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Interaction of Newcastle Disease Virus Strains of Different Virulence with Cell Receptors

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INTERACTION OF NEWCASTLE DISEASE VIRUS STRAINS OF DIFFERENT VIRULENCE WITH
CELL RECEPTORS

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A B S T R A C T

Interaction of NDV strains belonging to lentogenic, mesogenic and velogenic groups with cell receptors was studied using chicken red blood cell (RBC) system. A certain minimal (strain-specific) amount of the pre-adsorbed virus which splits a maximally possible (for a given strain) quantity of neuraminic acid (NANA) was found (a state of "enzymatic saturation"). The phenomenon interpreted as "crawling" of the adsorbed virions upon the RBC surface followed by "browsing" of RBC receptors and liberation of NANA was described. Neuraminidase (Nase) activity of the attached virus ("in situ Nase activity") as a factor providing both virion elution and "crawling" was quantitatively studied. The in situ Nase activity of all the strains used was determined quantitatively by (1) parameters of enzymatic kinetics (V_{max} , K_m , and K_m/V_{max}) and (2) parameters of enzymatic efficiency related to a certain quantity of the adsorbed virus. Usage of a specific inhibitor of viral Nase showed the enzyme involvement into the virus-induced hemolysis. Employment in the studies of avian paramyxoviruses (PMV) other than NDV revealed their diverse antigenic inter-connections with NDV. This permitted to suggest the presence of common antigenic determinants for a number of avian PMVs. The usage of modified dye uptake method for CPE measurement permitted to establish quantitative indices which appeared to be more sensitive than classic indications of the NDV pathogenicity. All the elaborated indications can be used in practice as in vitro markers of the in vivo virulence for diagnostics, selection of vaccinal strains, attenuation of virulent strains and vaccine control.

I N T R O D U C T I O N

Newcastle disease virus (NDV) displays a wide range of strains strikingly differing in their virulence starting from highly virulent epizootic (velogenic) strains causing absolute mortality and extending to avirulent (lentogenic) strains which cause no clinical symptoms (see reviews by Hanson, 1972, and Lancaster and Alexander, 1975). A remarkable phenomenon is that, according to the majority of the authors, all these different strains have no difference in antigenic properties of the viral glycoprotein entities possessing both hemagglutinin (HA) and neuraminidase (Nase) activities (HN glycoprotein, according to Choppin's terminology, Scheid et al., 1979). According to very few communications there are little differences among the strains detectable by kinetic neutralization test (Schloer, 1974; Schloer et al., 1975, Schloer, 1977; Pennington, 1978; Avery and Niven, 1979). In any case, this means that any factor(s) responsible for the NDV virulent properties is not connected with the presence (or absence) of a particular viral protein(s) making significant contribution to the virus HN antigenicity which could be inhibited by specific antibodies.

In connection with this, the present studies were undertaken on the basis of the following hypothesis. The strain-specific properties of NDV are associated with a specific kind of arrangement of viral envelope subunits, namely, the HN glycoprotein molecules and those possessing fusion activity (F), upon the virion surface. Particular arrangements may cause different steric effects - hindrance or facilitation - on various processes connected with virus-cell receptor interaction, namely, attachment of virions onto cell receptors, elution of the attached virions from the cell surface and the virion connected with (if not dependent on) in situ enzymatic action of the viral Nase, i.e. immediately on spot of the attachment of the virions to cell receptors. As a result of this in situ Nase activity free N-acetyl-neuraminic acid (NANA) must be split

from the cell receptors. Indeed, the in situ Nase activity on cell receptor substrate measured by direct determination of split NANA has been demonstrated in the influenza virus-red blood cell (RBC) system (Tsvetkova and Lipkind, 1968) as well as on influenza virus and NDV-chick embryo cell monolayer system (Lipkind and Tsvetkova, 1970, 1973; Tsvetkova and Lipkind, 1973).

However, during the conduct of the project we have met with the necessity to pursue two interesting and important leads which have arisen after analysis of the data obtained in our studies and the contemporary situation in the field. These topics were as follows:

1. The recently obtained data about antigenic relatedness between NDV and some other avian paramyxoviruses (PMV) which were isolated during the last decade were taken into account. This relatedness would suggest the same antigenic determinants in the structure of HN glycoproteins belonging to different PMV serotypes.

Impregnation of the NDV-specific antigenic determinants into the whole structure of the HN antigen belonging to different PMV serotypes would display an unlooked-for novel model for exploration of the main working hypothesis on which the whole project was based namely, correlation of the viral pathogenic properties with a specific kind of steric arrangement of HN subunits upon the virion envelope. This consideration came to our attention when we had already found clear correlation between some kinetic properties of the in situ Nase activity and the virulence of various NDV strains using virus-RBC experimental system. Involvement of the avian PMVs other than NDV but having the NDV-specific antigenic determinants in their HN antigen into the studies would much widen the experimental capacity of the proposed working hypothesis and be valuable and promising for further research on the problem under consideration.

M A T E R I A L S A N D M E T H O D S

Viruses. The virulent strains: Italien, Israel/67 and Israel/79; the mesogenic strain: Komarov, MAJ and Roakin, and the lentogenic strains: B1, LaSota, F146 and Queensland were used. All the strains were propagated in chicken embryonated eggs and were partially purified and concentrated as described previously (Rivet et al., 1980).

The following avian PMV reference viruses (designated together with abbreviations) were used:

PMV-2/chicken/California/Yucaipa/56	Yucaipa
PMV-3/turkey/Wisconsin/68	Ty/Wisc
PMV-3/parakeet/Netherlands/449/75	Pk/Neth
PMV-4/duck/Hong Kong/D3/75	D3/HK
PMV-6/duck/Hong Kong/D199/77	D199/HK
PMV-7/dove/Tennessee/4/75	Dove/Tn
PMV-8/goose/Delaware/1053/76	Goose/Del
PMV-9/duck/New York/22/80	Duck/NY
PMV-?/rock pigeon/Otaru/76	Pigeon/Ot

Cells. (a) RBC of 3-months-old chickens, known to be free of NDV and NDV-neutralizing antibodies, were used. The RBC were kept in Alsever solution at a concentration of 50% and were stored at 4°C for no more than 5 days. Before use, cells were washed three times with saline (0.15 NaCl, pH 7.2) and were resuspended to the appropriate concentration. Cells were diluted according to cell counting and hemoglobin determination after complete lysis of the cells with 0.03 M NH₄OH.

(b) Cell culture: Secondary chick embryo cell monolayer culture was used. Cells were grown in 25 cm² Falcon flasks on M199 (Hanks) medium containing 0.5% lactalbumin hydrolysate, 0.025 M Hepes buffer pH 7.4 and 10% calf serum.

Adsorption. Virus-RBC mixture pools corresponding to any combination of RBC and virus concentrations were kept in ice bath on a shaker. Samples were taken at intervals and immediately centrifuged in an Eppendorf centrifuge at 4°C for 1 minute at 12 000 rpm. Residual (non-adsorbed) virus was determined in supernatants and the quantity of adsorbed virus was quantitated by subtracting the titer of the residual virus (in supernatants after adsorption) from the titer of the input virus (before adsorption).

Elution. Virus-RBC mixture pools corresponding to various amounts of the adsorbed virus were prepared according to the above-described procedure, centrifuged at 1500 rpm (IEC-CENTRA-7R) at 4°C, supernatants were collected for determination of the residual virus and replaced by saline up to the initial volume. These cell-virus mixtures were then transferred immediately into a shaking water bath at 37°C. Aliquots from each pool were taken at intervals, cooled in an ice bath, centrifuged in an Eppendorf centrifuge for 1 minute at 4°C and the quantities of the eluted virus were determined in supernatants.

In situ neuraminidase activity. The in situ Nase activity was determined by detection of free NANA in the same supernatant samples obtained during the above-described experiment on determination of virus elution. The NANA content was detected in supernatants after elimination of hemoglobin accumulated there as a result of hemolysis proceeding concomitantly with the virus elution (Rivetz et al., 1980) by thiobarbituric acid assay (Warren, 1959) in modification of Aminoff (1961).

Hemagglutination assay. Since all the quantitative determinations were based on the hemagglutination titration and the precision of this method is rather low, the following modifications were used: Each sample was titrated in double using

various initial dilutions before two-fold serial dilutions, namely: 1;2, 1:3, 1:5, 1:5, 1:7, 1:9 and 1:11. End-point was determined as the highest dilution (within the whole set of the dilutions) which gave 100% hemagglutination pattern with no traces of hemagglutination pattern in the successive well. Since the performance of such test for each sample was time-consuming, the system of crosses designating a percentage of HA pattern was used (Horsfall and Tamm, 1953) which was adjusted in the way to correspond to the results obtained by titration using a set of various initial dilutions (Lipkind et al., 1973).

Neuraminidase inhibition (NI) test was performed according to the "Advanced Laboratory Technique for Influenza Diagnosis" (Palmer et al., 1975).

Statistical treatment of HI and NI titer values. It was found that although there were considerable fluctuations in values of HI and NI titers in various experiments the fluctuations of the corresponding values of cross reactivity (differences between the values of homologous and heterologous HI and NI titers) were substantially less expressed. Therefore, the statistical treatment was based on the latter values of the cross reactivity which designated as a difference d between homologous and heterologous titers expressed in $\log_2$. The Student's probability value P for the found differences (d) for a given number of experiments n was calculated. The reliability of the d values was accepted to be positive if it was in agreement with the 2nd Fischer's criterion of probability (95% probability, $P=0.95$). In the case of the absence of the cross reactivity between certain viruses the d was expressed as infinity (∞).

The same principles of the statistical treatment (Spiegel, 1961) were used in the cases of two-side asymmetric cross reactivity (cases displaying one-side asymmetry evidently did not need any statistical treatment). In this case the cross reactivity between compared viruses X and Y was considered to be asymmetric if the difference d_1 (when anti- X serum against both the viruses was used) was different from d_2 (when anti- Y serum against both the viruses was used).

Vital dye. Neutral red (NR) was kept as a stock solution of 0.1% in bi-distilled water. Prior to the experiment, the dye solution was diluted to the appropriate concentration (usually 0.01%).

