hemolytic assays (12, 19). All properdin titrations were performed by the zymosan assay (19).

Bacteria.—The major part of these studies employed *Shigella dysenteriae* (ATCC 9665), obtained as strain No. 377 from the culture collection of the Department of Microbiology, Western Reserve University. This strain had the typical biochemical characteristics of the species and was agglutinated by *Shigella* Group A (Shiga) antiserum.

In addition, some other strains of a variety of species were obtained from this and other culture collections, from freshly isolated human stool and blood cultures, and from the blood of rats and mice that had died following the intravenous injection of zymosan. Difco bacto tryptose blood agar base and Difco bacto brain heart infusion broth were used for preparing slant, plate, and liquid media cultures. Each strain was maintained by monthly subculture on agar slants which, after inoculation, were incubated at 37° for 18 hours and then stored at 4°.

Preparation of Bacterial Suspensions.—The suspensions of organisms used in the bactericidal tests were prepared either from 5 hour broth cultures or from 18 hour slant cultures incubated at 37°. The broth cultures or suspensions in ABA buffer made from the slant cultures were diluted with ABA or barbital buffer until the turbidity matched that of a $BaSO_4$ comparison tube which was known to be equivalent to 5×10^8 viable *Sh. dysenteriae* per ml. These suspensions were then further diluted 20,000-fold to give suspensions containing approximately 25×10^3 organisms per ml. which were used in most of the bactericidal tests. The diluted suspensions were held in an ice bath and used within 30 minutes of preparation.

Determination of Bactericidal Activity.—The reagents under test for bactericidal activity were distributed in 16 x 100 mm. cotton-plugged tubes held in an ice bath. The total volume of test mixture was 0.5 ml. of which 0.48 ml. was occupied by the reagents under test and 0.02 ml. by the diluted bacterial suspension. After addition of bacteria, mixtures were incubated in a 37° water bath, for 90 minutes unless otherwise stated, and viable counts were then made by spreading 0.1 ml. of each test mixture uniformly over the surface of an agar plate. When higher concentrations of bacteria were employed, the samples were serially diluted by 10-fold steps, and 0.1 ml. of each dilution plated. The plates were incubated overnight at 37°. Colonies were then counted and the viable count obtained. In most experiments ABA buffer was used as the control medium for comparison with known bactericidal reagents, such as serum, etc. After it had been established that the viable count in ABA buffer did not change during the time of the test, the percentage of organisms killed by or surviving exposure to serum, etc., was calculated on the basis that there was 100 per cent survival in ABA buffer.

In occasional screening experiments viable counts were determined by making serial dilutions of the bacterial suspension with a bacteriological loop standardized to hold 0.005 ml. Plates were inoculated by spreading a loopful of the dilution over one-third of the surface, three dilutions being used for each count: 1:1, 1:200, and 1:40,000. Colony counts were made after incubating for 18 hours at 37°. This method allowed an estimate to within a factor of 5- or 10-fold (±1 log unit) of suspensions containing 2×10^2 to 1×10^8 organisms per ml.

Bactericidal activity is reported either as the bactericidal index, *i.e.* $\left(\log \frac{\text{initial viable count}}{\text{final viable count}}\right)$, or as the percentage of organisms killed, *i.e.*, $100\left(1 - \frac{\text{final viable count}}{\text{initial viable count}}\right)$.

EXPERIMENTAL RESULTS

1. The Reduction of Bactericidal Activity by Reducing the Properdin Content of Serum.—

Method.—The bactericidal activity of serum and of RP_b prepared from the serum on three randomly chosen, unrelated organisms: *Shigella dysenteriae*, *Bacillus subtilis*, and *Staphylococcus albus* was determined using suspensions from 18 hour slant cultures. ABA buffer was used as a control. Serum or RP_b and bacteria were incubated at 37° for 4 hours. Viable counts were made by the loop method.

The initial experiment was designed to determine whether the bactericidal activity of serum was diminished when the serum was converted into RP_b. For this purpose three morphologically different organisms were chosen at random: *Sh. dysenteriae*, *B. subtilis* and *Staph. albus*. The results of this experiment are summarized in Table I. It is apparent that both the strain of *Sh. dysenteriae* and *B. subtilis* were destroyed by serum, and that the depleted properdin concentration in RP_b coincided with a decreased bactericidal effect. The control ABA buffer had no activity. The strain of *Staph. albus*, on the other hand, was not affected by either serum or RP_b, but was rapidly killed in the buffer.

TABLE I

Comparison of the Bactericidal Activity of Serum, RP_b, and ABA Buffer on Three Unrelated Organisms

Bacterium	Bactericidal index		
	Serum	RP_b	ABA buffer
Sh. dysenteriae	>3.6	2.8	0.0
B. subtilis	>2.0	0.5	0.5
Staph. albus	0.0	0.0	>2.3

RP_b had less bactericidal activity for the *B. subtilis* than for the *Sh. dysenteriae*, suggesting that the former might be a better test organism than the latter. However, evenly dispersed suspensions and reliable viable counts were difficult to obtain with *B. subtilis* because of the tendency of the organism to grow in chains. For this reason *Sh. dysenteriae* was chosen as the test organism for further studies of the bactericidal effect. Microscopic and macroscopic inspection of these organisms showed that they lysed following incubation with either serum, or RP_b and added properdin. This clearly shows that the properdin system is indeed bactericidal.

2. The Restoration of Bactericidal Activity to RP_b by the Readdition of Properdin.—

Method.—Properdin (4 units per ml.), serum, RP_b, RP_b to which properdin had been added (final properdin concentration: 4 units per ml.), and ABA buffer were used as test reagents.

To 1.42 ml. of each of the various reagents, 0.08 ml. of a *Sh. dysenteriae* suspension containing 5×10^5 organisms per ml. was added, and the mixtures incubated at 37°. Aliquots were taken during incubation at 0, 30, 60, 90, and 150 minutes, and viable counts made.

