AN ALTERNATIVE MECHANISM FOR THE PROPERDIN SYSTEM*

By ROBERT A. NELSON, JR., M.D.

(From the Department of Microbiology, Yale University Medical School, and The Howard Hughes Medical Institute, New Haven)

(Received for publication, May 21, 1958)

In 1954 Pillemer and his associates (1) described experiments which indicated the presence in normal serum of a protein, named properdin (P), to which was ascribed a basic role in varied areas of academic and clinical interest. This protein was stated to combine with zymosan (Z) at temperatures only above 15° and in the presence of both Mg^{++} and certain components of complement (C′). At 37° the Z·P complex was stated to inactivate the third component of C′ (C'_3) without fixing the other components of C′, provided that Mg^{++} was present. From subsequent work (2) properdin has assumed a position of importance in explanations for the mechanism of natural immunity against bacteria and viruses, and for the defect in the immune mechanism following irradiation. In view of the reactivity of this substance in a variety of apparently unrelated phenomena, a re-examination of certain elementary aspects of the properdin system seemed indicated in order to definitely establish the specific nature of this hypothetical protein. In the present study this has been approached through a detailed investigation of fixation of the components of C′ in guinea pig serum in order to learn the mechanism of inactivation of the third component by Z as compared with inactivation by certain classical antigen-antibody complexes.

While the evidence supporting the concept of properdin as a single substance was persuasive, it seemed equally reasonable that many of the characteristics of properdin could be explained on the basis of low levels of antibody (Ab) in normal serum capable of reacting with zymosan or related substances in conjunction with certain components of C′. However, as emphasized initially by Pillemer, there was no evidence for Ab to zymosan, and certain characteristics of properdin, *e.g.* its apparent lack of serological specificity, the requirement for Mg^{++} and for C′, and the unique aspects of reactivity at 15°, were not compatible with classical notions of Ab reactivity.

In view of the evidence available at that time (November, 1955) an investigation was initiated to test the following two postulates. First, the zymosan-properdin complex is composed of varying proportions of three complexes, Z·Ab, Z·Ab·$C'_{1,4}$, and

* Reports of this work were presented at the Connecticut Valley branch of the Society of American Bacteriologists in October, 1956, and at a Conference on Complement, Walter Reed Institute for Research in March, 1957.

$Z \cdot Ab \cdot C'_{1,4,2}$, dependent upon the time and temperature involved in preparation and purification; and, second, the purported selective inactivation of C'_3 by Z reacting with whole serum was misconstrued in the properdin studies owing to the expression of residual units of C′ components in terms of percentage rather than of reactive units. Since by the usual technique the number of reactive units of C'_3 in whole serum represents only $\frac{1}{15}$ to $\frac{1}{20}$ the number of reactive units of $C'_{1,4}$, an equivalent loss of both components would appear as more significant for loss of C'_3 than for $C'_{1,4}$. In addition, the loss of only 100 to 400 units of $C'_{1,4}$ would be masked within the error of measurement (±50 per cent) inherent in the classical technique for titration of components.

From this hypothesis and from recent studies by Cowan (3) on the neutralization of bacteriophage by normal serum has evolved the concept that the "natural" Ab's in different sera have varying avidity for different polysaccharide antigens and that with Ab of low avidity C′ may serve to strengthen the union between Ab and antigen. Thus, under certain conditions C′ would appear to be necessary for the "combination" of Ab with antigen, perhaps analogous to the model proposed by Dacie *et al.* with "incomplete" hemolytic antibody (4).

Since zymosan has been employed extensively as the model for studies on properdin, most of the experiments here described concern: (*a*) evidence for the presence in normal serum of Ab which combines with Z; and (*b*) measurements of fixation of $C'_{1,4}$ and of C'_3 by Z reacting with whole serum. It should be noted as a limitation in this study that most of the latter have been performed with guinea pig C′ since this represents the only species which had been studied thoroughly with regard to quantitative measurements of C'_3 and to the exact requirements for divalent cations and for temperature during fixation. Presumptive evidence has been obtained for the formation and "decay" of the complex $Z \cdot Ab \cdot C'_{1,4,2}$ in this complex reacted with C'_3 in the presence of ethylenediaminetetraacetate (EDTA)[1] added to bind Ca^{++} and Mg^{++}, but lost this ability to react with C'_3 after incubation at 37°. The results with Z have been compared with measurements of C′ fixation by *Diplococcus pneumoniae* and by washed precipitates of a complex consisting of bovine serum albumin–rabbit antiserum (BSA–anti BSA). While not extensive, the latter results are interpreted as indicating that under controlled experimental conditions, approximately equivalent ratios of units of C'_3 to $C'_{1,4}$ are fixed in all three systems. Finally, initial experiments have been performed with purified properdin which indicate that this material contains significant amounts of what is interpreted to be classical Ab.

EXPERIMENTAL

I. Preliminary Experiments.—Initial evidence for Ab in normal serum which reacts (or cross-reacts) with Z came from experiments on immune

[1] EDTA, ethylenediaminetetraacetate.

adherence (I-A)[2] of Z with human erythrocytes. A brief description of two early experiments follows:—

Experiment 101755.—A pool of fresh serum from normal guinea pigs was "passed" through amberlite IRC-50 to remove Mg^{++} and Ca^{++}. A sample of non-passed serum was heated at 56° for 45 minutes. Reaction mixtures were prepared containing 0.5 ml. of washed zymosan[3] with: (*a*) veronal buffer with Ca^{++} and Mg^{++} (VB^{++}); (*b*) heated serum and VB^{++}; (*c*) fresh "passed" serum and "passed" veronal; and (*d*) fresh "passed" serum and VB^{++}. After 30 minutes at 37° the zymosans were washed and mixed with human erythrocytes. Upon microscopic examination, 85 per cent of the particles from mixture (*d*) were found to be adherent to the erythrocytes, while essentially none were adherent from mixtures (*a*), (*b*), and (*c*).

Since evidence to date indicates that both Ab and C′ are required for I-A (6), these results suggested that the normal serum contained Ab which combined with Z to fix C′ and induce susceptibility to I-A. In assays with pneumocci and with BSA-anti BSA, it has been found that I-A will occur with serum diluted to contain only about 0.01 μg. Ab nitrogen (N). In subsequent assays with guinea pig serum, 1 ml. of a $\frac{1}{500}$ dilution sensitized Z to react in I-A, suggesting that undiluted serum contained amounts of Ab approximately 5 μg. Ab N. An absorption of the Ab from serum at 0° in the relative absence of divalent cations was performed next.

Experiment 111455.—To 1 gm. of Z, washed with "passed" VB, was added 25 ml. of cold, fresh, and "passed" serum from a normal human being. After agitation by hand for 30 minutes at 0°, the mixture was centrifuged, and the supernatant serum absorbed with another 1 gm. of Z for 30 minutes at 0°. The zymosan was removed by centrifugation at 20,000 R.P.M. for 30 minutes. A sample of "non-passed" serum was heated at 56° for 60 minutes. Four mixtures were made of Z suspended in VB^{++} with: (*a*) VB^{++} only; (*b*) heated serum; (*c*) fresh, absorbed serum; and, (*d*) heated serum *plus* fresh absorbed serum. The absorption of fresh serum at 0° in the absence of Mg^{++} and Ca^{++} resulted in complete loss of ability to induce I-A. While serum heated at 56° similarly was non-reactive, the combination of heated serum with fresh absorbed serum resulted in restoration of I-A reactivity.