Measurement of NR uptake by infected cells. Cells were inoculated with the virus in a multiplicity of 50 PFU per cell and adsorption period was 1 hour at 4°C. Unadsorbed virus was removed by three successive washings with cold PBS. Four ml of maintenance M199 medium supplemented with 0.025 M Hepes buffer, pH 7.4 and 2% calf serum, were added and the cells were incubated at 37°C. At intervals, 1 ml of 0.01% neutral red was added to each of 4 flasks and the cells were incubated in the dark (to avoid photo-oxidative effects) for 30 minutes (pulse). The quantity of the accumulated NR was determined immediately after pulse. The maintenance medium from 2 of 4 flasks was removed, the cells were washed with PBS pH 7.2 and 5 ml of ethanol-citrate Sørensen I buffer, pH 4.2 (50:50 v/v) solution were added (extraction of the intracellular dye). The quantity of the bound NR was determined in the other 2 flasks. After removing of the maintenance medium and washing with PBS, 5 ml of fresh maintenance medium without NR were added to the cells which were then incubated for 1 hour at 37°C in the dark (chase). In order to take into account NR contained in the cells detached from the flask surface, the maintenance medium was centrifuged, pellet was washed with PBS and added by the ethanol-citrate Sørensen buffer solution for the dye extraction. Thus, the NR was determined both in the cells remaining in the monolayer culture and in the cells detached and floating in the medium, the total of extracted dye being determined spectrophotometrically at 540 nm. The NR uptake was expressed as the specific dye accumulation and binding, i.e. NR accumulated or bound per cell protein.

Protein content was determined both in cell monolayer and in the detached cells after NR extraction. Treatment of the cells by ethanol-Sørensen solution was shown not to interfere with the protein determination by Lowry method (1951).

Lactate dehydrogenase (LDH) leakage from cells. LDH leakage determination was performed in parallel flasks. At different times post infection, the media were collected and the cells were washed and scrapped from the surface by the aid of a policeman rubber. LDH activity was determined in the medium and in cell homogenate. The complete reaction mixture contained: 0.05 M phosphate buffer, pH7.5, 0.3 micromoles of sodium pyruvate, 0.16 micromoles NADH and enzyme in a final volume of 1 ml. The reaction was conducted at room temperature and the activity was determined spectrophotometrically by measuring the decrease in the adsorbancy at 340 nm.

CPE microscopic registration. The evaluation included changes in cell shape, presence of intracellular granulation and vacuolization, percentage of the cells of the monolayer as a whole and the estimation of degree of cell detachment. The estimation was done arbitrarily using cross system.

R E S U L T S

1. Kinetics of the virion attachment to RBC receptors in the case of NDV of different virulence.

The first step was to determine optimal conditions for virus adsorption, elution and in situ Nase activity. In this respect, two points were of importance. The first was the necessity to use an amount of RBC which would provide a total virus-adsorbing surface sufficient for accumulation of detectable quantity of the split NANA in the corresponding experiments. Second, valid quantitative comparison of the strains in elution properties and, especially, in in situ Nase activity could be done only if all the strains would have the same conditions of pre-adsorption. As far as the adsorbing capacities of different strains would differ, the assumption was that such a condition would be defined as a state of "physical saturation" of the total

receptor-containing surface capacity of RBC taken in experiment.

In order to achieve both the above conditions the following experiments were carried out. In the first experiment the dependence of virus attachment on RBC concentration was studied. The experiment was carried out using 10-minute-period of adsorption and two 10-fold differing virus concentrations: 80 and 800 HA units per ml. Adsorption was measured as a percentage of the adsorbed virus related to the quantity of the input virus. It can be seen (Fig. 1) that the adsorption of all the strains increases along with increasing RBC concentration. However, in the case of four strains - B1, F146, Israel/67 and Israel/79 - 100% adsorption was not achieved. For all the strains, 15% RBC concentration seems to be optimal since (a) at this concentration the virus adsorption was maximal for those strains which were fully adsorbed and (b) in the case of the strains which showed less than 100% adsorption there was no additional adsorption along with the further increase of RBC concentration. Thus, this experiment has shown some strain-specific differences in the completeness of virus adsorption. RBC concentration of 15% was chosen for further experiments.

The next experiment was to study kinetics of the adsorption of the viruses onto 15% RBC suspension. Two ten-fold differing virus concentrations - 140 and 1400 HA units per ml - were used. The amount of the adsorbed virus was also expressed as the percentage related to the amount of the input virus. The results (Fig. 2) showed very fast kinetics of adsorption of all the strains during the first 5-10 minutes. Six out of the ten strains used in the studies reached 100% adsorption. The adsorption of two of the rest of four strains - Israel/67 and Israel/79 - did not reach 100% in spite of the further incubation, and in the case of two other strains - B1 and F146 - there was a significant decrease in the quantity of the adsorbed virus. Significant decrease of the quantity of the attached virus was observed also in the case of adsorption of Israel/67 and Israel/79 strains at high virus concentration.

Taking into account these strain-specific differences, in the further experiments the adsorption period of 20 minutes was used as optimal for all the strains except B1, F146, Israel/67 and Israel/79 for which 10-minute adsorption period was used.

The aim of the next experiment was to achieve the state of "physical saturation" of total RBC surface by attached virions. Correspondingly, the dependence of adsorption on input virus concentration was studied. One can see that this experiment also shows some strain-specific differences (Fig. 3). Saturation conditions were achieved only in the case of Komarov strain. Four strains including all the virulent ones (Italien, Israel/67, Israel/79) and mesogenic strain MAJ showed linear dependence up to the value of adsorption corresponding to the input concentration of 3200 HAU/ml. Four other strains seem to show divergence of the dependence curves from linearity; this may mean that the "saturation" of the RBC adsorbing capacity becomes influencing.

These results together with the results of the previous experiments showed strain-specific differences in the virus capacity to attach to RBC receptors. However, it seemed that the state of "physical saturation" which we considered as an obligatory condition for further comparison of different strain is not achievable (with exception of Komarov strain). As far as Italien, Israel/67 and Israel/79 are concerned, it can be assumed that further increase of input virus concentration would cause the saturation. However, the curves corresponding to F146, Queensland, Roakin and LaSota, which show divergence from linearity suggest that these curves reflect not only the process of attachment but also detachment. This was also demonstrated in the case of B1 and F146 strains and less evidently in the case of Israel/67 and Israel/79 strains (Fig. 2). It is very probable that the absence of full adsorption shown in the experiment concerning the dependence of adsorption on RBC concentration is also due to the detachment process in the case of these strains (Fig. 1). Therefore, we came to

the conclusion that the condition of "physical saturation" of total adsorbing capacity of RBC cannot be used as a common criterion for the comparison of all the strains in the further studies.

The experiments, concerning the *in situ* Nase activity, helped to find the necessary criterion (Fig. 4).

2. Kinetics of elution at 37°C of the virions pre-adsorbed on RBC at 4°C.

In the next series of the experiments we studied the kinetics of the elution of the pre-adsorbed virus. For each strain, different amounts of adsorbed virus varying by 2-3 orders of magnitude were used. The results are presented in Fig. 5. First, it is clearly seen, that in the case of high concentrations of the adsorbed virus in all the strains there is a very fast kinetic of elution reaching 100% of virus liberation within 5 minutes. The characteristics of the elution kinetic curves were not changed until the lowest amount (20-60 HA units per sample for different strains), when the curves appeared to be quite different. At this low concentration there was no elution of the attached virus during the first 15-60 minutes and only then eluted virus began to appear in the supernatant. These data lead to two main conclusions: (a) in a large concentration range of pre-adsorbed virus the kinetics of the elution of the virus do not differ significantly, (b) however, if the density of the pre-adsorbed virus upon the RBC surface is less than a certain critical point the elution begins with a considerable delay.

3. Kinetics of accumulation of neuraminic acid split from RBC receptors by eluting virion Nase (*in situ* Nase activity). Correlation between kinetics of elution and kinetics of the *in situ* Nase activity.

First, it was necessary to study whether the elaborated conditions were optimal for detection of the free NANA split as a result of the *in situ* Nase activity. Essentially, it was the same experimental arrangement used before

(Fig. 3), with split NANA being detected after incubation of RBC loaded with virus at 37°C for 3 hours. The NANA was determined in supernatant buffered saline which was added at the same volume as that used in the virus adsorption experiments. As can be seen (Fig. 4), first, significant amounts of the split NANA were detected with all the strains. This means that the range of experimental conditions was suitable for the detection of the NANA split from the RBC receptors as a result of the in situ Nase activity of the attached virus. Second, there were strain-specific differences in the quantities of split NANA which were evident even at 200 HAU of virus per sample. Third, in most of the strains, a maximal quantity of the split NANA was achieved at 500-1000 HAU and further increase of adsorbed virus was not followed by the concomitant increase of the NANA quantity. The latter result leads to the following conclusions: a certain minimal amount of virus is sufficient to split the maximal quantity of NANA which can be split by this virus strain, and this is a strain-specific property. This observation was considered as a basis for formulating a state of "enzymatic saturation", as opposed to the state of "physical saturation".

In the light of the concept of "enzymatic saturation" the above conclusion about kinetics of elution depending on the density of pre-adsorbed virus can be correlated with the kinetics of the in situ Nase activity. Namely, a drastic difference in the shape of the elution curves along with the increase of the density of the pre-adsorbed virus can be described as follows:

After temperature shift from 4°C to 37°C viral Nase starts splitting NANA, breaking the bonds holding the virions upon the cell surface. In the case of a large amount of the pre-adsorbed virus, most of the NANA-containing bonds are broken as a result of a simultaneous enzymatic action of all the adjacently adsorbed virions which, thus, get free of the linkage and detach from the RBC surface. In the case of small amount of the pre-adsorbed virus the virions are scattered

upon RBC surface at a relatively long distance between each other. Therefore, just after viral Nase splits the bonds holding the virions, these virions immediately get held by NANA-containing bonds which had not yet been split by the adjacent pre-adsorbed virions. While the viral Nase continues splitting the newly formed bonds, further bonds continue to hold the virions. Such a process can be imagined as a "crawling" of the attached virions upon the cell surface (Burnet, 1952; Buzzell and Hanig, 1958) followed by "browsing" of the NANA-containing cell receptors. As a result of such crawling the number of NANA residues drops to a certain critical point when the remaining not yet destructed cell receptors cannot hold anymore the attached virions upon the cell surface. The virions then detach from the cell surface and this is recorded as the beginning of the elution. On the other hand, if the amount of the pre-adsorbed virus is above this critical point, so that most of the neighbouring cell receptors are split by the adjacent attached virions, the kinetics of the elution in a large range of concentrations of pre-adsorbed virus resemble each other (Fig. 5). Therefore, the split NANA must be detected in spite of the lack of the elution, being due to the in situ Nase activity of the "crawling" virus. This was evidenced by the experiment on direct determination of the split NANA (Fig. 6) which was detected in the same supernatant samples in which the eluted virus was determined (Fig. 5). The curves display kinetics of accumulation of split NANA in the case of the same sets of quantities of the pre-adsorbed virus for all the strains used. First of all, it is clearly seen (Fig. 5) that there is evident accumulation of the free NANA in the case of the lowest quantity of the pre-adsorbed virus, the highest rate of the NANA accumulation being observed mainly during the first 20 minutes when no eluted virus was detected (Fig. 5).

