Hemagglutinating and Fusogenic Activities of Newcastle Disease Virus: Studies on Receptor Binding Specificity and pH-induced Conformational Changes

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Vaccinal and wild strains of Newcastle Disease virus (NDV) were analyzed for cell receptor binding and fusogenic biological properties associated with their HN (hemagglutinin-neuraminidase) and F (fusion protein) surface structures respectively. The evaluation of the biological activities of HN and F was carried out respectively by determination of hemagglutinating titers and hemolysis percentages, using erythrocytes from various animal origins at different pH values. Significant differences in hemagglutination titers for some strains of NDV were detected, when interacting with goose, sheep, guinea-pig and human "O" group erythrocytes at neutral pH. Diversity of hemolysis percentages was observed between different NDV strains at acid pH. These analysis were developed to evaluate particular aspects of the actual influence of the receptor specificity and pH on the receptor binding and fusogenic processes of Newcastle Disease viruses.

Newcastle Disease viruses (NDV) are RNA viruses that present glycoproteins inserted in their envelope structure, playing important roles on different steps of the infectious process. These viruses exhibit HN (hemagglutinin-neuraminidase) and F (fusion protein) glycoproteic spikes as their surface structures which are responsible for cell receptor binding (HN), cleavage of cell receptors (HN) and fusion (F) processes, during the virus replication cycle (Marsh & Helenius 1989, Marsh & Pelchen-Matthews 1993, Vainiopää & Hyypiä 1994).

HN structures of NDV which exhibit receptor-binding activity on gangliosides during the virus-cell adsorption process, have been studied by hemagglutination test. In analysis using influenzavirus as model, the amount and quality of sialic acids receptor residues which vary among erthrocytes from different animal origins, show influence on the virus-cell receptor binding process, where the crucial conformational changes of HA structures are pH-dependent (Markwell et al. 1986, Katz & Webster 1988, Phyalla et al. 1988, Marsh & Helenius 1989, Marsh & Pelchen-Matthews 1993).

F glycoproteins, which are responsible for the fusion process, exhibit biological activities after cleavage to F_1 and F_2 enzymatic activity (eg. trypsin). In this process, pH-independent fusion occurs after insertion of the hydrophobic moiety of F_1 into the target membrane, which is *in vitro* revealed by hemolysis reaction, when erythrocytes are used as cellular model (Marsh & Helenius 1989, Marsh & Pelchen-Matthews 1993, Stegmann 1994).

Analysis on the influence of cell receptors and pH on the adsorption and hemolysis processes have been developed, using non purified or non standardized preparations of viruses from different families (Clark & Nagler 1943, Winslow et al. 1943, Muller & Stanley 1944, Chu 1948, Clavell & Bratt 1972).

The aim of this study was to analyze different purified and standardized NDV strains for particular aspects of their hemagglutinating and hemolytic activities, using different parameters. The pH was analyzed as interfering factor on hemagglutination and fusion processes.

MATERIALS AND METHODS

Viruses - Lentogenic (B1 and La Sota) and velogenic (SO-93)* virus samples were inoculated into the allantoic cavity of 10 day-old chicken embryonated eggs and incubated for 48 hr at 37°C.

*Wild sample isolated in Brazil which was provided by Dr Ary Monteiro de Souza, Depto de Virologia, Empresa Brasileira de Agropecuária, RJ.

The allantoic fluids were harvested and clarified by centrifugation at 3.500 x g for 30 min at 4°C. The virus samples were 50 x concentrated (rediluted in Phosphate Buffer Solution-PBS pH 7.0 after ultracentrifugation at 80.000 x g for 60 min), purified in 40% potassium tartrate 5% glycerol continous density gradient by ultracentrifugation at 100.000 x g for 120 min. The virus bands were collected, diluted (1:10) in PBS pH 7.0 with 2mM EDTA and centrifuged at 4°C again for 60 min at 80.000 x g. The pellets were rediluted using PBS-EDTA and stored at -20°C until utilization (Couceiro et al. 1994). Evaluation of the protein content of the virus samples was processed by the Bradford method (Bradford 1976) for the obtainment of standardized concentrations of virus proteins to develop hemagglutination and hemolysis assays.

