

## THE PROPERDIN SYSTEM AND IMMUNITY

### VI. THE INACTIVATION OF NEWCASTLE DISEASE VIRUS BY THE PROPERDIN SYSTEM\*, †

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The inhibition of Newcastle disease, influenza A and B, and mumps viruses by a labile component present in normal serum has been described previously (1). In addition to complement another component of normal serum, distinct from complement, was required for viral inhibition. Inactivation of virus by this inhibitor was temperature-dependent, appeared to require calcium, and under usual circumstances the virus did not dissociate spontaneously from the inhibitor. These findings were in agreement with those of other investigators (2-4) and characterized the heat-labile inhibitor of normal serum as a mechanism distinct from antibody (1-4) or heat-stable (Francis) inhibitors of viral hemagglutination (2-4).

The requirements and kinetics of the interaction of the properdin system with zymosan (5, 6) and certain erythrocytes (5, 7, 8) bore a resemblance to the requirements and kinetics of the inhibition of virus by the heat-labile component of serum (1). Therefore, preliminary experiments were carried out to determine whether the properdin system had any viral inhibiting properties. Such inhibiting properties were found (9). Since that time, it has been shown that the properdin system has a variety of activities including the lysis of certain erythrocytes (7, 8) and the destruction of various bacteria (10) and protozoa (11).

The purpose of this paper is to describe in detail the experiments which indicate that the properdin system can inactivate Newcastle disease virus (NDV); that such viral inactivation equates with the other described activities of the properdin system; and that the inhibition of hemagglutination by Newcastle disease virus (NDV) is partially reversible.

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### Nomenclature

The nomenclature used throughout is similar to that of all previous papers in this series (5, 7, 10, 12, 13). The abbreviations in the text, figures, and tables are designated in the section on materials and methods. The term RP is used in a generic sense to describe serum rendered deficient in properdin alone.

### Materials and Methods

#### Diluents.—

*Barbital Buffer.*—Barbital buffer was prepared as previously described (12, 14). When noted, magnesium and calcium were added in concentrations of  $1.5 \times 10^{-4}$  M and  $4.2 \times 10^{-4}$  M respectively.

*Citrate Buffer.*—2.5 per cent sodium citrate was used.

*Phosphate Buffer.*—Isotonic saline buffered at pH 7.2 with 0.01 M phosphate was employed.

*Tryptose Phosphate Broth.*—Difco bacto-tryptose phosphate broth, containing penicillin (50 units per ml.) and streptomycin (0.5 mg. per ml.) was used as the diluent in all infectivity studies.

#### Zymosan.—

This insoluble polysaccharide complex of yeast cell walls was prepared as previously described (12, 15). The zymosan employed in this study met criteria previously established for the preparation of reagents (12).

#### Human Serum Reagents.—

*Fresh Human Serum.*—Blood was drawn from healthy donors and the serum separated and stored as previously described (10). Occasional sera were encountered that contained heat-stable inhibitor; this inhibitor resisted heating at 56°C. for 30 minutes and when tested was sensitive to culture filtrate of *Vibrio cholera* (RDE) prepared as previously described (16). Five to 10 per cent of fresh sera tested had agglutinin titres of 1:8 or higher against chick red cells. Heat-stable inhibitor of viral hemagglutination and chick red cell agglutinins interfered with the studies of the serum heat-labile inhibition presented in this paper and therefore, sera containing either of these factors were discarded.

*Properdin.*—Highly purified human properdin dissolved in barbital buffer (5, 12), was used throughout. The properdin concentration of the purified material and of test reagents was determined by the zymosan assay (12).

*Serum Deficient in Properdin (RP).*—The preparation of serum deficient in properdin (RP) for a reagent has been described previously (5, 12), and it has been pointed out that not all sera can be satisfactorily depleted of properdin alone by a single adsorption with zymosan (5, 6, 10, 12, 13). A double adsorption with zymosan was found necessary to deplete serum of properdin satisfactorily for bactericidal studies (10). For these experiments RP was prepared either in a manner identical with that for bactericidal studies (10) or by triple adsorption with zymosan. For a variety of reasons presented and discussed elsewhere (10, 12) only 10 to 20 per cent of serum samples yielded satisfactory RP.

*Serum Deficient in Components of Complement.*—Reagents lacking individual complement component activity were prepared from fresh human serum and characterized by standard methods (12, 14): R1, the reagent lacking C'1, and R2, the reagent lacking C'2, by dialysis against acetate buffer (pH 5.6, ionic strength 0.02); R3, the reagent lacking C'3, by incubation with zymosan at 37°C.; R4, serum lacking C'4, by incubation with hydrazine. In one experiment partially purified C'3, prepared from human plasma fraction III (Cohn), was kindly supplied by Dr. Florence Roth (17).

Heated serum, inactivated at 56°C. for 30 minutes, lacking C'1, C'2, C'3, and properdin, was used as a general control for all experiments.

*Resin-Treated Serum.*—Serum was rendered deficient of magnesium and calcium with cation exchange resin, amberlite IRC-50 in the sodium cycle, by methods previously described (10, 18). Such resin treatment of serum has been found to reduce the concentration of magnesium and calcium to less than  $1 \times 10^{-6}$  M without inactivating complement or properdin.