These results demonstrated that I-A with Z requires both a relatively heat-stable component of normal serum which was removed by absorption of serum at 0° in the relative absence of divalent cations, and a heat-labile component of normal serum, acting only in the presence of Ca^{++} and/or Mg^{++}. Tentatively, the two components were interpreted to be Ab and C′ reacting in the classical fashion. Subsequent experiments showed that the relative heat stability of Ab varies with the species from which the normal serum was obtained, and that the ease with which Ab may be removed by absorption in the cold in the presence of EDTA varies with the individual serum employed

[2] I-A, immune adherence.

[3] Zymosan for these early experiments was supplied by Dr. D. Rowley. Zymosan, lots 5B-171, 6B-382, 6B-383, and 6B-14, was purchased from Standard Brands Inc., for subsequent experiments.

and presumably is dependent upon the avidity as well as the total content of Ab in the serum.

II. Agglutination.—It was soon found that sera from a variety of animals and human beings would induce agglutination of Z particles (Fig. 1), but

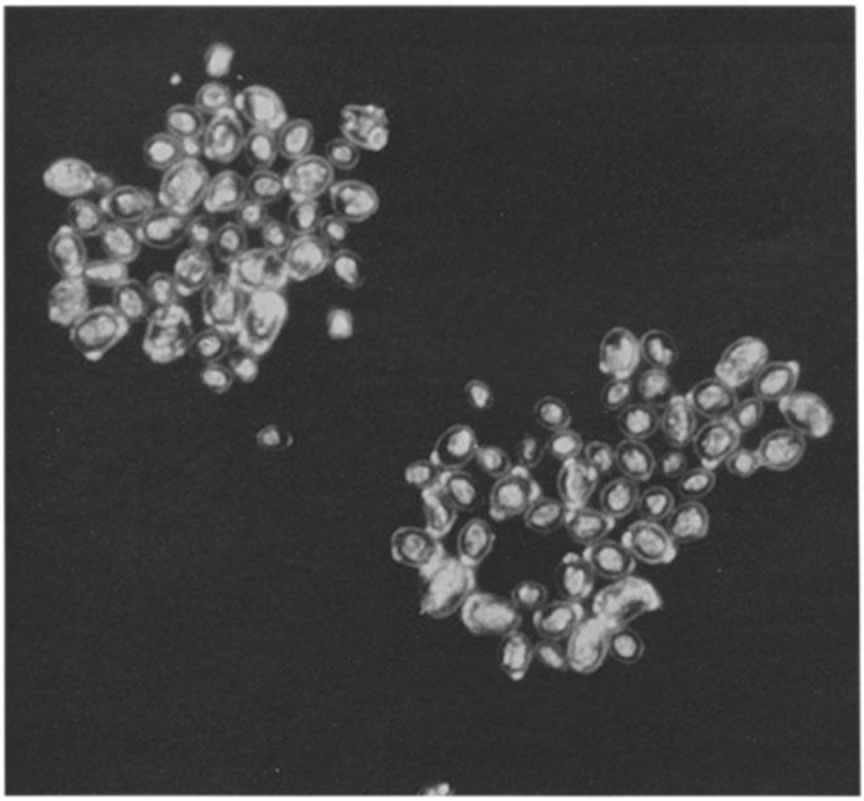

FIG. 1. Darkfield photomicrograph of agglutinated zymosan granules. The mixture contained zymosan, EDTA, a $\frac{1}{16}$ dilution of normal serum K.H., and was incubated at 2°C. overnight.

usually only at low dilutions of serum. Care was taken to exclude the possibility that agglutination would be caused by properdin as defined by Pillemer, *i.e.*, all mixtures were incubated at 0 to 2° and in the presence of 0.01 M EDTA (final concentration). Nonetheless, the agglutinating reactivity was found in fraction $III_{1, 2, 3}$ of the serum protein after treatment of a human serum by the Cohn method 10 (7). The agglutinating reactivity of several sera was unchanged after removal of C′ by a heterologous antigen-Ab complex; *i.e.*, BSA-anti BSA. In addition to this evidence for Ab in normal sera,

Z has been shown to increase Ab levels when injected into rabbits or guinea pigs.

Experiment 083056.—A stock suspension of Z containing 10 mg. per ml. was washed 3 times in 0.02 M EDTA and parallel 2-fold dilutions were made to 1/32 in EDTA at 2°. Duplicates of 0.5 ml. of each suspension were mixed with 1.0 ml. of cold undiluted serum from a rabbit injected 12 times each with 10 mg. Z. The rabbit serum had been "decomplemented" previously by treatment with a washed precipitate containing 2.2 mg. antibovine serum albumin N. Duplicates of 1/1 and 1/2 Z without serum were included as controls. The mixtures

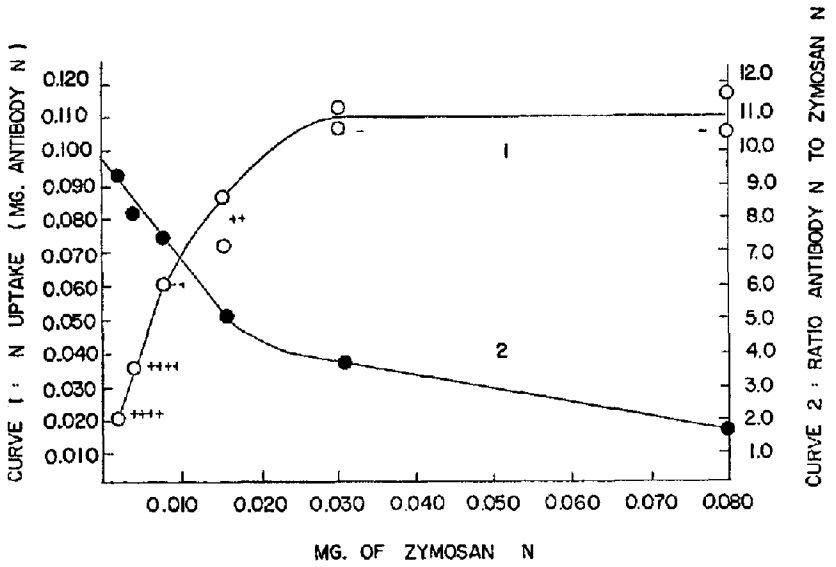

FIG. 2. Uptake of N by zymosan in quantitative agglutination with serum from a rabbit injected with zymosan. Tests for residual agglutinating activity in the supernatant serum are recorded for each mixture.

were rotated (about 6 R.P.M.) at 0 to 2° for 24 hours. Marked agglutination of Z occurred in all mixtures with serum. The mixtures were centrifuged and the supernatant fluids were tested for residual agglutinating activity by addition of 1.0 ml. containing 100 μg. of fresh Z. The deposited particles were washed 4 times and examined for N by a modified micro-Kjeldahl method using a fine capillary tip for titration with HCl.

A plot of the N uptake is shown in Fig. 2 and a summary of N uptake and agglutination titers of sera from this rabbit and of other sera is shown in Table I. As yet no insight is available into possible dissociation of Ab from Z. Therefore the N values obtained to date are considered as representative of minimal Ab per milliliter of serum. On the other hand, it may be that other serum components containing N will combine with particulate substances such as zymosan granules under the circumstance outlined herein. Nonetheless, it

seems highly unlikely that any material other than Ab would invoke the variety of immunological phenomena as described in the present study.

