(Table II). A few strains appeared to be similar in one cross: 11914 antisera with RO virus (6.4; 6.7) and quite different when the reciprocal cross was used, RO antisera with 11914 virus (6.7; 4.7). Sometimes the neutralization titer appeared to be greater using a heterologous serum than a homologous one (11914 antisera and RO virus) (6.4; 6.7). Strain 11914 with which this occurred most frequently has been found to protect chickens better against challenge with certain other strains than against itself(3). The sensitiveness of individual sera in detecting antigenic differences varied considerably, (Table II), but in general, chicken and rabbit antisera were equally sensitive in detecting differences among strains of NDV. The study of the antigenic diversity of strains of Newcastle disease viruses by the serum neutralization test while still in preliminary stages has revealed differences of diagnostic importance, and raises a question of the feasibility of using a single vaccine in all situations.

Summary. Seventeen heterologous combinations of Newcastle disease virus and antiserum have been used in serum neutralization tests. Of these combinations 13 exhibited a difference of greater than 1.0 log between the neutralization of the homologous and the heterologous virus by prepared antiserum. A difference of 5.0 logs was observed between 2 strains.

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Hemolysis of PR8-Virus Sensitized Erythrocytes.* (20755)

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Microbial components may be roughly separated into 2 general groups with respect to their ability to agglutinate erythrocytes of various species. One, consisting of various viruses and bacterial products, exhibits the well-known Hirst phenomenon or direct hemagglutination(1,2); and the other, consisting usually of polysaccharide components of various bacteria, sensitizes erythrocytes of various species to agglutination by antisera homologous to the adsorbed microbial components(3-5). In the case of the former group(6) and of the latter group(5,7-10) it has been found by several workers that following the addition of antiserum homologous with the adsorbed microbial component, the addition of complement (C′) brings about lysis. This type of hemolytic system, therefore, differs from the usual complement-fixation system in that the antibody is directed not toward the erythrocyte but to the adsorbed component.

One phase of a related problem in which we are interested directed our attention to a study of the conditions under which the influenza virus could be involved in the lysis of erythrocytes. A survey of the literature available to us failed to reveal any reports upon the lysis of influenza virus-sensitized erythrocytes under the influence of C′, in spite of the generally accepted procedure of inactivating sera before use in the hemagglutination-inhibition test(11). This report will describe some results obtained from such a study.

Methods. Veronal buffer, pH 7.4(12) was

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used as diluent in all hemolytic procedures, and 0.85% NaCl was used in all hemagglutination (HA) tests. Erythrocytes were collected from several individuals of the same species in Alsever's solution and stored as such at 4°C. For use, erythrocytes were washed 3 times in the appropriate diluent and pooled from several individuals. Sharp and Dohme Lyo-complement was used as the source of guinea pig C′ and C′ from other species was collected and stored as serum at —20°C and used within a few days. The egg-adapted departmental strain of the PR8 influenza virus was used throughout this study, representing a pool of chorio-allantoic fluid (CAF) virus possessing an original hemagglutinating titer of 1:2,560(11). HA and hemaggluation-inhibition (HAI) tests were performed as described by the Expert Committee on Influenza(11), except that a 0.25% suspension of erythrocytes was used. Antiviral serum was prepared in rabbits and used as a pool of sera collected from several immunized rabbits and stored at —20°C. Normal rabbit serum was prepared in a similar fashion from non-immunized rabbits. All sera and C′ were subjected to multiple adsorptions with appropriate erythrocytes sufficient to remove naturally occurring antibodies before use in tests. Hemolytic titrations generally followed the procedure of Kabat and Mayer(12) and Mayer *et al.*(13). K values (50% hemolytic end-points of C′) were determined by plotting curves on log-log paper, following the equation of von Krogh (12), and taking the x-values where values of $y/1 - y = 1.0$. $1/n$ values, or the slopes, were also measured from the curves. y values (% hemolysis) were calculated from optical density (O.D.) determinations, using a Coleman Universal spectrophotometer at 550 mμ, which wavelength was selected on the basis of spectral-transmittance curves of hemoglobin from chicken erythrocytes.

Results. In a preliminary study it was found that guinea pig C′ did not lyse several species of erythrocytes, including those of the chicken, which had been sensitized only with virus. On the other hand it was found that chicken erythrocytes which had been sensitized with virus at 4°C and washed, and in the presence of anti-PR8 rabbit serum were lysed by guinea pig C′. Such findings resemble those of Fisher and Keogh(6) who worked with a hemagglutinin from *Hemophilus pertussis*.