*Virus.*—

Newcastle disease virus (Hickman strain) was chosen as the test agent because antibody to it, which would have interfered with the test procedures, is rarely found in human serum. Newcastle disease virus (NDV) was propagated in the allantoic sac of 10 day embryonated chick eggs. Eggs were inoculated with 0.2 ml. of a  $10^{-6}$  dilution of NDV infected allantoic fluid and incubated at 35°C. for 40 hours. Following chilling, the eggs were harvested for allantoic fluid. Generally the allantoic fluid containing the NDV was dialyzed against 80 volumes of barbital buffer for 12 to 24 hours in the cold (4°C.) before use. Such pools had hemagglutination titres of 1:256 to 1:1024 and infectivity titres of  $10^{9.7}$  EID<sub>50</sub> per ml.

For certain experiments dialyzed virus was rendered deficient in magnesium and calcium by resin treatment in the same manner as the resin treatment of serum.

In one experiment influenza A and B viruses (PR8 and Lee strains), cultivated in chick embryos in a manner similar to that described for NDV, were used.

*Chicken Red Cells.*—

Chicken red cells stored in Alsever's solution, or as a "stabilized 5 per cent suspension" (Cappel Laboratories, West Chester, Pennsylvania) were suspended to a 0.5 per cent concentration in barbital, citrate, or phosphate buffer on the day of use.

*Storage.*—

All serum, reagents, properdin, and virus were quick frozen and stored at  $-70^{\circ}\text{C}$ . until the day of use. During test procedures, unless otherwise mentioned, all reagents and mixtures were kept chilled at 4°C.

*Glassware.*—

All glassware used was chemically cleaned in sulfuric acid-dichromate solution and rinsed thoroughly with distilled water.

*Determination of Viral Inhibition and Activity.*—

It has been shown previously (1, 2) that the complex of virus and heat-labile inhibitor is stable, and that the virus does not dissociate freely from the inhibitor, as is the case with Francis' inhibitors (2, 19, 20). Chick cell agglutinins and other factors in human serum may under the conditions of "constant serum, varying dilutions of virus" and the converse "constant virus, varying dilutions of serum" titrations interfere with, and often obliterate the end point of hemagglutination. All titrations to determine the degree of viral inhibition were carried out as follows: Equal volumes of test serum or serum reagent and virus were mixed together and incubated at 37°C. for 30 minutes. The residual quantity of active virus was then titrated by preparing dilutions of the virus-serum mixture, and testing these for hemagglutinating or infectious activity.

*Hemagglutination Titration.*—

Double dilutions of serum-virus mixture in 0.4 ml. of buffer were made. To each dilution and to an aliquot of the undiluted serum-virus mixture an equal volume (0.4 ml.) of 0.5 per

cent chicken red cells was added. The reagents were mixed by shaking, and after incubation at 4°C. for 60 minutes the tubes were read for hemagglutination patterns. The highest dilution showing definite hemagglutination was recorded as the end point. The hemagglutination titre is the reciprocal of this dilution. The inhibitory activity of the serum or reagent is reported as an "inhibition index." The inhibition index is the ratio between the hemagglutination titre of the control (buffer or heated serum) and the test reagent; *i.e.*,  $\frac{(\text{control titre})}{(\text{reagent titre})}$ . Since unity is the least index obtainable by this computation (when the control and test reagent titres are identical), it should be remembered throughout that an inhibition index of one by hemagglutination indicates *no* inhibition.

*Infectivity Titrations.*—

Ten-fold dilutions of the serum-virus mixture were made in tryptose-phosphate broth. 0.2 ml. of each dilution was inoculated into the allantoic sac of 10 day embryonated chicken eggs. Four eggs were used for each dilution. Following 40 hours' incubation, the eggs were chilled and the allantoic fluid harvested. The presence of virus in the allantoic fluid was determined by hemagglutination titrations and the EID<sub>50</sub> calculated (21). Inhibitory activity is reported as an "inhibition index," defined as the ratio between the EID<sub>50</sub> of the control and the test reagent:  $\frac{(\text{control titre})}{(\text{reagent titre})}$ . In infectivity titrations this is expressed as the logarithm of the inhibition index calculated by subtracting the log of the titre in the reagent from the log of the titre in the control; *i.e.*, log EID<sub>50</sub> in control minus log EID<sub>50</sub> in reagent.

EXPERIMENTAL

*The Properdin Requirement for Viral Inhibition by Human Serum*

The initial experiment was designed to determine whether properdin was an essential component for the inhibition of viral activity by serum.

Equal volumes of NDV and serum, heated serum (56°C. for 30 minutes), RP, RP to which properdin was added (5 units per ml.), properdin alone in barbital buffer with added magnesium and calcium (5 units per ml.), or barbital buffer with magnesium and calcium alone (control) were mixed and incubated at 37°C. for 30 minutes. Following incubation, viral activity was determined by hemagglutination titrations. A similar experiment using infectivity titrations to determine viral activity was also performed.

The findings (Table I) indicate that properdin was necessary for the inhibition of virus by the heat-labile factors of normal serum. Fresh serum inhibited virus as determined by both hemagglutination and infectivity titrations. The inhibitor was heat-labile, since heated serum gave little or no inhibition. When serum was rendered properdin deficient (RP) there was a distinct reduction in viral inhibition. The readdition of properdin to RP restored the inhibitory activity. Properdin alone, however, was never inhibitory.