Erythrocytes - Human "O" group (Rh+), guinea-pig, sheep and goose erythrocytes were used. Erythrocytes were collected in Alsever solution and stored at 4°C (Pinto et al. 1994). After washing for three times, they were adjusted at 10% concentration (v/v) in saline (0.15 M NaCl). Afterwards those 10% erythrocyte suspensions were diluted in adequate buffer for each test (Pinto et al. 1994).

Hemagglutination test - Virus samples were diluted serially in equal volumes (25 µl) for saline (0.15 M NaCl) and to each dilution of virus was added 25 µl of 1% mammalian or 0.5% goose erythrocyte suspension at pH values ranging from 5.0 to 8.6. Mammalian and goose erythrocyte suspensions at pH values ranging from 5.0 to 8.6 were prepared in Michaelis barbital buffer (Pinto et al. 1994). Each assay was developed in triplicates for twice, the geometrical average of the titers representing its final titer. The titer of each assay was reported as the reciprocal of the highest virus dilution responsible for complete hemagglutination at 4°C (Pinto et al. 1994).

Hemolysis test (Lenard & Miller 1981) - Hemolysis activity of NDV samples was determined in a total volume of 1.5 ml in capped tubes, containing 0.1 ml of human "O" group (Rh+) erythrocyte suspensions (10%), 0.1 ml of each virus sample at two standardized protein concentrations (1 and 5 µg/ml) and 1.3 ml of Sorensen's phosphate buffer (Pinto et al. 1994) at different pH values. The capped tubes were incubated for 60 min in cold bath (0-4°C), which was followed by incubation for 60 min at 37°C with frequent mixing. After incubation, the tubes were centrifuged for 10 min at 600 x g and the amount of released hemoglobin (Hb) in the supernatant was estimated by absorbance (Ab) measurement, using a Perkin Elmer spectrophotometer at 590

nm. Each assay was developed in triplicates for twice, considering the arithmetical average of the data (Ab) as the final result of each type of experiment. The hemolysis level of each assay (Pinto et al. 1994) was determined by comparison of the measured Ab with that of 1.0 ml of erythrocyte suspension in 1.4 ml of buffer solution (spontaneous hemolysis) and that of 1.0 ml of erythrocyte suspension in 1.4 ml of deionized water (total hemolysis).

Evaluation of different NDV samples for hemagglutinating activity at neutral pH, using erythrocytes from different animal origins - The virus samples were diluted in PBS with posterior addition of erythrocyte (mammalian or goose) suspensions at neutral pH (7.0). The technical procedures for hemagglutination were those described above.

Evaluation of NDV samples for hemolysis activity at acid pH -The NDV samples were diluted to 1 µg/ml protein concentration in PBS and they were analyzed by hemolysis test, which was developed such as previously described, with utilization of erythrocytes in Sorensen's phosphate buffer (pH 5.0). Erythrocytes in deionized water (total hemolysis) and erythrocytes at the different pH values (spontaneous hemolysis) were used as controls.

Evaluation of B_1 strain of NDV for hemolysis activity after variation of pH (5.0 - 6.0) - The sample (5 µg/ml protein concentration) showing the lowest hemolysis percentage at pH 5.0 was exposed at pH variation, using 1N NaOH, as indicated below:

pH variation: 5.0 - 6.0
pH 5.0 - pH 6.0 (for 5 min)
NaOH

Evaluation of the hemagglutinating activity at large pH range, using that B_1 strain of NDV that exhibits the lowest hemagglutinating titers at neutral pH - The sample was diluted in PBS with posterior addition of erythrocyte (sheep or goose) suspensions at pH values ranging from 5.0 to 8.6, which were prepared in Michaeli's barbital buffer (Pinto et al. 1994). The technical procedures for hemagglutination were those described above.

