

The Contribution of Sialic Acid to the Surface Charge of the Erythrocyte*

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Under the conditions of physiological pH and ionic strength, the erythrocytes from many animal species exhibit a negative electrophoretic mobility (1), and thus have a negative surface charge. Attempts to describe this surface charge in molecular terms have been made from studies of electrophoretic mobility under varying conditions of pH and ionic strength (2-4). Furchgott and Ponder (5) and others (6) have found the erythrocyte to be isoelectric near pH 2.0 and have attributed its negative surface character to phosphoric acid groups of the cephalins. Recent studies (4) comparing the cation-binding behavior of erythrocytes and phospholipids have similarly been interpreted in terms of phospholipid phosphate, notably phosphatidic acid and monophosphoinositide (7).

However, these studies have failed to consider the work of Hanig (8) and Ada and Stone (9) who demonstrated that influenza virus and the neuraminidase of *Vibrio cholerae* greatly reduce erythrocyte mobility. Klenk and Uhlenbruck (10) have shown that the enzyme acts upon erythrocyte stroma by releasing *N*-acetylneuraminic acid, which they crystallized. It is now apparent that neuraminidases catalyze the hydrolysis of the glycosidic linkage of sialic acid (11) in glycoproteins.¹

The present work was carried out to determine the relationship between sialic acid and the intrinsic negative charge of the erythrocyte. Preliminary work (12, 13) indicated that the reduction in erythrocyte mobility due to enzymatic action could be interpreted in terms of the removal of sialic acid and hence of a carboxyl group of pK near 2.6. The situation is analogous to that of the α_1 -glycoprotein,¹ bovine submaxillary mucoprotein (14), and fetuin (15). In a recent paper, Cook, Heard, and Seaman (16) describe a similar study with human erythrocytes. The interpretation of the erythrocyte surface charge by Bateman *et al.* (2), who treated guinea pig erythrocytes with influenza virus, was based on earlier work of Curtain (17) and no longer appears plausible.

In the present study it was found that all the erythrocyte sialic

acid of several animal species is present on the membrane surface and 95 to 100% of it can be removed by neuraminidase action. It is apparent for erythrocytes of most species that the negative surface charge is primarily due to the carboxyl group of sialic acid rather than the phosphate groups of phospholipids. Furthermore, it was found that the theoretical equations, which derive surface charge from calculation of mobilities, underestimate the true potential at the surface shear.

EXPERIMENTAL PROCEDURE

Erythrocytes—For each experiment, erythrocytes from a single animal were used except in the case of chickens, where blood from three or four birds was pooled. The pig, lamb, and calf blood, which was obtained at a local slaughter house, was collected into 5% sodium citrate solution. Human group O, Rh-positive erythrocytes² were collected into 5% sodium citrate by antecubital vein puncture. The blood was centrifuged immediately after collection, and the erythrocytes washed three times in 10 volumes of isotonic buffer, pH 7.2, which was 0.135 M sodium chloride and 0.02 M sodium phosphate. The buffy coat was removed in each case. The washed erythrocytes were used within 48 hours or discarded. All procedures were performed at 0-4° unless otherwise specified.

Preparation of Stroma—The washed erythrocytes, 100 to 300 ml, were brought to a known volume with an approximately equal volume of isotonic buffer, and an aliquot was taken for cell count and hematocrit. The solution was slowly diluted to 6 liters with distilled water. The pH was carefully adjusted with 0.1 N HCl between pH 5 and pH 6 where the stroma formed a heavy precipitate which settled to the bottom of the container on standing 5 hours. The clear supernatant fluid was decanted. This procedure was repeated (at least five times) until the supernatant fluid showed no visual trace of hemoglobin. The stroma was carefully collected by centrifugation, diluted with distilled water to a known volume, and stored at 2°. In some cases, where the stroma was not of uniform texture, the suspension was mixed in a Waring Blendor for 2 minutes in the cold. Aliquots were taken as desired for study and stroma volume determination.

Preparation of Enzymes—The pneumococcal neuraminidase was prepared from broth cultures of *Diplococcus pneumoniae*.³ The enzyme assayed between 10,000 to 15,000 units per ml by the electrophoretic assay that is described below and was free of pro-

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³ E. H. Eylar, O. V. Brody, and J. L. Oncley, in preparation.

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¹ E. H. Eylar and J. L. Oncley, in preparation.

teolytic and glycosidic activity. *Vibrio cholerae* neuraminidase was prepared from broth cultures as described by Madoff, Eylar, and Weinstein (18). The enzyme preparations were frozen and stored at -20° .

