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Destruction of Virus Hemagglutination Inhibitor of Egg-White by Newcastle Disease Virus.* (21968)

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The viruses of mumps, influenza and Newcastle disease cause agglutination of certain mammalian red blood cells (RBC) by connecting these cells and forming a lattice-like matrix. If the ratio of virus and RBC is controlled, the reaction can be observed visually. In most instances the virus elutes or dissociates from the RBC after an indefinite time following mixing of the virus and red cells. The virus-treated cells are no longer capable of being "rebridged" by the virus but the virus retains its capacity to act again on untreated cells(1).

It is now known that normal ferret serum, serum from other animal species(2), a variety of tissue suspensions(3), ovalbumin(4), cow's milk(5), etc., will inhibit viral hemagglutination (HA). Inhibitor present in all of these substances seems to be very similar in composition to the "virus receptor" substance of the surface of erythrocytes which is commonly regarded as mucoprotein(6,7). Viruses of the mumps-Newcastle-influenza group can enzymatically destroy the inhibitor common to these materials. The exact role of this enzymatic action in infection has not been determined. One hypothesis is that firm adsorption to the cell surface is needed to allow the virus to enter the cell.

Since so many variations in characterized properties, *e.g.*, pathogenicity, heat stability of the hemagglutinin, of strains of Newcastle disease virus (NDV) exist, a study of the enzymatic activity of several strains of this virus seemed warranted. The following studies have utilized crude egg-white as the substrate or HA inhibitor in determining the mucinase activity of NDV.

Materials and methods. Viral strains. A repository for strains of NDV, maintained in the Department of Veterinary Science, University of Wisconsin, with the cooperation of the Agricultural Research Service, U. S. Department of Agriculture and the Newcastle Disease Technical Committee of the North Central States Region, has more than 80 strains, including 1 from Australia, 8 from Canada, 2 from Mexico, 1 from Japan, 8 from Europe and the remainder from the United States. All repository strains and the N strain of Dinter(8) were tested for their ability to destroy egg-white inhibitor (EWI). For the rate studies, however, only 7 strains were employed: Vic-Australia-1932, B1, Hert-Eng-1933, Milano-Italy-1945, GB-Texas-1948, (H)Najarian-Ohio-1948 and Roakin-NJ-1946. The Lee strain of influenza B virus was used as the indicator virus in the hemagglutination-inhibition (HI) tests. White Leghorn chick-

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en eggs from a commercial hatchery were used. As far as could be determined, these eggs came from flocks not vaccinated or infected with Newcastle disease.

Viral pools. Newcastle disease and N viruses were prepared by inoculating 10 or 11-day-old chicken embryos, intraallantoically, with 0.05 ml of a 10^{-2} dilution of stock, allantoic-fluid virus. Inoculated eggs were incubated at 98 to 99°F and candled 2 to 3 times daily. From embryos that had died after 24 hours, allantoic fluids were harvested and pooled. If the fluids were to be used within 4 to 5 days, they were stored at 4°C; otherwise at −20°C. The Lee strain of influenza B virus was prepared by inoculating 10 to 11 day-old, embryonated eggs, intraallantoically, with 0.05 ml of a 10^{-2} dilution of stock, allantoic-fluid virus. After further incubation at 98 to 99°F for 65 hours, the living embryos were chilled overnight and allantoic fluids harvested and handled as described for the other viruses. Calcium chloride ($CaCl_2$) 1 g, sodium chloride (NaCl) 9 g, boric acid (H_3BO_3) 1.203 g, and sodium tetraborate ($Na_2B_4O_7 \cdot H_2O$) 0.052 g were dissolved in 1 liter of distilled water, pH 7.0 ± 0.2. *Hemagglutination tests.* Starting with a 1 to 5 dilution of allantoic-fluid virus, serial 2-fold dilutions were made in 0.25 ml volumes of physiological saline contained in 13 x 75 mm tubes. Chicken RBC, 0.25 ml of a 1% suspension in physiological saline, were thoroughly mixed with each dilution. The test was read after 30 to 45 minutes at room temperature. The endpoint, 1 HA unit, was the highest dilution of virus showing 1+ agglutination. One plus HA was not evident in most instances since the last tube to show agglutination was usually 4+. Consequently, most 1+ values were obtained by interpolation, *e.g.*, if the highest dilution of virus showing agglutination was the 1 to 320 dilution and the degree of agglutination was 4+ the 1+ value was 320 plus three-fourths the difference between 320 and the next dilution, 640. The result is a value of 1 to 560 for 1+ agglutination.