In order to find out whether there are strain-specific differences in the in situ Nase activity this property should be expressed in some quantitative form.

We defined the in situ Nase activity as a quantity of NANA split by a certain amount of the per-adsorbed virus per a certain time unit. As has been shown (Figs. 5 and 6), the accumulation of NANA is connected with either elution or "crawling" of the pre-adsorbed virus depending on its amount. Therefore, in order to normalize conditions for all the strains to be compared we used two main indications defining the efficiency of the in situ Nase activity.

1. E_{es} - is the efficiency of the in situ Nase activity referring to that amount of the pre-adsorbed virus which provides the "enzymatic saturation" (es) i.e. a minimal quantity of the attached virus which splits a maximal quantity of NANA (HA_{es}). In such conditions all the pre-attached virions elute simultaneously and the "crawling" is negligible (if any). The in situ Nase activity can be expressed by the equation:

$$E_{es} = \frac{NANA_{es}}{HA_{es} \times t} \quad (I)$$

where $NANA_{es}$ is a maximal quantity of the split neuraminic acid which can be reached in the case of a given virus strain at an appropriate time t ; HA_{es} is a minimal amount of the pre-attached virus which is sufficient to split $NANA_{es}$.

2. E_c - is the efficiency of the in situ Nase activity referred to the amount of the "crawling" virus during the initial period after the temperature shift when no eluted virus is detected. It can be expressed by the equation:

$$E_c = \frac{NANA_c}{HA_c \times t} \quad (II)$$

where $NANA_c$ is a quantity of the neuraminic acid split by the "crawling" virus at an appropriate time t after the temperature shift; HA_c is that amount of the pre-adsorbed virus which is below some critical concentration providing its elution.

As to the E_{es} , the main difficulty is the exact experimental determination of this minimal amount of the adsorbed virus which is sufficient to provide the conditions of the "enzymatic saturation" (HA_{es}). However, the experimental data (Fig. 5) present a set of various concentrations of the pre-adsorbed virus for each strain and the HA_{es} value appears to be within the limits of these concentrations. This gives grounds for the attempt to try an analytical determination of HA_{es} .

Essentially, the experimental data reflect the dependence of the rate (velocity) of the in situ Nase activity on the enzyme concentration, which is indirectly expressed through the amount of the pre-adsorbed virus measured by the HA determination. This, in fact, is a particular case of Michaelis-Menten kinetics (Ainsworth, 1977) when both components of the system are immobilized: the substrate is represented by RBC receptors immobilized upon cell plasma membrane and the enzyme entities are immobilized upon the rigid virion envelope. Since it was not clear a priori that our case could be adequately described by Michaelis-Menten kinetics, its applicability had to be determined.

(According to the Michaelis-Menten theory, the kinetics of an enzymatic reaction are described by the equation:

where E is an enzyme, S is a substrate, ES is an enzyme-substrate complex, P is a reaction product and K_1, K_2 and K_3 are the corresponding kinetic constants. In conformity with our case, E is the Nase of the pre-adsorbed virus - HA_{es} (I) or HA_c (II), S is a maximal quantity of NANA which can be split by the virus from a fixed amount of RBC receptors, and P is a quantity of the NANA actually split as a result of the in situ Nase activity of either

eluting (I) or "crawling" (II) virus.

In fact, the above-proposed E_{es} indication correlates with the velocity of the enzymatic reaction (which, in general, depends on the concentrations of both the substrate and the enzyme), when the substrate concentration is fixed (RBC concentration) and the concentration of the enzyme is a changing parameter (different concentrations of the pre-adsorbed virus). In accordance with this, we considered the quantity of the NANA split during five minutes after beginning of the elution as the velocity of the in situ Nase activity and, hence, plotted $1/\text{NANA}$ versus $1/\text{HA}$ (Fig. 7), according to Lineweaver-Burk plot (1934). Figure 7 shows the Michaelis-Menten kinetics plotted by simple plot as well as Lineweaver-Burk plot. The latter seems to display a certain strain-specific correlation between the steepness of the regression lines and the virulence. However, a quantitative comparison of the strains for finding strain-specific differences in the in situ Nase activity could be done only by means of numerical quantitative indications.

4. Elaboration of the quantitative in vitro markers of virulent properties of NDV strains.

On the bases of the above-described data and in accordance with the computations, the following indications have been calculated (Table 1):

1. $V_{\max}$ - a maximal velocity of an enzymatic reaction when the enzyme sites are saturated with the substrate and, hence

$$V_{\max} = K_3 [E_T] \quad (2)$$

where E_T is a total enzyme concentration (in our case this is an amount of the attached virus) and K_3 is a constant of the rate of the product (P) liberation (1) also called a turnover number. Thus, $V_{\max}$ can be expressed as a maximal quantity of NANA which can be split from the given constant amount of RBC receptors. The above-described conditions of "enzymatic saturation" provide the realization of the $V_{\max}$ value while corresponding only to a minimal amount of the enzyme needed for the maximal liberation of NANA (HA_{es} in our case).

2. K_m - Michaelis constant which is expressed as:

$$K_m = \frac{[E] \times [S]}{[ES]} = \frac{K_2 + K_3}{K_1} \quad (3)$$

3. K_m/V_{max} is an indication which can be considered as a very specific one for a given enzyme-substrate complex because it combines all the three kinetic constants of enzymatic reaction (3). Since nothing is known about relationships between K_1 , K_2 and K_3 in our system, the value of K_m/V_{max} which involves K_3 through V_{max} (2) is not just an arithmetic expression of the "known" two values K_m and V_{max} but it has an independent meaning.

Analysis of the values of all the three above-described kinetic indications (Table 1) shows as follows:

V_{max} does not seem to correlate with the virulence. K_m shows a clear correlation with the virulence: the higher the virulence, the higher the K_m . (The mesogenic strain Roakin seems to be the only exclusion standing close to the lentogenic viruses). The K_m/V_{max} indication also shows correlation with the virulence: the higher the virulence, the higher K_m/V_{max} . (The mesogenic Komarov strain seems to be an exception being close to the velogenic group).

The next task was to find out the values for the above-described expressions of E_{es} - enzymatic efficiency in conditions of "enzymatic saturation" (I) and E_c enzymatic efficiency in conditions of "crawling" (II). As to the latter indication, the values for $NANA_c$ and HA_c needed for the calculation of E_c can be directly taken from the experimental data (Figs. 5 and 6) because the absence of elution was quite sufficient proof that the respective HA_c values correspond to the state of "crawling". As to the E_{es} indication, the values of HA_{es} were determined from the corresponding Lineweaver-Burk plots by means of drawing two regression lines: one including 3 points respective to the low concentrations of the attached virus and the other including 2-3 points corresponding to the high concentrations of the attached virus. In the cases of three strains the Eadie-Hofstee plot (Gardiner and Ottaway, 1969) turned out to be a more appropriate

way for the HA_{es} estimation and in two cases (Queensland and LaSota) both the Lieweaver-Burk and Eadie-Hofstee plots did not help to estimate HA_{es} .

The final indication to be calculated was E_{K_m} which can be defined as the enzymatic efficiency of the attached virus whose amount is equal to K_m :

$$E_{K_m} = \frac{NANA_{K_m}}{HA_{K_m} \times t}$$

where $HA_{K_m} = K_m$, $NANA_{K_m}$ is the quantity of the NANA corresponding to the HA_{K_m} and $t = 5$ min.

Analysis of the values of the three above-proposed indications of the in situ Nase efficiency shows as follows (Table 1):

The in situ Nase efficiency in the state of "enzymatic saturation" (E_{es}) in the cases where it was determined shows correlation with the virulence: at least, there is an evident difference between the velogenic group (low efficiency) and all the other viruses for which HA_{es} was succeeded to be calculated (high efficiency).

Indication of the in situ Nase efficiency in the state of "crawling" (E_c) shows less expressed correlation with the virulence. Only mean data between marginal lentogenic and velogenic groups are in the limits of the region of significance ($P = 95.2\%$).

Indication of the in situ Nase efficiency per amount of the attached virus equal to the Michaelis constant (E_{K_m}) shows a clear correlation with the virulence.

5. Studies on the possibility of the existence of Burnet's RBC receptor gradient for different strains of NDV.

The performance of experiments in attempt to apply the concept of receptor gradient to NDV strains according to the proposed research plan meets with an obstacle: virus-induced hemolysis which proceeds simultaneously to the action of viral neuraminidase upon RBC receptors. In order to overcome this difficulty, we tried different approaches:

a) This approach was based on results that have been obtained by Clavell

and Bratt (1972) showing that only a small fraction of virus population has the capacity to lyse RBC. During hemolytic interaction these virions are losing this activity. In an attempt to isolate the non-hemolytic virus population it was found that: i) after one hemolytic reaction cycle, the virions still retain a part of the hemolytic activity which is sufficient to disturb in the receptor gradient experiments; ii) In addition, along with the decrease in the hemolytic activity there is a partial loss of the neuraminidase activity.