Removal of Sialic Acid from Erythrocytes—In order to remove adsorbed plasma proteins, the erythrocytes were washed 8 to 10 times with the isotonic phosphate sodium chloride buffer (see "Experimental Procedure"). The packed red cells were finally suspended in an approximately equal volume of sodium chloride-phosphate buffer, pH 6.4, ionic strength, 0.16. The total volume was accurately determined, and an aliquot was removed for cell count and hematocrit determination. Usually the total volume was near 100 ml and the hematocrit near 0.4.

The red blood cell suspension was incubated at 37° after addition of pneumococcal or *V. cholerae* neuraminidase. When the *V. cholerae* neuraminidase was added, cacodylate buffer which contained 0.002 M calcium acetate, pH 6.4, ionic strength 0.15, was used instead of phosphate buffer. At appropriate times an aliquot, usually 25 ml, was removed and immediately cooled to 0° by swirling in a Dry-Ice alcohol bath in order to inhibit enzyme activity. Approximately 1 ml of the aliquot was added to 25 ml of cold isotonic sodium chloride and the mixture was centrifuged. These red cells were further washed three times and used for electrophoresis. The rest of the solution was centrifuged and the supernatant solution decanted for sialic acid determination. Some hemolysis always occurred, particularly on incubation over $\frac{1}{2}$ to $1\frac{1}{2}$ hours. The hemoglobin released did not exceed 1% of the total available as measured by absorption at $540\text{ m}\mu$ in a Beckman DU spectrophotometer. The calf erythrocytes were especially resistant to hemolysis, whereas chicken erythrocytes were most susceptible.

For the sialic acid determination, an aliquot of the supernatant solution was evaporated to dryness in a vacuum rotator at $35-40^{\circ}$. Absolute ethanol was added and the solution again evaporated to dryness. Water was added and the solution was centrifuged to remove the denatured hemoglobin. The sialic acid in the clear supernatant fluid was measured by the Warren and resorcinol methods.

Standard *N*-acetylneuraminic acid was added to a suspension of human erythrocytes at 37° . After incubation for 3 hours, the concentration in the supernatant solution was found to be within 3% of the initial value, and showed that the erythrocyte membrane is impermeable to sialic acid. In addition, when *N*-acetylneuraminic acid was carried through the evaporation procedure given above, it was completely recovered.

Incubation of erythrocytes at 37° for 3 hours did not release measurable sialic acid into the supernatant solution.

Release of Sialic Acid from Stroma—Aliquots from the stroma suspension were made 0.05 N with H_2SO_4 . The solution was heated at 80° for 1 hour. After cooling, the solution was neutralized with an equal volume of 0.1 N Na_2HPO_4 and centrifuged. The supernatant solutions were colorless. Sialic acid determinations were carried out directly on the supernatant solutions.

When neuraminidases were used, the stroma was incubated with shaking at 37° . The volume of the supernatant fluid was determined by centrifugation in Wintrobe tubes. When stroma from 20 ml of packed erythrocytes was incubated with 500 units of neuraminidase (pneumococcal), the total sialic acid was released within $\frac{1}{2}$ hour. The pH was set at 6.0 with 0.05 M sodium phosphate buffer.

Electrophoretic Measurements—The erythrocyte mobility was

measured by direct microscopic observation in a microelectrophoresis apparatus with a rectangular cell oriented in the "lateral" position (19). The apparatus is shown schematically in Fig. 1. The advantages of this design are numerous. Contamination with heavy metal ions was minimized by the use of Ag-AgCl electrodes in saturated KCl which were displaced by a long buffer path from the cell. The entire apparatus was thermostated to $\pm 0.1^{\circ}$, and calculation of mobility could be performed over the range of 0 to 37° . Convection currents were normally insignificant so that particles with a very small mobility could be measured accurately. Bubbles were easily flushed from the system. The apparatus was cleaned with strong acid. Monochromatic green light was used. A minimum of 10 to 15 ml of solution was required.

The microscope was fitted with a $22\times$ KsCel Leitz objective, working distance, 1.6 mm, and a $10\times$ Spenser eyepiece provided with a reticule.

Preliminary measurements showed that the distribution of erythrocyte mobilities with depth gave the expected symmetrical parabola where the depth is the distance along the optical axis which is intersected by the two faces of the cell. Red cell mobilities were measured at the first stationary level only, $0.211 \times$ depth. The depth varied between 600 to $800\ \mu$ from cell to cell.