III. Fixation of $C'_{1,4}$ and of C'_2.—Patterned upon the experiments of Levine and Mayer (8) an attempt has been made to measure precisely the residual $C'_{1,4}$ after treatment of guinea pig serum with Z only; with Z presensitized

TABLE I

Agglutination Titration and Measurement of Antibody N Uptake by Quantitative Agglutination Assays with Zymosan and Sera from Normal Human Beings and Animals. Increase of Antibody in Sera from Animals Injected with Zymosan

Species of serum	Agglutination titer	μg. N* per ml. serum	Species of serum	Agglutination titer	μg. N* per ml. serum
	per ml. serum			*per ml. serum*	
Human			Guinea pig		
P.B.	8	5.7	Pool Y 080856	6	2.7
			Pool C 062256	8	2.3
S.C.	48	14.0	Pool C 011157	4	4.5
K.H.	128	23.5	No. T preinjection‡	10	
			No. T postinjection‡	250	
K.C.	32	7.7			
			Rat		
A.M.	48	7.7			
			Pool C 062256	24	10.1
E.F.	24	11.0			
			Rabbit		
D.N.	16				
			No. 2 preinjection‡	10	3.7
R.N.	16		No. 2 postinjection‡	250	110.0
			No. 3 preinjection‡	10	
D.G.	32		No. 3 postinjection‡	250	

* Control preparations of 1.0 mg. of zymosan showed an average of .0177 mg. nitrogen: .0180 mg. (7/11/56); .0178 mg. (8/30); .0184 mg. (9/6); .0194 mg. (10/22); .0194 mg. (10/31); and .0144 mg. (11/29). Blank spaces indicate that measurements were not done.

‡ Titration for agglutination performed with 5-fold dilutions of serum instead of usual 2-fold dilutions.

with Ab and washed; with the washed precipitate of BSA-anti BSA; and, with *D. pneumoniae*, type XIII. Certain theoretical considerations have been modified in order to employ a reasonably simple technique applicable to the large number of serum assays required in the present investigation.

In brief, the assay consisted of two stages. First, in order to form the complex $EAC'_{1,4}$ a suspension of sensitized sheep erythrocytes (EA)[4] was exposed at 0° for 4 hours to dilutions of the serum under examination. Then, to each

[4] EA, suspension of sheep erythrocytes sensitized with rabbit amboceptor.

tube was added diluted C'y (hereafter referred to as $C'_{2,3}$ for clarity) reagent and lysis was allowed to proceed at 37°. The $C'_{2,3}$ reagent was prepared as in reference 8 in except that only one absorption with bovine albumin–anti-albumin containing 4.4 mg. Ab N was necessary to remove all hemolytic activity. The resultant $\frac{1}{10}$ dilution of $C'_{2,3}$ reagent was preserved at −35°. In preliminary assays with $EAC'_{1,4}$, 0.5 ml. of this reagent produced 79.2 per cent lysis when diluted $\frac{1}{160}$. In the present assays 0.5 ml. of a $\frac{1}{20}$ dilution was used to provide a large excess.

Provisional Method.—Dilutions of the serum were made at 0° in veronal buffer containing 0.1 per cent crystalline bovine serum albumin (SAVB[5]) and 0.00015 M Ca^{++}, but no Mg^{++}. Sheep erythrocytes were sensitized with rabbit amboceptor[6] in SAVB-Ca^{++} and standardized with the Beckman DU spectrophotometer so that 1.0 ml. diluted to 15 ml. with water resulted in an optical density (O.D.) of 0.340 at 414 mμ. Mixtures containing 1.0 ml. of serum dilution and 1.0 ml. of EA were shaken by hand intermittently during 4 hrs. at 0°. At that time, the stock $\frac{1}{10}$ dilution of $C'_{2,3}$ reagent in SAVB-Ca^{++} was diluted $\frac{1}{2}$ in SAVB-Ca^{++} containing 0.005 M Mg^{++}, and warmed to 37°. 0.5 ml. of $\frac{1}{2}$ $C'_{2,3}$ was added to each of the 2.0 ml. of EA-serum mixtures and to controls containing EA only to yield a final 0.0005 M Mg^{++}, and brought to 37° as rapidly as possible. Each was shaken frequently for 60 minutes at 37°. 5.0 ml. of cold saline was added to each and the mixtures centrifuged. The degree of lysis was determined by analysis of the supernatant fluids for oxyhemoglobin at 414 mμ. In all assays the units of $C'_{1,4}$ represent the reciprocal of the serum dilution producing 50 per cent lysis of EA in the presence of "excess" $C'_{2,3}$ reagent.

Two separate assays were required for most measurements. A preliminary experiment with 2-fold dilutions of serum served to locate the range of hemolysis, but as a rule only one dilution produced lysis between 10 and 90 per cent. A second assay was performed with smaller dilutions made by the parallel technique, to locate precisely the dilution yielding 50 per cent lysis. With the high dilutions of serum giving $C'_{1,4}$ activity, it has been found that total reactivity with EA was not always complete even after 4 hours at 0°. Therefore the erythrocytes were not washed before addition of the $C'_{2,3}$ reagent. The depressing effect of low temperature on the rate of combination of $C'_{1,4}$ confirms the results in (9) and would seem pertinent in theoretical consideration of the optimal temperature of 16° for the formation of the so called Z·P complex.

Experiment 081556 and 081856.—Two essentially similar experiments were performed. 3 mg. of washed Z were mixed for 2 hours at 2° with 1.5 ml. of decomplemented rabbit immune serum, diluted $\frac{1}{5}$ in 0.01 M EDTA. The rabbit serum was estimated to contain 33 μg. Ab N.

Reaction of guinea pig serum with Z and with Z Ab: 2 samples of 1.5 ml. each of fresh serum were mixed respectively with 3 mg. of washed Z and with 3 mg. of washed Z·Ab for 17 hours at 2°. A third sample of 1.5 ml. of serum has handled similarly as a non-treated con-

[5] SAVB, veronal buffer containing 0.1 per cent crystalline bovine serum albumin.

[6] The rabbit antiserum was supplied by Dr. H. J. Rapp of Johns Hopkins University and was used at a dilution calculated to yield about 1,000 antibody molecules per erythrocyte.

trol. In experiment 081856 a fourth sample of the guinea pig serum was included as another untreated control. The three sera were centrifuged twice to remove Z and then were tested for residual $C'_{1,4}$ using 2-fold dilutions of serum in the first experiment and varying volumes of a dilution of each serum in the second. Although the variation in volume in the latter interfered with the accuracy of the measurement in that all points in the plot of C′ concentration against lysis did not fall on a straight line, the results were clear regarding the relative reactivity of the 3 serum samples. The results of both experiments are included in Table II.

TABLE II

*Measurements of Residual $C'_{1,4}$ in 1.5 Ml. of Guinea Pig Serum Absorbed 17 Hours at 2° with 3 Mg. of Zymosan and with 3 Mg. of Zymosan Presensitized with Rabbit Immune Serum**

Guinea pig serum pool 080856‡	Experiment 081556			Experiment 081856		
	Residual $C'_{1,4}$			Residual $C'_{1,4}$		
	Units per ml.	Units lost	Per cent loss	Units per ml.	Units lost	Per cent loss
Unabsorbed control	2460	—	—	2140	—	—
" "				2140	—	—
Absorbed with zymosan	1940	520	21.2	1540	600	28.0
" " zymosan-anti-zymosan complex	904	1556	63.1	750	1390	64.9

* The rabbit immune serum was "decomplemented" by treatment for 48 hours at 2°C. with a washed precipitate consisting of 0.08 mg. bovine serum albumin nitrogen and 0.58 mg. antibovine albumin nitrogen. For formation of the Z-Ab complex 1.5 ml. of a 1/5 dilution of decomplemented serum was mixed with 3 mg. of Z at 0° in 0.01 M EDTA. This dilution contained 33 μg. Ab N.

‡ Subsequent experiments were interpreted to mean that this pool of normal guinea pig serum contained between 2 and 3 μg. of antibody N per ml. which was capable of combining with zymosan particles in the presence of EDTA.

