ELECTROKINETIC STUDIES ON ERYTHROCYTES TREATED WITH MODIFIED INFECTIOUS BRONCHITIS VIRUS

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INTRODUCTION

Infectious bronchitis virus (IBV) normally does not cause hemagglutination. When modified with trypsin, the virus agglutinates erythrocytes of chickens and turkeys only. The hemagglutination is attributed to a modification of the virus to permit adsorption to the surface of the erythrocytes.

The surface of the erythrocyte is a macropolyanion that imparts a net negative electric charge. When treated with virus, some of the ionizable groups of the erythrocyte surface preferentially adsorb opposite ions supposed to be present on the surface of the virus particle, thereby reducing the surface charge density of the erythrocyte and hence its electrophoretic mobility. Electrophoresis offers a means by which precise determination of this physical entity on a biological surface can be made.

This study was undertaken to ascertain the electrophoretic mobility of erythrocytes before and after treatment with IBV and trypsin-modified IBV.

LITERATURE REVIEW

Electrophoresis

Electrophoresis is the movement of charged particles suspended in a liquid medium, under the influence of an applied electric field (Overbreek and Lijklema, 1959). The electrophoretic mobility, v, is calculated from the electrophoretic velocity (V) per unit field strength (X) (Abramson et al., 1942) as follows:

$v = V/X$, expressed in μ per sec. per volt per cm.

The field strength X is determined according to Ohm's law and is dependent upon the specific conductance of the solution (Ks), the cross sectional area of the cell (q) in cm^2, and the current (I) in amperes as follows:

$$X = I/q\ Ks.$$

The microscopic method of electrophoresis deals with the direct observation of microscopically visible particles as they migrate in the electric field. Four types of electrophoresis cells are available and each of them claim some advantage over the others: (1) Northrop-Kunitz wide, flat, rectangular, horizontal type (Northrop-Kunitz, 1925; Abramson, 1929a); (2) Vertical flat cells (Abramson et al.,

1942; Ponder and Ponder, 1955; Angers and Rottino, 1961); (3) Lateral flat cell (Hartman et al., 1952); (4) Cylindrical cell (Bangham et al., 1958b, Seaman and Heard, 1961). In all the flat type of cells, when the particles are suspended in a suitable medium and the current is applied there is an electroosmotic flow of the liquid along the surfaces of the cell wall and the liquid returns through the center. Therefore, it is only at the two stationary levels, which are intermediate to the center and the interior surfaces of the cell, that the liquid is motionless. For accurate measurement of electrophoretic mobility, the location of the stationary level with respect to the depth of the cell must be determined. Smoluchowski's equation (0.211X or 0.789X), when X is the depth of the cell, may be used only when the cell width is great compared to the depth. Otherwise, Komagata's correction (Stationary level = $0.5 + 0.2887\sqrt{1 + \frac{1.255}{A}}$) where A is the ratio of the cell width to the depth must be used (Bull, 1951).

The use of the micro-electrophoresis cell has been of considerable help for investigation of the surface phenomena related to biological substances. Besides the knowledge of the surface charge density on the erythrocytes in normal (Abramson, 1929b; Furchgott and Ponder, 1941; Bateman and Zeliner, 1956; Angers and Rottino, 1961) and disease conditions (Rottino and Angers, 1961), bacteria, spermatozoa and submicroscopic substances adsorbed on to

the surface of the carrier particles can be studied (Brinton and Lauffer, 1959). Submicroscopic substances such as proteins, lipoproteins, antibodies, detergents, and viruses can be adsorbed on to the surface of microscopically visible particles such as glass, quartz, collodion, carbon, mineral oil, ferric oxide, ion exchange resins, silica gel, aluminum oxide and even air bubbles. The electrophoretic mobility of a particle covered with adsorbate is characteristic of the adsorbed material and not of the particle. Uniformity of results depends upon complete covering of the particle with the adsorbate (Miller et al., 1944).

Erythrocyte

The surface of the erythrocyte offers a unique tool for observation of many biological phenomena. The cell stroma has an inner fibrous layer and an outer layer of plaques (Moskowitz and Calvin, 1952) consisting of long, thin rods composed of a lipid-protein-carbohydrate complex (Hiller and Hoffman, 1953) which serves as the receptor to which certain viruses may become attached. The lipid-rich protein fraction is also a potent inhibitor of PR-8 influenza indicator virus (Howe, 1951).

Depending on the pH and ionic strength of the suspending medium, the erythrocyte has a specific electrophoretic mobility characteristic of the species (Abramson and Moyer, 1930; Abramson et al., 1942). Furchgott and Ponder (1941) found that the electrokinetic behavior of human

erythrocytes is mainly associated with the lipid fraction of the cell stroma. The phosphoric acid group of cephalin may be mainly responsible for such behavior. Protein does not have any significant role in the electrophoretic mobility of erythrocytes. According to Bangham et al. (1958a) phosphate groups on lipids are responsible for the negative electric charge on sheep erythrocytes. Heard and Seaman (1960) and Seaman and Heard (1960) consider the surface structure of the human erythrocyte to be a macropolyanion imparting a definite electric charge which can be modified by opposite ion association and possibly by adsorption of hemolysate. The authors attribute the negative charge on the erythrocyte to be due principally to a carboxyl or sulfate group or a mixture of both.