In all experiments in which infectivity was used as an index of viral activity, a reduction in viral inhibition could be demonstrated following the removal of properdin from serum in the preparation of RP. Experiments in which hemagglutination was employed as an index of viral activity were not as uniformly satisfactory. In almost every instance in which the parent serum and RP were

tested simultaneously, there was considerably less viral inhibition by RP. Many sera adsorbed two times with zymosan, however, still inhibited hemagglutination by NDV to a significant degree. Readdition of properdin to those adsorbed sera having the highest inhibition indices did not result in an increase of viral inhibition—probably because of the large amount of properdin not adsorbed by zymosan (*vide infra*). Another group of zymosan-adsorbed sera had no viral inhibitory activity and failed to inhibit hemagglutination by NDV after properdin was added—complement had probably been reduced in these sera (10). The remaining adsorbed sera fulfilled all criteria for a satisfactory RP: They had markedly reduced viral inhibitory activity and on addition of

TABLE I  
*The Requirement for Properdin in the Inhibition of Virus*

Test reagent	Test procedure			
	Hemagglutination		Infectivity	
	Titre	Inhibition index*	Log EID <sub>50</sub>	Log inhibition index†
Serum	4	64	5.3	4.2
Heated serum	256	1	9.5	0.0
RP	32	8	8.5	1.0
RP with added properdin	4	64	5.0	4.5
Properdin alone	256	1	9.5	0.0
Barbital buffer	256	1	9.5	0.0

$$* \text{ Inhibition index} = \frac{\text{control titre}}{\text{reagent titre}}$$

$$† \text{ Log inhibition index} = \log \text{EID}_{50} \text{ control} - \log \text{EID}_{50} \text{ test reagent.}$$

properdin the viral inhibitory activity was restored to levels comparable to that of the original serum.

It was concluded from these experiments that properdin was necessary for the action of the heat-labile viral inhibitor in normal serum. Properdin, however, was not effective alone; additional factors present in serum were needed. It was also apparent that maximal removal of properdin in the preparation of RP affected these other factors in serum (probably complement) necessary for the system. Less complete removal of properdin, while sparing these factors, did not necessarily produce a decrease in inhibitory activity, because of the residual properdin. Therefore, in the preparation of an RP satisfactory for studies of viral inhibition, a compromise was necessary; an RP was required in which properdin was removed maximally but which still retained satisfactory serum cofactor levels. Such requirements are not unique, and have also been found in the study of the other activities of the properdin system (10).

*The Relationship between Properdin Concentration and Viral Inhibition*

Previous experiments suggested that properdin was necessary for the inhibition of virus, but the amount of properdin required for complete inhibition was small. Therefore, experiments were carried out to determine the quantitative relationship between properdin concentration and the degree of viral inhibition.

Constant volumes of increasing dilutions of properdin were added to aliquots of an RP to give final concentrations of properdin ranging from 0.008 to 8 units per ml. of RP. The capacity of each reagent and appropriate controls to inhibit NDV was measured by the hemagglutination technique.

TABLE II  
*The Relationship between Properdin Concentration and Viral Inhibition*

Test reagent	Properdin concentration <i>units per ml.</i>	Hemagglutination titre	Inhibition index*
Serum	4-6	4	64
RP	—	32	8
Heated RP (56°C.)	—	256	1
RP + properdin	8	4	64
" " "	2	8	32
" " "	0.5	8	32
" " "	0.125	4	64
" " "	0.031	4	64
" " "	0.008	32	8

$$* \text{Inhibition index} = \frac{\text{control titre}}{\text{reagent titre}}$$

The initial experiment utilized hemagglutination as an index of viral activity (Table II). The parent serum had an inhibition index of 64 units. RP alone had some inhibitory activity. Heated RP had no antiviral effect. The addition of 0.008 units of properdin per ml. of RP did not increase the inhibition above that found in the RP alone. The addition of 0.03 units of properdin per ml. of RP, however, restored the inhibitory activity to the level seen in the original serum. The addition of increasing amounts of properdin did not further enhance the degree of inhibition. The demonstration that as little as 0.5 per cent of the original serum concentration of properdin induced maximal inhibition emphasizes the fact previously noted that almost complete removal of properdin in the preparation of RP was required to abolish viral inhibitory activity by serum.

The assay of inhibition by hemagglutination titrations under the conditions used was sensitive only between levels of 50 and 98 per cent inhibition. The use of infectivity titrations to determine viral inhibition was sensitive with the

method employed when over 90 per cent of the virus was inactivated. Similar experiments using infectivity titrations were therefore carried out to determine whether the degree of viral inhibition could be quantitatively related to properdin concentration when larger quantities of properdin were employed.