The mobility was computed from the equation $U = ADk/IT$ where U is the electrophoretic mobility, A is the cross-sectional area, D is the distance traveled by the erythrocyte in time T , k is the specific conductance, and I is the current. All mobility measurements were corrected to water at 25° by multiplying by 1.28, the relative viscosity of the phosphate-sodium chloride-sucrose buffer.

Neuraminidase activity was measured in the following manner. To 1.5 ml of phosphate sodium chloride buffer, ionic strength 0.22, pH 6.4, or cocadylic acid-NaOH buffer, pH 6.4, 0.5 M in total cacodylate, or ethylenediaminetetraacetate buffer, ionic strength 0.125, pH 6.4, was added 0.5 ml of enzyme solution. Extensively washed and packed group O, Rh-positive human erythrocytes, 0.1 ml, were added to each tube. The contents were mixed by inversion, and the tubes were incubated for 20 minutes. At this time, the contents of each tube were dispersed in 20 ml of 0.155 M sodium chloride prechilled to 2° . The red cells were centrifuged, washed once, and finally suspended in electrophoresis buffer. Mobility measurements were made at 25° . The electrophoresis buffer contained 0.201 mole of sucrose, 0.0059 mole of Na_2HPO_4 , 0.0108 mole of NaH_2PO_4 , and 0.0441 mole of NaCl per liter, ionic strength 0.072, pH 6.4.

One unit of neuraminidase activity was defined as the amount of enzyme required to reduce the erythrocyte mobility by 50%. Under the conditions of the assay, a straight line relationship between the enzyme concentration and the mobility was found in up to 60% reduction in mobility for human erythrocytes. Samples prepared for electrophoresis contained approximately 0.2 to 0.4% erythrocytes by volume and were run in duplicate. For each sample, six randomly chosen cells were timed in two directions, by reversing the direction of the field, in order to reduce convection effects. Generally the values in either direction agreed within 10 to 20%. From day to day, mobility values for a particular sample agreed within 5%.

The isoelectric point of neuraminidase-treated erythrocytes was determined by measuring the mobility in phosphate-sodium chloride buffers at different pH values. The pH was adjusted