At a physiological pH and ionic strength there is no positive grouping and evidence for the phosphate groups being mainly responsible for the net negative charge is circumstantial.

Madoff and Eyler (1961) reported that the negative charge on the erythrocyte is mainly due to sialic acid of varying degree in different species of mammals. N- glycolylneuraminic acid is present in the stroma of the erythrocytes of the sheep, pig, horse, and ox (Klenk and Uhlenbruck, 1958) and N-acetylneuraminic acid is in the stroma of erythrocytes of man and chicken (Klenk and Lempfrid, 1957; Klenk and Uhlenbruck, 1958). There is a linear relationship between the percentage of total N-acetylneuraminic

acid released and the per cent reduction in the electrophoretic mobility or total surface charge on human erythrocyte (Eyler et al., 1961).

The erythrocyte behaves unusually and differently to a variety of substances of animal and plant origin. Gelatin, egg albumin, casein, hemoglobin, and fibrinogen do not adsorb to the surface of the erythrocyte and its electrophoretic mobility is not affected (Abramson et al., 1942). According to Abramson and Moyer (1930), hemolysis caused by chloroform, freezing and thawing, and water does not significantly alter the mobility of the erythrocytes of rabbits. Rottino and Angers (1961) however, reported that hemolysis due to ruptured cells, chronic inflamatory diseases and malignant neoplastic diseases alters the electrophoretic mobility of human erythrocytes. Trypsin reduces the electrophoretic mobility of human erythrocyte by 25% (Ponder, 1951), and a glycopeptide containing sialic acid is released from the human erythrocyte (Cook et al., 1960).

Agglutination of erythrocytes by extracts from plant (Netter, 1956) and animal tissues (Stone, 1946), higher fungi (Fahey, 1954), bacteria (Netter, 1956), rickettsia and pleuro-pneumonia-like organisms (Fahey, 1954) has long been established. Endowed with an antigen and receptor on its stroma, the erythrocyte is agglutinable by complementary structures in human sera and some viruses, respectively.

That influenza virus agglutinates chicken erythrocytes was a significant step forward in virology (Hirst, 1941; McClelland and Hare, 1941). Hirst (1942a, 1942b) demonstrated that influenza virus adsorbed to the chicken erythrocyte and formed bridges between adjacent erythrocytes with resulting agglutination. After a reaction period dependent upon time and temperature, the virus eluted from the surface of the erythrocyte. It was soon discovered that hemagglutination was not a characteristic of all viruses and that not all erythrocytes from mammals or birds were agglutinable. Different mechanisms of hemagglutination are involved and viruses can be classified under several groups (Hirst, 1959).

Direct Hemagglutination

1. Arthropod-borne viruses

The virus particle itself is possibly the hemagglutinin (Sabin and Buescher, 1950) and very specific conditions are necessary for hemagglutination. Japanese B, Western equine, yellow fever, and dengue viruses are some known agents of the group that have a wide difference in antigenicity. They are not known to contain any enzymatic activity (Anderson, 1959).

2. Lipoportein hemagglutinins

The hemagglutinin is distinctly separable from the virus particle. There are two sub-groups: (a) Pox viruses that include vaccinia, variola, and ectromelia; (b) Psittacosis-lymphogranuloma venerum group including meningo-

encephalitis virus. Lecithinase inactivates the hemagglutinin which may be a lipoprotein.

3. Echo viruses and Coxsackie B_3

The hemagglutinin is associated with the virus particle which elutes from the surface of the erythrocyte. The receptors of this group of viruses are different from those of the myxoviruses. Hemagglutinin is active against human type O erythrocytes.

4. Adenoviruses

More than 18 different types of viruses of this group specifically agglutinate erythrocytes from different species of animals.

5. Miscellaneous group

Encephalomyocarditis viruses agglutinate only sheep erythrocytes. Pneumonia virus of mice agglutinates mouse and hamster erythrocytes. The hemagglutinin appears to be a part of the virus particle.

The murine poliomyelitis (GDVII strain) virus has a hemagglutinin which agglutinates human erythrocytes at low temperature.

The polyoma virus of mice, an oncogenic virus, agglutinates erythrocytes from many mammalian species.

6. Myxoviruses

Mumps, Newcastle disease virus, fowl plague, and strains of influenza virus are included in this group. The

hemagglutinin is associated with the virus particle containing the enzyme, neuraminidase.

The interaction of influenza virus and the erythrocyte is similar to the formation of an enzyme-substrate complex (Hirst, 1942a). The initial attachment is inoic in nature and the hydroxyl group of a polysaccharide chain present on the substrate is responsible (Buzzell and Hanig, 1958). The virus finally elutes from the erythrocyte due to the action of neuraminidase on the receptor of the erythrocyte. According to Hanig (1948), there is a decrease of the net surface charge of the human erythrocyte and the electrophoretic mobility which may be attributed to any of the following: (1) loss of charged substances from the cell surface, (2) change in the spatial configuration of charged groupings on the cell surface, or (3) adsorption by the erythrocyte of charged elements of the solvent. Hanig also concluded that the reduced electrophoretic mobility was a result of the destruction of the virus receptors on the erythrocyte surface. Bateman et al. (1956) have attributed the reduced electrophoretic mobility to the formation of additional free positively charged groups on the surface of erythrocytes.