Properdin was added to aliquots of an RP in concentrations ranging from 0.1 unit to 20.0 units per ml. The parent serum, heated serum, RP, aliquots of RP to which varying concentrations of properdin were added, properdin alone (5 units per ml. in barbital buffer with added

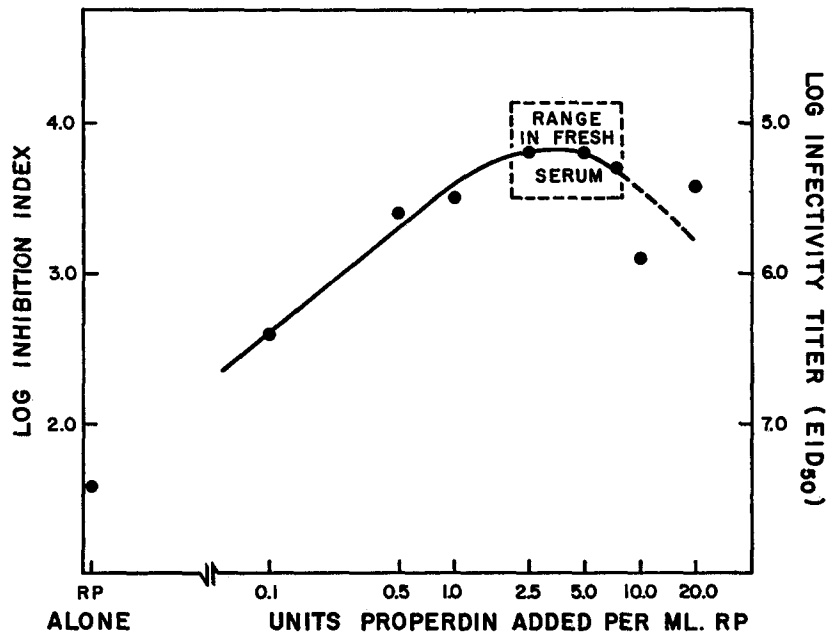


FIG. 1. The relationship between properdin concentration and the quantity of virus inhibited.

magnesium and calcium), heated RP, and barbital buffer alone (with magnesium and calcium) were incubated with an equal volume of NDV and the quantity of virus inhibited determined by infectivity titrations.

The results presented (Fig. 1) summarize the geometric means of data obtained from two comparable experiments. In these experiments heated serum, heated RP, and properdin alone were non-inhibitory. The parent sera had a log inhibition index of 3.8; the RP was significantly less inhibitory, having a log inhibition index of 1.6. The addition of as little as 0.1 unit of properdin per ml. of RP gave a distinct increase in the quantity of virus inhibited. There was a progressive increase in viral inhibition as properdin was added until a concentration of 2.5 units per ml. was reached. The inhibition index at this proper-

din concentration was similar to that of the parent serum. Increasing amounts of properdin above this concentration did not increase viral inhibition. Indeed, the data suggest that excessive amounts of properdin may interfere with the antiviral effect.

The increase in viral inhibition with increments of properdin up to 2.5 units per ml., while quantitative, was not a first order reaction. By extrapolation<sup>1</sup> the residual activity of the RP (log inhibition index of 1.6) would be equivalent to approximately 0.01 unit per ml. of properdin. Since the parent sera contained between 4 and 8 units per ml., it can be estimated that the preparation of RP removed more than 99.8 per cent of the properdin present in the fresh serum. The inability of properdin in excess of 2.5 units per ml. to increase viral inhibition suggests that factors other than properdin in the serum may be limiting.

These findings are similar to those reported for the quantitative aspects of the bactericidal activity of the properdin system (10); in the bactericidal studies also, excessive properdin concentrations interfered with the activity of the system.

#### *The Requirement for the Components of Complement in Viral Inhibition*

In all the previous experiments it was apparent that serum factors in addition to properdin were also necessary for, and could limit, viral inhibition. Experiments were, therefore, carried out to determine the requirement for the components of complement in viral inhibition by normal serum.

Serum reagents deficient in single components of complement were prepared. R1, R2, R3, and R4 were each added to aliquots of NDV and the quantity of virus inhibited in each mixture ascertained by hemagglutination and by infectivity titrations. Similarly, the inhibitory activity of the parent sera, and of the reagents with added properdin was determined. In one experiment properdin and partially purified C'3 (17) were added to R3 in a similar type of procedure.

All four components of complement were needed for viral inhibition (Table III) R1, the reagent lacking C'1, R2, the reagent deficient in C'2, and R4, the reagent in which C'4 had been inactivated, without, or even with properdin added, had essentially no inhibitory activity. R3, the serum rendered deficient in C'3, alone retained some inhibition in the infectivity test. The addition of properdin to R3 did not significantly increase viral inhibition. The addition of C'3 to the R3 with added properdin, however, resulted in the inactivation of significantly greater amounts of NDV. The R3 was not totally deficient in C'3; approximately 10 per cent of the C'3 was left in the reagent during preparation. It is probable that the residual inhibition in this reagent was, therefore, due to

<sup>1</sup>log inhibition index =  $3.6 + \log$  (properdin concentration); for concentrations of properdin in this RP up to 2.5 units per ml.



the traces of C'3 and properdin that had not been removed. The recombination of R1 and R2, restored all components to the system, and likewise restored the antiviral effect.