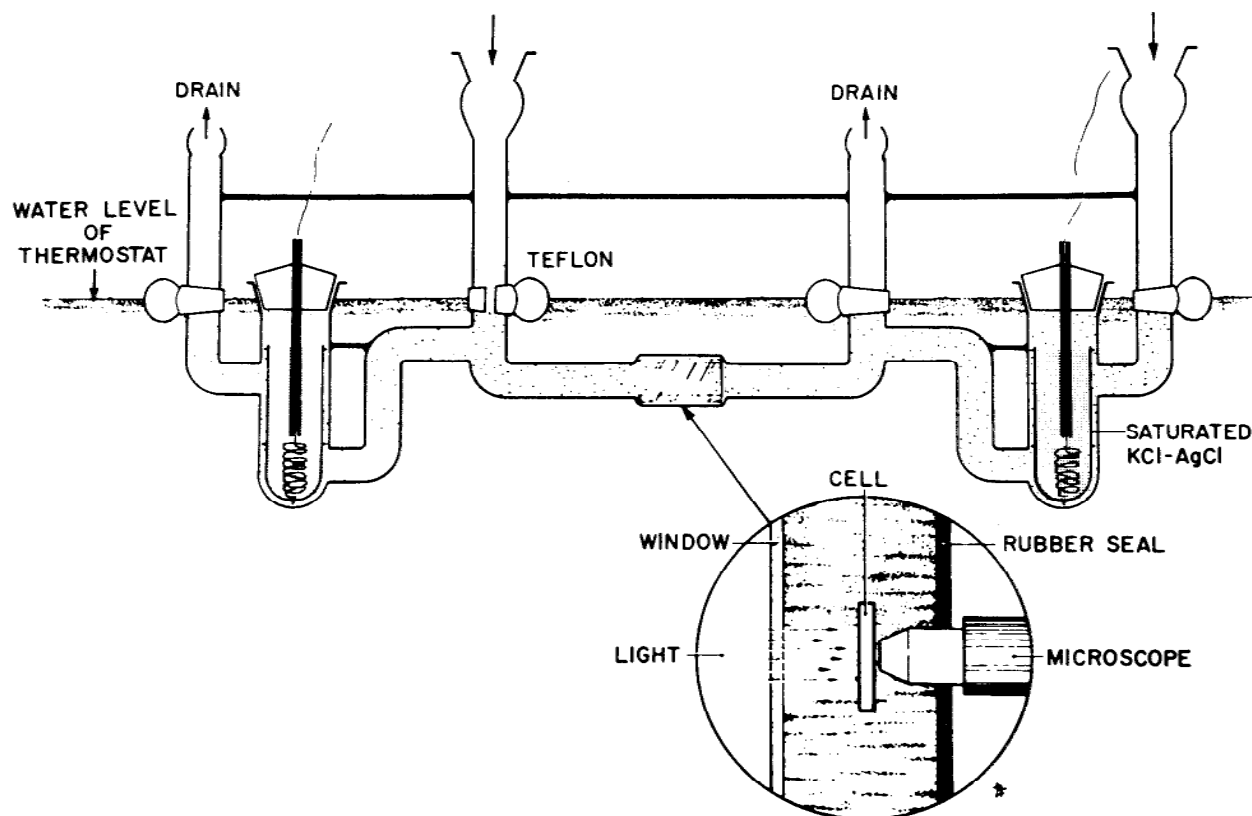


FIG. 1. A schematic view of the microelectrophoresis apparatus

with H_3PO_4 , NaH_2PO_4 , Na_2HPO_4 , and NaOH , and the ionic strength was kept near 0.07. The pH was measured in a Cambridge pH meter which was standardized with pH 4.00 and pH 7.00 buffers (Beckman). The conductivity was measured with a standard bridge in a cell with constant approximately 0.1.

To 20 ml of electrophoresis buffer, 0.1 ml of packed red cells was added. Within 1 minute, the first mobility measurements were made. The pH of the mixture did not vary more than 0.1 pH unit during the mobility measurement.

The effect of formaldehyde was ascertained by preparing the buffers in 0.5% formaldehyde. The stability of normal and neuraminidase-treated erythrocytes was determined in the higher pH range with and without formaldehyde. Two samples each of normal and enzyme-treated cells were allowed to stand at 4° for 2 days at pH 8.6; one sample of each contained 1% formaldehyde. In all 4 cases, the mobilities changed by less than 10%.

Assays—The sialic acid was measured by both the resorcinol (20) and Warren methods (21). Standard *N*-acetylneuraminic acid was prepared by the procedure of Svennerholm (22) from Cohn Fraction IV-4 of human plasma. Similarly *N*-glycolyl neuraminic acid was prepared from porcine submaxillary mucoprotein. Both standards gave a single spot on paper chromatography in butyl acetate-acetic acid-water, 3:2:1, and pyridine-butanol-water, 6:4:3. The sialic acids were detected on paper with the *p*-dimethylaminobenzaldehyde spray (23).

In order to determine whether the erythrocytes contained other types of sialic acid, stroma was incubated with neuraminidase and the supernatant solution was adsorbed and eluted from a small column, 1 × 3 cm, of charcoal-Celite equilibrated with water. The sialic acid, about 0.5 to 1.0 mg, was eluted with 10 to 30% ethanol. *N,O*-Diacetylneuraminic acids of the type described

by Blix *et al.* (24) were not found on paper chromatography. Because of the low sensitivity of the spray, small amounts could have escaped detection.

The *N*-glycolyl determinations were made by the procedure of Klenk as described by Whitehouse and Zilliken (25). The sialic acid sample was first purified by column chromatography with Dowex 50-X8H⁺ cycle and Dowex 1-X8 formate cycle.

RESULTS

The behavior of erythrocytes of horse, lamb, human, calf chicken, and pig was compared with respect to electrophoretic mobility and maximal response to neuraminidase. The enzyme action was judged complete when no further change in erythrocyte mobility was found. At pH 6.4, ionic strength 0.072, all the cells are negatively charged. The results in Table I show that horse cells exhibit the highest mobility whereas those of the chicken and pig are lowest. For a given species, the mobilities of erythrocytes from different animals vary within 7%. The mobility of human erythrocytes, corrected to the viscosity of water at 25°, is -16.4×10^{-5} cm² per volt per second, and falls in the range given by others (3, 26, 27) of -15.4 , -16.8 , and -14.6×10^{-5} cm² per volt per second at ionic strength 0.072.

The neuraminidase treatment reduces the mobility of each type of erythrocyte, most noticeably in the case of the human, and least in that of the horse. The cells still retain a negative mobility under the given conditions, however. Further evidence of a pronounced change in the surface character is revealed by the titration curves shown in Fig. 2. In each case the isoelectric point is raised. Except for the horse, the isoelectric point of the erythrocytes is raised from the region of pH 2 to 3 (4) to pH 4.5 to 5.5. Reorientation of surface groups is not apparent for

the pH range of 3.5 to 9 since the shapes of the curves for the normal and treated cells are similar.