Analysis of that B_1 strain of NDV analized by hemagglutination and hemolysis experiments, using electron microscopy - NDV virus particles of that purified B_1 strain used in experiments were observed by electron microscopy, showing the influence of neutral pH, concentration and purification processes on the original virus structures. A negative combined (4% phosphotungstic acid and uranyl acetate for one min each one) staining technique was developed as described by Fonseca et al. (1984) using a Phillips 301 transmission electron microscope.

RESULTS AND DISCUSSION

NDV preparations were analyzed for their hemagglutinating and hemolytic activities, using cells from different origins as targets, while ambient pH was used as interfering factor on these processes.

A variation in the hemagglutinating activity of NDV (B_1, La Sota and SO-93 strains) at neutral pH could be observed by the utilization of goose, sheep, guinea-pig and human "O" group erythrocytes (Fig. 1), that might be suggestive of the existence of different subpopulations or variants of virus particles in the same virus preparation. Variants with higher affinity for determined cell surface structures, which could be selected during the virus propagation process.

Influenza viruses show a known standard behavior due to the importance of the receptor specificity, during the hemagglutinin-cell receptor interaction process. In parallel experiment, A/PR/8/34, sample of influenza viruses exhibited a very significative variation of hemagglutinating titers (from 256 to 4096) due to the presence of specific cell receptors on membranes of avian and mammalian erythrocytes (ESS Couceiro, JNSS Couceiro & MC Cabral, unpublished results). The propagation of that sample in embryonated chicken eggs selected a virus preparation with higher affinity to sialic acid alpha-2,3Gal-containing receptors (Rogers & D'Souza 1989) which are found in higher percentage on avian (goose) cells (hemagglutinating titer equal to 4096). Lower hemagglutinating titers were observed to mammalian cells as sheep (512), guinea-pig (1024) and human

(256) erythrocytes (ESS Couceiro, JNSS Couceiro & MC Cabral, unpublished results), which exhibit an higher level of sialic acid alpha-2,6Gal-containing receptors (Rogers & D'Souza 1989, Couceiro et al. 1993).

However the same level of variation was not observed when paramyxoviruses were utilized as model. The variation was not significant for the SO-93 sample of NDV (titers from 64 to 128) as can be observed in Fig. 1, when erythrocytes from different animal origins were used. The results present the preparations of B_1 and La Sota lentogenic samples of NDV as the most heterogeneous mixtures of paramyxovirus variants (hemagglutinating titers varying from <2 to 256), also exhibiting higher hemagglutinating titers than that SO-93 velogenic sample. The cell receptors for paramyxoviruses (NDV) are expressed by different complex gangliosidic structures as GD1a, GT1b and GQ1b (Markwell et al. 1986, Haywood 1994), which could be more plentiful on cell surfaces of erythrocytes originated from specific animal origins. The results from hemagglutination assays obtained with sheep, goose, guinea-pig and human "O" group erythrocytes showed the occurrence of variability in the amount and specificity of erythrocytes receptors for paramyxovirus, as described by Martone et al. (1973). Thus, it can be considered that the majority of the NDV samples presented virus particles that were able to recognize more efficiently those cellular gangliosidic receptors exhibited on guinea-pig and human "O" group erythrocytes better than on those from other origins. 60% homology level at aminoacid level between different influenza virus strains for receptor binding HN structures could also explained these differences (Vainionpää & Hyypiä 1994).

The existence of those different variants in a particular virus preparation could be explained by mutations or selection, during the replication process in the host system. Those mutations can originate a predominance of certain types of virus variants with surface spikes exhibiting receptor affinity for erythrocytes from specific origins at different pH values, exhibiting configurations or characteristic types of cell receptors (Markwell et al. 1986, Rogers & D'Souza 1989). However, Muller and Stanley (1944) demonstrated that a minor effect of pH that occurred over the 6.0-8.0 pH range. Domingo et al. (1983) and Steinhauer et al. (1989), during viral genome analysis, could demonstrated that the majority of the RNA virus populations have a high number of sites of possible mutations, which originate heterogenicity of phenotypes in virus preparations.