The previous experiment indicated that RP_b had less bactericidal activity than serum. If this were due to the loss of properdin alone, the readdition of

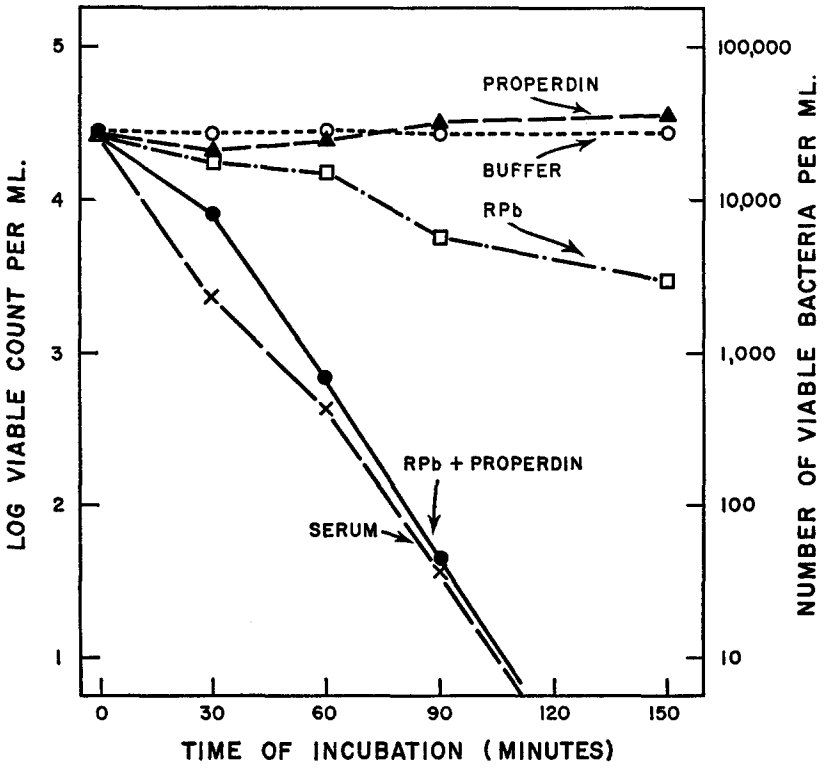

FIG. 1. The bactericidal activities of properdin, RP_b, buffer, serum, and of RP_b with added properdin on *Sh. dysenteriae*.

properdin to RP_b should restore the bactericidal activity. This hypothesis was tested experimentally and the results are shown in Fig. 1.

Properdin alone, or buffer, showed no bactericidal effect. Within the limits of experimental accuracy the viable count during incubation in these agents remained constant.[1] RP_b alone showed slight bactericidal effect; over the 150 minute period, there was a decrease of one log (tenfold) in the viable count.

[1] In additional experiments it was shown that properdin alone in concentrations up to 400 units per ml. had no bactericidal activity.

RP_b to which properdin had been readded, however, showed a marked bactericidal activity which was essentially identical to that of serum. In both these reagents the viable count dropped rapidly during incubation. By 90 minutes the number of viable organisms had decreased three logs (1000-fold). While the majority of the organisms were killed within 90 minutes, a relationship exists between the rate of bactericidal activity and properdin concentration. At certain properdin levels small differences in the properdin concentration greatly change the rate of bacteriolysis. These experiments will be presented in detail in a subsequent publication.

These experiments clearly demonstrated that the bactericidal activity of RP_b was considerably less than that of the serum from which it was prepared. The readdition of properdin to RP_b restored (almost completely) the bactericidal effect. Properdin alone was non-bactericidal. Thus, while properdin was essential, certain factors in RP_b were apparently also necessary for the bactericidal action of serum.

3. The Relationship between Bactericidal Activity and Concentration of Properdin in RP_b.—

Method.—0.4 ml. RP_b and 0.08 ml. of varying concentrations of properdin were mixed, and 0.02 ml. *Sh. dysenteriae* suspension added. Following incubation, viable counts were performed.

The experiment, the results of which are shown in Fig. 2, was designed to determine the minimum concentration of properdin that would give a perceptible restoration of bactericidal activity to RP_b. The RP_b itself showed no bactericidal action. However, the addition of as little properdin as to give a final concentration of 0.01 unit per ml. showed definite bactericidal effect. 50 per cent of the organisms were destroyed with only 0.04 unit of properdin per ml. Maximal activity was obtained between 1 and 10 units of properdin per ml. Increasing the concentration of properdin above this level, which is that found in normal serum, did not result in any increase in bactericidal activity. In fact, a large excess of properdin was inhibitory; the concentration of 60 units of properdin per ml. was less bactericidal than the concentration of 1 unit per ml.

Further evidence of this inhibition of the bactericidal phenomenon by excessive concentrations of properdin was obtained in other experiments. It was shown best when the test serum or RP_b was diluted. For example, serum diluted 1:3 in ABA buffer killed 90 per cent of *Sh. dysenteriae* suspension in 30 minutes at 37°, whereas the identical serum killed only 5 per cent of the organisms when the same dilution was made in the same buffer containing 100 units of properdin per ml.

4. The Similarity of Purified Properdin and Serum in Restoring Bactericidal Activity to RP_b.—

Method.—Either serum containing 4 units of properdin per ml. or purified properdin in buffer at 4 units per ml. was added in amounts varying from 0.01 to 0.08 ml. to 0.4 ml. RP_b and the volume of each mixture made up to 0.48 ml. with buffer. To each mixture 0.02 ml. of *Sh. dysenteriae* suspension was added and, following incubation, viable counts made.

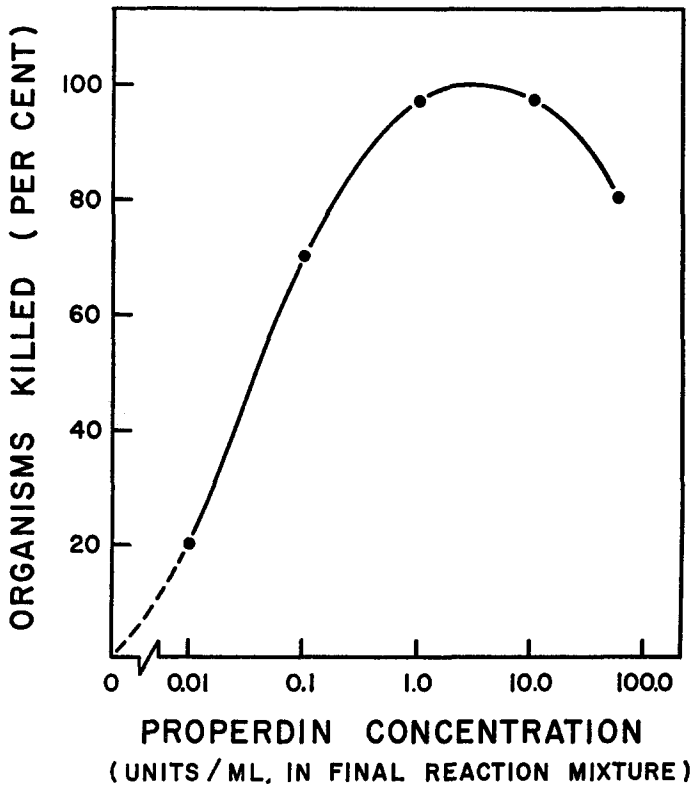

FIG. 2. The progressive restoration of bactericidal activity to RP_b by the addition of increasing amounts of properdin.