The ionic strength 0.072 was chosen because it falls in the range of maximal stability (3) and is low enough to give accurate readings for cells with a small mobility. As mentioned by many authors (1-3), it was qualitatively observed that some species of erythrocytes exhibited marked instability as the pH was lowered. The enzyme-treated cells appeared in every case to be more stable. For example, at pH 3.1 the mobility of normal human erythrocytes changed from a negative value to a large positive value within 2 minutes and in some cases agglutinated. No hemolysis was apparent. Neuraminidase-treated cells have a reproducible mobility at this pH for over a span of 5 minutes. Horse erythrocytes showed this same phenomenon at pH 4.0. This instability is more drastic than the reversible instability noticed by Bateman *et al.* (2) which occurred above pH 5.0 when pH or ionic strength was lowered.

In order to evaluate the negative surface charge, two equations were used. If the erythrocyte is assumed to be spherical, and

that $\kappa a \gg 1$, then

$$U\eta = \sigma(1/\kappa + a_i) \tag{1}$$

where U is the mobility in cm^2 per volt per second, η is the viscosity in poises, σ is the surface charge density in electrostatic units per cm^2 , a is the radius of curvature of the erythrocyte, and κ is the Debye-Hückel function in cm^{-1} . At 25° in water, $\kappa = 0.327 \times 10^8 \sqrt{\Gamma/2}$ where $\Gamma/2$ is the ionic strength. The radius of the counterion, a_i , was assumed to be 2.67 Å (sodium ion).

For ionic strength 0.1 to 0.01, Equation 1 has been shown to describe satisfactorily the behavior of the human erythrocyte (26). In Table II, the charge density was computed from the mobility for each cell species assuming that Equation 1 is applicable at $\Gamma/2 = 0.072$. The total surface charge was found by multiplying the charge density by the area. The charge per erythrocyte is greatest in the case of the chicken and human, which are the largest cells.

The more commonly used relationship, the flat plate model, is expressed as:

$$\sigma = \frac{1}{1 + \kappa a_i} \left[\frac{NDkT}{2000\pi} \sum_i C_i \left\{ \exp\left(\frac{-z_i e \zeta}{kT}\right) - 1 \right\} \right] \tag{2}$$

where N is the Avogadro number = 6.02×10^{23} , D is the dielectric constant of water, 78, at 25° , k is the Boltzman constant = 1.38×10^{-16} , T is the absolute temperature, C_i and z_i are the concentration and valency of the i th ion, e is the electronic charge = 4.8×10^{-10} e.s.u., and ζ is the zeta potential = $4\pi U\eta/D$ for $\kappa a \gg 1$. The zeta potential is the potential at the surface of shear.

Although objections (26) have been made on theoretical and experimental grounds to the application of Equation 2 to the erythrocyte, it was found to give values of σ that were only 5 to 10% above those of Equation 1.

The total erythrocyte sialic acid is located in the stroma since no sialic acid was detected in the supernatant solution after

TABLE I

Maximal change in erythrocyte mobility and isoelectric point resulting from neuraminidase action

Species	$U\eta$ ($\text{cm}^2/\text{volt}/\text{sec.} \times 10^5$)*		% Reduction in mobility	New pI
	Normal	Enzyme-treated		
Horse	-19.8	-15.8	20	3.7
Lamb	-16.9	-4.3	74	4.6
Human	-16.4	-1.0	94	5.5
Calf	-14.4	-2.3	84	4.5
Chicken	-12.8	-3.6	72	5.0
Pig	-11.8	-3.9	67	

* At pH 6.4, ionic strength 0.072.

