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The Inactivation of Newcastle Disease Virus Hemolysin by Antiserum and High-Energy Electrons.* (24296)

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Previous studies on the hemolysin of Newcastle Disease Virus (NDV) have shown that this property is influenced by the physical treatment of the virus(1), is inhibited by calcium ions(2), and is dependent on pH and the temperature of incubation(3). Radiation studies on NDV(4) have shown that there are about 15 hemolysin units per virus and that the form of these units is probably a flat plate 100-140 Å in radius and 20 Å thick. The experiments described here are concerned with the mechanism of action of the NDV hemolysin. The results of Dulbecco *et al.* (5) and Rubin and Franklin(6) indicate that study of virus neutralization by antibody may yield valuable information on structure and components of the virus. Inhibition of the hemolysin by antiserum and inactivation by high-energy electrons were studied.

Methods. The 87909 strain of NDV was used throughout these studies. High titer virus stocks were prepared by inoculating fertile chicken eggs on the tenth day of incubation with about 10^7 NDV particles. After 30 hours of incubation the infected eggs were chilled to 4°C and the allantoic fluid was harvested. Infected allantoic fluid was then centrifuged for 30 minutes at 30,000 g in a Servall SS-1 centrifuge at 4°C. The virus deposit was resuspended in isotonic saline containing 2% sodium citrate and stored at 4°C until used. *Virus assays.* In the hemagglutinin assay, serial 2-fold dilution of virus in citrate saline were added to .2 ml of a 4% suspension of washed chicken red blood cells (rbc). The highest dilution of virus showing agglutination was scored as the endpoint. All virus samples to be used for hemolysin assays were first dried in citrate saline and resuspended in the same medium. This drying is similar in effect to freezing and thawing and increased the hemolysin titer of the virus preparations about 50-fold. Hemolysin titrations were performed in two ways. The *total hemolysis assay* was adapted from Granoff and Henle(1). 1.0 ml of a virus suspension was placed in a test tube with 1.0 ml of washed rbc in citrate saline (pH 7.2). This mixture was incubated at 37°C for one hour, then 1.0 ml of cold phosphate buffered saline (pbs) was added to stop the reaction. Rbc were removed by centrifugation and optical density of the supernatant fluid was measured at 540 mμ. One tube containing rbc but not virus was used to balance the spectrophotometer and thus eliminate the effect of spontaneous hemolysis. In another tube distilled water was added to give 100% hemolysis. The percent hemolysis for each sample was then calculated from the optical densities. The *hemolysis rate assay* is a modification of the total hemolysis assay. 1.0 ml aliquots of a virus suspension were mixed with 1.0 ml of a 1% suspension of washed rbc and incubated at 37°C. The hemolysis reaction was allowed to proceed in different tubes for 1, 2, and 3 minutes, then stopped by addition of 2 ml of cold pbs. Rbc were removed by centrifugation and optical density of the supernatant fluid was measured at 540 mμ. Rate of he-

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TABLE I. Total Hemolysis Assay.

Relative virus concentration	Optical density	% hemolysis
1.0	.420	100%
.8	.400	96
.6	.370	88
.4	.322	77
.2	.240	57
.1	.188	45
.08	.160	38
.06	.138	34
.04	.107	26
.02	.078	19
.01	.046	11
Distilled water	.420	100

Virus preparation contained about 1000 hemagglutinin units/ml before dilution.

molysis was determined by plotting percent hemolysis at each time and calculating percent hemolysis per minute.

Neutralization by antiserum. 1.0 ml of a diluted chicken antiserum against NDV (containing about 400 hemagglutinin inhibiting serum equivalents/ml) was added to 1.0 ml of a virus suspension and incubated for one hour at 37°C. Aliquots of this mixture were then tested for hemagglutinin and hemolytic activity by the methods described above.

Electron irradiation. Virus samples for irradiation were prepared by pipetting .05 ml of high-titer virus on ½ inch diameter round glass cover-slips. These samples were then dried in a vacuum desiccator. Dry samples were irradiated in vacuum with various doses of 1.0 mev electrons which were produced by a High Voltage Engineering Van de Graaff generator. Electron beam currents were measured with a microammeter and electron beam areas were determined by irradiating a piece of osilid paper placed in the sample position and measuring the exposed area of the paper. From the known beam area, beam current, and duration of irradiation, the dose given each sample was calculated in electrons/cm^2. Irradiated samples were resuspended in citrate saline and assayed as described above.