Since purified properdin was obtained by the interaction of the serum factor with zymosan, followed by elution, precipitation, solution and concentration (1), an experiment was performed to determine whether the purified material restored bactericidal activity to RP_b in a manner comparable to properdin in its natural state in serum. The results (Fig. 3) show that normal serum and a solution of purified properdin, each having the same properdin concentrations (4 units per ml.), gave virtually the same bactericidal response when varying amounts of each were added to constant amounts of RP_b. Thus

the removal of properdin from serum and further purification did not apparently alter its activity in the bactericidal system.[2]

5. The Preparation of RP_b from Different Serum Pools by Double Adsorption with Zymosan.—

Method.—Pools of different individuals' serums were adsorbed once with zymosan at 17° for 75 minutes and centrifuged. An aliquot of each supernatant (RP_1) was taken for testing

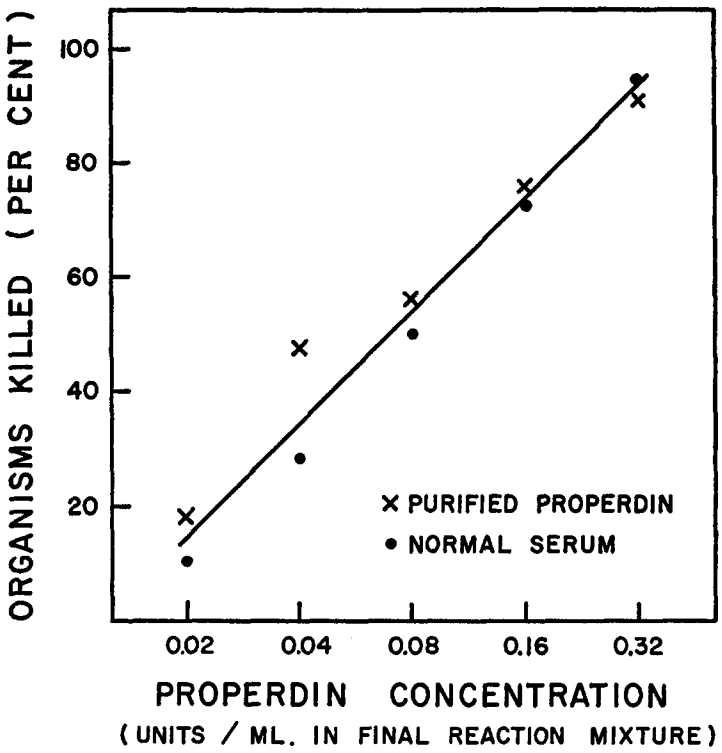

FIG. 3. Comparison of purified properdin and normal serum, both containing 4 units of properdin per ml., in restoring bactericidal activity to RP_b.

and the remainder of each pool adsorbed with zymosan a second time for 30 minutes at 37° making an RP_b. Aliquots of the serum pool, RP_1, RP_b alone, and RP_b with properdin re-added in a concentration of 2 units per ml., were tested for bactericidal activity against *Sh. dysenteriae.*

Early experiments suggested that preparations of RP_b differed both in the degree of reduction of bactericidal activity (compared with the original serums),

[2] In addition it has been shown that bovine properdin and human properdin derived from serum, and human properdin derived from placental extracts have identical activity.

and in the extent of restoration of bactericidal activity by identical amounts of properdin. In Table II are summarized the bactericidal activities of RP prepared from various serum pools, adsorbed once (RP_1) or twice (RP_b), and the ability of the preparations of RP_b to be restored by properdin in the bactericidal system. All of the serum pools initially had a bactericidal index of 3.5 or greater. The RP_1 had a decreased, but still considerable, bactericidal index. Only after the second adsorption in the preparation of RP_b, did a pronounced fall in the bactericidal index occur. The readdition of properdin to RP_b resulted in a significant increase in the bactericidal index. However only one RP_b, upon the readdition of properdin, approached the bactericidal activity of the parent serum. Pool 5 is representative of the type of RP_b suitable for studying the mechanism of the bactericidal action of the properdin system and for the assay of properdin. The general experience of this labora-

TABLE II

Bactericidal Activities of Pools of Serum, of RP_1, and RP_b with and without Added Properdin

Serum sample	Bactericidal index			
	Untreated serum	RP_1	RP_b	RP_b + properdin
Pool 5 (2 serums)	5.5	5.3	0.3	5.0
Pool 8 (4 serums)	4.3	3.8	0.1	2.0
Pool 10 (4 serums)	4.7	2.3	0.2	3.5
Pool 20 (8 serums)	5.0		0.7	2.5
Pool 25 (6 serums)	3.5	1.4	0.1	1.0

tory, both with viral inhibition and the inactivation of C'3 by zymosan, indicates that only 10 to 20 per cent of serums are suitable for preparing satisfactory RP.

6. The Demonstration of Residual Properdin in RP Prepared by a Single Adsorption with Zymosan (RP_1).—

Method.—A singly adsorbed RP (RP_1) was prepared as for the standard zymosan assay of properdin (1, 2). An aliquot of this was converted to RP_b by a second adsorption. Varying amounts of the initial serum (properdin concentration 4 units per ml.) and RP_1 were added to the RP_b, and the bactericidal index of the resultant mixtures determined.