Hemagglutination-inhibition test. Starting with a 1 to 100 dilution of EWI solution, serial 2-fold dilutions were made in 0.25 ml volumes of physiological saline contained in 13 x 75 mm tubes. Five HA units of indicator virus contained in 0.25 ml were mixed with each dilution. After 30 minutes at room temperature, 0.25 ml of a 1.0% suspension of chicken RBC was added to each tube and the HA patterns ascertained 45 minutes later. The endpoint was considered as the last tube to show complete inhibition of agglutination. *Egg-white inhibitor or ovomucin.* The "thick" albumin of fresh, White Leghorn eggs served as the source. Stock ovomucin was a 5% solution in calcium borate, buffered saline. Inhibitory activity was determined by HI testing using 5 HA units of heated Lee virus as the indicator antigen. One HI unit was considered as the smallest quantity of inhibitor required to completely inhibit the activity of 1 HA unit of the heated, Lee strain of virus. *Indicator virus.* Allantoic fluid from eggs infected with the Lee strain of influenza B virus was heated at 56°C for 30 minutes. This heated virus has the capacity to combine with inhibitor but not the ability to destroy it. Consequently, heated Lee virus serves well as a sensitive indicator for inhibitor titrations.

Results. In exploratory experiments employing 7 strains of NDV, each was effective in inactivating EWI substance. Subsequently, the entire collection of NDV strains in the repository and the N strain of Dinter(8) were tested for their ability to destroy EWI. Tests were conducted by adding 20 HA units of each virus to 1 ml of a 1:100 dilution of raw egg-white and incubating the mixture at 37°C for 15 hours. The reaction was terminated and virus activity eliminated by heating the mixture at 65°C for 30 minutes. The content of each tube was assayed for EWI content by an HI test employing heated, Lee strain of influenza B virus as the indicator. With each strain of NDV 20 HA units completely inactivated the inhibitor contained in 1 ml of 1:100 dilution of egg-white.

A series of experiments was designed to study certain kinetic features of the reaction between EWI and NDV. Strains Vic, Hert-E, Milano, GB, Roakin, Najarian and B1 were selected for the first experiment in which the rate of inactivation of 1280 HI units of EWI by each strain was determined as a function of its HA concentration. The reaction was car-

ried out at 37°C for 90 minutes after which time the reaction was stopped by heating as indicated. The content of each tube was assayed for residual EWI by HI testing with heated, Lee B indicator virus. Under the conditions of the experiments, the inactivation rates differed among strains and was dependent on the virus concentration within strains. For the Hert-E, Milano and B1 strains, only 32 HA units were required to completely destroy the inhibitor whereas 64 units of the Najarian and GB strains and 128 units of the Vic and Roakin strains were required.

The next experiment was to determine, as a function of time, the rate of inactivation of 1280 HI units of EWI by various quantities of 6 strains of NDV. The strains employed were the same ones used in previous experiments with the exception of the Najarian strain. Three quantities of each virus suspension were used, 256, 128 and 64 HA units. The virus and EWI mixtures were incubated at 37°C, and at 5 minute intervals for 30 minutes, a sample of each mixture was removed and heated at 65° C for 30 minutes. The residual content of EWI was assayed as in the previous experiment. The Milano, Hert-E and B1 strains not only inactivated the EWI most rapidly but behaved similarly, except for less activity with the 64 HA units concentrations with Hert-E. The inactivation rates of GB and Vic strains were similar but somewhat slower than of the Milano, Hert-E and B1. The Roakin strain did not inactivate any EWI within the 30 minute test period. In all cases where inactivation occurred, the rate of inactivation of EWI corresponded directly to the HA concentration of the virus.