- b) We used Ca^{++} that is known to inhibit hemolysis. At a large range of Ca^{++} concentrations (2 to 20 mM) there were no significant changes in neuraminidase activity compared to that in the absence of Ca^{++} . However, the hemolysis was reduced by no more than 65%.
- c) Instead of intact erythrocytes we used resealed ghost preparations. These preparations showed receptor activity and it was possible to demonstrate the attachment of NDV and the action of neuraminidase on receptors. Moreover, using ghost preparations and a number of virus strains we could show differences in the kinetics of neuraminidase activity on the receptors, which resembled in part to those shown on intact RBC. Nevertheless, experiments that were performed to study the possible existence of the receptor gradient gave unclear results. Since the resealed ghost is much smaller than the intact cell, the amounts of the bound virus and split NANA may be not sufficient and not constant for proper detection.
- d) We used the original approach of Burnet, employing receptor destroying enzyme (RDE) from Vibrio cholerae. Preliminary experiments showed significant differences in the quantity of virus that is attached to erythrocytes by different strains of NDV after treating the cells with RDE (at several concentrations and incubation times). Moreover, it was also possible to demonstrate different quantities of NANA released when those virus-treated RBC complexes were transferred to 37°C. These preliminary results may indicate the possibility that such a gradient of receptors exists among NDV strains.

6. Studies on the functional significance of viral Nase.

The studies were composed of the two parts. The 1st one concerns conclusions derived from the experiments on the in situ Nase activity. These conclusions will be considered in the Discussion. The 2nd part, however, concerns especial studies on functional relationship between hemolytic and the in situ Nase activities. These studies were based on the found phenomenon of loss of the hemolytic activity of NDV during interaction with chicken erythrocytes accompanied by a partial loss of the in situ activity of viral neuraminidase, and hence, a delay in the elution of the attached virions from cell surface. The reduction in both activities showed a similar dependence in virus concentration: Both activities decreased with the increase of the amount of virus involved in the interaction and in temperature (no reduction at 4°C). The reduction in these activities occurred without any loss of the infectivity and the ability to agglutinate erythrocytes. Analysis of the kinetic properties of the enzyme after hemolytic interaction, in an in vitro system using soluble substrates, indicated that the kinetic parameters (K_m and V_{max}) of neuraminidase were altered. Interaction of the virus with fetuin (NANA-containing glycoprotein) did not result in any change in the kinetic constants of viral enzyme. Moreover, the in situ activity of the enzyme and the hemolytic activity did not change either. When the virus reacted with glutaraldehyde-fixed cells, that lost their capacity to bind the viral F glycoprotein (responsible for cell fusion and hemolysis), the results were similar to those obtained with normal cells.

These results might indicate that the enzyme inactivation is not due to the enzyme-substrate reaction, but probably to an interaction with cell membrane. Second, these results can probably account for the finding reported by Clavell & Bratt (1972) that the loss of hemolytic activity is not a result of hemolysis itself, but is due to a particular interaction with the cell; the cessation of the hemolytic activity might be associated with neuraminidase inactivation.

In order to study the possibility of a functional relationship between the hemolytic activity and the in situ neuraminidase activity of NDV and thereby to elucidate further the role of the enzyme in the early stages of virus infection, we employed a specific inhibitor of neuraminidase. 2-deoxy-2, 3-dehydro-N-acetyl neuraminic acid (DDNANA) is an analog of NANA known to inhibit specifically neuraminidase in an in vitro system. We first examined whether the attachment of the virus to RBC is affected by DDNANA. It was found that the inhibitor does not affect the adsorption of the virus on erythrocytes at a large range of concentration (10^{-6} to 10^{-2} M). This is an additional evidence that the hemagglutinating and neuraminidase activities belong to distinct sites at the same glycoprotein entities. We, next, examined the effect of the analog on the NDV-RBC complex following transfer to 37°C . The following results were obtained:

- 1) The in situ activity of neuraminidase is less sensitive to the inhibitor than the activity in vitro, using fetuin as a substrate (5×10^{-4} M and 64×10^{-5} M DDNANA for 50% inhibition respectively).
- 2) Elution of the virus is sharply inhibited at low DDNANA concentrations (by 90% at 10^{-5} M).
- 3) The hemolytic activity responds to the analog in a peculiar manner: at low DDNANA concentrations hemolysis increases along with the increase of the amount of the analog added; at a concentration where neuraminidase activity is inhibited by more than 60% (2×10^{-3} M), the hemolytic activity begins to drop sharply.
- 4) The hemolytic activity, as well as the neuraminidase activity, is reversed after the inhibitor is washed out.
- 5) Preliminary experiments indicate that by addition of excess of partially purified neuraminidase (from Vibrio cholerae), the hemolytic activity can be reversed.

The results of the experiments with the neuraminidase inhibitor along with occurrence of the parallel decrease in both activities during the interaction with RBC indicate that

viral neuraminidase has a role in the process of hemolysis induced by the virus. Moreover, based on these results we may suggest a mechanism where only a partial activity of neuraminidase is sufficient to initiate the hemolytic activity while, on the other hand, the neuraminidase action on cell receptors results in elution of the attached virus from cell surface, which, therefore, "competes" with the hemolytic activity.

7. Antigenic inter-relationships between different avian PMVs. Revealing of NDV-specific antigenic determinants in other avian PMV serotypes.

The full scale HI as well as NI cross reaction tests using 10 representative avian PMVs covering 8 of 9 established PMV serotypes, were carried out.

The results supported some of the previously made conclusions about the antigenic relationships between different serotypes of avian PMVs and revealed some other relationships. The overall data presented at Tab.2-5 & Fig.9 can be summarized in two main points:

1. There are various diverse antigenic connections between different established PMV serotypes, being one-side asymmetric in some cases, so that the whole network of interconnections is very complicated.

2. The antigenic connections found by means of HI test in some cases differ from those found by NI test.

As it can be seen (Figure 9), by HI test NDV has antigenic interrelationships with 7 representatives of avian PMVs belonging to 6 different serotypes and at NI test it has interrelationships with 6 representatives belonging to 5 different serotypes. This means that HN antigen of these PMVs has common antigenic determinants with NDV.

On the basis of these results a combinatorial mathematical model describing the experimentally found diverse antigenic interconnections is determined by the specific combinatorial sets of antigenic determinants, some of them being serotype-specific

and the others being common with some other avian PMV serotypes. The suggested model is based on the postulates concerning PMV virion structure; bifunctional organization of PMV HN glycoprotein, its amount per virion and a mechanism of antibody-caused inhibition of its functional activities; a definition of an antigenic determinant as an elementary unit inducing and reacting only with a homologous type of antibodies.

The suggested model through cooperating with mathematically expressed different definitions of antigenic kinship describes some experimental phenomena connected with interserotype antigenic relationships, especially, a long-ago-known but unexplainable phenomenon of asymmetric cross reactivity.

8. A quantitative approach for measurement of cytopathic effect caused by NDV strains differing by virulence.

Virus-induced cytopathic effect (CPE) is an expression of virus pathogenicity displayed on the level of virus-cell culture interaction. According to widely accepted views, the CPE represents morphological damage which can be registered by light microscopy observation. Therefore, the CPE estimation is based on a qualitative description of observed alterations both in the cells and in the cell monolayer as a whole. Such an approach being suitable when used for virus titration and neutralization ("all or none" principle of registration) is not satisfactory for estimation and comparison of virus strain virulence. Indeed, the quantitative estimation of the CPE is reduced to that concerning (a) intensity of cell destruction and (b) dynamics of the destruction development. Both the criteria have no strict quantitative parameters.

The approach elaborated here and based on the usage of vital dye uptake was to find out quantitative criteria of the CPE estimation.

The method of measurement of the vital dye uptake for registration of the virus-induced CPE was first suggested by Finter (1969) and it was then modified by Pidot (1971)

and McManus (1976). However, their procedures included long exposure of the cells to the dye and hence the registration was done according to the same "all or none" principle. The usage of the method for the quantitative approach could be valid if the following consideration was taken into account: since the vital dye is present only in living cells and is released outside as far as the cells die there should be a regular connection between the presence of the vital dye in the cells and a degree of the cell "vitality" meaning that cell damage should cause certain changes in the vital dye uptake. Since the exact determination of the vital dye uptake is well developed (Finter, 1969; Pidot, 1971; McManus, 1976) the approach would present a method for quantitative measurement of CPE.

However, preliminary studies on the measurement of neutral red (NR) uptake have revealed a complicated mechanism of NR-cell monolayer culture interaction. Namely, it was found that the dye uptake which is a general notion consists of at least three different processes being in dynamic equilibrium: accumulation of the NR in the exposed cell, binding of the dye to certain intracellular sites and release of the dye outside the cell. It was shown that quantities of accumulated, bound and released dye can be determined separately.

The report presents the results concerning the application of the elaborated method of dye uptake measurement to estimation of CPE induced by lentogenic, mesogenic, and velogenic NDV strains.

Figure 10 presents the results on NR uptake, CPE microscopic evaluation, LDH activity and intracellular protein content in cells infected by the highly velogenic NDV Italian strain.

Initial sign (+) of CPE (formation of gaps between adjacent cells) could be seen at 8 hours p.i. (Fig.10C). More definite CPE (++) could be seen at 10 hours p.i., where the gaps increased and at certain areas cell orientation began to change from a parallel array to centripetal arrangement. At 12 hours p.i., the involvement of the whole monolayer in the process could be seen (+++). At this stage the cells became vacuolized. At 14 hours p.i. there was a full CPE development (++++). Significant decrease in the specific NR binding was found at 6 hours p.i. while the accumulation of the dye began to decrease 2 hours later (Fig.10B). The reduction in both NR binding and accumulation stopped at 14 hours p.i., where the decreases were about 90% and 70%, respectively, in comparison to uninfected cells. Intracellular protein content began to decrease significantly at 12 hours p.i.; at 16 hours p.i. about 40% of the protein were still bound to cells (Fig.10A). A significant leakage of LDH was recorded at 10 hours p.i., the rate of the further leakage being exponential (Fig.10A). The curves obtained (Fig.10B) permit the expression of the effect of the virus infection on both NR accumulation and binding in the form of two indices:

a) the time p.i. of the beginning of significant decrease in dye uptake; b) the time p.i. when 50% decrease in dye uptake is reached. Tab.6 & Fig.11 summarize the data on the virus-induced decrease of NR uptake expressed by means of the above indices in the case of six strains of NDV of differing virulence.