The results from Table show that the hemolytic activity by NDV is a pH-independent process (Lenard & Miller 1981, Junankar & Cherry 1986,
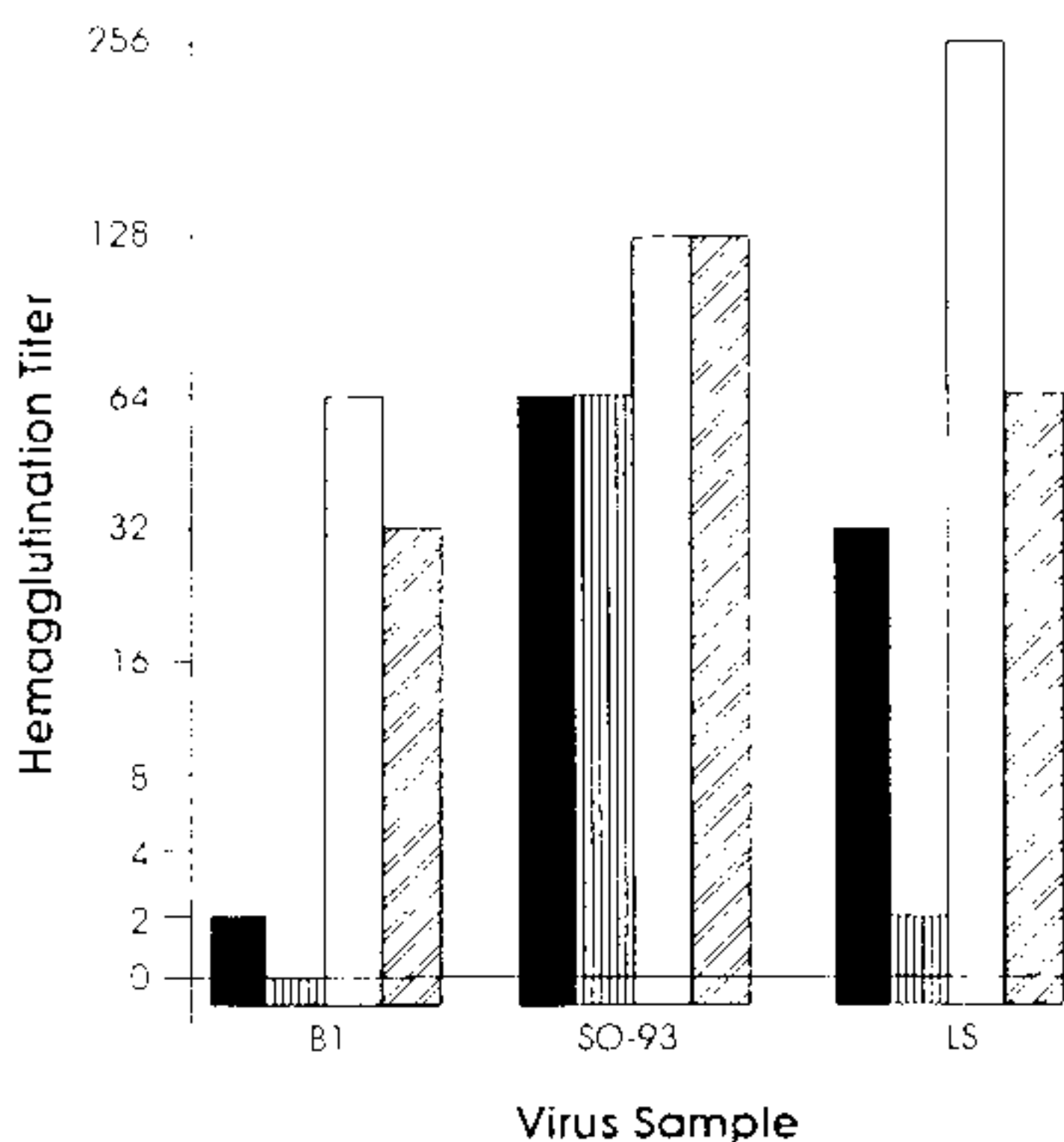

Fig. 1: titers of hemagglutination activity of B_1 SO-93, LS strains of Newcastle Disease viruses, using goose ■, sheep ▥, guinea-pig □ and human "O" group ▨ erythrocytes at neutral pH (7.0).