In further studies it was found that C′ from the guinea pig, dog, rabbit, turkey, chicken, duck, and human were all active in lysing PR8-sensitized chicken erythrocytes in the presence of anti-PR8 rabbit serum. All of the various species of C′, as well as the anti-PR8 rabbit serum, were previously adsorbed with chicken erythrocytes, and the human C′ required additional adsorption with centrifuged PR8 virus because it was found to contain PR8 antibodies.

Preliminary studies of optimal conditions for this hemolytic procedure led us to accept the following constant conditions, some of which would possibly vary with different pools of virus and antivirus: One volume of washed, packed chicken erythrocytes was sensitized at 4°C for 90 minutes with 100 volumes of virus diluted 1:10. The sensitized erythrocytes were then washed twice at 4°C and resuspended in veronal buffer to a final concentration of approximately 1.4%. The density of this suspension was further adjusted so that 1.0 ml, when completely hemolyzed with 6.0 ml distilled water, gave an O.D. of 0.50 ± 0.2. Equal volumes of this sensitized suspension and the appropriate antivirus dilution were thoroughly mixed and allowed to stand for 10 minutes at room temperature. Two ml portions of this mixture were pipetted into a series of tubes, to which varying volumes of a constant C′ dilution were pipetted and the total volume in each tube brought to 7.0 ml with veronal. The tubes were stoppered, incubated at 37°C in a water bath for one hour with mixing at 10-minute intervals, centrifuged, and the O.D. values read on the supernatants. Plotted points were averages of duplicate tube readings. Other conditions of this procedure, including controls, have been discussed at length elsewhere(12,13).

Fig. 1 shows the results of a typical titration. It can be seen that K values generally increase with an increase in the dilution of antivirus. The exception is the 1:10 dilution

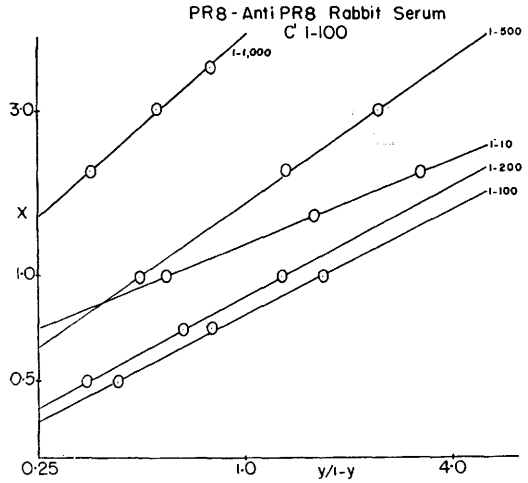

FIG. 1. Hemolysis of PR8 virus-sensitized chicken erythrocytes in the presence of variable antivirus dilutions (1:10 to 1:1000) and C′ volumes. Log-log scale. x equals ml of C′ and y the % hemolysis in the von Krogh equation: $x = K\left(\frac{y}{1-y}\right)^{1/n}$, where K is the 50% hemolytic unit of C′ and 1/n is the slope of the curves.

of antivirus which shows an inhibition of C′ activity resembling the Neisser-Wechsburg phenomenon. More interesting is the increase in slopes of the curves with increase in antivirus dilution. It is apparent that the 1:1,000 dilution of antivirus contains insufficient antibody to sensitize over some 50% of the cells to hemolysis by volumes of C′ permissible in our procedure. One might expect this curve to increase sharply beyond this last point, which finding has been substantiated in other experiments. In order to be able to plot points on both sides of the point of 50% hemolysis (y/1 — y = 1.0), antivirus dilutions of 1:500 or less were used thereafter.

The increase in slope with corresponding increase in antivirus dilution noted in Fig. 1 and Table I is not typical of hemolytic curves involving chicken erythrocytes sensitized with anti-chicken erythrocyte rabbit serum (Table II). It may be seen in Table II that the slopes are quite constant over antibody (hemolysin) dilutions from 1:10 to 1:1,000.

Control experiments were set up, following exactly the same titration procedure described above, except that: a) Pooled CAF from non-infected embryos was used to treat the erythrocytes in place of CAF virus. Such treated red cells were then mixed in separate titrations with both anti-PR8 rabbit serum and pooled serum from non-immunized rabbits, and b) virus sensitized erythrocytes were titrated with pooled serum from non-immunized rabbits. No hemolysis occurred in either a) or b).