DISCUSSION

The present studies have shown a correlation between the virulent properties and strain-specific variations in in situ Nase activity of different NDV strains expressed by different parameters. This general conclusion seems to demonstrate the

validity of the hypothesis upon which the study was based. The hypothesis is connected with the fact that a notion of "strain" in the case of NDV means a stable line characterized mainly by virulent properties meaning neither a serologically distinguishable type, nor a subtype, nor even a serological variety. Therefore, in the limits of the hypothesis any proposed virulence-bearing factor is not associated with an antigenically determinable portion in the HN glycoprotein, but with a specific mosaic arrangement of the HN and F subunits in the viral envelope. The differences among the strains are realized through a degree of "steric congruence" between the mosaic arrangement pattern of the HN spikes upon the virion surface and a mosaic pattern of NANA-containing receptor clusters upon the cell surface (Weiss, 1974), in particular, that of RBC (Bächi et al., 1977). Particular degrees of the congruence between both the mosaic patterns may cause either steric hindrance or steric facilitation for the action of Nase enzymatic sites of the HN molecules upon RBC NANA-containing receptors in situ. The outcome of this interaction is realized by accumulation of NANA split as the result of this in situ Nase activity.

In accordance with this, the main effort of the studies was directed to determining appropriate conditions permitting a quantitative comparison of the in situ Nase activity of all the strains used. During these studies, however, some unexpected phenomena were found. First, in the case of B1, F146, Israel/67 and Israel/79 NDV strains full adsorption of the virus at 4°C upon RBC was not achieved (Fig. 1) and, moreover, a sharp decrease in the amount of the adsorbed virus was registered (Fig. 2). This can be explained by detachment of the adsorbed virus (Fig. 2) which was observed and described as "cold elution" or non-enzymatic elution (without destruction of RBC receptors) for influenza virus (Hirst, 1949; Tamm, 1954; Barua and Fauconnier, 1959) as well as for NDV (Sagik and Levine, 1957; Spalatin et al., 1970). Thus, some strain-specific differences between NDV strains in their capacity for the adsorption on RBC were found, but there was no correlation between this capacity and the virulence.

Together with this the state of "physical saturation" could not be achieved for all the strains. Therefore, we postulated the idea of "enzymatic saturation" as a common equalizing parameter for the studied NDV strains. This parameter is the measurement of the minimal (strain-specific) amount of adsorbed virus which splits a maximally possible (for a given strain) quantity of NANA. The situation is displayed in Figure 8. This postulate was proven to be valid by the experiment on the dependence of the quantity of the split NANA on the amount of the adsorbed virus: after a critical amount of the adsorbed virus, additional virus did not result in further liberation of NANA. From this fact the following conclusion emerges: enzymatic action of the pre-adsorbed virus extends from an adsorbed virion radially at a certain distance from the attachment point. The situation is as if the NANA residues in a certain (strain-specific) proximity to the spot of attachment are attracted to the enzymatic sites of the adsorbed virus. It can be explained either by (1) a mechanical flexibility of RBC surface which can be bent so that the NANA-containing receptors could get in contact with the virion enzymatic sites, or by (2) a mobility of the NANA-containing receptors as a result of fluidity (Singer and Nicolson, 1972; Edidin, 1972) or by (3) a certain motion (agitation) of the adsorbed virions around the attachment spot. The latter possibility has much in common with the explanation of the phenomenon of splitting additional quantities of NANA from RBC receptors by a non-eluting influenza virus which was described by means of the hypothesis of "legs of compasses" (Tsvetkova and Lipkind, 1968). This hypothesis was also based on the idea about a kind of agitation of a virion which is anchored to RBC surface by a Nase-resistant bond (fixed "leg") while another (mobile) "leg" is a Nase-sensitive bond which is split and formed repeatedly and, thus, the adsorbed virion is as if moving ("crawling") around the adsorption spot. On the other hand, the whole situation is compatible with the idea of an "at distance" interaction between the adjacent virions during the process of elution of the virus pre-adsorbed upon RBC (Lipkind and Urbakh, 1974), this idea being based on a mathematical

model approach (Lipkind and Urbakh, 1983). The idea of "action at the distance" was first suggested by Sagik and others (1954), but their hypothesis considered the adsorbed virions as firmly fixed at the points of initial adsorption, contrary to Burnet's hypothesis (1952) of migration ("crawling") of the adsorbed virions upon RBC surface resulting in "browsing" receptors. In our experiments there was accumulation of free NANA in the case of the lowest amount of the pre-adsorbed virus when no eluted virus was detected (Fig. 5). This unambiguously indicates that the proposed "crawling" process is connected with the intensive in situ Nase activity of the adsorbed virus, resulting in the accumulation of the NANA split from native RBC receptors. The amount of NANA accumulation is not proportional to the amount of the pre-adsorbed virus. Moreover, in most of the strains after a certain critical point the further increase of the amount of the pre-adsorbed virus was not followed by splitting of any additional quantity of NANA. These results are in full agreement with the proposed concept of "enzymatic saturation".

The in situ Nase activity was shown to be submitted to the main equations of the Michaelis kinetics (Fig. 7). This permitted the establishment of its quantitative parameters which were of the two sorts: (a) kinetic ones ($V_{\max}$, K_m and $K_m/V_{\max}$) and (b) those related to the efficiency of the activity expressed per a certain quantity of the virus: either per amount of the adsorbed virus in the case of "enzymatic saturation" (HA_{es}), or per amount of the "crawling" virus (E_c), or per amount of the adsorbed virus equal to the K_m (E_{K_m}). Comparison of the kinetic parameters has shown no significant differences between various strains in $V_{\max}$ while there are significant differences in K_m and $K_m/V_{\max}$ (Table 1). Namely, the values of the two latter parameters increase along with the virulence indexes, and the difference is statistically significant especially if velogenic strains are compared with lentogenic ones. The interpretation may be that the high K_m value in the case of velogenic strains means a weak binding of the enzyme-substrate (ES) complex (Eq. 1) of the in situ Nase activity

and, respectively, the low K_m of lentogenic strains means a strong binding. Similar interpretation can be applied to the case of K_m/V_{max} parameter: the property of virulence correlates with weak affinity of the enzyme-substrate complex in the in situ Nase activity.

These results are to be compared with the data on K_m of Nases of different NDV strains studied in vitro using N-acetyl-neuramin-lactose as a substrate (Alexander et al., 1970). In the latter study no difference in K_m between various NDV strains differing in virulence was found. The correlation between the virulence and K_m and K_m/V_{max} parameters found in our studies relates to interaction of Nase of adsorbed virions with cell receptor-bound NANA, this being more compatible with conditions of natural infection.

As to the parameters concerning the efficiency of the in situ Nase activity, their correlation with the virulence was even more evident. First, the amount of the pre-adsorbed virus providing "enzymatic saturation" (HA_{es}) increases along with the virulence. The interpretation of this fact is that since in the case of lentogenic strains a smaller amount of adsorbed virus is needed for the maximally possible liberation of NANA, the in situ Nase activity should be higher. This means that the area of spreading of the enzymatic activity from any adsorbed virion around the spot of its attachment per time unit is larger. Hence, in the limits of the above-described considerations the movement or "crawling around" of the adsorbed lentogenic virions should be more active. This is fully expressed by the respective parameters of the efficiency of the in situ Nase activity related to different amounts of the pre-adsorbed virus: that minimal amount which is sufficient for the "enzymatic saturation" (E_{es}); that providing "crawling" conditions without elution (E_c); and that equal to K_m . All the three parameters have shown correlation with the virulence. Besides, the comparison between the values of the E_{es} and E_c parameters which are related to the maximal and minimal amounts of the pre-adsorbed virus, respectively, shows that the latter is considerably higher. This means that the in situ Nase activity of the "crawling" virus is much higher than that of the eluting virions and this is true for all the strains irrespective of their virulence. However,

if the values of the E_c and E_{K_m} parameters are compared, there is a certain connection with the virulence. Namely, the values of these two parameters do not differ significantly in the case of lentogenic strains, in the case of mesogenic strains the difference can be assumed but it is not significant, and in the case of velogenic strains the difference is more apparent although statistically it is still not significant (Table 1). This can be interpreted in such a way that in the case of lentogenic and, perhaps, mesogenic strains, the amount of the pre-adsorbed virus equal to K_m is close to that amount providing the conditions of "crawling", while in the case of velogenic strains the amount of the pre-adsorbed virus exceeds that providing only "crawling" conditions and the elution process also takes place.

Thus, the enzyme kinetic parameters as well as its efficiency indications have shown that the lentogenic strains have a higher in situ Nase activity. This is in agreement with some results which showed reverse relationship between Nase activity and some virus-induced manifestations of pathogenicity. In earlier studies with influenza virus it was suggested that influenza virions with less active Nase caused more cytopathological changes in tissue culture (Choppin, 1963; Choppin and Tamm, 1964) and were more pathogenic in vivo (Smith and Cohen, 1956) if compared to virions containing a more active Nase. The mumps strains with more efficient Nase caused little cytopathic effect and no cell fusion in cell culture while the strains with less active Nase caused extensive cell fusion (Merz and Wolinsky, 1981), more expressed cytopathic effect in cell and organ cultures (Wolinsky et al., 1976), and were more neuroinvasive and neurovirulent (Wolinsky and Stroop, 1978). Inhibition of Sendai virus Nase activity increased its hemolytic activity (Neurath et al., 1972), and the ability of different NDV strains to elute from cells was inversely related to their ability to cause fusion from without (Bratt and Gallaher, 1972). Finally, when Nase deficient mutants of NDV and its revertants became available (Smith and Hightower, 1982) the similar regularity was found: pathogenic properties of the Nase-deficient mutants were not less expressed than those of the wild parents (Smith and Hightower, 1983).