Marsh & Pelchen-Matthews 1993). Different strains of NDV exhibited high hemolysis activity at acid pH (5.0), which was too similar between SO-93 (33.07%) and La Sota (40.72%) samples. However, that B_1 sample exhibited low hemolytic activity at that pH value (1.86%). Moreover, Table shows that B_1 strain of NDV, which is usually weakly hemolytic at acid pH, exhibited a higher hemolytic activity (47.6%) when it was immediately exposed to neutral pH variation, although the pH-independent character of the paramyxovirus-cell fusion process is known. This observation could the explained by dissociation between virus particles with better exhibition of fusogenic sites (F) on virus membranes (Edwards et al. 1983, Haywood 1994, Stegmann 1994).

The structure responsible for fusion process developed by structures as influenzavirus hemagglutinin (HA), a fusion peptide, presents high homology. However analysis on receptor binding and fusogenic characters of influenzavirus strains shows variability which is explained by low homology of non fusogenic sequences of HA structures. Structures of H3N2 (X-31 strain of A/Aichi/68) and H2N2 (A/Japan/305/57) strains of influenza virus exhibited clear differences when analyzed by hemagglutination and hemolysis assays. HA structures of X-31 binds to sialoglycoproteins causing aggregation in the viral membrane, self-aggregation that inhibits those receptor binding (hemagglutination) and fusogenic (hemolysis) processes (Puri et al. 1990).

B_1 sample could be a HN variant between NDV samples analyzed, showing aggregation of HN structures in the viral membrane at acid pH which did not occur for La Sota and SO-93 strains. A possible explanation for low hemolysis could be

that fusogenic process was inhibited by aggregation of HN structures, affirming a dependence between HN and the fusogenic process developed by F structures which exhibit high homology at aminoacid level (Hu et al. 1992, Vainionpää & Hyypiä 1994).