Ada and Stone (1950), Stone and Ada (1952) reported that the reduction in electrophoretic mobility after treatment of human erythrocytes with myxoviruses and receptor destroying enzyme (RDE) was in close approximation to the "receptor gradient" proposed by Burnet et al. (1946).

However, Newcastle disease and swine influenza viruses in allantoic fluid deviated from their normal position in the receptor gradient, indicating that there may be some inhibitors in the allantoic fluid for these viruses, and that the loss of agglutinability was not a direct function of the total residual electric charge on the erythrocyte surface.

Receptor Destroying Enzyme

Receptor destroying enzyme (RDE) (Burnet et al., 1946; Burnet and Stone, 1947) is an induced (adaptive) exo-enzyme of Vibrio cholerae that has played an important role for a better understanding of some of the biological concepts underlying the virus-erythrocyte receptor reaction, inhibition of hemagglutination, and inactivation of both receptor and inhibitor by virus. This enzyme of V. cholerae is now known as neuraminidase (Gottschalk, 1957). It is defined as the specific α - glycosidase cleaving the α - ketosidic linkage joining the potential keto group of a terminal N-acylated neuraminic acid to an adjacent sugar residue in a disaccharide, trisaccharide, or polysaccharide (Gottschalk, 1958). Neuraminidase can be prepared in crystalline form (Schramm and Mohr, 1959; Ada and French, 1959). The enzyme can also be prepared from a variety of microorganisms such as Clostridium welchii, Clostridium tertium, Pseudomonas fluorescens, Pseudomonas pyocyanus, Pseudomonas stuzeri, Lactobacillus bifidus, and Diplococcus pneumoniae.

The myxoviruses possess α - neuraminidase and the enzyme, assumed to be present in patches on the virus surface, seems to be associated with the virus hemagglutinin (Gottschalk, 1960). Howe et al. (1961) suggested that myxovirus hemagglutinin and neuraminidase have separate identities.

Burnet (1942) proposed a "receptor gradient" or the order in which the myxoviruses may be graded according to their receptors. According to Hirst (1959), there are two hypotheses to explain the receptor gradient: (1) several different kinds of receptors are on the erythrocyte surface and different kinds of viral enzymes may be involved although there is yet no evidence for such multiplicities, and (2) accessibility of similar receptors on the erythrocyte surface for viral enzymatic action. This does not explain the situation where the hemagglutination titer is not a direct function of the total residual charge on the erythrocyte surface. Inhibitors (Ada and Stone, 1950), or mucoprotein receptor substances, having different configurations (Howe et al., 1961) may be responsible.

Inhibitors

Francis (1947) reported that influenza virus heated at 56 C for 30 minutes agglutinates erythrocytes but does not elute from them. Francis identified this as "indicator virus." Normal serum strongly inhibits hemagglutination by indicator virus but not non-heated virus. The inhibitors

identified thus far are mucoproteins (Hirst, 1959) and some mucolipids (Rosenberg et al., 1956; Rosenberg and Chargaff, 1958). Mucoproteins are defined as conjugated proteins with multiple hexosamine containing oligosaccharides or small polysaccharides as the prosthetic groups. The prosthetic groups are covalently linked to the protein core (Gottschalk, 1952, 1954).

Inhibitory muscoproteins are present in a variety of sources such as human and rabbit serum, ovarian cysts, sheep salivary gland (McCrea, 1948, 1952a), normal human urine (Tamm and Horsfall, 1952), tissue extract (Hirst, 1959), sputum mucoid and brain mucoid (Howe et al., 1961), meconium from infants (Curtain et al., 1953; Pye, 1955; Zilliken, et al., 1957), and erythrocyte stroma (Howe, 1951; Howe et al., 1957; McCrea, 1953b).

All these known inhibitors, however, lose their activity when treated with intact myxoviruses, RDE (neuraminidase), trypsin, or periodate (0.001M) (Gottschalk, 1960). Sialic acid, a group name for acylated neuraminic acids, is released from the inhibitors through the action of neuraminidase. Neuraminic acid is the basic unsubstituted structure $C_9H_{17}O_8N$, common to all the inhibitors.

Not all sialo-mucoproteins are inhibitory. To qualify as a virus hemagglutinin inhibitor, the mucoprotein must have a substrate for neuraminidase, and compete successfully with the receptor on the cell surface for the virus. The successful competitor, either the erythrocyte receptor or

the mucoprotein, exerts the more attractive force and has the relatively greater number of functional groups for the virus particle (Gottschalk, 1960).

Mucolipids from ox brain (Folch et al., 1951) and human brain have a low degree of inhibitory capacity towards PR-8 (Rosenberg et al., 1956; Rosenberg and Chargaff, 1958) and PR 301 (Howe et al., 1961) indicator virus.

Indirect Hemagglutination

Indirect hemagglutination (Lennette, 1959) is due to the action of specific immune serum on erythrocytes treated with tannic acid and then coated with viral or rickettsial antigen. Tanned sheep erythrocytes to which herpes simplex virus has been adsorbed agglutinate with specific immune serum (Scott et al., 1957).

Brown et al. (1962) have reported that the horse erythrocyte can be modified with tannic acid to give an indirect hemagglutination test for two strains of IBV.