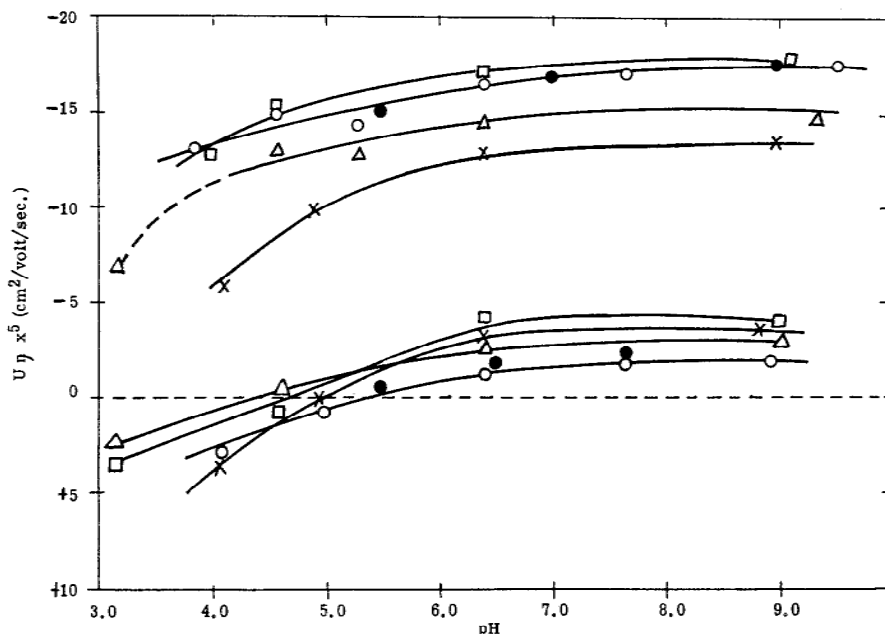


FIG. 2. Variation of electrophoretic mobility with pH for human (●), calf (Δ), lamb (□), and chicken (×) erythrocytes as well as human erythrocytes (●) treated with formaldehyde. The upper curves represent the normal cells, the lower curves the neuraminidase-treated cells. A phosphate-saline-sucrose buffer, ionic strength 0.07, was used.

TABLE II

Calculation of number of electronic charges per erythrocyte from electrophoretic mobility (pH 6.4, ionic strength 0.072) by Equation 1

Species	σ (e.s.u./cm ²)	Area (μ^2)*	Charge/erythrocyte
Horse	4230	85	7.5×10^6
Lamb	3610	62	5.0
Human	3500	163	11.9
Calf	3070	95	6.1
Chicken	2730	225	13.3
Pig	2520	105	5.5

* The areas for the human and lamb red blood cells were taken from Reference (28). The areas for horse, calf, and pig red blood cells are an average value computed empirically from the volume, and the equation area = $8.04 r^2$ where r is the long radius given in the same reference.

For chicken red blood cells, the area was computed from the volume which was determined by cell count and hematocrit.

TABLE III

Determination of number of sialic acid molecules per erythrocyte and availability to neuraminidase

Species	Sialic acid, $\mu\text{g.}$, released/ml. of packed cells			Molecules sialic acid $\times 10^{-6}$ /cell surface
	From stroma		From cells	
	Acid*	Enzyme	Enzyme	
Horse	131	74	67	5.9
Lamb	113	105	110	7.6
Human	134	130	141	24.0
Calf	114	107	119	10.0
Chicken	57	59	62	12.9
Pig	48	46	45	5.6

* Hydrolysis of stroma in 0.05 N H₂SO₄ for 1 hour at 80°.

TABLE IV

Types of sialic acids in erythrocyte stroma

Species	% N-Acetyl	% N-Glycolyl
Chicken	95-100	0-5
Human	95-100	0-5
Lamb	58	42
Calf	40	60
Pig	34	66
Horse	5-10	90-95

hemolysis and centrifugation. The results in Table III show that nearly equivalent amounts of sialic acid are liberated by neuraminidases from stroma or intact erythrocytes. The molecular weights of 310 and 324 were used for *N*-acetylneuraminic and *N*-glycolylneuraminic acids, respectively. In every case but the horse, the enzymatically released sialic acid coincides with the total erythrocyte sialic acid which was determined by mild acid hydrolysis. It should be noted that for a given species, sialic acid values showed as much as a 15% variation from animal to animal. The ratio of molecules of sialic acid to the surface area of the cell (Column 4) was computed with an average of the en-

zyme and acid values for sialic acid content. For the horse, only the enzyme value was used. The sialic acid figures shown in Table III represent the results from the Warren determination. The resorcinol method gave somewhat higher figures than were expected in the case of the horse, pig, sheep, and cow, although the correction for the difference in absorbance between *N*-glycolylneuraminic and *N*-acetylneuraminic acid was made (20). The type of sialic acid (Table IV) is seen to vary considerably from nearly 100% *N*-acetylneuraminic acid for the chicken and human to nearly 100% *N*-glycolylneuraminic acid for the horse. The same ratios were found in samples obtained by neuraminidase action or weak acid hydrolysis which suggests that the type of grouping attached to the nitrogen has little influence on the activity of the neuraminidase. Although no *N,O*-diacetylsialic

TABLE V

Comparison of chemical and electrical changes that occur with neuraminidase treatment of erythrocytes

Species	Charges lost $\times 10^{-6}$ /erythrocyte	Sialic acid molecules* removed $\times 10^{-6}$ /erythrocyte	Degree of roughness† in A
Horse	1.5	5.9	<20
Lamb	3.4	7.6	20-40
Human	10.6	24.0	20-40
Calf	5.0	10.0	20-40
Chicken	9.4	12.9	50-100
Pig	3.4	5.6	50-100

* Equals the total sialic acid for each species except the horse (see Table III).