The third experiment was designed to ascertain the rate of inactivation of EWI by strains of NDV as a function of EWI concentration. The strains of NDV were the same as employed in the previous experiment. Three concentrations of EWI, 1280, 640 and 320 HI units, were used and 128 HA units of each strain of virus were employed. The virus and EWI mixtures were treated exactly as described in the previous experiment. Hert-E, Milano and B1 gave similar inactivation patterns. The patterns of Vic and Roakin were similar while that of GB was intermediate between the two groups. For all strains, the rate of inactivation was fastest with the 320 HI units of EWI. The rates of inactivation with 640 and 1280 HI units approximated each other but were slower than the 320 HI unit concentration.

TABLE I. Capacity of NDV* in Allantoic Fluid Containing 0.4% Formalin to Destroy Egg-White Inhibitor.

NDV strain	Time in days after treatment with formalin					
	3	4	9	14	21	28
Vic	++†	—	—	—	—	—
Hert-E	++	++	++	++	++	±
Milano	++	++	++	++	++	—
GB	++	++	++	++	++	++
Roakin	++	++	++	—	—	—
Naj	++	++	++	++	++	++
B1	++	++	++	++	++	++
Md-II	++	++	++	++	—	—
Hert-M	++	++	++	++	++	±
Ky-50	++	++	++	++	++	++
Lederle	++	++	++	++	++	++
RO	++	++	++	++	++	—
Wagner	++	++	++	++	++	±
Appleton	++	++	++	++	++	++
CG	++	++	++	++	++	++
ViPol	++	++	++	++	++	++

* All strains of unformalinized NDV destroyed egg-white inhibitor after storage at 5°C for 25 days.

† —, ±, ++ = no, partial and complete destruction, respectively, of egg-white inhibitor in 1:100 dilution of egg-white.

The final experiment was concerned with the stability of the mucinase property of allantoic-fluid NDV when exposed to 0.4% formalin. Sixteen strains were employed. At 3, 4, 9, 14, 21 and 28 days after adding the formalin and storing the mixtures at 4°C, the ability of each strain to destroy EWI was tested. This was accomplished by adding 0.25 ml of formalinized, allantoic-fluid virus to a 1:100 dilution of ovomucin. After overnight incubation at 37°C, the mixtures were heated at 65°C for 30 minutes. The residual EWI determinations are recorded in Table I. All strains except Vic possessed mucinase activity at 4 days after formalinization and at 14 days Roakin had lost mucinase activity. The Md-II strain did not inactivate egg-white inhibitor after 21 days. After 28 days, RO was inactive, while Hert-E, Hert-M and Wagner were only partially active.

Discussion. The mucinase activity of the mumps-Newcastle-influenza group of viruses is

widely recognized. If viral mucinase has any biological significance, it remains to be defined. Many hypotheses have been proposed but none have been definitely proved. However, it is well established that viruses are intracellular parasites which must invade and enter a susceptible host cell before multiplication can occur. This immediately raises the question of the mechanism of penetration of the host cell and the relation of inhibitors to the infectious process.

Almost all cells of the body, especially those of the respiratory tract, and except those of the skin, are protected by a mucous secretion. Since the viruses mentioned above have, in the main, an affinity for respiratory tract tissues, mucinase may have as its function the destruction of this protective material in order to clear the way for the attachment of the virus to the host cell preparatory to its penetration.

Strains of NDV are known to vary markedly from one another in properties such as pathogenicity, heat stability of the hemagglutinin and neurotoxicity. For some strains these properties have been well defined. However, no one has reported any mucinase studies of the various strains of NDV with the purpose of possibly relating them to some of the known properties. This purpose prompted, in part. the studies presented in this paper.

All strains of the repository were able to destroy the hemagglutination inhibitor of hen's egg-white. After the preliminary experiment, 7 strains of NDV markedly different from each other were selected for determination of the rate of the reaction. Despite the fact that these studies were performed in a crude fashion since neither the enzyme nor the substrate were separated and purified from their native sources, the results obtained appear susceptible to logical explanation. The first concern was with the rate of inactivation of EWI as a function of virus concentration. The 7 strains employed fell into 3 groups: (1) Hert-E, Milano and B1; (2) Najarian and GB; (3) Vic and Roakin. Group 1 inactivated the inhibitor rather quickly while the other 2 were progressively less active. With the exception of the Najarian virus, these 7 strains were used in other studies of inactivation rate as follows: (1) Rate of inactivation of 1280 HI units of EWI by various quantities of NDV as a function of EWI concentration. For each study, the 6 strains fell into 3 groups which are the same groups as described for the initial rate study. The greater the quantity of virus (enzyme), the faster was the inactivation rate. For the inhibitor concentration studies, the results were slightly different since the 320 HI units were inactivated in all cases at a faster rate than the 640 and 1280 units, which were inactivated at about the same rate. Whether the slower rate for the larger concentrations of the inhibitor was due to higher viscosity of the crude substrate solution is not known. There was also observed in all of these experiments a lag period whose duration depended on the concentration of the enzyme.