It can be concluded from these experiments that for viral inhibition, as for the lysis of bacteria or erythrocytes (7, 8, 10), all four components of complement are required in addition to properdin. The effect of the system can be

TABLE III  
*The Requirements for Complement in the Inhibition of Virus*

Test reagent	Component known to be absent					Inhibition index	
	P	C'1	C'2	C'3	C'4	From hemagglutination*	From infectivity
Parent serum						16	3.0
R1	+	+				1	0.0
R1 + properdin		+				1	0.1
R2			+			1	0.5
R2 + properdin			+			1	0.5
R3	+			+		1	1.2
R3 + properdin				+		1	1.4
R3 + C'3 + properdin						Not done	2.5
R4					+	1	0.1
R4 + properdin					+	1	0.1
R1 + R2						8	Not done

\* Inhibition index =  $\frac{\text{control titre}}{\text{reagent titre}}$ ; control titre = 128.

† Log inhibition index =  $\log \text{EID}_{50}$  in control -  $\log \text{EID}_{50}$  in test reagent.  $\log \text{EID}_{50}$  in control = approximately 9.0.

‡ This preparation of R3 was not entirely free of C'3; between 30 and 40 units (10 per cent of the original concentration) of C'3 per ml. were present in the reagent at the concentration used.

limited by a deficiency in any one of these four components, and when susceptible to experimentation the readdition of the missing component can be shown to restore inhibitory activity.

#### *The Cation Requirement for Viral Inhibition*

For all reported activities magnesium is an absolute requirement for the properdin system. A series of experiments were performed, therefore, to see if this was the case for the inhibition of Newcastle disease virus.

To 0.4 ml. aliquots of resin-treated serum was added 0.04 ml. of a solution containing either  $m/10$  barium, calcium, cobalt, copper, iron, magnesium, manganese, or potassium. The cation solutions were made from the most soluble chloride salt available. To each was added 0.4 ml. of dialyzed and resin-treated NDV. Controls containing resin-treated serum and cation alone,

and NDV with cation alone were tested in parallel. Similar experiments were performed using resin-treated RP, and resin-treated RP with added properdin.

Resin treatment of serum completely removed the heat-labile viral inhibitory activity. This activity could be restored by the readdition of magnesium or

TABLE IV  
*The Cation Requirement for the Inhibition of Virus*

Cation added to reagent	Inhibition index*
Barium	1
Calcium	1
Cobalt	1
Copper	1
Iron	2?
Magnesium	32
Manganese	32
Potassium	1
Resin-treated serum alone (no added cation) . . . . .	1

\* Inhibition index =  $\frac{\text{control titre}}{\text{reagent titre}}$ . Inhibition index of serum before resin treatment = 32.

Hemagglutination titre of dialyzed and resin-treated NDV: (control) = 128.

TABLE V  
*The Cation Requirement for the Inhibition of Virus by Properdin in RP*

	Cation added		Inhibition index*
	Calcium	Magnesium	
Fresh serum	+	+	16
" "	0	0	1
RP (serum lacking properdin)	+	+	1
RP with added properdin	0	0	1
" " " "	+	0	1
" " " "	0	+	16
" " " "	+	+	16

\* Inhibition index =  $\frac{\text{control titre}}{\text{reagent titre}}$ ; control titre = 128.

manganese (Table IV). Barium, calcium, cobalt, copper, and potassium had no effect; the inhibitory activity did not return on the readdition of these cations. Since the addition of ferrous salts resulted in the formation of a precipitate in the serum-virus mixture following incubation, the inhibition index of serum with added iron is in doubt. No significant increase in inhibitor titre was found with added iron. The cations in the concentrations used did not

inhibit NDV by themselves, nor did they alone cause sufficient agglutination of the chicken red cells to interfere with the experiments.

In order to show specifically the requirement for magnesium in the properdin system, additional studies were made using resin-treated RP with and without added properdin (Table V). These data add further evidence that magnesium but not calcium was an essential component of the properdin system in the inhibition of virus. Furthermore, the results indicate clearly that calcium did not enhance the inhibition attained with magnesium alone.

TABLE VI  
*The Cation Requirement for the Inhibition of NDV, Lee, and PR 8 Virus in Guinea Pig Serum*

Virus added to resin-treated serum	Cation added		Inhibition index*
	Calcium	Magnesium	
NDV	0	0	1
	+	0	1
	0	+	4
	+	+	4
LEE	0	0	1
	+	0	1
	0	+	16
	+	+	16
PR8	0	0	1
	+	0	1
	0	+	16
	+	+	16

\* Inhibition Index =  $\frac{\text{control titre}}{\text{reagent titre}}$ . Control titre: NDV = 512; Lee = 128; PR8 = 1024.

The data of Ginsberg and Horsfall (1) suggested that calcium rather than magnesium was required for viral inhibition. Much of their data were obtained using guinea pig serum with Newcastle disease or influenza viruses. Therefore, further experiments were carried out using resin-treated guinea pig serum and dialyzed, resin-treated NDV, and influenza A and B virus (PR8 and Lee strains).

Procedures identical with the above were carried out using resin-treated guinea pig serum and dialyzed, resin-treated NDV, Lee, and PR8 viruses. In these experiments magnesium and calcium only were added. In all experiments viral activity and inhibition were determined by hemagglutination titrations.

It was apparent again from these experiments (Table VI) that magnesium, not calcium, was required for viral inhibition. Calcium neither restored in-

hibitory activity nor enhanced the inhibition induced by magnesium. Such results were obtained consistently; in every sample tested magnesium but not calcium restored inhibitory activity to resin-treated serum. There is thus a discrepancy between this and previous reports (1, 2).