During incubation of the mixtures of Z with pooled guinea pig sera there were losses of 520 and 600 units of $C'_{1,4}$. With the present titration technique this is significant. Despite the large number of units lost, it is to be noted that these represent only 21 and 28 per cent of the total units in untreated serum. In similar experiments not shown, the fixation of $C'_{1,4}$ in both human and guinea pig sera by Z was inhibited by 0.004 M EDTA. In experiments described below it will be seen that the fixation of between 200 and 600 units of $C'_{1,4}$ is accompanied by a loss of 49 to 100 per cent of C'_3. This evidence would serve to support our original contention that the fixation of $C'_{1,4}$ and C'_3 may attain a ratio approaching unity when experimental conditions are adjusted to facilitate fixation of C'_3, in particular with limited Ab in a reaction of antigen with concentrated serum at temperatures near 37°.

Employing the method of Rapp (10), measurements of residual C'_3 have been made after treatment of guinea pig serum with Z, with *D. pneumoniae*, and with the washed precipitate of BSA-anti BSA.

Method.—(*a*) The complex $EAC'_{1,4,2}$ was formed at 0° by mixing for exactly 20 minutes 2.0 ml. of whole guinea pig serum, as C′, with 10.0 ml. of EA standardized to yield an O.D. of 0.680 at 541 mμ. The treated erythrocytes were washed once and incubated for 4 hours at 0° to permit lysis of any cells which fixed all C′ components, but to retard by the low temperature, in so far as possible, the decay of $EAC'_{1,4,2}$ to an "inactive" complex. The erythrocytes were washed in SAVB, without divalent cations, were resuspended to 8 times original volume in SAVB containing 0.0005 M EDTA, and were restandardized spectrophotometrically to yield an O.D. of 0.680 at 414 mμ.

(*b*) 0.5 ml. of cold $EAC'_{1,4,2}$ was pipetted into two samples of 1.5 ml. of guinea pig serum (unabsorbed control and absorbed test samples) diluted in cold 0.0005 M EDTA but prewarmed to 37° just before addition of $EAC'_{1,4,2}$. The mixtures were shaken by hand for 60 minutes at 37°, and then brought to 7.5 ml. with cold saline. The supernatant fluids were assayed for oxyhemoglobin spectrophotometrically at 414 mμ. C'_3 units were expressed as the reciprocal of the dilution of serum giving 50 per cent lysis of $EAC'_{1,4,2}$.

From the foregoing experiments it was clear that Z would fix about 500 to 600 units of $C'_{1,4}$ while fixing about 100 to 150 units of C'_3 when mixed with fresh guinea pig serum. However, as numerically the $C'_{1,4}$ units in guinea pig serum were about 15 to 20 times the number of C'_3 units, the *percentage* loss of $C'_{1,4}$ was low. As it was well known that essentially all the $C'_{1,4}$ could be removed from guinea pig serum with classical antigen-antibody complexes containing large amounts of Ab, it was deemed of prime importance to measure the relative quantities of $C'_{1,4}$ and C'_3 fixed when the Ab was present in small amounts more comparable to the postulated levels in normal sera which react with Z.

Experiment 011657.—0.5 ml. of a 5 per cent suspension of washed *D. pneumoniae* type XIII, was centrifuged in each of 5 tubes. To the packed deposits and to a control without pneumococci was added 1.0 ml. of fresh guinea pig serum which had been absorbed once with pneumococci for 30 minutes at 0° to reduce natural Ab. After thorough mixing, 0.5 ml. of dilutions in $SAVB^{++}$ of a rabbit anti-pneumococcal serum containing 2.6 mg. of Ab N per ml. was added as follows: (*a*) $\frac{1}{5}$ dilution containing 260 μg. Ab N; (*b*) $\frac{1}{50}$ dilution containing 26 μg. Ab N; (*c*) $\frac{1}{500}$ dilution containing 2.6 μg. Ab N; (*d*) no antiserum added, *i.e.*, a control with pneumococci only; (*e*) neither antiserum nor pneumococci added, *i.e.*, a control for C′ titration. After frequent mixing for 60 minutes at 37°, the tubes were cooled to 0° and the pneumococci removed by 2 centrifugations. The residual titers of $C'_{1,4}$ and of C'_3 were determined as described above.

The residual $C'_{1,4}$ and C'_3 titers from 5 mixtures are listed in Table III. Despite the absorption of the guinea pig serum with pneumococci, there remained enough sensitizing substance to remove 240 $C'_{1,4}$ units, *i.e.*, about 8.6 per cent, from the serum exposed to pneumococci alone for 60 minutes at 37°. At the same time, the reaction with pneumococci which resulted in a loss of 240 units of $C'_{1,4}$ also resulted in a loss of 94 units of C'_3, *i.e.*, about 87 per cent. Only traces of C'_3 remained in the sample containing extra rabbit pneumococcal Ab. Thus, the ratio of fixation of $C'_{1,4}$ and C'_3 by the pneumococcus was remarkably similar to that produced by Z. The removal of properdin by certain pneumococci has been described (2), although it was

stated that inactivation of other components of C′ occurred. The present results would suggest that with serum containing small amounts of what is presumed to be natural Ab, fixation of C′ components is essentially the same with Z and with pneumococci. With serum containing more than 20 μg. of natural Ab N to whatever antigen might be employed, it would be anticipated that moderate to marked depletion of $C'_{1,4}$ would occur.

In order to investigate further the premise that limited Ab was responsible for the apparent preferential fixation of C'_3 and to avoid the problem of natural antibody in the guinea pig C′, experiments were performed with washed specific precipitates formed at equivalence and diluted to contain small

TABLE III

Measurements of $C'_{1,4}$ and C'_3 in Guinea Pig Serum Treated with D. pneumoniae, Type XIII, for 60 Minutes at 37°

Guinea pig serum pool 011157	Residual $C'_{1,4}$			Residual C'_3*		
	Units per ml.	Units lost	Per cent loss	Units per ml.	Units lost	Per cent loss
Unabsorbed control	2790			108		
Absorbed with pneumococci	2550	240	8.6	12	94	87
Absorbed with pneumococci and rabbit anti-serum						
0.26 μg. Ab N	2330	460	16.5	8	100	92.5
2.6 " " "	2220	570	20.4	8	100	92.5
26.0 " " "	1050	1740	62.4	5	103	95.4
260.0 " " "	0	2790	100	3	105	97.4

* Titers of less than 10 were calculated on the basis of lysis produced by 1.0 ml. of a $\frac{1}{10}$ dilution of serum.

amounts of Ab N. Although it was recognized that fixation of C′ may be less efficient per unit of antibody when C′ is mixed with the washed precipitate rather than when C′ is present during the formation of the antigen-Ab complex, the results were considered mainly for the ratio of fixation of $C'_{1,4}$ to C'_3 for any given amount of precipitate rather than for the amounts of components fixed per μg. of Ab N.

Experiments 090156 and 012557.—An immune precipitate containing 4.40 mg. anti-BSA was prepared during 48 hours at 2° in the presence of 0.01 M EDTA. The precipitate was washed and diluted to contain 440, 44, and 4.4 μg. per ml. 0.5 ml. of each was pipetted into 6 ml. tubes and deposited during centrifugation at 20,000 R.P.M. for 20 minutes. 1 ml. of fresh guinea pig serum was added to each precipitate and to a control tube without precipitate, and mixed for 60 minutes at 37°. The precipitates were removed by centrifugation and the supernatant fluids were assayed for residual $C'_{1,4}$ and C'_3 as outlined above.

Results from 2 preliminary trials with BSA-anti BSA are summarized in Table IV. Although some variations occurred in the 2 experiments, which

were performed 4 months apart, results were similar to those obtained with Z and with pneumococci. While the percentage loss of C'_3 was significantly higher than the percentage loss of $C'_{1,4}$, the loss of reactivity units of $C'_{1,4}$ was 3 to 8 times the loss of C'_3.