In Experiment 5 (Table II) it was shown that the single adsorption of serum by zymosan in the preparation of RP_1 did not completely deplete the bactericidal activity. Yet the same adsorption rendered the serum inactive as a source of properdin for the inactivation of C'3 by zymosan. By definition (1) a 50 per cent reduction of the C'3 titer with zymosan under standard con-

ditions requires 0.5 unit of properdin. However, in the bactericidal activity as little as 0.04 unit may kill 50 per cent of the organisms (Experiment 3). The bactericidal phenomenon, therefore, is on occasion more than 12 times as sensitive an index of the properdin concentration as the inactivation of C'3 by zymosan in the standard assay. In addition, with properdin concentrations of 1 unit per ml., the bactericidal activity may have reached an actual maxi-

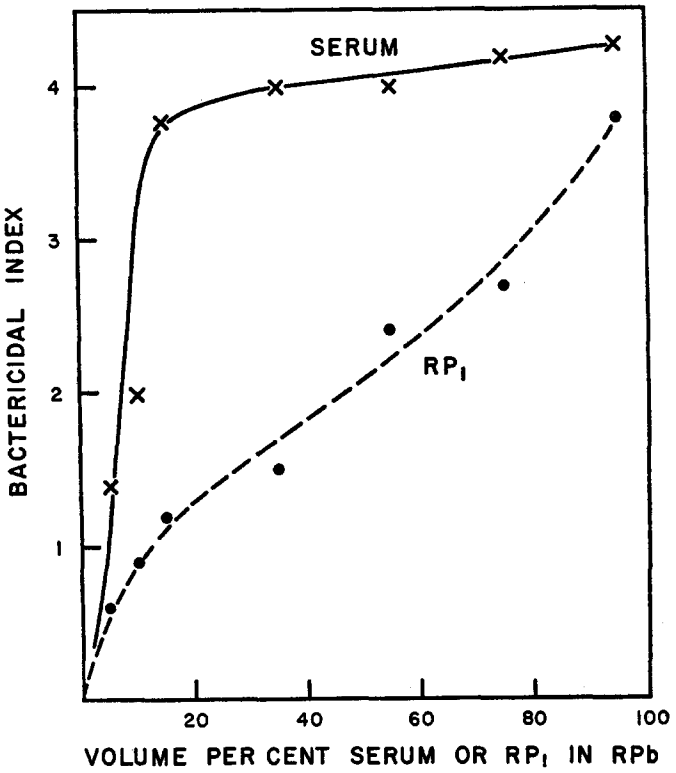

FIG. 4. Bactericidal activity of mixtures in varying proportion of (*a*) RP_I and RP_b, and (*b*) normal serum and RP_b.

mum. It was therefore considered likely that the residual bactericidal activity in the singly adsorbed RP (RP_I) was due to residual properdin which, although insufficient to be detected by the zymosan assay with the inactivation of C'3, was sufficient to show bactericidal activity.

This hypothesis is supported by the results given in Fig. 4 which show that serum was approximately 6 times more effective than RP_I in restoring bactericidal activity to RP_b. The serum contained 4 units of properdin per ml. Calculation suggests that the RP_I contained approximately 0.7 unit of proper-

din per ml. While this is an insufficient quantity for accurate measurement of the inactivation of C'3 by zymosan, it is close to the concentration required for maximal bactericidal activity. Confirmation of the presence of properdin in RP_1 was obtained by the demonstration of properdin in an eluate from the zymosan which had been used in the second adsorption during the preparation of RP_b.

7. The Effect of the Preparation of RP_b on Complement Components of Serum.—

Method.—Complement components were titrated by standard methods on samples of untreated serum and of serum subjected to the first and second adsorption by zymosan in the preparation of RP_b.

TABLE III

Serum sample	Reagent	Complement component titer				Bactericidal index*
		C'1	C'2	C'3	C'4	
Pool 5	Serum	7680	960	640	960	5.5
	RP_1‡	7680	960	480	960	5.3
	RP_b	7680	960	320	640	0.3
	RP_b + properdin	7680	960	320	640	5.0
Pool 20	Serum	10,240	1920	480	1920	5.0
	RP_b	10,240	1920	120	1920	0.7
	RP_b + properdin	10,240	1920	120	1920	2.5

* Against *Sh. dysenteriae*; from Table II.

‡ Single adsorption with zymosan.

Table II shows that the bactericidal activity could not always be satisfactorily restored to all preparations of RP_b by the readdition of properdin. Since it is known that the properdin system requires complement, an experiment was carried out to show the effect of the preparation of RP_b on the titers of the four components of complement. The results are tabulated in Table III. In both pools the second adsorption decreased the titer of C'3; in one instance to half, and in another to a quarter of the initial serum value. In addition, the C'4 titer of pool 5 was also reduced. Occasionally decreases in the titer of C'1 and C'2 were also noted. Since, as will be shown in Experiment 9, all components of complement are necessary for the bactericidal activity of the properdin system, it seems likely that the failure of certain RP_b preparations to return to normal bactericidal levels following the readdition of properdin is due to the partial inactivation of one or more components of complement.

8. The Effect of Dilution on the Bactericidal Activity of Serum, RP_b and RP_b Plus Properdin.—

Method.—Serum, RP_b and RP_b to which properdin had been readded in a concentration of 5 units per ml. were each mixed in varying proportions with ABA buffer, and the bactericidal index of the resultant mixtures determined by incubating 0.9 ml. of each dilution with 0.1 ml. of a suspension of *Sh. dysenteriae* containing 5×10^8 organisms per ml. Viable counts were made by the loop method after 4 hours at 37°.

Further evidence that the bactericidal activity of the properdin system can be limited by certain factors in RP was obtained by measuring the bactericidal index of varying dilutions of serum, RP_b, and RP_b to which properdin had been added in concentrations adequate to give optimal bactericidal effect in all dilutions. The results (Table IV) indicated that a decrease in the bactericidal index of RP_b reconstituted by the addition of properdin occurred

TABLE IV

Effect of Dilution (in ABA Buffer) on the Bactericidal Activity of Serum, RP_b and RP_b with Properdin

Reagent	Bactericidal index				
	Volume per cent of reagent in final mixture				
	90	70	50	30	0
Serum	6.5	6.5	5.0	0.9	0.0
RP_b	0.0	0.4	0.3	0.0	0.2
RP_b + properdin, (5 units/ml.)	—	2.0	1.4	0.0	0.0

with dilution in a manner similar to that of serum, and that when the concentration of either fell below 30 per cent, the bactericidal activity was barely, if at all, demonstrable. At this dilution the mixtures still contained more than 1 unit of properdin per ml. which has been shown (Fig. 2) to be sufficient to give optimal bactericidal effect. Thus it was concluded that under these circumstances the concentration of factors other than properdin in the RP_b rather than the concentration of properdin itself had become limiting.