Infectious Bronchitis Virus

Infectious bronchitis virus, Tarpeia pulli (Merchant and Packer, 1961; von Rooyen, 1954), is the etiological agent of infectious bronchitis of chickens. The virus is a sphere with a diameter of 65 mμ to 135 mμ (Reagan et al., 1950); Reagan and Brueckner, 1952; Nazerian, 1960). It exists in two phases, the thermolabile D phase and the thermostable O phase (Singh, 1960). The optimum pH for stability is 7.8. The isolectric point is about pH 4.05

(Cunningham and Stuart, 1947). The approximate density of the virus is 1.15 (Buthala, 1956).

The virus can readily be cultivated in the chicken embryo. The virulence of the virus for chicken embryo is increased through serial passage (Cunningham, 1957).

The Beudette strain or egg-adapted strain of IBV can be cultivated in chicken embryo kidney cells, chicken embryo fibroblasts (Spring, 1960), chicken liver and heart cells (Fahey and Crawley, 1956), and in the isolated chorioallantoic membrane (Ferguson, 1958; Ozawa, 1959).

Infectious bronchitis virus in allantoic fluid does not cause hemagglutination. Modification of the virus with trypsin induces agglutination (Corbo and Cunningham, 1959) of erythrocytes from turkeys and from chickens older than three weeks. The same receptors on the chicken erythrocyte may be involved with both influenza (PR-8) and infectious bronchitis virus (Muldoon, 1960). The hemagglutinin is associated with the virus particle (Nazerian, 1960). Specific inhibition of hemagglutination of anti-IBV serum has not been accomplished. Inhibition also occurs with normal chicken serum (Corbo and Cunningham, 1959; Muldoon, 1960). Trypsin, sodium or potassium periodate (0.9 to 0.01 M), zymosan, and RDE do not remove inhibitors of the virus hemagglutinin present in normal and immune sera (Muldoon, 1960).

Trypsin-modified IBV only adsorbs to and agglutinates chicken erythrocytes according to studies with the indirect

fluorescent antibody technic (Stultz, 1962), and electron microscopy (Nazerian, 1960).

Trypsin-modified IBV in allantoic fluid does not precipitate with its antibody in agar-gel-medium whereas non-trypsinized infectious bronchitis virus precipitates with its antibody (Tevethia, 1962).

Brown et al. (1962) have reported that horse erythrocytes can be modified with tannic acid to give an indirect hemagglutination test for two strains of IBV.

MATERIALS AND METHODS

Viruses

Infectious bronchitis virus, 41 (IBV-41), the repository code for the Massachusettes strain, Newcastle disease virus (NDV) and PR-8 strain of influenza virus were used.

The viruses were cultivated in ten-day-old embryonating chicken eggs, inoculum 0.1 ml per egg. The allantoic fluid from the IBV-infected embryo was harvested 72 hours postinoculation whereas NDV and PR-8 influenza virus were harvested 48 hours postinoculation. The pooled viruses were stored at -70 C in screw cap vials. At the time of use they were thawed at room temperature, centrifuged at 1400 g for ten minutes, and the supernatant fluid was removed and used for experimental purposes. All cultures were tested for bacteriological sterility in Brewer thioglycolate medium (Difco).

Normal Allantoic Fluid

Normal allantoic fluid (NAF) was collected from thirteen-day-old embryonating chicken eggs to serve as the control for the viruses contained in allantoic fluid.

Erythrocytes

Blood was obtained from a Single Comb White Leghorn cockerel and from a turkey by cardiac puncture. Human type O blood and blood from a cow and horse were obtained by venipuncture.

The blood was collected in tubes containing 1 ml of a 2% (w/v) sodium citrate solution for each 6 ml of blood. The blood was centrifuged immediately and the plasma was removed. One volume of the packed erythrocytes was then washed three times by centrifugation for ten minutes per wash, using about thirty volumes of saline solution for each wash. After the last wash, the saline was removed from the packed erythrocytes which were stored at 4 C for as long as four days. At the time of use, the erythrocytes were diluted to an appropriate concentration with buffer for a particular experiment.

Diluents

Saline, sodium chloride 0.85% (w/v) in double distilled deionized water, and Bacto (Difco) hemagglutination buffer (pH 7.35 $\pm$ 0.05) were used.

Sorensen's 0.067M phosphate buffer (Clark, 1925) at pH 7.35 was also used. Dextrose (dehydrated) 1% (w/v), was added to prevent hemolysis during electrophoresis.

Trypsin

Bacto-trypsin (Difco 1:250) 1% in double distilled deionized water was incubated for 15 minutes at room

temperature, passed through a Seitz EK filter and the filtrate was stored at -30 C until used.

Eggwhite Trypsin Inhibitor

A one per cent solution (w/v) of egg white trypsin inhibitor (ETI) (California Corporation for Biochemical Research) in double distilled deionized water was stored at -30 C until used.

Receptor Destroying Enzyme

A lyophilized sample (Behringwerke, Marburg-Lahn, Germany) was reconstituted to volume in double distilled deionized water and serial two-fold dilutions were prepared with calcium borate buffered saline solution (Burnet and Stone, 1947).

Electrophoresis Apparatus

A Northrop-Kunitz flat, horizontal type electrophoresis cell apparatus (Arthur H. Thomas Co.) was used.

The electrical circuit consisted of a VOKAM power pack (Shandon Scientific Co., Ltd., London) that can convert AC to DC with a variable potential from 0 to 400 volts and a current strength varying from 0 to 80 milliamperes. In the experiments, 100 volts with a current strength of 5 milliamperes were used.