Any explanation of such inverse relationship is inseparably connected with the much discussed problem of functional significance of myxovirus Nase and, in particular, its role in the expression of viral pathogenicity. Without going into comprehensive discussion on this problem which is still far from being completely resolved (Choppin and Scheid, 1980), one can find that there is at least one indisputable inference: carbohydrate moiety of glycoproteins of both ortho- and paramyxoviruses normally do not contain NANA residues due to the viral Nase activity (Klenk et al., 1970a, 1970 b; Klenk and Choppin, 1970). On the other hand, Nase deficient mutants of influenza virus (Palese et al., 1974) and NDV (Smith and Hightower, 1982) in non-permissive conditions do contain NANA, and virions thus possessing receptors for themselves form large aggregates which limit the spread of the virus (Palese et al., 1974), elution of virions from RBC is impaired, and the rate of virion attachment to cell culture decreases (Smith and Hightower, 1983). Trimming of the NANA residues by means of external Nase treatment restored both the impaired elution rate and the rate of attachment to the cell culture (Smith and Hightower, 1983). Removal of the HN glycoprotein by selective proteolysis caused conversion of non-fusing mumps virus infections to fusing infections with respective development of cytopathology (Merz and Wolinsky, 1983). Hence, at least one role of the virus Nase is to promote the efficient spread of the virus (Palese et al., 1974; Choppin and Scheid, 1980; Nagai et al., 1976; Klenk et al., 1982; Smith and Hightower, 1983). However, as far as NDV different strains are concerned, the data on Nase activity are rather contradictory. Namely, Alexander and co-workers (1970) found no difference in virion Nase activity towards a soluble substrate in vitro between NDV strains differing by virulence while McNulty and co-workers (1975) found the Nase activity in velogenic strains compared to lentogenic ones to be significantly higher. However, definite data on inverse relationship between Nase activity and cytopathic manifestations can be explained as a consequence of a relative duration of association of the progeny virions with the plasma membranes of adjacent

infected and still uninfected cells, resulting in an increased cell fusion potential (Rott and Klenk, 1977; Huang et al., 1980; Choppin and Scheid, 1980, Merz and Wolinsky, 1981b, 1983).

The results shown at Fig. 9 have a certain relationship to the problem concerning association of HA and Nase activities with the same or different functionally active sites of the HN glycoprotein molecule. This has become to be an intriguing question since that time when the association of both the HA and Nase activities with the same glycoprotein entity was discovered (Scheid et al., 1972; Scheid and Choppin, 1973; Tozawa et al., 1973; Shimizu et al., 1974). Specifically the question became to be whether these two activities are bound to topologically distinct regions of the HN molecule, and it has remained unanswered. On one side, HA and Nase activities of NDV and Sendai HN can be uncoupled genetically (Smith and Hightower, 1980, 1982; Portner, 1981) which is consistent with the concept that they occupy different sites on the HN glycoprotein. However, the functionally active sites of these two activities (enzymatic sites in the case of Nase and sites involved in attachment to cell receptors in the case of HA) may be distinct from the corresponding antigenic determinants recognized by the antibodies, this having been shown for Sendai virus (Örvell and Grandien, 1982) as well as for NDV (Nishikawa et al., 1983). On the other side, polyclonal monospecific antisera to HN have shown inhibition of both HA and Nase activities (Seto et al., 1974, Merz et al., 1981a). Therefore, it seemed that analysis by means of a panel of monoclonal antibodies against HN glycoprotein could be much more suitable to solve the problem. However, the results obtained by means of monoclonal antibodies turned out to be rather ambiguous. On one hand, it has been shown that some of the monoclonal antibodies against Sendai virus HN were active in the HI test with some of the Sendai virus antigenic variants but inactive in the NI test and vice versa (Portner, 1981). On the other hand, it has been shown that although the anti-HN

monoclonal antibodies are directed against distinct regions of the HN molecule none of them inhibited exclusively either HA or Nase activity of HN of parainfluenza type I virus (Yewdell and Gerhard, 1982), that of Sendai virus (Orwell and Grandien, 1982), and that of NDV (Nishikawa et al., 1983; Hoshi et al., 1983; Russell et al., 1983). In general, views have arisen about principal limitations of the approach using monoclonal antibodies for discriminating between the presence of one or two active sites on the HN molecule (Lubeck and Gerhard, 1981; Yewdell and Gerhard, 1982; Iorio and Bratt, 1983). These limitations are connected with: (a) steric effects (likely to act in the HI test) when an antibody may bind to a region on the HN molecule that is distant from the HA site but interfere with the hemagglutination; (b) allosteric effects connected with conformational modifications which may spread through a wide region of the HN molecule from a distant point of an antibody binding and overlap with other epitopes (Yewdell and Gerhard, 1982;) (c) a phenomenon of non-specific binding of some of monoclonal antibodies with some homologous cross-reacting determinants on unrelated antigens (Roering et al., 1980; Lane and Koprowski, 1982; Iorio and Bratt, 1983); (d) the dependence of antigenicity revealed by monoclonal antibodies to HN glycoprotein on disulfide bonding which may mean that the HN antibodies are directed against determinants of a conformational nature rather than primary amino acid sequences (Iorio and Bratt, 1983). All these circumstances may greatly reduce the resolution of the approach.

Taking into account the above considerations, we are inclined to consider our data as more unambiguous for judging about the possible distinct location of HA and Nase antigenic sites on the HN glycoprotein molecule. In connection with this a more particular question is raised, namely, whether (a) the HA and Nase active sites are dispersed homogeneously (or at random) on the HN molecule body, or (b) the sites of

each kind are clustered separately in the distinct parts of the HN molecule. If the latter is the case certain hypothetical monospecific anti-HA and anti-Nase sera would cause an independent binding, and, hence independent HI and NI inhibition patterns in the case of either antisera. If the former is the case the cross inhibition of both the activities by any monospecific antiserum would be expected. The found discrepancies between the results of the cross reactivity obtained by HI and NI tests in our experiments (Tables 3 and 5, Figure 9) are in favour of the hypothesis about separate topological (polar) location of HA and Nase antigenic sites. In particular, this means that if two (or a number of) compared viruses display antigenic relationship(s) the cross reactivity may relate independently to either HA or Nase topologically distinct antigenic sites within the same HN molecule. Concerning the evolutionary aspect, it means that, irrespective of any conceivable mechanism of evolutionary pathways, the HA and Nase sites should undergo independent antigenic drift which involves also the proposed conserved portion containing common antigenic determinants causing the observed cross reactivity (Figure 12). This can explain the cases when antigenic relationship between the compared viruses by HA antigenic determinants exists while that concerning Nase antigenic determinants does not. In fact, a conclusion about independent antigenic drift of HA and Nase antigens in the case of influenza was made in 1968 (Schulman and Kilbourne, 1968) and now it is a truism for multisegmented genome-containing influenza viruses but for PMV HN glycoprotein possessing both the activities this conclusion is not trivial. First, very little is known, in general, about antigenic drift in PMV case, especially about interdependence between HA and Nase antigenic drift. Secondly, the above conclusion may have important meaning in respect of topological distribution of functionally different HA and Nase antigenic sites on HN molecule.

Thus, the general conclusion is that the HA and Nase activities of avian PMVs are bound to two different domains of antigenic sites upon HN glycoprotein molecule and the sites of either domain can have independent relatedness to the corresponding sites of another PMV strain. That means that there are two different sets of antigenic

determinants related to either HA or Nase in each virus, some of the HA and Nase determinants going in parallel to each other while the others behaving independently in the cross reactivity.

Comparison of the methods of estimation of NDV-induced CPE has shown some advantages of the vital dye uptake method. First, a significant decrease in NR binding (6 hours p.i.) and accumulation (8 hours p.i.) occurred earlier than definite CPE manifestation (10 hours p.i.) and full CPE development (14 hours p.i.) could be registered (Fig. 1B and 1C). The other methods of following up the CPE manifestation like LDH leakage and protein content also appeared to be less sensitive. Another advantage of the dye uptake method is the usage of a single criterion of registration (vital dye uptake) instead of different cytopathological phenomenon taken into account for CPE evaluation, such as intracellular granulation and vacuolization, disorientation, cell rounding, fusion etc. Such a criterion permits precise spectrophotometric measurement which is decisively advantageous in comparison to microscopic observations. Two different characteristics of the dye uptake were used: a) intracellular accumulation of NR as a result of incubation with the dye in the medium (pulse) and b) intracellular binding of NR determined after an incubation of the "pulsed" cells with medium without the dye (chase).

The intracellular NR-binding property turned out to be more sensitive to virus infection than NR accumulation. The dye uptake method permits the establishment of a number of indices: (a) time p.i. of the beginning of the significant decrease in NR accumulation and binding; (b) time p.i. when 50% of the decrease is reached and (c) time p.i. when the maximal decrease of dye uptake is reached (not shown in this report). The use of these indices have shown considerable differences between two mesogenic strains - Komarov and Roakin. The first appeared to be closer to the velogenic strains. This difference could not be demonstrated by comparison of the strains according to classic indications of pathogenicity (MDT, ICPI, IVPI) together with mean plaques diameter (Rivetz, 1980).

The final question of interest is whether the parameters proposed here which correlate with the virulence are only formal in vitro markers or whether they are causal factors of the virus-induced in vivo pathogenicity. An artificial cell system such as RBC does not permit any definite conclusions about functional role of Nase as a virulence factor. Therefore, we are inclined meanwhile to consider the correlations between some quantitative parameters of the in situ Nase activity and the virulence as mere in vitro markers of the in vivo virulence rather than actual factors of pathogenicity.

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COOPERATION

Cooperation with Dr. G. M. Schloer was done in the form of discussions on the objectives of the project and then on the results obtained during the studies. This was done mainly by correspondence. Besides, there was extensive exchange by views and discussions during a short visit of the Principal Investigator to Plum Island Animal Disease Center in June 1981 (This was arranged during the trip of the Principal Investigator to USA in the limits of the other BARD project; I-42-79, "Ecology of animal influenza in Israel"). Some possibilities for more intensive collaboration including mutual experiments performed in both collaborating institutions were suggested and discussed with Dr. Schloer, the Cooperating Investigator and Dr. J. J. Callis, Director of the USDA Plum Island Animal Disease Center.

In April-May 1982 there was the 2nd visit of the Principal Investigator to the laboratory of Dr. G. M. Schloer in the limits of the present BARD project. That time mutual experiments concerning NDV-RBC receptor interactions (kinetics of elution and hemolytic activity) were performed using technical approach elaborated in Kimron Veterinary Institute and some NDV strains isolated in USA. The results were in agreement with the previously obtained data. In June 1982 Dr. G. M. Shloer visited our laboratory and became acquainted with some experimental procedures. All the results obtained up to that time were thoroughly discussed. Some regulations from the governments of USA and Israel restricting exchange of NDV strains prevented us from more close cooperation on the basis of experimental work.