Although it is tempting to speculate upon the increasing 1/n values as being a reflection of the combination of virus and antivirus in variable proportions, the possibility of interaction between some normal CAF component and anti-PR8 rabbit serum influencing the slopes was considered. Therefore, a titration identical with that carried out for Fig. 1 and Table I was performed, with the exception that antivirus was previously adsorbed with a sedimentable component from normal pooled CAF. Approximately 18 ml anti-PR8 rabbit serum (diluted 1:5) was adsorbed with sediment from 76 ml pooled normal CAF obtained by centrifugation at 24,000 G at 4°C for one hour. Such adsorption did not appreciably alter the change in 1/n values.

Since both the sedimentable and more soluble components of CAF influenza virus are known to fix C′ with their respective antibodies, the nature of the component responsible for this hemolytic reaction was studied.

TABLE I. K and 1/n Values of Hemolytic Curves of Virus-Antivirus Sensitized Chicken RBC. C′ dilution 1:25.

Antivirus dilution	K (ml)	1/n
1:10	.33	.36
1:100	.21	.58
1:200	.26	.62
1:500	.57	.85
1:1000	1.52	.87

TABLE II. K and 1/n Values of Hemolytic Curves of Hemolysin-Sensitized Chicken RBC. C′ dilution 1:50.

Hemolysin dilution	K (ml)	1/n
1:2	.26	.15
1:10	.19	.28
1:100	.47	.27
1:500	.62	.27
1:1000	1.02	.28
1:5000	2.50	.78

TABLE III. Activity of Virus Components Separated by Centrifugation.*

CAF virus	HA titer	Hemolytic procedure: 100% C′ titration, unit of C′	50% C′ titration K (ml)	50% C′ titration 1/n
Before centrifugation	1:200	.05†	.96	.62
Supernatant	1:400	>.20	—‡	—
Sediment	1:10	.05	.48	.33

* CAF virus centrifuged at 24500 G at 4°C for 90 min.

† ml C′ (1-5) required for 100% lysis.

‡ Insufficient hemolysis with C′ volumes used to enable plotting significant curve.

TABLE IV. K and 1/n Values of Hemolytic Curves of Inactivated Virus-Antivirus Sensitized Chicken RBC. C′ dilution 1:25.

Antivirus dilution	K (ml)	1/n
1:10	.66	.38
1:100	.36	.55
1:200	.65	.66

Pooled PR8 virus was centrifuged at 24,500 G at 4°C for 90 minutes. The upper half of the supernatant and the sediment resuspended in the bottom half of the supernatant fluid were both studied for hemagglutinating activity and activity in the hemolytic reaction. In the hemolytic titrations, both 50% and 100% hemolysis end-points of C′ were determined. 50% end-points were determined as described above and 100% end-points were determined following the same general protocol, except that 0.2 ml volumes of 1.0% suspension of sensitized erythrocytes were mixed with 0.2 ml volumes of antivirus (diluted 1:100), varying C′ volumes from 0.01 to 0.2 ml were added, and total volumes brought up to 1.0 ml with veronal buffer. Table III shows that activity of the virus in this hemolytic reaction follows that of the hemagglutinating particle.

The possibility that elution of virus, under our conditions of incubation at 37°C, might be responsible for the change in slope noted in Fig. 1 was investigated. The same procedure for hemolytic titrations was followed with the exception that virus, which had been inactivated at 56°C for 30 minutes, was used. This inactivation was found to reduce the HA titer only slightly but to markedly inhibit elution at 37°C. Table IV shows that such inactivation did not appreciably alter the change in slopes from those with non-inactivated virus (Table I). This might indicate that the lytic process had begun soon after the C′ was added at 4°C or before much virus had eluted at 37°C.

Summary. A hemolytic system is described, which involves the lysis of virus-antivirus sensitized chicken erythrocytes by C′ and apparently depends upon specific interaction of virus and antivirus. Hemolytic curves resemble the usual erythrocyte-hemolysin hemolytic curves except that the slopes of the curves in the former increase upon dilution of antivirus. Activity of CAF virus in such a system apparently follows that of the hemagglutinating particle. Inactivation of the virus at 56°C for 30 minutes, which markedly inhibited elution at 37°C, had no appreciable effect upon such hemolysis by C′ in terms of the change in slopes. Changes in 1/n values were also not appreciably altered by previous adsorption of antivirus with a sedimentable component from pooled normal CAF.

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