9. The Complement Requirements for the Bactericidal Activity of the Properdin System.—

Method.—Serum was rendered deficient in one or more components of complement by a variety of methods. To some of the reagents so prepared, the missing component was readded in the form of a purified preparation. To those reagents in which properdin might have been deficient, additional properdin was added. In each case the bactericidal activity of the reagent or mixture was determined by incubating 0.4 ml. of the reagent mixed with 0.08 ml. of buffer or properdin (5 units per ml.) and 0.02 ml. *Sh. dysenteriae* suspension.

All four components of complement as well as properdin were necessary for bactericidal activity; a reagent deficient in any one or more of the four components was devoid of bactericidal effect (Table V). When the missing component of complement was restored to the reagent, the mixture became bactericidal. R1, the reagent deficient in C'1, and R2, deficient in C'2, alone were non-bactericidal. When the two reagents were combined, the resultant mixture containing all four components of complement was again bactericidal. That it did not regain the full antibacterial activity of whole serum can be

TABLE V

Bactericidal Activity of Treated Human Serums and Serum Fractions Variously Lacking Properdin and Components of Complement

Reagent or treatment of serum	Constituents of properdin system known to be absent	Organisms killed with	
		Reagent alone	Reagent plus properdin
		per cent	*per cent*
Serum	None	98	
56°, 30 min. serum	C'1, C'2, C'3, P	0	0
R1	C'1, P	0	0
R2	C'2	0	
R1 + R2*	None	82	
R1 + C'1‡	None	91	97
C'1	C'2, C'3, C'4	0	
R3	C'3, P	0	0
R3 + C'3‡	None	78	99
C'3	C'1, C'2, C'4	0	0
R4	C'4	0	
CF serum	C'2, C'4	0	
SK serum	C'2, C'4	0	

* Equal volumes.

‡ 2 volumes + 1 volume respectively.

fully explained by the fact that the mixture was, in respect to serum, diluted twofold by the process of preparation. The readdition of C'1 to R1 similarly restored the bactericidal effect, as did the readdition of C'3 to R3 (the reagent lacking C'3), while the C'1 and C'3 alone were entirely non-bactericidal. In both these instances the restored mixtures were partially deficient in properdin (the R1 by the precipitation of properdin in the dialysis, the R3 by the treatment with zymosan). The further addition of properdin increased the bactericidal activity of both mixtures.

These experiments allow the conclusion that in the bactericidal action of properdin, as in all of its other reported activities, all components of complement are required.

10. The Magnesium Requirement for Bactericidal Activity.—

Method.—The bactericidal activity of mixtures consisting of 0.15 ml. of resin-treated serum and 0.33 ml. of barbital buffer or barbital buffer containing different concentrations of magnesium (or other cations), and 0.02 ml. *Sh. dysenteriae* suspensions was determined. As a control, to detect any possible bactericidal effect of the cation alone, resin-treated serum heated

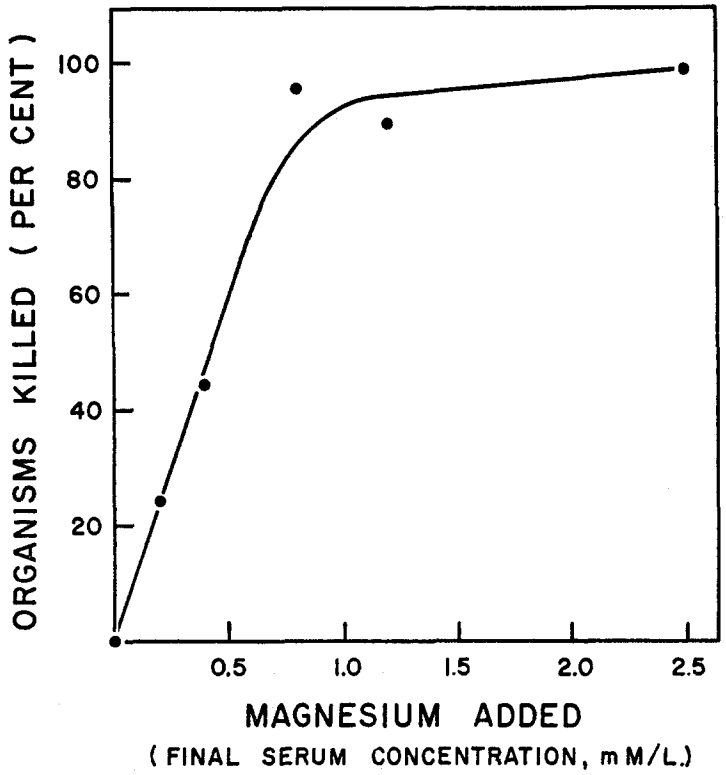

FIG. 5. The ability of magnesium ions to restore bactericidal activity to resin-treated serum.

at 56° for 30 minutes to destroy complement and properdin was used in an identical manner and run in parallel for each determination.

To test the requirement for magnesium in the bactericidal activity of serum, serum rendered deficient of cations other than sodium was prepared by treatment with ion exchange resin. Varying amounts of magnesium were then added to this resin-treated serum (Fig. 5).

The resin-treated serum alone was non-bactericidal, but as increasing amounts of magnesium were readded, the bactericidal activity was progres-

sively restored. Maximal bactericidal effect was obtained with a magnesium concentration similar to that of normal serum. The effect of magnesium was not due to direct toxic activity, for the addition of the cation to heated serum gave a completely non-bactericidal mixture.