Results. Table I gives data for the total hemolysis assay. Percent hemolysis rises with increasing virus concentration and approaches a maximum of 100% hemolysis at a virus concentration of about 1000 hemagglutinin units/ml. This result is similar to that obtained by Granoff and Henle(1), but differs from that obtained by Sagik and Levine(3), who found that percent hemolysis decreased with increasing virus concentration at pH 5.9-7.7. The latter result was probably due to the fact that their virus preparation contained sufficient calcium ion to inhibit the hemolysis process. A plot of the data from Table I was used as a standard curve for total hemolysis assay.

The percent hemolysis for a given virus concentration increases linearly with time for the first 5 minutes of incubation, then begins to level off. The slope of the straight line portion of this curve is proportional to the virus concentration over a wide range of values (Table II). In the hemolysis rate assay, the percent hemolysis per minute was measured and a plot of the data of Table II was used as a standard curve for the assay.

Fig. 1 shows the effect of antiserum on hemagglutinin and hemolysin of NDV. The percent of active virus hemagglutinin or hemolysin remaining after incubation with antiserum is plotted *vs.* the antiserum concentration in serum equivalents. One serum equivalent is the antiserum concentration which reduces the virus titer to 37% of the controls (5) and corresponds to a concentration of one antibody molecule per virus particle, assuming neutralization to be a monomolecular reaction. Both of these curves show the same rate of neutralization and are "single hit," *i.e.* 1 antibody molecule is sufficient to inactivate either property.

Inactivation of the NDV hemolysin with 1.0 mev electrons using the hemolysis rate assay is shown in Fig. 2. The percent hemolytic activity is plotted *vs.* the electron dose. This single hit inactivation curve may be represented by the equation

TABLE II. Hemolysis Rate Assay.

Relative virus concentration	% hemolysis at 1 min.	2 min.	3 min.	% hemolysis/min.
100	19	37	51	19
84	15	25	46	15
67	10	24	30	11.3
50	8	20	30	11
33	6	11	16	6
17	2.8	4	6	2.3

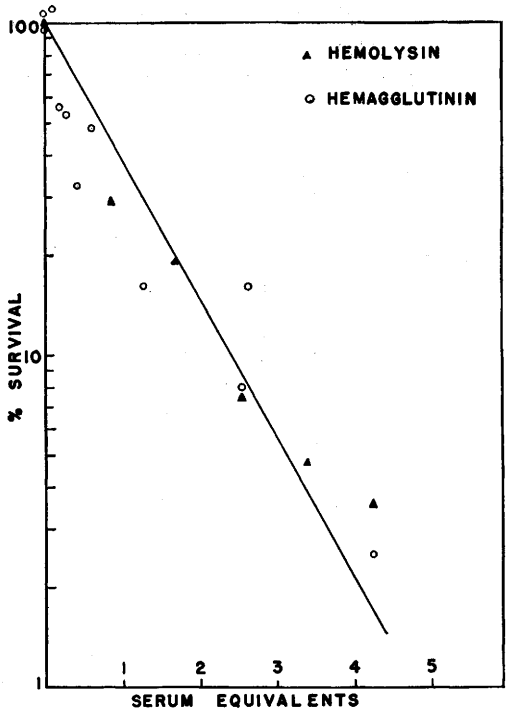

FIG. 1. Inhibition of NDV hemagglutinin and hemolysin by antiserum.

$$N/N_0 = e^{-VD}$$

where N/N_0 is the surviving fraction after an electron dose D, and V is the radiation sensitive volume of the hemolysin unit. The inactivation volume found is 4×10^{-19} cm^3 which corresponds to a molecular weight of 310,000 for the hemolysin unit. For details of the calculation of molecular weights from radiation data see Pollard *et al.*(7).

Inactivation of the NDV hemolysin by 1.0 mev electrons using the total hemolysis assay is shown in Fig. 3. The multiple hit inactivation curve may be represented by the equation

$$N/N_0 = 1 - (1 - e^{-VD})^M$$

where M is the number of hemolysin units per virus. From the slope of the curve in Fig. 3,

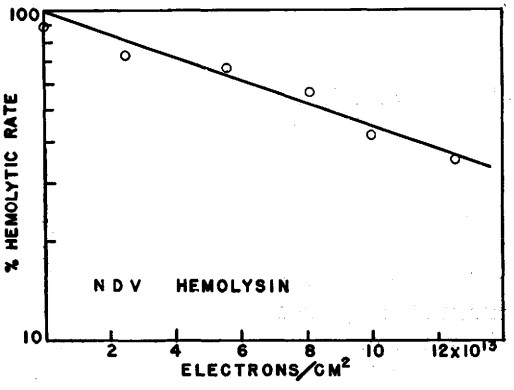

FIG. 2. Inactivation of NDV hemolysin by 1.0 mev electrons using hemolysis rate assay.