BENEFIT TO AGRICULTURE

Scientific contribution of the studies is based on high importance of two general problems on which the whole research is based: a) virus-cell receptor

interaction, and b) nature of virulence and expression of virus pathogenic properties. Importance of both the problems, is related to the prime attribute of the viruses as infecting agents, namely, their ability to cause disease or death. Usage of NDV in the study increases significance of the problems since a) namely in the case of NDV there is a lack of knowledge related to virus-cell interaction as compared with other myxoviruses, b) NDV is a unique model including a wide range of antigenically, morphologically and structurally indistinguishable strains which highly differ by their virulent properties

Diverse antigenic interconnections between avian PMVs including NDV permitted to arrange taxonomic subdivision within avian PMV group.

Immediate agricultural contribution of the results is due to the fact that NDV is a continual threat for any poultry-raising country and any knowledge concerning the virulent properties of this pathogen can be of practical importance. Establishment of new in vitro markers of the in vivo virulence can be of immediate use for diagnostics, selection of vaccinal strains, attenuation of virulent strains and vaccine control.

Involvement into the studies of avian PMVs other than NDV but showing antigenic cross reactivity with it permitted to suggest the presence of common antigenic determinants for a number of avian PMVs affecting poultry. This may give a new approach for the development of polyvalent vaccines against several avian PMVs.

The elaboration of dye uptake method modification for CPE measurement permitted to establish quantitative indices which appeared to be more sensitive than classic indications of NDV pathogenicity, and can be of practical use.

LIST OF PUBLICATIONS

1. Rivetz, B., Shihmanter, E., and Lipkind, M. (1980).
A quantitative approach for the measurement of cytopathic effect caused by NDV strains differing by virulence.
Proceedings of the 2nd International Conference on the Impact of Viral Diseases on the Development of African and Middle East Countries, Nairobi, Kenya, Dec. 1980.

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Interaction of Newcastle disease virus with red blood cell receptors.
Israel Journal of Medical Sciences 16: 482 (Abstracts of the Annual Meeting of the Israel Society for Microbiology, 1980).
3. Rivetz, B., and Lipkind, M. (1981).
Elution from red blood cells and in situ neuraminidase activity of NDV different strains.
Proceedings of the Regular Meeting of the European Association against Virus Diseases, Sterling, Scotland, Aug. 1981.
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Archives of Virology (In press).
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Antigenic relationships between avian paramyxoviruses. I. Quantitative characterization based on hemagglutination inhibition and neuraminidase inhibition tests.
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Archives of Virology (In press).
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Antigenic relationships between avian paramyxoviruses. III. A combinatorial mathematical model of antigenic kinship.
Archives of Virology (In press).
9. Rivetz, B., and Lipkind, M. (1985).
A quantitative approach for measurement of cytopathic effect caused by NDV strains differing by virulence.
Journal of General Virology. (Submitted).

of NDV strains with pathogenicity indexes.

Viruses	V _{max}	K _m	K _d /V _{max}	H _{ves}	E _{es} x 10 ⁵	E _c x 10 ⁵	E _{K_m} x 10 ⁵	Pathogenicity indexes		
								MDT	ICPI	IVPI
Queensland	0.132	55	419	-	-	33.0	24.0	∞	0.16	0
LaSota	0.216	41	190	-	-	56.7	52.7	103	0.19	0
Fl46	0.115	29	252	277	8.30	28.5	39.6	168	0.20	0
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M ± SE	0.154±0.022	41.7±5.3	287±48	-	-	39.4±6.2	38.8±5.9			
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Roakin	0.117	55	465	435	5.38	17.3	21.3	69	1.42	0
Konarov	0.114	90	785	714	3.19	31.0	12.7	69	1.46	0-0.20
Maj	0.149	83	560	714	4.17	25.0	17.9	67	1.51	0.57
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M ± SE	0.127±0.008	76.0±7.6	603±67	621±66	4.25±0.45	24.4±2.8	17.3±1.8			
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italien	0.187	158	848	3640	1.03	24.8	11.8	50	1.86	2.81
Israel/67	0.109	100	916	2800	0.78	20.0	10.9	42	1.89	2.63
Israel/79	0.082	125	1527	2562	0.64	10.7	6.5	46	-	-
<hr/>										
M ± SE	0.126±0.022	127.7±11.8	1097±153	3001±231	0.82±0.08	18.5±2.9	9.7±1.1			
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Statistical significance	P _{L-M}	-	97.6	97.9	-	88.4	97.1			
	P _{M-V}	-	97.6	95.2	99.9	99.9	73.6	97.4		
	P _{L-V}	-	99.6	99.3	-	95.2	99.2			

* Mean value with standard error (M ± SE)

** P is per cent of confidence or level of significance.
95% confidence meaning 0.05 level of significance was accepted in these studies as the region of significance.

P_{L-M} - level of significance between compared indications related to lentogenic and mesogenic strains.

P_{M-V} - the same values related to mesogenic and velogenic strains.

P_{L-V} - the same values related to lentogenic and velogenic strains.

Table 2. Indices of vital dye uptake in the case of various strains of NDV differing by virulence

Strain	Virulence	Time p.i. of Definite CPE Manifestation (++)	Time p.i. of Full CPE devel. (++++)	Beginning of NR binding	Decrease of NR accumulation	Time p.i. of NR binding	50% decrease NR accumulation
Italian	Velo.	10*	14	6	9	10-11	12-13
Israel 1967	Velo.	12	16	8	12	14	19
Komarov	Meso.	15	16	10	14	15	17
Roakin	Meso.	16	24	14	24	22	26
LaSota	Lento.	-	No CPE**	-	No decrease**	-	No decrease
Queensland	Lento.	-	No CPE	-	No decrease	-	No decrease

* Hours p.i.

** Observed during 24 hours p.i.

Table 3. Cross reaction hemagglutination inhibition test. A typical experiment.

V I R U S E S									
ANTISERA									
NDV	Yucaipa	Ty/Wisc	Pk/Neth	D3/HK	D199/HK	Dove/Tn	Goose/Del	Duck/NY	Pigeon/Ot
NDV	<u>640</u> ^a	< ^b	20	80	40	<	40	20	80
Yucaipa	<	<u>320</u>	<	<	40	<	80	<	10
Ty/Wisc	80	<	<u>160</u>	160	40	<	<	10	20
Pk/Neth	40	<	80	<u>640</u>	10	40	80	160	80
D3/HK	20	<	20	40	<u>640</u>	20	<	20	40
D199/HK	<	40	<	<	<u>640</u>	<	10	<	20
Dove/Tn	20	10	40	20	20	<u>320</u>	40	20	320
Goose/Del	<	<	<	<	<	<	<u>240</u>	<	10
Duck/NY	10	<	10	20	<	10	10	<u>160</u>	20
Pigeon/Ot	20	10	20	40	<	80	40	20	<u>320</u>

^aHI titres are expressed as the reciprocal of the highest dilution of serum causing inhibition of 8 HA units of virus (Palmer et al., 1975).

^bHI titre less than 10

Table 4. Comprehensive cross reaction hemagglutination tests. Statistically treated mean data from all the experiments.

V I R U S E S										
ANTISERA	NDV	Yucaipa	Ty/Misc	Pk/Neth	D3/HK	D199/HK	Dove/Tn	Pigeon/Ot	Goose/Dei	Duck/NY
NDV	0 n=2	∞ n=2	3.91 ⁺ 0.37 ^a n=5 P>0.99	3.00 ⁺ 0.30 n=6 P>0.99	3.72 ⁺ 0.85 n=6 P>0.99	∞ n=2	5.37 ⁺ 0.43 n=5 P>0.99	3.21 ⁺ 0.43 n=5 P>0.99	5.30 ⁺ 0.30 n=4 P>0.99	3.26 ⁺ 0.57 n=5 P>0.99
Yucaipa	∞ n=2	0	4.24 ⁺ 0.45 n=3 P>0.99	∞ n=4	∞ n=2	3.57 ⁺ 0.37 n=4 P>0.99	5.30 ⁺ 0.70 n=2 P>0.92	4.92 ⁺ 0.70 n=3 P>0.98	2.87 ⁺ 0.13 n=3 P>0.98	∞ n=4
Ty/Misc	2.98 ⁺ 0.44 n=5 P>0.99	∞ n=2	0	1.37 ⁺ 0.21 n=6 P>0.99	2.18 ⁺ 0.47 n=5 P>0.99	∞ n=2	∞ n=5	1.75 ⁺ 0.31 n=4 P>0.99	∞ n=3	4.44 ⁺ 0.63 n=3 P>0.99
Pk/Neth	3.08 ⁺ 0.48 n=3 P>0.98	∞ n=3	1.75 ⁺ 0.64 n=6 P>0.96	0	4.07 ⁺ 1.13 n=5 P>0.98	∞ n=2	3.81 ⁺ 0.60 n=6 P>0.99	2.05 ⁺ 0.52 n=8 P>0.99	3.17 ⁺ 0.32 n=6 P>0.99	2.19 ⁺ 0.56 n=7 P>0.99
D3/HK	4.58 ⁺ 0.43 n=5 P>0.99	∞ n=2	2.95 ⁺ 0.66 n=4 P>0.99	5.52 ⁺ 0.60 n=4 P>0.99	0	∞ n=2	5.58 ⁺ 0.72 n=3 P>0.99	4.06 ⁺ 0.78 n=4 P>0.99	∞ n=2	4.80 ⁺ 0.76 n=4 P>0.99
D199/HK	∞ n=2	4.71 ⁺ 0.79 n=4 P>0.99	∞ n=2	∞ n=2	∞ n=3	0	∞ n=2	5.04 ⁺ 0.96 n=2 P>0.91	6.10 ⁺ 1.60 n=2 P>0.98	∞ n=2
Dove/Tn	3.29 ⁺ 0.22 n=5 P>0.99	4.36 ⁺ 0.34 n=2 P>0.92	3.30 ⁺ 0.79 n=4 P>0.97	3.35 ⁺ 0.20 n=6 P>0.99	3.14 ⁺ 0.70 n=4 P>0.98	3.50 ⁺ 0.50 n=2 P>0.91	0	0.05 ⁺ 0.36 n=6 P>0.10	2.81 ⁺ 0.28 n=4 P>0.99	3.37 ⁺ 0.29 n=6 P>0.99
Pigeon/Ot	3.61 ⁺ 0.43 n=5 P>0.99	5.16 ⁺ 0.53 n=3 P>0.98	4.19 ⁺ 0.69 n=4 P>0.99	3.00 ⁺ 0.34 n=8 P>0.98	5.00 ⁺ 0.93 n=4 P>0.99	6.01 ⁺ 0.01 n=2 P>0.99	2.40 ⁺ 0.22 n=6 P>0.99	0	3.17 ⁺ 0.35 n=5 P>0.99	3.72 ⁺ 0.33 n=6 P>0.99
Goose/Dei	∞ n=2	6.08 ⁺ 1.13 n=3 P>0.99	6.66 ⁺ 0.80 n=4 P>0.99	5.88 ⁺ 0.18 n=6 P>0.99	∞ n=2	∞ n=2	6.09 ⁺ 0.22 n=4 P>0.99	4.71 ⁺ 0.53 n=5 P>0.99	0	7.16 ⁺ 0.18 n=4 P>0.99
Duck/NY	4.02 ⁺ 0.13 n=5 P>0.99	∞ n=4	3.52 ⁺ 0.82 n=4 P>0.98	3.65 ⁺ 0.43 n=7 P>0.99	4.16 ⁺ 0.70 n=4 P>0.99	∞ n=2	4.14 ⁺ 0.68 n=6 P>0.99	3.25 ⁺ 0.42 n=6 P>0.99	4.20 ⁺ 0.26 n=4 P>0.99	0