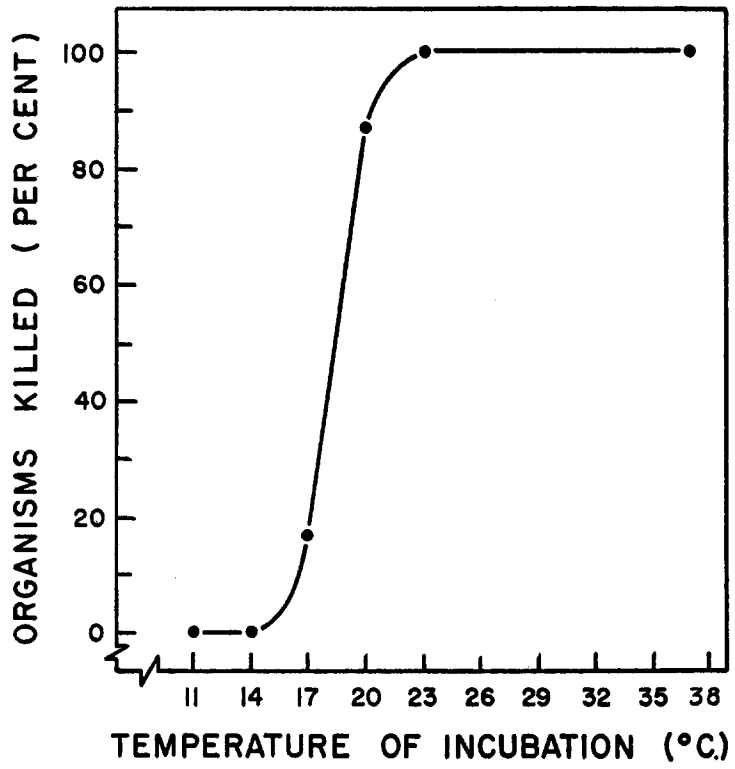

FIG. 6. The effect of temperature of incubation on the bactericidal activity of serum.

The magnesium requirement was specific. Calcium was completely ineffective in restoring bactericidal activity. In addition to calcium and magnesium, 22 other metal ions of the alkali, alkaline earth and heavy metal groups were tested. Of these none restored bactericidal activity to resin-treated serum in physiologic concentrations. Cobalt, manganese, and iron would, in concentrations between 10^{-2} to 10^{-3} M, restore the bactericidal activity. Such concentrations, being so much greater than those occurring normally in serum, preclude the possibility of these ions taking part in the bactericidal reactions of serum.

11. The Temperature Requirements of the Bactericidal Activity of the Properdin System.—

Method.—Duplicate mixtures containing 1 ml. of serum and 0.04 ml. of *Sh. dysenteriae* suspension were incubated for 80 minutes at various temperatures and viable counts determined and compared with the count at zero time. Pipettes cooled at 4° were used to remove the samples for counting from each mixture, and each sample was then diluted 50-fold in saline at 4° before plating.

The inactivation of C'3 by zymosan is known to be temperature-dependent (1). An experiment (Fig. 6) was designed to determine the influence of temperature on the bactericidal activity of serum. Bactericidal action did not occur at temperatures below 17°. At 17° slight bactericidal activity could be deter-

TABLE VI

The Susceptibility or Resistance of Various Strains of Bacteria to the Properdin System

Species	No. of strains		
	Susceptible	Resistant	Unknown
Shigella	3		3
Salmonella	3	2	3
Escherichia coli	4	1	3
Aerobacter aerogenes		2	
Proteus	3	3	1
Pseudomonas	3	1	
Paracolobactrum	2	3	
Bacillus subtilis	2	2	
Total	20	14	10

mined, at 20° it was marked, and above 23° complete bactericidal action was present.

The temperature dependence of the bactericidal activity of human serum is thus identical with the inactivation of C'3 by zymosan. Neither occur at temperatures below 17°, both attain maxima at temperatures above 20°.

12. The Action of the Properdin System on Other Bacteria.—

Method.—44 strains from a variety of genera were tested for sensitivity to the bactericidal action of serum, RP_b, RP_b with properdin readded, properdin alone, and ABA buffer. Bacterial viability was determined as before.

Experiments presented above demonstrated that two unrelated organisms were sensitive to the properdin system. Other strains tested were shown to fall into three distinct groups (Table VI): (*a*) Twenty strains which were

killed by fresh serum and by RP_b to which properdin was readded, but which were viable in RP_b alone, were classified as susceptible; (*b*) Fourteen strains which were viable in serum and in RP_b with added properdin were considered resistant; (*c*) Ten strains which were killed in RP_b alone, as well as in serum and in RP_b with readded properdin, but which were viable in properdin alone and in ABA buffer, were classed as unknown.

Each strain gave consistent results when tested against a variety of samples of serum and RP_b. However, with some strains sensitivity depended upon the type of culture used in preparing the bacterial suspension. Suspensions prepared from 5 hour broth cultures of such strains were more sensitive than suspensions made from 18 to 24 hour slant cultures. It was also apparent that while sensitivity to the properdin system may have been consistent for each strain, it was a property of the strain not of the species. Most genera or species contained both resistant and sensitive strains. Further work is in progress along these lines.

13. The Effect of Antibody on the Bactericidal Activity of the Properdin System.—

Method.—Serum was obtained from two normal rabbits and two rabbits immunized against *Sh. dysenteriae*. The agglutinin titers of the sera were measured by standard procedures. Bactericidal tests were made by adding 0.08 ml. of varying dilutions of each rabbit serum to tubes containing 0.42 ml. of RP_b previously inoculated to contain approximately 1.6×10^3 *Sh. dysenteriae* per ml. Similar dilutions of the rabbit sera were added in 0.08 ml. quantities to 0.42 ml. of RP_b inoculated to contain approximately 1.0×10^3 *E. coli* per ml. Both the *E. coli* and *Sh. dysenteriae* strains had been previously found sensitive to the properdin system. As a control 0.08 ml. of the rabbit sera, heated to 56° for 30 minutes, was added to similar mixtures of RP_b and bacteria. All were incubated at 37° for 90 minutes. Viable counts were made and the per cent of organisms killed calculated for each serum dilution. The amount of each serum needed to kill 50 per cent of the organisms in the inocula was then estimated graphically.

Because some human serums might contain antibody against strains of the organisms used in experimental studies, it was felt warranted to investigate the effect that antibody, artifically induced in an experimental animal, might have on the bactericidal activity of the properdin system. For this purpose two rabbits were immunized with the *Sh. dysenteriae* strain used in all the previous experiments. The serums from these rabbits and two normal rabbits were tested for bactericidal activity against the same strain of *Sh. dysenteriae* and also a strain of *E. coli*. Both organisms had previously been found to be sensitive to the properdin system. To insure the presence of sufficient amounts of the factors other than properdin needed for bactericidal activity the test mixtures were made using small amounts of the rabbit serum mixed with large amounts of human RP_b previously shown satisfactory for bactericidal studies.