The activity groups into which these strains fell appear subject to correlation with only one known property *i.e.*, the rate of their elution from red cells. There is no apparent correlation between mucinase activity and pathogenicity for chickens since 2 highly pathogenic strains, Hert-E and Milano and a non-pathogenic strain, V1 constituted the group with the greatest mucinase activity.

A study dealing with the stability of mucinase to 0.4% formalin indicated this enzymatic activity was fairly stable.

Summary. Studies were made on the presence and mucinase activity of Newcastle disease virus and of the stability of this enzyme function to formalin. (1) All strains of Newcastle disease virus studied possessed mucinase activity although the rates at which they inactivated egg-white inhibitor differed greatly. (2) The rate at which egg-white inhibitor was inactivated was dependent on enzyme (virus) concentration. The concentration of egg-white inhibitor also affected the rate of its inactivation. (3) The rate of inactivation by Newcastle disease strains of virus could be correlated only with one known property—its rate of elution from red blood cells. (4) Newcastle disease virus mucinase was fairly stable to formalin.

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Importance of Optimum Amounts of Virus to Demonstrate Neutralizing Antibody Rise in Convalescent Poliomyelitis Sera.* (21969)

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(Introduced by L. Emmett Holt, Jr.)
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A study of 38 patients infected with poliomyelitis viruses showed a 5-fold or greater increase in the homologous antibody titer in 35 (92%). This finding was at variance with that of other workers(1,2), who postulated that the rise in neutralizing antibodies occurred so rapidly that often no increases in titer were demonstrable. Thus Miller and Wenner, using a virus concentration of 32 $TCID_{50}$† in their neutralization tests, observed a 10-fold rise in homotypic antibody in only 3 of 14 patients(2). Tests for homologous antibody were carried out in only 7 patients, none of whom showed a similar rise. Melnick and his associates, with a virus inoculum of 100 $TCID_{50}$, reported rises of homotypic antibody in 15 (37%) of 41 patients from whom poliomyelitis viruses had been isolated(1). Homologous antibody formation was not studied by these workers.

This report presents evidence that the demonstration of increases of homologous antibody in patients infected with poliomyelitis viruses is related to the dose of virus used in the neutralization test.

Materials and methods. Data are derived from 38 patients studied in New York City in 1953, a non-epidemic year.‡ Their stools were collected within 2 weeks of onset of illness and after concentration in the Spinco high speed centrifuge, yielded poliomyelitis viruses as follows: Type 1, 25 patients; Type 2, 2 patients; Type 3, 11 patients. All but 4 showed some degree of weakness or paralysis. Serum specimens were collected at frequent intervals and tested for neutralizing antibodies against the patient's own virus and the 3 prototypes. To be reported here are the results of tests carried out on mixtures of homologous virus and serum obtained usually in the 1st or 2nd week of illness, in the 3rd or 4th week, and lastly at about the 12th week. Trypsinized monkey kidney cells grown in tissue culture in glass tubes§ and examined microscopically for cytopathogenic changes constituted the test object for isolation of virus and for neutralization tests. Neutralization tests were carried out usually with serial 4-fold dilutions of serum, inactivated at 56°C for 30 min. Serum dilutions were mixed with an equal vol-

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† $TCID_{50}$—Tissue culture infective doses, 50% end point.

‡ We are grateful to the staffs of Willard Parker, Mt. Sinai, Babies', and Flower-5th Avenue Hospitals for supplying material for this investigation.

§ For the past year tubes containing cultures of monkey kidney cells supplied by Microbiological Associates, Inc., Bethesda, Md., have been used.