The data of Ginsberg and Horsfall (1) were obtained by depleting serum of calcium and magnesium using dialysis and the addition of citrate. The depletion of calcium in this manner may render complement unstable (18, 22). However, the removal of magnesium (and calcium) would not be complete. It is entirely possible that sufficient magnesium was present in their system to allow inhibition upon the readdition of enough calcium to protect and maintain the complement content of the serum. The addition of magnesium would not afford such protection, and thus complement might have been inactivated when magnesium alone was added. Since the properdin system requires complement, in addition to properdin, the inactivation of complement in a calcium-deficient system could explain their results. The ion exchange resin used in the present studies removes cations more rapidly and efficiently, thereby diminishing the danger of inactivation of complement.

The data presented here indicate that magnesium is a requirement for the inhibition of virus by the properdin system. In this requirement the inhibition of virus is entirely similar to all other reported activities of the properdin system. Of the other cations tested, manganese alone could replace magnesium in the system. The concentration of manganese required precludes the possibility that this metal has any significance in the inhibition of virus when such inhibition occurs naturally in serum.

#### *The Thermal Dependence of Viral Inhibition*

In contrast to the interaction of antigen and antibody, the optimal interaction of the properdin-zymosan complex with C'3 requires temperatures of incubation above 15°C. (5). The characteristics of the thermal requirement for viral inhibition were therefore determined by the following experiments.

Tubes containing 0.4 ml. of virus and 0.4 ml. of serum were incubated for 30 minutes at temperatures ranging from 0°C. to 45°C. Twofold dilutions were then made in cold 2.5 per cent citrate buffer using chilled pipettes. Chicken red cells in cold citrate buffer were added. As a control, parallel tubes containing heated serum and virus, and barbital buffer and virus were treated similarly. Viral activity was determined by hemagglutination titrations.

In a series of such experiments (Fig. 2) inhibition of virus never occurred at temperatures below 10°C. Between 10°C. and 20°C. no inhibition or occasional slight inhibition could be demonstrated. At temperatures above 20°C. viral inhibition invariably occurred, maximal inhibition appearing uniformly between 30°C. and 40°C. Temperatures above 45°C. inactivated the virus, making determinations at higher temperatures impossible.

These data indicate that the thermal requirement for viral inhibition is similar to that of the other manifestations of the properdin system (5, 10).

*The Effect of the Inhibition of Virus on the Components of the Properdin System*

While components of complement are needed for the interaction of properdin with substances such as zymosan and certain polysaccharides, the interaction does not necessarily inactivate the component required (13). In the classic

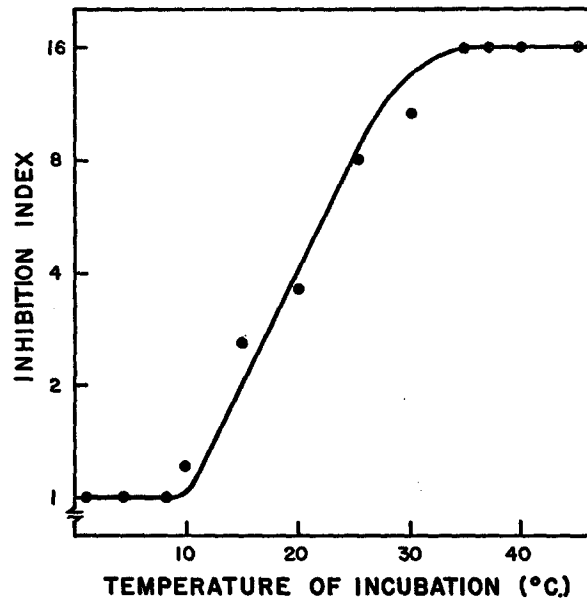


FIG. 2. Effect of temperature on viral inhibition (hemagglutination inhibition test).

inactivation of complement by zymosan, C'3 alone of the four components is destroyed (5, 15). At 15°C. to 17°C. the interaction of properdin with zymosan does not even inactivate C'3 (5), and certain preparations of zymosan and some polysaccharides, while combining avidly with properdin, do not inactivate C'3 at higher temperatures (up to 37°C.) (12, 13). The data of previous investigators indicated that the inactivation of virus by the heat-labile inhibitor in normal serum removed inhibitor without affecting complement (1, 2).

In order to ascertain the effect of the inhibition of virus on the properdin system, properdin, complement, and complement component titres were determined on serum following incubation with virus and were compared with similar determinations on serum incubated with allantoic fluid.

Equal volumes of dialyzed NDV or normal allantoic fluid and serum were mixed. Following incubation (37°C. for 30 minutes), the inactivation of the virus was determined by hemag-

glutination titrations and the properdin titres of the serum incubated with NDV and of the serum incubated with normal allantoic fluid were determined by zymosan assay (12) and bactericidal activity (10). Similarly complement and complement component titres were measured. Controls using heated serum and barbital buffer were used in parallel.