IV. The Intermediate Complex $Z \cdot Ab \cdot C'_{1,4,2}$.— An integral part of the alternative hypothesis presented herein concerns the formation of a complex $Z \cdot Ab \cdot C'_{1,4,2}$ and the reactivity of this complex with C'_3 in the absence of Ca^{++} and/or Mg^{++}. Conditions for formation of a similar complex with the

TABLE IV

Measurements of $C'_{1,4}$ and C'_3 in Guinea Pig Serum Treated with Varying Quantities of a Washed Precipitate of Bovine Serum Albumin and Rabbit Antiserum

Guinea pig serum	Residual $C'_{1,4}$			Residual C'_3		
	Units per ml.	Units lost	Per cent loss	Units per ml.	Units lost	Per cent loss
Unabsorbed control 011157*	2250	—	—	120	—	—
Absorbed with precipitate						
2.2 μg. Ab N	2350	+100	+4.4	110	10	8.3
22 " " "	2050	200	8.9	58	62	51.7
220 " " "	84	2166	96.2	2	118	98.2
Unabsorbed control 080856‡	1630	—	—	103	—	—
Absorbed with precipitate						
10 μg. Ab N	1620	10	0.6	64	39	37.6
20 " " "	1280	350	21.4	53	50	48.6
40 " " "	990	640	39.2	27	76	73.8

* In Experiment 012557, the precipitates reacted with serum for 60 minutes at 37°.

‡ In Experiment 090156, residual $C'_{1,4}$ was measured after reaction for 17 hours at 2°, and residual C'_3 was measured after 60 minutes at 37°.

classical sheep hemolytic system have been described (9). Preliminary experiments have been performed with Z and with pooled fresh guinea pig serum which indicate that a complex will form in less than 30 minutes at 0° in the presence of Ca^{++} and Mg^{++} which, when washed and mixed at 37° with guinea pig serum containing EDTA, will remove C'_3 to about the same extent as control mixtures containing Z and guinea pig serum with Ca^{++} and Mg^{++}.

Experiment 011257.—Five suspensions were prepared each containing 5 mg. of Z in 5 ml. $SAVB^{++}$. Step 1: At 0°, 5 ml. of a fresh pool of guinea pig serum were added to each of four suspensions of Z for varying times, *i.e.*, 180, 120, 60, and 30 minutes, respectively. No serum was added to the fifth suspension. All preparations were centrifuged at 0° and washed 3 times in cold, "passed" SAVB. Step 2: 1.5 ml. of prewarmed, pooled guinea pig serum (as C'_3) containing 0.005 M EDTA was added to each of the 5 cold deposits of Z granules and to a control tube without Z. The mixtures were incubated for 60 minutes at 37° and then cooled to 0°. The Z was removed by centrifugation. Step 3: The supernatant sera were diluted in cold

SAVB^{-} containing 0.0005 M EDTA and were titrated for residual C'_3 as outlined above with $EAC'_{1,4,2}$.

At step 2, two additional controls were included to show the expected reactivity of Z with C'_3 in fresh guinea pig serum in the presence of Ca^{++} and/or Mg^{++} as described by Pillemer (1). 1.5 ml. of guinea pig serum (no EDTA) was added to 5 mg. of Z and to a control tube without Z, and incubated at 37° for 60 minutes. Both were subsequently handled as outlined in step 3.

TABLE V

Residual C'_3 after Reaction of Guinea Pig Serum with Zymosan Compared with Residual C'_3 after Reaction of Guinea Pig Serum in the Presence of EDTA (C'_3) with Zymosan and with Washed Zymosan Pretreated at 0°C. to Form the Theoretical Complex $Z \cdot Ab \cdot C'_{1,4,2}$

I. Controls—Guinea pig serum with Ca^{++} and Mg^{++} reacted with zymosan

Guinea pig serum at 37° 60 min.	Residual C'_3		
	Units per ml.	Units lost	Per cent loss
Zymosan	<2	>98	>98
Control—no zymosan	83	—	—

II. Guinea pig serum as C'_3 with EDTA, reacted with treated zymosans

Pretreatment of Z with guinea pig serum to form $Z \cdot Ab \cdot C'_{1,4,2}$	Residual C'_3		
	Units per ml.	Units lost	Per cent loss
0° 30 min	10	90	90.0
0° 60 "	<3	>97	>97
0° 120 "	<3	>97	>97
0° 180 "	<3	>97	>97
Z control, no pretreatment	96	4	4.0
C'_3 control, no zymosan	100	—	—

The residual C'_3 reactivities with $EAC'_{1,4,2}$ are shown in Table V. As expected in the control mixture, 5 mg. of Z removed 98 per cent of the C'_3 reactivity from fresh guinea pig serum. Only 4.0 per cent of C'_3 reactivity was removed by Z when EDTA was present in the guinea pig serum. In striking contrast to the failure of Z to react with C'_3 in the presence of EDTA, all samples of Z pretreated with serum at 0° and then washed, removed 90 per cent or more of C'_3 reactivity.

The ability of Z to form a complex at 0° which will react with C'_3 in the absence of Mg^{++} and Ca^{++} is remarkably similar to the formation of $EAC'_{1,4,2}$ and its reactivity with C'_3 as in the sheep erythrocyte hemolytic system. Since preceding experiments indicate a loss of $C'_{1,4}$ when Z reacts with guinea pig serum at 0° (Table II) the present experiment is suggestive that C'_2 also is fixed in the classical fashion.

The complex presumed to be $Z \cdot Ab \cdot C'_{1,4,2}$ differs from the usual definition

of Z·P in two ways: 1, it is formed at 0°, while Z·P is usually prepared at 15 to 17°; and 2, it will inactivate C'_3 in the absence of Mg^{++}, while Z·P requires Mg^{++} for reaction with C'_3. However, it is known with the sheep cell system that the complex $EAC'_{1,4,2}$ is unstable and it has been found that a loss of reactivity with C'_3 occurs rapidly at temperatures significantly above above 0° (5). A portion of complexes at sites on the erythrocytes, however, progress only to the stage $EAC'_{1,4}$ during the 20 minute interval ordinarily employed for formation of $EAC'_{1,4,2}$ and it has been shown that these sites will accept C'_2 in the presence of Mg^{++} and will then fix C'_3 with subsequent lysis.

Since Z·P is formed at 15 to 17°, it might be expected also that a portion of the sites (S) $S\cdot Ab\cdot C'_{1,4,2}$ on the zymosan granule would, under certain circumstances, "decay" before contact with, and inactivation of C'_3 in the two step properdin assay. However, in the assay technique for the reaction of Z·P with C'_3 as employed by Pillemer it is to be noted that both additional $C'_{1,4}$, and additional C'_2 are available in the reagent termed R·P and therefore would be available for the conversion of S·Ab to $S\cdot Ab\cdot C'_{1,4}$ to $S\cdot Ab\cdot C'_{1,4,2}$ and of $S\cdot Ab\cdot C'_{1,4}$ to $S\cdot Ab\cdot C'_{1,4,2}$ provided Mg^{++} were available. The experiments cited below were designed to determine the stability of the intermediate complex by quantitative measurements of reactivity with C'_3.

Experiment 011457 et seq.—Initial experiments were performed to detect "decay" of the presumed complex $Z\cdot Ab\cdot C'_{1,4,2}$ formed as just described. Complexes formed at 0° for 60 minutes by admixture of 5 mg. of Z with 5 ml. of guinea pig serum retained their ability to deplete more than 96 per cent of C'_3 in 1 ml. of guinea pig serum in the presence of EDTA even after incubation for 60 minutes at 37°. This indicated either that decay of the complex did not occur or that there was a great excess of sites on the Z granules in the state, $S\cdot Ab\cdot C'_{1,4,2}$, and that decay still left a number adequate to reach with C'_3 in 1 ml. of serum.