The optical system consisted of a monocular microscope with a 4 mm 0.66 NA high dry objective and 10X eye piece with a micrometer disc. A Reichert Viewscope was mounted

over the eye piece for ease of viewing the specimen. The micrometer disc lines projected at the level of the electrophoresis cell were 33.33 μ apart. This measurement was made by superimposing the image of the disc upon the rulings of a hemocytometer placed at the level of the electrophoresis cell.

The zinc electrodes were cleaned with water to remove the deposition formed on them after being used four to five times. The electrodes were used with a saturated zinc sulfate solution prepared in deionized water.

The electrophoresis cell was thoroughly washed with acid cleaning solution, rinsed with double distilled water, and coated with gelatin solution and then with the buffer to be employed before each individual test. The two stationary levels of the electrophoresis cell were determined after the interior of the cell was coated with one per cent gelatin in water (Abramson et al., 1942). Measurements made at the two levels were similar. After several preliminary electrophoretic tests in which the measurements were essentially the same, it was more convenient to make readings at only one of the two levels. Usually ten electrophoretic tests were made of each sample and the results were averaged. There was little difference in the measurements for each sample when the direction of the current was reversed. Tests were performed first with normal erythrocytes.

The electrophoretic mobility, v, of the erythrocyte is expressed as the velocity per electrical field strength (X) in volts per cm.

$$v = \text{velocity}/ X = \frac{\text{distance traveled}}{\text{time}} / X$$

$$X = I/ q K_s ,$$

where I = current in amperes; q = cross sectional area of the electrophoresis cell in cm^2; and K_s = specific conductivity of the buffer.

The following were the constants for the experiments:

$$I = 0.005 \text{ amperes}$$

$$q = 0.12 \text{ cm}^2$$

$$K_s = 0.0045 \text{ ohms}^{-1} \text{ cm}^{-1}.$$

$$X = \frac{0.005 \text{ amps}}{0.12 \text{ cm}^2 \times 0.0045 \text{ ohm}^{-1} \text{ cm}^{-1}} = 9.26 \text{ volts/cm.}$$

A typical example for v = velocity/X =

$$\frac{10.85 \mu \text{ sec}^{-1}}{9.26 \text{ volts cm}^{-1}} = 1.17 \mu \text{ sec}^{-1} \text{ volt}^{-1} \text{ cm}^{-1}$$

Hemagglutination

Modification of IBV by trypsin was based on the procedure described by Muldoon (1960). Allantoic fluid containing the virus was thawed at room temperature, centrifuged at 1400 g for 10 minutes and the supernatant fluid was collected. To two volumes of the supernatant fluid, one volume of 1% trypsin was added. After the mixture

was incubated in a water bath at 56 C for 30 minutes, one volume of 1% ETI was added and the mixture was incubated at room temperature for at least 15 minutes.

The procedure for the hemagglutination test was the same for all viruses (Cunningham, 1960). Serial two-fold dilutions of the virus were prepared in hemagglutination buffer. To each of a series of 12 x 75 mm tubes was added 0.25 ml each of diluted virus, saline solution, and 0.5% erythrocytes. The tubes were shaken for about 10 seconds and then incubated for 1 hour at room temperature. The hemagglutination titer expressed as HA units was the reciprocal of the highest dilution of the virus in which hemagglutination was complete.

Treatment of Erythrocytes with Virus

All the erythrocytes were used with trypsin-modified IBV, but only chicken erythrocytes were used for NDV and PR-8 influenza virus. The ratio of virus to erythrocytes and the optimum time for the establishment of equilibrium were based on the electrophoretic mobility of the treated erythrocytes.

The following was established as the standard procedure. To 0.25 ml of packed erythrocytes was added 5 ml of virus containing 100 HA units per 0.25 ml and the mixture was incubated for four hours at room temperature. The erythrocytes were then washed with buffer for a particular experiment and a final suspension of 0.1% erythrocytes was made.

Control samples of erythrocytes were prepared in the same manner except that buffer was used in place of the virus suspensions or other reagents. Chicken and turkey erythrocytes were treated with non-trypsinized IBV, NAF, and NAF treated with trypsin and egg white trypsin-inhibitor (NAF + T + ETI) in the same manner as for trypsin-modified IBV. The treatment of the erythrocytes with these reagents also served as a control as they did not cause hemagglutination.

Treatment of Erythrocytes with Trypsin

Erythrocytes were treated with trypsin in the same manner as they were treated with the viruses. When four volumes of trypsin were added to one volume of packed erythrocytes and incubated for 30 minutes at 37 C the same result was obtained as at room temperature for four hours.

Chicken and turkey erythrocytes were treated with trypsin, washed, and then treated with trypsin-modified IBV, NDV, PR-8 influenza virus, RDE, non-typsinized IBV, NAF, and NAF treated with trypsin and ETI. The erythrocytes were washed finally and resuspended in the appropriate buffer.

Treatment of Erythrocytes with RDE

To 0.25 ml of packed erythrocytes was added 5 ml of RDE diluted in calcium borate buffered saline to contain 100 RDE units per ml. The mixture was incubated at 37 C for four hours. The erythrocytes were then washed and some

of these erythrocytes were further treated with PR-8 influenza virus and trypsin-modified IBV.