The results of such experiments are summarized in Table VII. Following incubation of serum and virus, there was significantly less properdin available in the serum mixed with virus than in the serum to which allantoic fluid had been added. The sera incubated with allantoic fluid contained an average of 8 units of properdin per ml.; the sera incubated with virus, 5 units of properdin per ml. The total hemolytic complement and complement component titres in both groups of sera were slightly less than usually found in fresh serum, particularly with regard to C'1 and C'2. This decrease was probably related to the well known instability of complement, especially C'1 and C'2, when diluted serum is incubated, and was believed to represent the slight spontaneous

TABLE VII  
*The Effect of the Inhibition of Virus on Properdin and Complement in Serum*

Test mixture	Titre of components of the properdin system after incubation, units per ml serum					
	P	C'	C'1	C'2	C'3	C'4
Serum + NDV	5	76	280	200	280	960
Serum + normal allantoic fluid	8	72	360	160	280	880

Hemagglutination titre of NDV = 1024; inhibition index of serum = 32.

deterioration of complement that would be expected following the incubation of any diluted serum. The complement (C') and complement component (C'1, C'2, C'3, and C'4) titres of the sera mixed with NDV or normal allantoic fluid, however, were not significantly different.

It can be concluded that the inhibition of virus, which requires properdin, rendered a significant amount of properdin unavailable. On the other hand, while all components of complement are also required, the inhibition of virus did not affect them, or else affected them to such a slight degree that the changes were not detectable by the techniques employed.

*The Restoration of Hemagglutinating Activity to Previously Inactive Virus-Serum Mixtures by Prolonged Incubation with Cation Exchange Resin*

The similarity between the requirements for the interaction of virus or zymosan with the properdin system and the demonstration that, following inhibition of virus, available properdin concentrations are decreased, suggested that virus might interact with properdin in a manner analogous to the

interaction of properdin with zymosan or polysaccharide. Properdin can be dissociated from zymosan (5). Previous experiments (1) suggested that when serum-virus mixtures containing inactivated virus were exposed to citrate for prolonged periods of time, there resulted a restoration of some hemagglutinating activity. Experiments were therefore designed to determine whether the exposure of serum-virus mixtures to cation exchange resin would result in the release or restoration of viral activity.

Equal volumes of fresh or heated serum and NDV were incubated at 37°C. for 30 minutes and the hemagglutination titre of the residual virus in the mixture determined. To each serum-virus mixture an equal volume of cation exchange resin, amberlite IRC-50 in the sodium cycle, was added and aliquots of the mixtures reincubated, at 37°C. for 30 to 90 minutes;

TABLE VIII  
*Effect of Incubation with Cation Exchange Resin on Inhibition of NDV by Fresh Serum*

Reaction mixture*	Incubation with resin		Hemagglutination titre
	37°C.	22°C.	
	<i>min.</i>	<i>hrs.</i>	
Fresh serum and NDV	—	—	64
	90	—	64
	90	18	256
Heated serum and NDV	—	—	1024
	90	—	512
	90	18	512

\* Previously incubated at 37°C. for 30 minutes.

at 4°C. for 18 hours; or at room temperature (22°C.) for 18 hours after incubation at 37°C. for 90 minutes. Suitable controls without resin were treated in a similar manner. During incubation, the resin was intermittently remixed with the serum-virus mixture by gentle inversion. Following the entire procedure, the degree of viral activity was again measured. All determinations of viral activity were made by hemagglutination titrations.

The result of one such experiment is summarized in Table VIII. Incubation of serum-virus mixtures with cation exchange resin for short periods of time (at 37°C. for 30 to 90 minutes) or for long periods of time in the cold (18 hours at 4°C.) did not influence the hemagglutination titre of the mixture. Incubation of serum-virus mixtures with resin at room temperature for 18 hours following incubation at 37°C. did, however, result in a significant restoration of hemagglutinating activity. Incubation of suitable controls, with or without resin, in a similar manner had no effect on the hemagglutination titre of such mixtures.

In a series of six such experiments, comparable and significant release of virus was obtained under these conditions. At no time, however, was complete reappearance of hemagglutinating activity induced. Generally only 10 to 50

per cent of viral hemagglutinating activity could be restored to the mixture in this manner. The limited reappearance of viral (hemagglutinating) activity has precluded satisfactory infectivity experiments. These data and the data previously obtained (1) do, however, indicate that inhibition of viral activity, as evidenced by hemagglutination at least, was reversible to a certain extent under these conditions.

#### DISCUSSION

It is apparent from the experiments described here that the properdin system can inactivate Newcastle disease virus. Inhibition of virus requires all the known constituents of the properdin system: properdin, all four components of complement and magnesium. The removal of any one of these results in a loss of inhibition; replacement of the missing constituent restores antiviral effect. These findings identify the properdin system as a single system capable of viral inhibition, lysis of certain erythrocytes (7, 8) and bacteria (10) and destruction of protozoa (11).