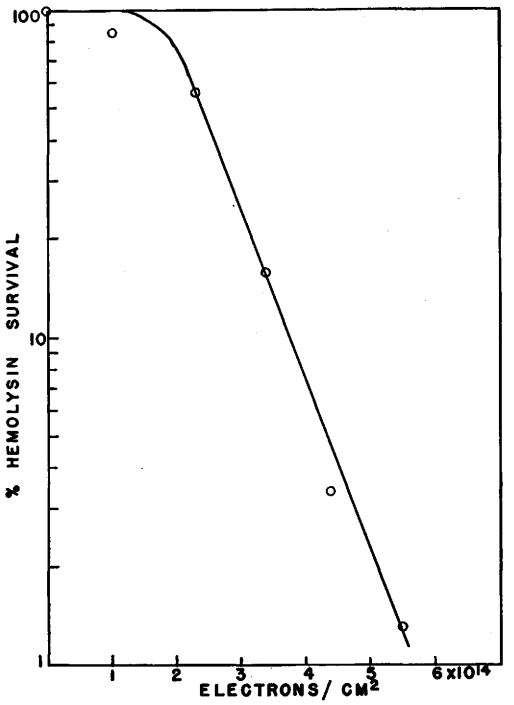

FIG. 3. Inactivation of NDV hemolysin by 1.0 mev electrons using total hemolysis assay.

$V = 4.8(10)^{-19}$ cm^3 which corresponds to a molecular weight of 370,000, and average value of multiplicity in these experiments was 14 ± 5. A similar result was obtained in previous studies on the NDV hemolysin(4) which indicated that there are 15 ± 3 hemolysin units per virus.

Discussion. 1.0 mev electrons produce ionizations along their paths about every 4000 Å. When an ionization occurs inside a hemolysin unit on the virus, chemical bonds in the molecule or molecules making up this unit are broken and the hemolysin unit is no longer able to function. From the shape of the curve in Fig. 3 it can be seen that electron inactivation of one or 2 hemolysin units does not destroy hemolytic activity, but that all 15 units

must be inactivated before this activity is lost. Thus only one intact hemolysin unit per virus particle is necessary for the virus to be able to lyse rbc.

In Fig. 2, reduction of rate of hemolysis by NDV is seen to be proportional to dose of radiation and is single hit. Rate of hemolysis depends on number of intact hemolysin units per virus particle and although a virus particle with only one intact hemolysin unit can lyse rbc, it does so at a much slower rate than a virus with 15 intact hemolysin units.

The results of neutralization of the NDV hemagglutinin and hemolysin by antiserum (Fig. 1) indicate that either process is inactivated by adsorption of one antibody molecule per virus particle. Since the NDV hemagglutinin is presumed to reside in two sites on opposite sides of the virus, it is not surprising that one antibody molecule adsorbed to one virus particle renders it unable to agglutinate rbc. It is, however, surprising that the hemolysin unit is inhibited by only one antibody molecule per virus particle. It is possible that the 15 hemolysin units are clustered together in such a way that the attachment of one antibody molecule to any one hemolysin unit makes it impossible for the other 14 to function. This seems unlikely since it has been estimated that about 10-20% of the total surface area of the virus particle is covered by the hemolsyin units(4). It is also possible that the virus possesses a single attachment site for the rbc which is not associated with the hemolysin units. Thus adsorption of one antibody molecule to this attachment might then inhibit hemolysis by preventing attachment of the virus to the red cell surface. Rubin and Franklin(6) observed that NDV possesses between 2 and 3 such attachment sites for the host cell although only one antibody molecule adsorbed to the virus particle will inhibit the process of infection and plaque formation. It would seem that NDV has several different attachment sites which serve different purposes and act on different cell receptors.

Summary. The NDV hemolysin is shown to be inactivated by a single antibody molecule per virus. Electron irradiation of NDV indicates that rate of hemolysis of rbc depends on number of intact hemolysin units per virus, whereas ability to lyse rbc, given sufficient time, depends on the presence of only one intact hemolysin unit per virus.

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Enhancement of Tetracycline Serum Levels with Glucosamine Hydrochloride as Adjuvant. Cross-over Studies in Beagle Dogs. (24297)

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In the effort to achieve greater serum concentrations of antibiotics after oral administration, most research has centered on chemical modification of the antibiotic or the use of adjuvants. As an example of the latter, Carlozzi has shown that serum levels of tetracycline* in man were enhanced after oral administration by the use of the amino sugar, D-glucosamine as an adjuvant(1). Data on serum concentrations after oral administration

* Registered trade mark of Chas. Pfizer and Co., for tetracycline is Tetracyn.