Figure 2 shows the receptor binding activity of B_1 HN structures which was inhibited at acid pH, showing positivity at higher values of pH. B_1 sample that showed the lowest hemagglutination titers at neutral pH was analyzed at large pH range.
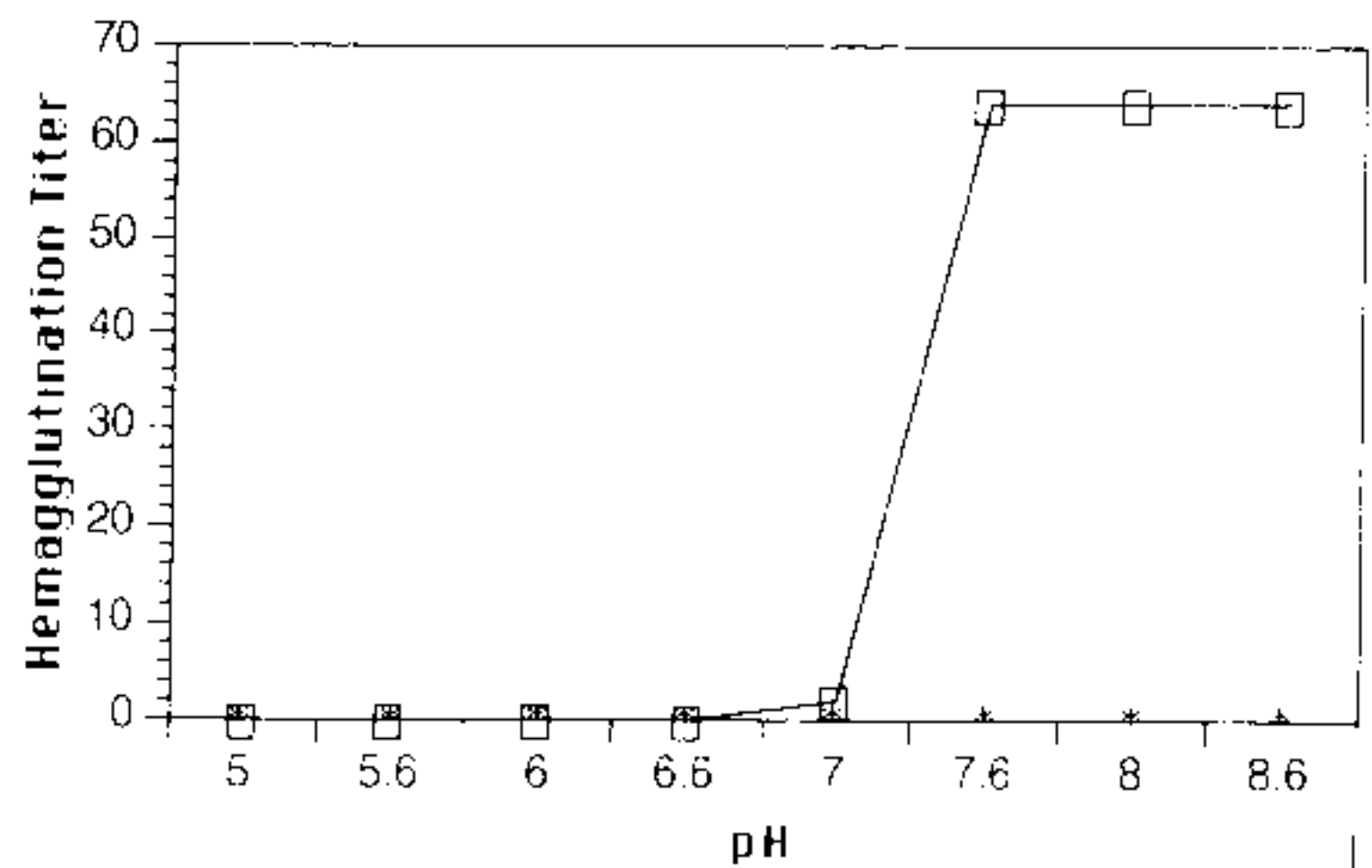

Fig. 2: titers of hemagglutination activity of the B_1 strain of Newcastle Disease viruses at different pH values, using sheep (*) and goose ⊐ erythrocytes.

TABLE

Hemolysis percentages exhibited for B_1, SO-93 and LS strains of Newcastle Disease viruses at different pH values at 5.0 - 6.0 pH variation [a]

Virus samples[b]	Hemolysis percentage at	
	pH	pH/variation
	5.0	5.0 - 6.0
B1	0.00	47.60
SO-93	33.07	ND
LS	40.72	ND

[a]: different pH values were adjusted and pH variations were obtained using Sorensen phosphate buffer (Pinto et al. 1994), 5M HCl and 1N NaOH, respectively
[b]: the virus samples were used at 5 μg/ml protein concentration
ND - not done

Fig. 3: micrography from the preparation of the B_1 strain of Newcastle Disease viruses at neutral pH, showing pleomorphism of non aggregated virus particles and a leakage (arrow) of an elongated virus structure (57.000 x 1 cm   172 nm).

The B_1 NDV was not recognized by sheep erythrocytes at various pH values higher than 7.6 (7.6 to 8.6) was revealed. Negative results were already demonstrated when using sheep erythrocytes and a non purified NDV velogenic sample (Chu 1948).

B_1 sample already analyzed as above by hemagglutination and hemolysis assays was observed by electron microscopy. The purification process and freezing-thawing cycles developed at neutral pH are shown as important steps to attain disaggregation and disruption of the pleomorphic virus structure, which induces leakages that are necessary for the liberation of hemoglobin with easy revealation of the virus-cell fusogenic process (Homma et al. 1976). The micrography from a non aggegated preparation of B_1 strain of NDV showed a leakage (arrow) that can be observed on an elongated virus particle close to an integral one (Fig. 3). Alterations that were certainly present in all virus preparations submitted at the concentration processes as those evaluated here.

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