† Based on the ionic strength-mobility curve for human erythrocytes and represents the radius of curvature which will cause the charges to coincide with the sialic acid lost.

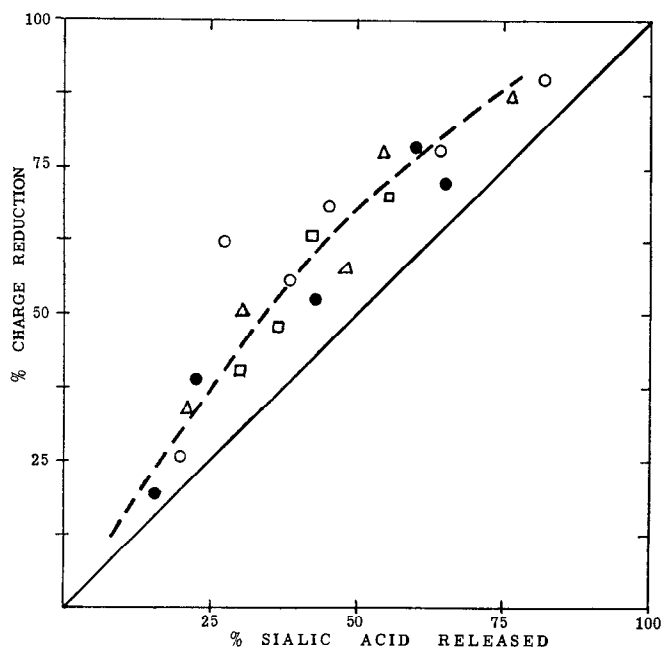


FIG. 3. Determination of the per cent change in charge and the per cent sialic acid removed from the erythrocytes of chicken (\square), calf (\bullet), lamb (Δ), and human (\circ) after limited neuraminidase action. The per cent change in charge was computed from Equation 1 or 2. The solid line is for a 1 to 1 relationship.

acids were found, it is possible that they are present in small quantities.

The human erythrocyte contains the largest amount of sialic acid, which is about twice the amount present in the chicken, horse, and calf cells. By comparison with Table II, it is clear that the number of sialic acid molecules exceeds the number of surface negative charges for the lamb, human, and calf.

In Table V, the change in charge is compared with the sialic acid removed. The change in charge was computed from the change in mobility given in Table I by Equations 1 or 2. In each case the number of sialic acid molecules removed exceeds the number of charges lost, particularly for the horse.

When the erythrocytes were treated with enzyme for limited periods of time, the correlation shown in Fig. 3 was found. The points fall above the line which is drawn for a 1 to 1 relationship, and it is evident that the per cent loss of charge exceeds by as much as 20% in some cases the per cent loss of sialic acid.

DISCUSSION

The results shown in Tables I and III reveal directly that neuraminidase removes the sialic acid from the intact erythrocyte and concomitantly reduces the electrophoretic mobility. Gottschalk (11) has demonstrated that the *Vibrio cholerae* neuraminidase is an α -glycosidase which cleaves the *O*-glycosidic linkage between sialic acid and galactose or *N*-acetylgalactosamine. The pneumococcal neuraminidase¹ shows a similar specificity by its action on the α_1 -glycoprotein from human plasma. The possibility of a cationic charge being produced as a result of enzyme action is thus remote. After titration of the urinary mucoprotein, Curtin (17) reported new cationic groups (pK 11) uncovered by neuraminidase action. Based on this report it has often been suggested (29, 30) that sialic acid is involved in a peptide or *N*-glycosidic linkage which produces an amino group on hydrolysis. The pH-mobility curves for human erythrocytes treated with formaldehyde in Fig. 1 show little change from the curves for untreated erythrocytes and reveal that no amino group has appeared because of neuraminidase action. It is clear therefore for each species of erythrocyte except the horse that sialic acid, by virtue of its carboxyl group, is primarily responsible for the negative surface charge.