In Table VII the agglutinin titers of the rabbit sera against *Sh. dysenteriae* are compared with the amount of the rabbit serum in the RP_b needed to kill 50 per cent of the organisms in the test mixtures. More than four to eight times as much immune serum as non-immune serum was needed to kill the same number of *Sh. dysenteriae*. In contrast, within the experimental accuracy of the method, there was no difference between the immune or non-immune serum in the bactericidal activity against *E. coli*. All four serums had been previously shown to have similar properdin contents by the zymosan assay. No bactericidal activity was noted in controls using heated rabbit serum with the RP_b.

In the conditions of this experiment properdin was the only limiting factor for bactericidal activity. The marked difference between the bactericidal activity against *Sh. dysenteriae* of the immune and non-immune sera contrasted

TABLE VII

The Effect of Antibody against Sh. dysenteriae on the Bactericidal Effect of the Properdin System Using Sh. dysenteriae and E. coli as Test Organisms

Rabbit serum	Agglutinin titer against *Sh. dysenteriae*	Amount of serum added to RP_b required to kill 50 per cent of organisms using	
		Sh. dysenteriae	*E. coli*
		ml.	*ml.*
Anti-*Sh. dysenteriae*	1:125	>0.08	0.07
	1:15	0.04	0.07
Normal	1:1	0.01	0.04
	1:1	0.01	0.06

with the absence of any difference in bactericidal action with *E. coli* suggests that bacterial antibody may in some cases interfere with, or block, the action of the properdin system against the organism to which the antibody has been formed.

DISCUSSION

It is clearly evident that the properdin system is a natural bactericidal mechanism of normal human serum. All the recognized constituents of the properdin system, namely, properdin, the four components of complement and magnesium are required for this bactericidal action. If any of these factors is missing, bactericidal activity is lost; when the factor is replaced, bactericidal activity is restored. These factors are also required for other recognized *in vitro* activities of the properdin system.

Properdin is entirely distinct from antibody by virtue of the special requirements for its activity. The interaction of antigen and antibody is not

temperature dependent; the properdin system functions only in a narrow temperature range. The interaction of antigen and antibody requires no cofactors; the interaction of properdin with zymosan, bacteria, virus, or abnormal red cells requires complement and magnesium. Antigen-antibody aggregates inactivate C'1, C'2, and C'4 and spare C'3. The combination of zymosan with properdin, while requiring complement, inactivates only C'3, sparing C'1, C'2, and C'4. Properdin is thus not an antibody in any acceptable sense.

The widespread occurrence of the properdin system in normal mammalian serum and the diversified nature of its activities affirm the concept of the properdin system as a factor in natural resistance. The studies presented here show that it acts on a wide variety of bacteria independent of taxonomic relationship. In addition it has been found active against viral and protozoan (21) agents and interacts with yeast (zymosan) and with other high molecular weight polysaccharides as well as having lytic activity against certain erythrocytes. It may thus be regarded as an "immune" mechanism in that it may render a host "safe" from infection. It is not, however, an "immune" mechanism within the meaning that it appears in response to a specific stimulus, and to react with a specific agent. The amount of properdin in normal serum is too small for it to be possible that the variety of activities is due to specific individual agents pooled in the preparation of the purified substance.

It is probable that the antibacterial activity of the properdin system is more general than would be interpreted from an uncritical evaluation of the data presented in Table VI. Although only 45 per cent of the strains tested were found susceptible to the system *in vitro*, it is possible that the 23 per cent which were non-viable both in serum and RP_b may also have been susceptible. Two hypotheses are tenable to support this suggestion: These strains of doubtful sensitivity may have in fact been extremely sensitive to the properdin system and traces of properdin remaining in preparations of RP_b may have been sufficient for bactericidal activity. Alternatively the serums may have contained traces of specific antibacterial antibody. The demonstration that 32 per cent of the strains were resistant to the properdin system *in vitro* does not preclude the possibility that these organisms may interact with the properdin system. The yeast from which zymosan is prepared, for instance, is resistant to the properdin system (22), yet the zymosan prepared from the yeast interacts with properdin. Similarly, strains of organisms equally susceptible both to serum and RP have been shown to interact with properdin by different techniques. The *E. coli* strains BV and CV, which are equally susceptible to serum and RP, have been shown by Rowley (23) to combine with properdin at 17° and to inactivate C'3 at 37°. The two strains of *Salmonella typhi* tested were similarly sensitive to serum and RP, yet the O-antigen of *S. typhi* is known to combine with the properdin system *in vivo* and *in vitro*

(11). Thus it is apparent that an organism may have the potentiality of interacting with the properdin system, without this potentiality being demonstrable by present methods of testing the bactericidal effect *in vitro*.

The recent demonstration of the interaction of a variety of complex polysaccharides and bacterial cell walls with properdin (11) suggests that the properdin system may, chemically, be relatively specific. It is likely that a distinct pattern of branching in the polysaccharide, forming the matrix of the cell wall, acts as specific receptor sites for the properdin system. In the more highly evolved bacterium, this cell wall is covered in varying degrees by antigenic envelopes and the receptor sites may thus be blocked in certain organisms. If this hypothesis were true then the removal of such blocking substances would expose the receptor sites for properdin, and the organism would become susceptible. An investigation of this hypothesis is in progress.

Serum deficient in properdin (RP) is essential to the study of the system and is, in this respect, as important as properdin itself. The system involves six known variables; properdin, four complement components, and magnesium. Five of these variables are contained in RP; any one of them can limit the system and the bactericidal effect. A satisfactory RP could be made in only 10 to 20 per cent of serums or serum pools, possibly because of the necessity for the maintenance of adequate levels of these factors. A double adsorption with zymosan in the preparation of RP_b to remove traces of properdin shown to be present after one adsorption was necessary. The effect of this adsorption on complement activity, a requirement for the system, has been described. Also, the effect of dilution on the activity of RP has been shown. All these factors constitute problems in the study of the properdin system and of its mechanism of action in bactericidal phenomena.