Formation of the Complex in a Later Trial.—9 suspensions each containing 5 mg. of Z were prepared in $SAVB^{++}$. Similarly to step 1 in the preceding experiment, 4 of these were each mixed with 4 ml. of fresh guinea pig serum, 4 others with 2 ml. of serum, and 1 with SAVB only as a control. After exactly 30 minutes at 0°, all preparations were washed twice at 0° and deposited by centrifugation. To one sample of deposited granules treated with 4 ml. of serum, to one treated with 2 ml. of serum, and to the untreated control, was added 1.5 ml. of prewarmed guinea pig serum (as C'_3) diluted ⅔ to contain 0.005 M EDTA. These mixtures were incubated for 75 minutes at 37° and the supernatant serum separated and preserved as above in step 2.

"Decay".—The remaining 6 batches of Z granules were suspended in about 2 ml. of $SAVB^{++}$ and incubated as outlined in Table VI. One sample pretreated with 4 ml. of serum was matched with one pretreated with 2 ml. The 3 pairs were incubated at 37° for 30, 60, and 90 minutes, respectively. Afterwards, the granules were diluted with 10 ml. of ice cold 0.005 M EDTA and then deposited by centrifugation at 0°. 1.5 ml. of prewarmed guinea pig C'_3 containing 0.005 M EDTA was added to each deposit and to a control tube without Z. After 75 minutes at 37°

the 7 samples were centrifuged twice at 0° and the supernatant sera were preserved for measurement of residual C'_3.

Residual C'_3.—The 10 supernatant sera were diluted in SAVB containing 0.005 M EDTA and titrated for C'_3 by admixture with $EAC'_{1,4,2}$ for 90 minutes at 37°. For the preparation of $EAC'_{1,4,2}$ in this experiment a modified procedure was followed. Recently, Rapp (11) described a method based on the observations of Da Costa Cruz and De Azevedo Penna in which whole serum is treated with dilute formaldehyde. The formalinized C′ is reacted with EA to give complexes presumed to be $EAC'_{1,4,2}$ and which show a marked sensitivity to C′ containing EDTA; *i.e.*, C'_3. In brief, the method consisted of the following: 3 ml. of guinea pig serum was incubated at 37° for exactly 40 minutes with 0.45 ml. of formaldehyde diluted 1/80

TABLE VI

Instability of the Theoretical Complex $Z \cdot Ab \cdot C'_{1,4,2}$. *Assay for Residual* C'_3 *after Reaction of Guinea Pig Serum Containing EDTA* (C'_3) *with Treated Zymosans*

Pretreatment of Z with guinea pig serum to form $Z \cdot Ab \cdot C'_{1,4,2}$	"Decay" at 37° of treated Z	Residual C'_3		
		Units per ml.	Units lost	Per cent lost
Z control, no pretreatment	None	433	8	1.8
0° 30 min. 2 ml.	None	280	161	36.5
" " " "	30 min.	372	69	15.6
" " " "	60 "	428	13	2.9
" " " "	90 "	433	8	1.8
0° 30 min. 4 ml.	None	207	234	53.1
" " " "	30 min.	391	50	11.3
" " " "	60 "	413	28	6.3
" " " "	90 "	394	47	10.6
C'_3 control—no zymosan	—	414	—	—

in saline. 12 ml. of EA, standardized spectrophotometrically to an O.D. of 0.680 at 541 mμ was next incubated with 2.4 ml. of the formalinized serum for 45 minutes at 30° in order to form the presumed complex $EAC'_{1,4,2}$. The cells then were washed at 0° and diluted in SAVB with 0.005 M EDTA to yield an O.D. of 0.680 at 414 mμ. The diluted and prewarmed supernatant sera above were mixed with 0.5 ml. of ice-cold $EAC'_{1,4,2}$ and incubated for 90 minutes at 37°. After dilution with 5.0 ml. of cold SAVB with EDTA, the mixtures were centrifuged and the degree of lysis calculated from spectrophotometric assays at 414 mμ for oxyhemoglobin in the supernatant fluids. It should be noted that treatment of EA with formalinized C′ results in a complex which shows significantly greater sensitivity to C'_3 than $EAC'_{1,4,2}$ formed at 0° for 20 minutes; *e.g.* compare C'_3 titers in untreated serum in Table VI with those in Tables IV and V.

The results in Table VI demonstrate the predicted decline in reactivity with C'_3 of the Z complexes after incubation at 37°. While these results are admittedly crude from the standpoint of precisely estimating the rate of decay, they show about a 57 per cent loss of reactivity with C'_3 of the complex formed with 2 ml. of C′ after "decay" of only 30 minutes, and a loss of

about 79 per cent of reactivity of the complex formed with 4 ml. of C′ after a similar period of "decay."

Thus, this evidence for the formation of a complex $Z \cdot Ab \cdot C'_{1,4,2}$ and for the decreased reactivity with C'_3 of this complex after incubation at 37°, presumably due to "decay" of sites in the state $S \cdot Ab \cdot C'_{1,4,2}$ emphasizes the similarities of Z reacting with C'_3 in normal serum and the classical reaction of antigen-antibody complexes reacting with C′ as exemplified by the immune hemolytic system. Further, and as predicted by the theory outlined initially, the requirement for Mg^{++} both in the formation of the $Z \cdot P$ complex and in the subsequent reaction of the $Z \cdot P$ complex with C'_3 may be interpreted as a requirement for the reactivity of C'_2 in the initial stage and for additional C'_2 to react with sites still in the state $S \cdot Ab \cdot C'_{1,4}$ for the subsequent reaction with C'_3. The second requirement for Mg^{++} would thus reflect varying degrees of "decay" of C'_2 sensitized sites on the Z granules.

V. Initial Experiments with Properdin.—Purified properdin (pool F) was kindly donated by Dr. L. Pillemer. Since this material was subjected to rather extensive purification procedures, it was quite impossible to anticipate its nature when considered in terms of the alternative hypothesis above that the $Z \cdot P$ complex represents $Z \cdot Ab$, $Z \cdot Ab \cdot C'_{1,4}$ and $Z \cdot Ab \cdot C'_{1,4,2}$. Three assays were employed: (*a*) the uptake of N by Z; (*b*) the agglutination of Z in the presence of EDTA; and, (*c*) the fixation of $C'_{1,4}$ by Z mixed with human and guinea pig serum.

Experiment 040257.—A preliminary assay showed that agglutination of 200 μg. of Z granules occurred in the presence of 0.01 M EDTA at dilutions stated to be equivalent to 2.5 units of properdin. Zymosan (lot 6B 382) was washed thoroughly in saline and portions containing 200 μg. were pipetted into each of 30 tubes. These were centrifuged and resuspended in groups of 5 tubes each at pH 5.6, 6.1, 6.4, 7.0, 7.3, and 7.5. A solution of properdin containing 5 units per 0.5 ml. in saline was diluted to contain 2.5, 1.25, and 0.625 units per 0.5 ml. For each series of dilutions and for each pH a control sample of Z without properdin was included. The 6 sets of mixtures were mixed and incubated overnight at 0°.

Macroscopic agglutination occurred similarly in each series; it was easily observed with 5 and 2.5 units, and detectable at 1.25 units. The results were confirmed by darkfield microscopy in which a moderate amount of agglutination was discernible in the mixtures containing 1.25 units of properdin. Thus, a conservative estimate of 2.5 units giving agglutination may be compared with the 1.0 unit giving fixation of C'_3 as defined by Pillemer.