Titration of RDE was done according to the procedure described by Burnet and Stone (1947). To two-fold serial dilutions of the enzyme, an equal volume (0.25 ml) of 1% chicken erythrocytes was added. The mixture was incubated at 37 C for 30 minutes. One drop (0.04 ml.) PR-8 influenza virus, containing 10 HA units, was added to each tube containing the RDE treated chicken erythrocytes. The tubes were shaken for a few seconds and then incubated for 30 minutes at 37 C. The end point was the highest dilution of the enzyme in which partial agglutination occurred. The units were expressed in RDE units.

Standard error of the electrophoretic mobility was calculated after Waugh (1943).

Formula: $\sigma_m = \frac{\sigma_x}{\sqrt{N}}$ where σ_m is the estimate of the standard deviation of the means, σ_x is the standard deviation of the samples and N is the number of observations in each experiment.

RESULTS

Erythrocytes from the horse, cow, and human did not agglutinate with trypsin-modified IBV and there was no reduction of their electrophoretic mobility. Erythrocytes from the chicken and turkey agglutinated with trypsin-modified IBV. Electrophoretic mobility was reduced from 1.17 to 0.92, a 21.4% reduction, for the chicken erythrocytes and from 1.27 to 1.05, a 17.4% reduction, for the turkey erythrocytes (Table 1).

TABLE 1.--Electrophoretic mobility of erythrocytes in 0.067M phosphate buffer at pH 7.35 before and after reaction with trypsin-modified IBV.

Source of erythrocyte	Agglutination by typsin-modified IBV	Electrophoretic mobility μ sec^{-1}volt^{-1}cm^{-1}		Per cent reduction of mobility
		Normal	trypsin-mod. IBV	
Chicken	+	1.17	0.92	21.4
Turkey	+	1.27	1.05	17.4
Horse	-	0.91	0.91	0
Cow	-	0.93	0.93	0
Human	-	1.31	1.31	0

Agglutination of chicken and turkey erythrocytes and reduction of electrophoretic mobility (Table 1) occurred only when trypsin-modified IBV was used. Non-trypsinized IBV, NAF, or NAF treated with trypsin and ETI did not cause hemagglutination or reduction of the electrophoretic mobility (Table 2).

Trypsin reduced the electrophoretic mobility of the chicken and turkey erythrocytes from 1.17 to 1.04, an 11.1% reduction, and from 1.27 to 1.22, a 3.9% reduction, respectively. There was no reduction of the electrophoretic mobility of these erythrocytes after treatment with a mixture of trypsin and ETI. Erythrocytes treated with trypsin and then with NAF, NAF treated with trypsin and ETI or IBV did not have any further reduction of electrophoretic mobility. When trypsin-treated erythrocytes were treated with trypsin-modified IBV the electrophoretic mobility was further reduced (Table 3).

The electrophoretic mobility of chicken erythrocytes, 1.17 was reduced to 0.92 μ $sec^{-1}volt^{-1}cm^{-1}$, a 21.4% reduction, after treatment with trypsin-modified IBV; to 0.68, a 41.9% reduction, after treatment with PR-8 influenza virus; to 0.53, a 54.7% reduction, after treatment with NDV and to 0.42, a 64.1% reduction, after treatment with RDE (Table 4). The HA titer of the trypsin-modified IBV was 2560; PR-8 influenza virus, 5120; and NDV, 640.

TABLE 2.--Electrophoretic mobility of erythrocytes in 0.067M phosphate buffer at pH 7.35 treated with different reagents.

Reagents	Agglutination	Electrophoretic Mobility μ $sec^{-1}volt^{-1}cm^{-1}$							
		Chicken Erythrocytes				Turkey Erythrocytes			
		Average	Range	σ_m*	Per cent reduction of mobility	Average	Range	σ_m*	Per cent reduction of mobility
None	-	1.17	1.20-1.15	0.010	0	1.27	1.30-1.25	0.012	0
IBV	-	1.17	1.21-1.15	0.011	0	1.27	1.30-1.25	0.012	0
NAF	-	1.17	1.20-1.15	0.010	0	1.27	1.30-1.25	0.012	0
NAF+T+ETI	-	1.17	1.21-1.15	0.011	0	1.27	1.30-1.25	0.012	0
IBV+T+ETI	+	0.92	0.94-0.91	0.009	21.4	1.05	1.11-1.03	0.014	17.4

* σ_m = Standard error of the mean.

TABLE 3.--Electrophoretic mobility of erythrocytes in 0.067M phosphate buffer at pH 7.35 treated with different reagents.

Reagents	Electrophoretic Mobility μ $sec^{-1}volt^{-1}cm^{-1}$									
	Chicken Erythrocytes					Turkey Erythrocytes				
	Aggl.	Average	Range	σm*	Per cent reduction of mobility	Aggl.	Average	Range	σm*	Per cent reduction of mobility
Normal Erythrocytes										
None	-	1.17	1.20-1.15	0.010		-	1.27	1.30-1.25	0.012	
Trypsin	+	1.04	1.06-1.02	0.009	11.1	+	1.22	1.25-1.20	0.011	3.9
Trypsin+ETI	-	1.17	1.20-1.15	0.010	0	-	1.27	1.30-1.25	0.014	0
Trypsinized Erythrocytes										
IBV	...	1.04	1.06-1.02	0.009	11.1	...	1.21	1.25-1.20	0.012	4.0
NAF	...	1.04	1.06-1.02	0.009	11.1	...	1.22	1.25-1.20	0.011	3.9
NAF+T+ETI	...	1.04	1.06-1.02	0.009	11.1	...	1.22	1.25-1.20	0.011	3.9
IBV+T+ETI	...	0.97	1.00-0.95	0.012	17.1	...	1.15	1.17-1.13	0.008	9.4

* σm = Standard error of the mean.