The differences between the properdin system and systems requiring antibody, which are apparent from the distinctly separable factors involved, have been previously discussed (5, 10). The differentiation of inhibitors for virus has also been reviewed by many authors (1-4). The studies presented here, which equate the properdin system with the heat-labile inhibition of virus however, permit further differentiation. The Francis type of inhibitor is recognized by heat stability, susceptibility to destruction by RDE, and eventual dissociation of virus from the inhibitor (2, 19, 20). This substance is a mucoprotein and inhibits hemagglutination, not infectivity, of the myxoviruses (2, 20). In contrast, antibody and heat labile inhibitor, which do affect infectivity, are classically distinguished from each other by the thermal instability of the latter (1-4). Furthermore, inhibition of virus by the properdin system, as shown here, requires complement but does not inactivate it; in contrast, inactivation of virus by antibody does not require complement, although when complement is present it may be "fixed" by the antigen-antibody combination. It is of interest that "fixation" of complement by antigen-antibody interaction induces the inactivation of C'1, C'2, and C'4; C'3 is not affected (22). C'3, which is inactivated by the interaction of properdin and zymosan at 37°C., has previously been shown to be necessary only for the lysis of specifically sensitized erythrocytes and bacteria in systems requiring antibody (23).

The properdin system contains six known variables, all of which are essential for the viral inhibitory effect of serum. Five of the variables, properdin and the four components of complement, are relatively unstable. They may lose activity on handling, storage, or heating, and may be inactivated by seemingly innocent procedures; for example, through activation of the plasmin system (18, 22). Under these circumstances, experimental difficulties would be expected to



occur, and it is not surprising, therefore, that differences exist in the literature concerning the role of complement for the inactivation of virus by the heat-labile inhibitor (1-4, 24). Some of these differences may be related to the protection or inactivation of complement by the technical procedures used; for instance, the destruction of complement by trypsin (1, 25). The removal of inhibitor by adsorption with *V. Cholera* extracts, which has been shown to be independent of RDE activity (2), may be similarly related to the interaction of properdin with endotoxin, polysaccharide or cell walls (13).

Viral inhibition results in a decrease in the amount of available properdin in serum without affecting the concentration of the components of complement in a measurable fashion. Thus it seems likely that virus and properdin interact, possibly in a stoichiometric manner similar to the interaction of properdin with zymosan or polysaccharide. The components of complement would then be considered as cofactors. The relationship between properdin concentration and viral inhibition is not inconsistent with this hypothesis of stoichiometric proportions. In addition, it has been demonstrated that there exists a quantitative relationship between magnesium concentration and viral inhibition by the properdin system (26), similar to that of the bactericidal activity (10). This interaction also suggests a stoichiometric relationship. When serum-virus mixtures containing inactivated virus were incubated for prolonged periods with citrate (1) or a cation exchange resin, dissociation of virus with hemagglutinating activity occurred. Since both these procedures decrease or remove available magnesium from the system, these findings become of interest. The dependence on magnesium for the initial interaction of virus and properdin, and the finding that conditions which remove available magnesium result in restoration of hemagglutinating activity, suggest that magnesium may be an integral part of the virus-properdin complex. That hemagglutinating activity never returns completely suggests that either the bond, if it exists, is extremely firm, or that further interaction with magnesium is prevented by a process such as steric hindrance. It is further possible that a certain proportion of the virus which reacted with the properdin system is irreversibly inactivated.

Heat-labile inhibitors of virus have long been recognized (1-4, 27, 28) and have on occasion been considered a "laboratory" nuisance" (4). The description of the properdin system as a single system with bactericidal as well as antiviral activities warrants speculation on the viruses that may be affected. The properdin system appears identical with the inhibitors described for mumps, influenza A and B viruses, as well as Newcastle disease virus (1-4), and has requirements similar to those of the factor which neutralizes vaccinia and variola viruses (27, 29). In addition to these animal viruses, we have recently been able to confirm the observations (30) that the properdin system inhibits at least one bacterial virus (*E. Coli* phage T2). Apart from these viruses which are inactivated in the absence of antibody, the occurrence of a heat-labile factor in serum which

potentiates the neutralization of Western equine encephalomyelitis, Rous sarcoma, dengue, and herpes simplex viruses (24, 31-35) has been described. This factor may also be the properdin system. The requirement for antibody does not preclude the participation of the properdin system; antibody, and the properdin system may act synergistically as has been found in the destruction of *Toxoplasma gondii* (11, 36).

There is no available evidence to indicate whether the properdin system has antiviral activity *in vivo*. Even though such viral inhibition may exist, the inhibition of virus would not be a general phenomenon. Many groups of viruses are notably resistant to inactivation by the properdin system; for example, fresh serum is conspicuously non-inhibitory for the polioviruses (37). Inhibition of virus may also resemble the lysis of bacteria in that susceptibility to the properdin system may be a property of the strain, not of the species (2, 10). Finally, although from a teleologic viewpoint it is unlikely that the properdin system exists in serum without function, the fact that mumps virus is readily inactivated *in vitro* by the heat-labile inhibitor of human serum (1), and yet viremia is an important aspect of the pathogenesis of mumps, presents an unresolved dichotomy.

#### SUMMARY

Detailed experiments are presented which indicate that the properdin system is an inhibitor of Newcastle disease virus. Viral inhibition required all known components of the properdin system: properdin, all four components of complement and magnesium; the removal of any one constituent resulted in a loss of inhibition; the replacement of the constituent restored antiviral effect. The inhibition of virus was temperature-dependent. The process of inhibition by serum resulted in a decrease in the amount of properdin available in the serum without any measurable effect on the components of complement. The prolonged incubation of inactive serum-virus mixtures with cation-exchange resin resulted in the restoration of some, but not all, of the hemagglutinating activity of the virus. The requirements of the properdin system and the implication of these findings were discussed.

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