²An upper figure in each cell is a mean value with a standard error of d which is expressed as a log₂ of the ratio between homologous and heterologous HI titres when namely antiserum was the same for the two compared viruses. n is a number of experiments for each pair of the compared viruses. P is the Student's coefficient of probability for the found difference d for a given number of experiments n; it is expressed as a percentage of probability in the limits of checking of "zero hypothesis" i.e. the probability of that the difference d itself differs significantly from zero. The reliability of the d values (its difference from zero) was accepted as significant if it was in agreement with the 2nd Fischer's criterion of probability, namely, 95% probability (P = 0.95). In the case of the absence of the cross reactivity between certain viruses the d value was expressed as infinity (∞).

Table 5. Cross reaction neuraminidase inhibition test. A typical experiment.

ANTISERA	VIRUSES						
	NDV	Yucaipa	Ty/Wisc	Pk/Neth	D3/HK	D199/HK	Dove/Tn
NDV	<u>880</u> ^a	<	22	128	86	>	14
Yucaipa	>	<u>410</u>	>	>	>	>	>
Ty/Wisc	>	>	<u>48</u>	52	>	>	>
Pk/Neth	78	>	410	<u>1280</u>	42	>	18
D3/HK	86	>	>	42	<u>560</u>	>	19
D199/HK	>	>	>	>	>	<u>230</u>	>
Dove/Tn	>	>	20	19	15	>	<u>300</u>

^aNI titres are expressed as the reciprocal of the highest dilution of serum causing 50% inhibition of Nase activity of the virus dose giving spectrophotometric reading equal to 0,850 OD₅₄₉ (Palmer et al., 1975).

^bNI titre less than 10.

Table 6. Comprehensive cross reaction neuraminidase inhibition tests. Statistically treated mean data from all the experiments.

VIRUSES										
ANTISERA	NDV	Yucaipa	Ty/Wisc	Pk/Neth	DJ/HK	D199/HK	Dove/Tn	Goose/Del	Duck/NY	Pigeon/Ot
NDV	0	∞ n=1	4.50 ⁺ 0.28 ^a n=3 P>0.99	2.26 ⁺ 0.22 n=3 P>0.99	3.27 ⁺ 0.49 n=3 P>0.99	∞ n=1	5.37 ⁺ 0.11 n=4 P>0.99	4.38 ⁺ 0.10 n=2 P>0.99	1.80 ⁺ 0.97 n=2 P>0.99	5.33 ⁺ 1.26 n=4 P=0.97
			∞ n=2	∞ n=2	∞ n=2	∞ n=2	∞ n=2	∞ n=2	∞ n=2	∞ n=2
Yucaipa	∞ n=2	0	∞ n=2	∞ n=2	∞ n=2	∞ n=2	∞ n=2	∞ n=2	∞ n=2	∞ n=2
Ty/Wisc	∞ n=2	∞ n=1	0	0.05 ⁺ 0.45 n=4	∞ n=1	∞ n=1	∞ n=2	∞ n=1	∞ n=1	1.61 ⁺ 0.13 n=4 P>0.99
Pk/Neth	5.04 ⁺ 0.56 n=4 P>0.99	∞ n=1	1.45 ⁺ 0.62 n=4 P>0.89	0	5.05 ⁺ 0.15 n=2 P>0.99	∞ n=1	5.61 ⁺ 0.31 n=3 P>0.99	3.49 ⁺ 0.38 n=2 P>0.99	3.27 ⁺ 0.12 n=2 P>0.99	5.08 ⁺ 0.49 n=3 P>0.99
DJ/HK	∞ n=2	∞ n=1	∞ n=2	∞ n=2	0	∞ n=1	5.09 ⁺ 0.45 n=3 P>0.99	7.25 ⁺ 1.29 n=2	3.07 ⁺ 0.38 n=2 P>0.99	∞ n=4
D199/HK	∞ n=1	∞ n=2	∞ n=1	∞ n=1	∞ n=1	0	∞ n=1	∞ n=1	∞ n=1	∞ n=2
Dove/Tn	∞ n=3	∞ n=2	4.21 ⁺ 0.28 n=2 P>0.99	4.32 ⁺ 0.13 n=2 P>0.99	4.60 ⁺ 0.29 n=2 P>0.99	∞ n=2	0	3.63 ⁺ 0.93 n=3 P=0.94	4.75 ⁺ 0.27 n=2 P>0.99	0.40 ⁺ 0.37 n=4 P>0.65
Goose/Del	∞ n=2	∞ n=2	∞ n=2	∞ n=2	∞ n=2	∞ n=1	∞ n=3	0	∞ n=2	5.64 ⁺ 0.21 n=2 P>0.99
Duck/NY	4.69 ⁺ 0.98 n=3 P>0.96	∞ n=2	2.31 ⁺ 0.74 n=2 P>0.91	2.27 ⁺ 0.43 n=3 P>0.98	3.27 ⁺ 0.33 n=2 P>0.99	∞ n=1	∞ n=4	5.16 ⁺ 1.35 n=2 P>0.92	0	4.33 ⁺ 0.80 n=2 P>0.94
Pigeon/Ot	4.62 ⁺ 0.09 n=2 P>0.99	∞ n=2	3.33 ⁺ 0.75 n=3 P>0.98	2.80 ⁺ 0.23 n=3 P>0.99	4.13 ⁺ 0.74 n=2 P>0.94	∞ n=2	-0.08 ⁺ 0.37 n=3	1.28 ⁺ 0.24 n=3 P>0.97	3.86 ⁺ 0.03 n=2	0

^aThe same designations as those for the Table 2.

Figure 1. Dependence of adsorption of NDV strains on RBC concentration.

Figure 2. Kinetics of adsorption of NDV strains on RBC.

Figure 3. Dependence of virus adsorption on the amount of input virus.

Figure 5. Dependence of the kinetics of virus elution on the amount of the adsorbed virus.

The experimental points of each curve are designated differently; numbers above the curves indicate amounts of the adsorbed virus in HA units.

Figure 6. Kinetics of liberation of free NANA split by the eluting virus in case of different amounts of the adsorbed virus.

Designations of the curves are the same as those for the Figure 5.

Figure 7. Michaelis-Menten kinetics of the in situ Nase activity displayed by linear plot (NANA vs. HA_{ads}) as well as Lineweaver-Burk double reciprocal plot ($1/NANA$ vs. $1/HA_{ads} \times 10^4$).

Figure 8. Schematic illustration of "enzymatic saturation" as well as physical saturation of RBC surface by adsorbed virions.

Nase determinant antigenic relationships

	symmetric and two side asymmetric	one side asymmetric
IDENTITY : $d = 0-1 \log_2$		
CLOSE RELATEDNESS : $d = 1-2 \log_2$		
MODERATE RELATEDNESS : $d = 2-4 \log_2$		
LOW RELATEDNESS : $d = 4-7 \log_2$		

Figure 9. Graph expression of antigenic relationships between avian PMV serotypes determined by HI and NI tests.

Left: inter-connections showing "identity" and close relatedness ($d = 0-1 \log_2$).

Middle: inter-connections showing moderate relatedness ($d = 2-4 \log_2$) are superimposed upon the previous picture.

Right: inter-connections showing low relatedness ($d = 4-7 \log_2$) are superimposed upon the previous picture.

Figure 10. CPE registration by different methods during the infection of chick embryo cells by a virulent NDV strain (Italian).

- A. Intracellular protein content (closed circles) and leakage of LDH from the infected cells (open circles) expressed as a percentage of the total cellular content (activity).
- B. Specific neutral red accumulation (closed circles) and specific neutral red binding after chase (open circles).
- C. CPE registration by light microscopy.

Figure 11. Kinetics of neutral dye uptake by cells infected with NDV strains differing by virulence.

Intracellular accumulation of neutral red - closed circles.
Neutral red binding (after chase) - open circles.

Figure 12. Hypothetical scheme of two kinds of subdivision of a HN-coding genome material into: (a) "common-to-all-the-avian-PMVs" and "serotype-specific" portions, and (b) "conserved" and "variable" portions.

Relative sizes of the portions are arbitrary, being roughly estimated for the "common-to-all-the-avian-PMVs" by the value of d from the present paper (Tables 2 and 4, Figure 1) and for the "variable" portion in the case of PMV-1 (NDV) by the values of d estimated from the paper of Alexander et al. (1984) and in the case of PMV-2 (Yucaipa-like) from the data of Lipkind and Alexander (in preparation).