Experiment 040457.—A solution of properdin containing 20 units per ml. in VB was "stabilized" at 0° for 60 minutes and then centrifuged at 10,000 R.P.M. for another hour at 0°. The clear supernatant fluid was removed for assay. One ml. aliquots were mixed in triplicate at 0° with 1 mg. portions of thoroughly washed Z in VB. Three samples of Z only, and two of properdin only, and the three reaction mixtures were rotated slowly overnight at 2°. All tubes were washed with saline by 4 centrifugations for 60 minutes at 0° and the deposits

transferred to digestion flasks. After overnight digestion, the usual micro Kjeldahl technique was employed for estimation of N.

An increase of approximately 5 μg. of N was noted in the Z preparations treated with 20 units of properdin. An average of 0.0244 mg. N (0.0226, 0.0242, 0.0264 mg.) occurred with Z mixed with properdin, while 0.0026 mg. was detected in both samples with properdin only, and an average of 0.0171 mg. (0.0160, 0.0190, 0.0164) was detected with Z only. These results are interpreted to mean that 1 unit of properdin is equivalent to about 0.25 μg. of N capable of combining with Z. It should be emphasized that this combination occurred in the absence of C′ and that additional cross-reacting Ab N may be present in properdin but fail to combine under these experimental limitations.

Experiments 011957 and 012457.—Preliminary measurements of fixation of $C'_{1,4}$ by Z were performed with human serum PB estimated to contain about 6 μg. Ab per ml. and with pooled guinea pig serum 011157 estimated estimated to contain about 5 μg. Ab N per ml. (see Table I.). During reaction of 2 mg. of Z with 2 ml. of PB serum there was a loss of only 7.35 per cent of $C'_{1,4}$ during overnight incubation at 0°. On the other hand, similar reactions were prepared with 1.5 ml. of guinea pig serum mixed with 5 mg. Z and with 5 mg. Z pretreated with 50 units of properdin and then thoroughly washed. After overnight incubation, the untreated serum was found to contain 1880 units of $C'_{1,4}$. There was a loss of 18.1 per cent of $C'_{1,4}$ in the serum treated with Z only, *i.e.*, a residual 1540 units; and a loss of 24.5 per cent of $C'_{1,4}$ in the serum treated with the presumed Z·P complex, *i.e.*, a residuum of 1420.

Experiment 041357.—The preceding experiments were repeated in certain respects with human serum PB and guinea pig serum 011157. Four reaction mixtures were set up with 1.5 ml. of each serum mixed at 37° for 90 minutes with the following: (*a*) SAVB only; (*b*) 3 mg. Z only; (*c*) 50 units properdin only; and, (*d*) 3 mg. Z and 50 units properdin. The residual $C'_{1,4}$ was measured with guinea pig $C'_{2,3}$ reagent as outlined above.

With the $C'_{1,4}$ in guinea pig serum incubated with SAVB only as a standard the following depletions of $C'_{1,4}$ occurred: with properdin, 0.35 per cent; with Z alone, 18.2 per cent; with Z plus properdin 25.0 per cent. These results were almost identical with those of Experiment 012457 outlined above. With human serum PB there was a decrease in residual $C'_{1,4}$ reactivity of 8.7 per cent in the serum mixed with Z alone and a decrease of 23.4 per cent in the serum mixed with Z plus properdin.

These results are interpreted to mean that enhanced depletion of $C'_{1,4}$ occurs in the presence of human properdin in both human and guinea pig serum when treated with Z. Extreme caution must be employed in the estimation of quantitative aspects of these data since unknown amounts of $C'_{1,4}$

in an unknown state of reactivity might be expected in purified properdin as interpreted by the alternative hypothesis outlined above despite the fact that no $C'_{1,4}$ reactivity could be detected in an assay with EA and guinea pig $C'_{2,3}$ reagent. Nonetheless, it is perhaps striking that 50 units of properdin, estimated to contain about 12 μg. N, enhanced fixation of approximately 230 units of $C'_{1,4}$ with human serum while Z alone reacting with the same serum, estimated to contain 6 μg. N, fixed about 140 units of $C'_{1,4}$. This parallelism failed to be evident with guinea pig serum. Z alone reacting with this serum, estimated to contain 5 μg. N, fixed 340 units, while the human properdin enhanced fixation to 460–470 units. No explanation for the discrepancy is immediately apparent.

DISCUSSION

Experiments with zymosan particles suggest that most sera from normal individuals contain antibody which is capable of sensitizing zymosan to react in immune adherence, immune aggregation, and complement-fixation. Measurements of nitrogen uptake at 0° in the presence of EDTA indicate antibody in variable but often small quantities.

In view of the existence of this Ab in normal serum used as a source of C′, the hypothesis has been offered that the properties ascribed to properdin may be explained on the basis of the classical concept of Ab acting in conjunction with certain components of C′. Thus it is possible to explain the chief characteristics of the properdin system without invoking the concept of a new discrete entity. The evidence here presented suggests that the complex of properdin with zymosan represents a mixture of $Z \cdot Ab$, $Z \cdot Ab \cdot C'_{1,4}$, and $Z \cdot Ab \cdot C'_{1,4,2}$. The proportion of sites on the Z granule with any of the three complexes would be expected to be dependent upon the time and temperature employed for the interaction of Z with fresh serum and upon the well recognized innate affinity of the Ab in any particular sample of serum for the species of C′ employed. Ancillary evidence to support this concept has come from assays which are interpreted to mean that purified properdin contains Ab. In the presence of EDTA and at 0° properdin agglutinated Z granules. Measurements of N uptake by Z indicated that 0.25 μg. N were contributed by solutions stated to contain 1 unit of properdin. Also, addition of purified properdin to mixtures of Z with guinea pig or human sera resulted in increased fixation of $C'_{1,4}$.

The supposedly preferential reactivity of zymosan with C'_3 (1, 2), upon which the concept of properdin was based, is rendered dubious when quantitative measurements of $C'_{1,4}$ are performed as outlined herein. Significant fixation of $C'_{1,4}$ occurred when guinea pig or human sera were treated with Z. With levels of Ab N ranging from 2 to 40 μg., about the same numbers of reactive units of $C'_{1,4}$ and of C'_3 units were fixed at 37° by zymosan, by *D. pneumoniae*, and by washed specific precipitates

of bovine albumin–anti-albumin. However, the $C'_{1,4}$ in untreated serum was effective at approximately 20 times the dilution showing C'_3 reactivity. We are left with the conclusion that it is this marked dissimilarity in reactive titer and the subsequent expression of fixation of components in terms of percentage rather than reactive units which led to the concept of an apparent preferential inactivation of C'_3 by zymosan and other substances grouped in the properdin system. The evidence that small amounts of antigen-antibody complex will fix significant amounts of C'_3 stands in contrast with the classical concept that C'_3 is not fixed in complement fixation reactions. Data to support the lack of fixation of C'_3 were obtained with dilute reaction systems in which the concentration of C'_3 was low. The present assays, as well as those employed in the properdin system, differed radically in that essentially undiluted complement was used. On the basis of the theory developed by Rapp (10) and from an analysis of the reactivity of C'_3 as outlined by Mayer (14), it might have been predicted that demonstrable fixation of C'_3 would have occurred in the present assays performed at 37° with undiluted complement.

The concept of Ab in serum from immunized persons reacting with C′ to produce specific biological effects is of course a cornerstone of immunology. Nonetheless the possibility that Ab in normal serum may play a similar role is occasionally overlooked and a new concept evoked to explain the observed phenomena. The present study is a logical outgrowth of a similar investigation in which evidence was obtained that the "phagocytosis promoting factor" of normal serum is natural antibody reacting with C′ and that it sensitizes starch granules to undergo phagocytosis (12). Both of these studies serve to emphasize the importance for a clear definition of the mode of origin and action of natural Ab. The specificity of Ab reacting with zymosan and other varied polysaccharides is under investigation. It seems apparent that the factor common to the various substances postulated to react in the properdin system is the polysaccharide content of the antigens employed to date. Such broad reactivity, or, more likely, broad cross-reactivity, is not incompatible with the concept immunological specificity. At the moment, it seems entirely reasonable that the spectrum of reactivity is a reflection of the wide distribution in nature of closely related polysaccharides, analogous to the findings of Heidelberger and Cordoba (13) for antigens of typhoid bacilli. This fact, coupled with the possible increased reactivity of cross-reacting Ab in the presence of C′, would provide a sound immunochemical basis for the numerous substances which react in the properdin system.