The relationship between bactericidal activity and properdin concentration in RP_b suggested that a bactericidal assay for properdin should be possible, and studies were carried out to determine this point. It was clear that apart from the difficulties of preparing a satisfactory RP_b, several other problems were involved in devising such an assay. The organism for the assay had to be carefully chosen. Antibody or bactericidal activities of serum other than the properdin system might interfere with the test. The test organism must be sensitive only to the properdin system in the test serum and to be insensitive to RP_b. The *Sh. dysenteriae* strain was a satisfactory test organism because specific antibody to it was encountered only rarely in this area. Suspensions of this organism were added to graded dilutions of test sample in RP_b, and the bactericidal activities of the mixtures were compared with those of a standard serum or a known properdin preparation. A second procedure was studied using a properdin-sensitive strain of *Proteus*. A suspension of this organism was incubated with graded dilutions of test sample in RP_b, and the property of nitrate reduction of the viable organism used to determine the bactericidal

effect. Further work is at present in progress to determine the accuracy, sensitivity and reproducibility of such assay techniques.

SUMMARY

Methods for the preparation and standardization of reagents suitable for studies on the bactericidal action of the properdin system are described. The preparation and properties of serum free of properdin (RP_b) are presented in detail because of the necessity for a suitable RP_b in these studies.

The properdin system is responsible for the bactericidal action of normal human serum against a variety of microorganisms. The present work shows that the removal of properdin from serum also removes bactericidal activity. Addition of properdin to properdin-deficient serum restores bactericidal activity. A quantitative relationship exists between the final properdin concentration and bactericidal activity against sensitive organisms. The possibilities of a bactericidal assay for properdin are discussed.

It is demonstrated that, in addition to properdin, the four components of complement (present in RP_b) are necessary for the destruction of properdin-sensitive bacteria. If any component is missing, bactericidal activity is lost; when the component is replaced, bactericidal activity is restored. Magnesium is also necessary for the bactericidal activity of the properdin system. Maximal bactericidal activity is obtained with magnesium concentrations similar to that of normal human serum (10^{-3} to 10^{-4} M). The bactericidal activity of the properdin system occurs only at temperatures above 15°.

Resistant strains have been encountered in species of bacteria sensitive to the properdin system. Resistance or sensitivity is a characteristic of the individual strain and not of the species.

The widespread occurrence of the properdin system in normal mammalian serum and the variety of bacteria destroyed by it suggest that the properdin system is a factor in natural resistance.

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BIBLIOGRAPHY

1. Pillemer, L., Blum, L., Lepow, I. H., Ross, O. A., Todd, E. W., and Wardlaw A. C., The properdin system and immunity. I. Demonstration and isolation of a new serum protein, properdin, and its role in immune phenomena, *Science*, 1954, **120**, 279.

2. Pillemer, L., The properdin system. *Tr. New York Acad. Sc.*, 1955, **17,** 526.
3. Hinz, C. F., Jr., Jordan, W. S., Jr., and Pillemer, L., Properdin: A specific hemolytic factor in paroxysmal noctural hemoglobinuria, *J. Lab. Clin. Med.*, 1954, **44,** 811.
4. Hinz, C. F., Jr., and Pillemer, L., The requirement for the properdin system in the hemolysis of human erythrocytes treated with tannic acid, *J. Clin. Inv.*, 1955, **34,** 912.
5. Wardlaw, A. C., Blum, L., and Pillemer, L., Bactericidal activity of the properdin system in human serum, *Fed. Proc.*, 1955, **14,** 480.
6. Wedgwood, R. J., Ginsberg, H. S., Seibert, R. H., and Pillemer, L., The inactivation of Newcastle Disease virus by the properdin system, *Am. J. Dis. Child.*, 1955, **90,** 508.
7. Rowley, D., Stimulation of natural immunity to *Escherichia coli* infections. Observations on mice, *Lancet* 1955, **1,** 232.
8. Ross, O. A., Moritz, A. R., Walker, C. J., Wurz, L., Todd, E. W., and Pillemer, L., Role of the properdin system in whole body irradiation, *Fed. Proc.*, 1955, **14,** 496.
9. Frank, E., Fine, J., and Pillemer, L., Serum properdin levels in hemorrhagic shock, *Proc. Soc. Exp. Biol. and Med.*, 1955, **89,** 223.
10. Ginsberg, H. S. and Horsfall, F. L., Jr., A labile component of normal serum which combines with various viruses. Neutralization of infectivity and inhibition of hemagglutination by the component, *J. Exp. Med.*, 1949, **90,** 475.
11. Pillemer, L., Schoenberg, M. D., Blum, L., and Wurz, L., Properdin system and immunity. II. Interaction of the properdin system with polysaccharides, *Science*, 1955, **122,** 545.
12. Kabat, E. A., and Mayer, M. M., Experimental Immunochemistry, Springfield, Illinois, C. C. Thomas, 1948.
13. Pillemer, L. and Ecker, E. E., Anticomplementary factor in fresh yeast, *J. Biol. Chem.*, 1941, **137,** 139.
14. Pillemer, L., and Ratnoff, O. D., Blum, L., and Lepow, I. H., The inactivation of complement and its components by plasmin, *J. Exp. Med.*, 1953, **97,** 573.
15. Lepow, I. H., Wurz, L., Ratnoff, O. D., and Pillemer, L., Studies on the mechanism of inactivation of human complement by plasmin and by antigen-antibody aggregates. I. The requirement for a factor resembling C'1 and the role of Ca^{++}, *J. Immunol.*, 1954, **73,** 146.
16. Lepow, I. H., unpublished procedure.
17. Roth, F. and Pillemer, L., unpublished procedure.
18. Lepow, I. H., Pillemer, L., and Ratnoff, O. D., The influence of calcium ions on the inactivation of human complement and its components by plasmin, *J. Exp. Med.*, 1953, **98,** 277.
19. Pillemer, L., Blum, L., Lepow, I. H., and Wurz, L., The properdin system and immunity. III. The zymosan assay of properdin, *J. Exp. Med.*, 1956, **103,** 1.
20. Pillemer, L., unpublished data.
21. Gronroos, P., The action of properdin in toxoplasma gondii. A preliminary report, *Ann. Med. Exp. et Biol. Fenniae*, in press.
22. Wardlaw, A. C., unpublished data.
23. Rowley, D., personal communication.