The electrophoretic mobility of chicken erythrocytes, 1.17, was reduced to 1.04, an 11.1% reduction, after treatment with trypsin. When the trypsin-treated erythrocytes were washed and then treated with trypsin-modified IBV, PR-8 influenza virus and RDE the mobilities were 0.97, 0.76, and 0.51, respectively or 17.1%, 35%, and 56.4% reductions, respectively (Table 4).

TABLE 4.--Electrophoretic mobility of normal and trypsin-treated chicken erythrocytes in 0.067M phosphate buffer at pH 7.35 treated with different reagents.

Reagents	HA titer	Electrophoretic Mobility μ $sec^{-1}volt^{-1}cm^{-1}$ Average	Range	σm*	Per cent reduction of mobility
		Normal erythrocytes			
None	0	1.17	1.20-1.15	0.010	0
PR-8	5120	0.68	0.71-066	0.014	41.9
NDV	640	0.53	0.55-0.51	0.006	54.7
RDE	0	0.42	0.45-0.40	0.011	64.1
IBV+T+ETI	2560	0.92	0.94-0.91	0.009	21.4
		Trypsin-treated erythrocytes			
None		1.04	1.06-1.02	0.009	11.1
PR-8	....	0.76	0.79-0.74	0.014	35.0
RDE	....	0.51	0.53-0.50	0.008	56.4
IBV+T+ETI	....	0.97	1.00-0.95	0.012	17.1

* σm = Standard error of the mean.

Receptor destroying enzyme reduced the electrophoretic mobility of chicken erythrocytes from 1.17 to 0.42, a 64.1% reduction. There was no further reduction of the electrophoretic mobility of the RDE-treated-erythrocytes with

PR-8 influenza virus or trypsin-modified IBV (Table 5).

TABLE 5.--Electrophoretic mobility of RDE-treated-chicken erythrocytes in 0.067M phosphate buffer at pH 7.35 treated with different reagents.

Reagents	Electrophoretic Mobility μ sec^{-1}volt^{-1}cm^{-1}			Per cent reduction of mobility
	Average	Range	σm*	
None	0.42	0.45-0.40	0.010	64.1
PR-8	0.42	0.45-0.40	0.010	64.1
IBV + T + ETI	0.42	0.47-0.38	0.112	64.1
Normal erythrocyte	1.17	1.20-1.15	0.010	0.0

* σm = Standard error of mean.

DISCUSSION

Hemagglutination by IBV in allantoic fluid can be induced with trypsin (Corbo and Cunningham, 1959; Muldoon, 1960). Hanig (1948) reported that PR-8 influenza virus reduced the electrophoretic mobility of human erythrocytes. Other myxoviruses such as mumps virus, NDV, and different strains of influenza virus and RDE also reduce the electrophoretic mobility of human erythrocytes (Ada and Stone, 1950). Trypsin-modified infectious bronchitis virus in allantoic fluid also reduced the electrophoretic mobility of chicken and turkey erythrocytes. Non-modified infectious bronchitis virus in allantoic fluid, NAF, and NAF treated with trypsin and ETI, did not agglutinate or reduce the electrophoretic mobility of chicken and turkey erythrocytes. Human type O, cow and horse erythrocytes were not agglutinable by trypsin-modified IBV or by non-modified IBV.

To explain the agglutinability of erythrocytes by trypsin-modified IBV on a physico-chemical basis, consideration may be given that the initiation of hemagglutination is primarily due to an attractive force. This force is electrostatic between the virus and the surface of the erythrocyte. Electrophoresis measures the charge density present on the surface of the erythrocyte. This density is

dependent upon the chemical properties and configuration of the outermost molecules of the erythrocyte membrane, and their interaction with the suspending medium. Non-modified IBV in allantoic fluid apparently does not have a substrate for adsorption to the surface of erythrocytes. Only chicken and turkey erythrocytes have thus far been reported to be agglutinable by trypsin-modified virus. This would indicate that two parameters are involved: (1) the substrate or receptor on the erythrocytes for the trypsin-modified IBV, and (2) trypsin susceptible substrate on IBV in allantoic fluid.

Neuraminidase destroys the receptors of trypsin-modified IBV and PR-8 influenza virus and a similarity of the receptor sites on chicken erythrocytes has been proposed (Muldoon, 1960). Neuraminidase reduced the electrophoretic mobility of chicken erythrocytes by 64.1% as compared to 72% as reported by Eyler et al. (1962). This difference may be due to the purity of the neuraminidase used, or to erythrocytes from different chickens, and also to the difference in pH of the buffers, (pH 6.4 vs pH 7.35). When RDE-treated chicken erythrocytes were treated with trypsin-modified IBV and PR-8 influenza virus there was no further reduction of the electrophoretic mobility.