The importance of other factors is evoked or re-emphasized by the present results. First, ignoring for the moment the variation in avidity of individual Ab reacting with the polysaccharide as well as the combining affinity of individual Ab for a particular species of C′, the sera examined for Ab N (Table I) showed considerable variation in content. From the results with guinea pig serum, it might be expected that the variable Ab content would explain the finding that only 20 per cent of human sera react with Z at 16° to deplete antibody with retention of more than 75 per cent of C′ components, *i.e.*, make a "suitable R·P"; and only 10 per cent of sera react with Z at 37° to deplete C'_3 with retention of more than 75 per cent of C'_1, C'_4, and C'_2, *i.e.*, make

a "suitable R·3" (2). Using the assays with zymosan and with *D. pneumoniae* as models, we would suggest that Ab may be absorbed from serum PB (5 to 6 μg. Ab N) with the loss of only a small percentage of $C'_{1,4}$ units. On the other hand, absorption of sera to remove Ab of higher content, *e.g.* SC (14 μg.) or KH (23 μg.), would be expected to fix significantly more $C'_{1,4}$ as well as C'_3. Such trials seem indicated but were beyond the scope of the present study.

Second, and of more academic import, is the obvious need for an accurate measurement of C'_2. To date and on theoretical grounds such measurements have not been undertaken owing to the postulated instability of sites in the state $EAC'_{1,4,2}$ (5) and $Z \cdot Ab \cdot C'_{1,4,2}$ (Table VI).

Finally, the importance of a clear definition of the nature of the substances involved in "natural resistance" is apparent from the varied and fundamental character of the phenomena currently under study. Observations extending from the neutralization of bacteriophage (15) through the lysis of abnormal erythrocytes (16) and an increase of natural resistance to certain bacteria (17) to studies on the resistance to malignancy (18) have implicated the properdin system. Nevertheless, it seems evident that not only the interpretation of the observed phenomena but also the direction and emphasis that future research should take would be influenced markedly by the concept presented herein of a mechanism involving natural antibody and complement components instead of a single and discrete new substance, properdin.

SUMMARY

Evidence is presented that phenomena ascribed to the properdin system may be explained in terms of classical antibody (Ab) in combination with three of the components of complement (C'), *i.e.*, C'_1, C'_4, and C'_2 respectively. The results suggest that the complex of properdin (P) with zymosan (Z) represents a mixture of $Z \cdot Ab$, $Z \cdot Ab \cdot C'_{1,4}$, $Z \cdot Ab \cdot C_{1,4,2}$, and $Z \cdot Ab \cdot C'_{1,4,2}$ in a decayed state.

The purported preferential reactivity of Z with the third component of C' (C'_3) is not supported by the present experiments in which Z was reacted with C' in both guinea pig and human sera. Approximately the same number of reactive units of $C'_{1,4}$ and of C'_3 were inactivated by Z, as well as by *D. pneumoniae* and a washed specific precipitate of bovine albumin–anti-albumin.

The latter evidence that small amounts of antigen-antibody complex fix significant amounts of C'_3 stands in contrast with the classical concept that C'_3 is not fixed in ordinary complement fixation reaction. The observed reactivity of C'_3 is explained on the basis of the present use of essentially undiluted serum as a source of C'_3 and of 37° as the temperature for fixation.

Ancillary data indicate that purified properdin contains Ab. In the presence of a chelating agent and at 0° properdin agglutinated Z granules. Meas-

urements of nitrogen (N) uptake by Z suggest that 0.25 μg. N were contributed by solutions stated to contain 1 unit of properdin.

The broad spectrum of reactivity or cross-reactivity of the Ab in normal serum is likely due to the wide distribution in nature of closely related polysaccharides. Further immunochemical studies are necessary to establish definitively the origin and mode of action in "natural resistance" of antibodies reactive with these polysaccharides.

The experiments on measurements of C′ components were designed after discussions with Dr. Manfred M. Mayer and Dr. Herbert J. Rapp of The Johns Hopkins School of Hygiene and Public Health, Baltimore. Their help is gratefully acknowledged.

Dr. J. C. Kelsey cooperated in the early experiments performed at the London School of Hygiene and Tropical Medicine. Dr. Harold C. Woodworth participated in the performance of several experiments at Yale University.

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, *Science*, 1954, **120**, 279.
2. Pillemer, L., *et al*, Conference on Natural Resistance to Infections, *Ann. New York Acad. Sc.*, 1956, **66**, 233.
3. Cowan, K. M., Interpretation of properdin levels determined by the phage neutralization technique, *Science*, in press.
4. Dacie, J. V., Crookston, J. H., and Christenson, W. N., "Incomplete" cold antibodies: Role of complement in sensitization to antiglobulin serum by potentially hemolytic antibodies, *Brit. J. Haematol.*, 1957, **3**, 77.
5. Mayer, M. M., Levine, L., Rapp, H. J., and Marucci, A. A., Kinetic studies on immune hemolysis. VII, *J. Immunol.*, 1954, **73**, 443.
6. Nelson, R. A., and Woodworth, H. C., Immune-adherence as measured by agglutination of erythrocytes. I. Conference on Complement, Washington, D. C., Walter Reed Institute Research, March, 1957.
7. Cohn, E. J., *et al.*, A system for the separation of the components of human blood, *J. Am. Chem. Soc.*, 1950, **72**, 465.
8. Levine, L., and Mayer, M. M., Kinetic studies on immune hemolysis. V, *J. Immunol.*, 1954, **73**, 426.
9. Levine, L., Mayer, M. M., and Rapp, H. J., Kinetic studies on immune hemolysis. VI, *J. Immunol.*, 1954, **73**, 435.
10. Rapp, H. J., A mathematical theory of immune hemolysis, Dissertation, Baltimore, Johns Hopkins School of Hygiene and Public Health, 1955.
11. Rapp, H. J., Mechanism of immune hemolysis, *Science*, 1958, **127**, 234.
12. Nelson, R. A., and Lebrun, J., The requirement for antibody and complement for *in vitro* phagocytosis of starch granules, *J. Hyg.*, 1956, **54**, 8.
13. Heidelberger, M. M., and Cordoba, F., Cross-reactions of antityphoid and antiparatyphoid B horse sera with various polysaccharides, *J. Exp. Med.*, 1956, **104**, 375.
14. Mayer, M. M., Studies on the mechanism of hemolysis by antibody and complement, *Prog. Allergy*, 1958, **5**, 215.

15. Van Vunakis, H., Barlow, J. L., and Levine, L., Neutralization of bacteriophage by the properdin system, *Proc. Nat. Acad. Sc.*, 1956, **42,** 391.
16. Hinz, C. F., Jordan, W. S., and Pillemer, L., The properdin system and immunity. IV, *J. Clin. Inv.*, 1956, **35,** 453.
17. Landy, M., and Pillemer, L., Elevation of properdin levels in mice following administration of bacterial lipopolysaccharides, *J. Exp. Med.*, 1956, **103,** 823.
18. Herbut, P. A., and Kraemer, W. H., The possible role of the properdin system in transplantable cancer, *Cancer Research*, 1956, **16,** 1048.