Because of the specificity of the neuraminidase action upon the sialic acids, it is of interest to note that the receptors on the chicken erythrocytes for trypsin-modified IBV and PR-8 influenza virus were completely removed by the

enzyme. Sialic acids on erythrocytes are mainly responsible for their electrokinetic behavior. Neuraminidase releases the following amounts of the total sialic acids present on the erythrocytes: N-acetylneuraminic acid, 95-100% in human and chicken; 40% in calf; 5-10% in horse; and N-glycolylneuraminic acid, 0-5% in human and chicken; 60% in calf; and 90-100% in horse (Eyler et al., 1962). The similarity of sialic acids on chicken and human erythrocytes explains the susceptibility of the receptors to the PR-8 virus or neuraminidase. This does not provide a sound basis for suggesting the similarity of receptors for PR-8 and trypsin-modified IBV when the agglutinability of different species of erythrocytes by these viruses is taken into account. Trypsin-modified IBV does not agglutinate human erythrocytes or reduce their electrophoretic mobility. It is possible that the substrate on chicken erythrocytes for both viruses is similar, but dissimilar on human erythrocytes. It may also be possible that trypsin-modified IBV produces an ionogenic change on chicken and turkey erythrocytes but not on mammalian erythrocytes. Further work should clear the point as to what groupings are present on the chicken erythrocytes but not on mammalian erythrocytes for reception of the trypsin-modified IBV.

Myxoviruses elaborate a-neuraminidase and through this enzyme they split sialo-proteins and thus reduce the electrophoretic mobility of erythrocytes. The primary attachment or adsorption of the virus, however, depends upon the specificity of the substrates available to it on the surface of the

erythrocyte. The agglutination titer of chicken erythrocytes by trypsin-modified IBV, NDV, and PR-8 influenza virus were 2560, 640, and 5120, respectively. After the virus-erythrocyte reaction, there was no direct quantitative relationship between the agglutination titer and the reduction in electrophoretic mobility. Either different substrates or enzymes may be involved for each or all of the viruses.

Trypsin reduced the electrophoretic mobility of chicken erythrocytes by 11%. This reduction may be attributed to the enzymatic action of trypsin as there was no non-specific adsorption of trypsin to the erythrocytes, as measured by the electrophoretic mobility, when trypsin was previously neutralized by ETI.

The electrophoretic mobility of trypsin-treated erythrocytes were reduced by trypsin-modified IBV, NDV, PR-8 influenza virus, and RDE but not by NAF, trypsin-modified NAF or non-modified IBV. Trypsin splits polypepdide chains and a "structural reorganization" (Seaman and Heard, 1960), or "structural loosening up" (Ponder, 1951) results on the surface of the erythrocyte. This structural reorganization does not apparently destroy the receptors (ionogenic groupings) on the erythrocytes for the viruses or for RDE to a great extent, but it does present a situation where some receptors are made inaccessible to these agents.

The trypsin susceptible substrate on the virus is of particular interest for a better understanding of the mechanism of the induction of hemagglutination and the nature of

the protein coat and hemagglutinin associated with IBV. Trypsin is neutralized with ETI after incubation of the virus and there is no residual non-specific adsorption of trypsin to the surface of the erythrocyte. This is especially clear as a mixture of trypsin and ETI do not alter the electrophoretic mobility of the normal erythrocyte. The change by trypsin may be such that either some inhibitors ordinarily present in the allantoic fluid that mask the hemagglutinin of the virus are destroyed, or there is some structural reorganization of the protein coat of the virus that exfoliates some ionogenic groups that favor attachment to the surface of the erythrocyte. Further research on the biochemistry and biophysics of the virus and the receptors may determine the nature of the hemagglutinin of IBV.

SUMMARY

1. Trypsin-modified IBV in allantoic fluid reduced the electrophoretic mobility of chicken and turkey erythrocytes from 1.17 to 0.92 μ $sec^{-1}volt^{-1}cm^{-1}$, a 21.4% reduction, and 1.27 to 1.05 μ $sec^{-1}volt^{-1}cm^{-1}$, a 17.4% reduction, respectively.

2. Human type O, cow and horse erythrocytes were not agglutinable and their electrophoretic mobility was not reduced by trypsin-modified IBV.

3. Infectious bronchitis virus in allantoic fluid not treated with trypsin, NAF, and trypsin-modified NAF did not affect the electrophoretic mobility of chicken and turkey erythrocytes.

4. Trypsin reduced the electrophoretic mobility of chicken and turkey erythrocytes from 1.17 to 1.04 μ sec^{-1} $volt^{-1}cm^{-1}$, an 11.1% reduction, and from 1.27 to 1.22 μ $sec^{-1}volt^{-1}cm^{-1}$, a 3.9% reduction, respectively.

5. When trypsin was neutralized with egg white trypsin inhibitor, there was no non-specific adsorption of trypsin to the surface of chicken erythrocytes and no reduction of the electrophoretic mobility.

6. Trypsin-modified IBV, PR-8 influenza virus or RDE reduced the electrophoretic mobility of trypsin-treated

chicken erythrocytes. Trypsin did not destroy the receptors for the viruses or RDE but brought about a structural reorganization on the surface of the erythrocyte that probably made some receptors inaccessible to these agents.

7. There was no direct relationship between the agglutinability and the reduction of electrophoretic mobility of the chicken erythrocytes due to trypsin-modified IBV, NDV, and PR-8 influenza virus.

8. The electrophoretic mobility of the RDE treated chicken erythrocytes was not appreciably changed when erythrocytes were further treated with trypsin-modified IBV or PR-8 influenza virus.

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