With the exception of the horse cells, the total sialic acid of the erythrocyte is located at the outer surface of the membrane since the amount liberated from the stroma by weak acid agrees with the amount released by enzyme from intact cells (Table III). The large size of the neuraminidase molecule³ precludes its entry into the interior of the erythrocyte. The total enzyme activity could be recovered in the supernatant fluid after completion of the reaction. Both types of sialic acid are liberated by the neuraminidase. The proportion of *N*-acetylneuraminic to *N*-glycolylneuraminic acid shown in Table IV reveals a wide variation with species.

A low estimate of surface charge computed from Equations 1 or 2 is suggested by comparison of Tables II and III which show that the total number of sialic acid molecules per erythrocyte is greater in some cases than the total surface charge at pH 6.4. The more critical comparison seen in Table V leads to the result that the number of sialic acid molecules released is much greater than the number of surface charges lost. For lamb, human, and calf the difference is approximately 100%, and even greater for the horse. These figures should coincide if the cell surface is similar to a protein surface, *i.e.* barring a change in configuration,

titration or removal of a charged constituent should produce a similar change in mobility. For example, when the 15 to 16 molecules of sialic acid are removed from the α_1 -glycoprotein (31), a reduction in mobility of about 2.3 units (corrected for anion binding) occurs for any pH above 4. Likewise, titration of 15 groups results in a reduction in mobility of 2.5 units.

The inadequacy of Equations 1 and 2 to evaluate accurately surface charge is illustrated by the following two possibilities.

1. Only the number of sialic acid molecules equal to the charges lost is located at the surface of shear; the others are buried so that they do not influence the mobility. This would be 1.5, 3.4, and 10.6×10^6 sialic acid molecules at the surface in the case of horse, lamb, and human erythrocytes. However, it is difficult to picture sialic acid molecules hidden in such a way that they do not influence the mobility, and yet are accessible to the enzyme. From Fig. 3 it is seen that all the sialic acid molecules appear to influence the mobility.

2. All the sialic acid molecules, in addition to other negative and positive groups, are located at the surface of shear. The excess negative charge would be given by σ . From Table III it is apparent that removal of the sialic acid molecules should produce a positive surface charge in each case but that of the horse. For example, removal of 24×10^6 sialic acid molecules from human cells would result in a charge of $+14 \times 10^6$. However, the actual change in charge is much less than the loss of sialic acid. In view of the similar slopes for a given species for the pH-mobility curves, it would not be appropriate to assume that the small change in charge arises from a major rearrangement at the surface.

Alternately, Brinton and Lauffer (32) have shown that if the cell does not have a smooth surface with infinite radius of curvature, *e.g.* for $\kappa a < 100$, Equations 1 and 2 are no longer valid and the correction factor of Henry, $f(\kappa a)$, must be applied. Assuming the mobility-determining structures to be spherical or cylindrical, they have computed for human erythrocytes that σ does not vary with ionic strength when a is 20 to 40 Å. An important outcome of this treatment is that for a near 20 to 40 Å, the value of σ is increased by 2 to 2.5 times over the value for $\kappa a \gg 1$. Examination of Table V for human erythrocytes shows that the sialic acid released is about 2.3 times the change in charge. It appears therefore that the electrophoretic estimate can be reconciled with the sialic acid data by assuming a smaller value of a . Supporting evidence for the smaller value of a comes from electron microscopic studies (33) of stroma, and virus hemagglutination studies (34) which suggest that the receptor material (containing the sialic acid) extends from the erythrocyte in long chains.

The radius of curvature required to make the charges coincide with the sialic acid lost, in the last column of Table V, can serve as a measure of the degree of "roughness" of the erythrocyte surface. Actually, the degree of roughness may be indicative of the radius of curvature of the virus receptor glycoprotein. Based on the ionic strength-mobility curve for human cells, the lamb and the calf fall in the 20 to 40 Å range also, whereas pig and chicken are in the 50 to 100 Å range. For horse erythrocytes, a would be smaller than 20 to 40 Å.

Another approach has been offered recently by Haydon (35), who developed expressions for calculation of σ from the zeta potential which are based on models that more closely approximate the actual cell membrane. When account is taken of the likely distribution of counter ions within the surface region, it is

found that Equations 1 and 2 should be corrected by a factor α which will *always increase* the value of σ . The results shown in Fig. 3 reveal that a precise 1 to 1 relationship does not exist at any fraction of charge reduction. In other words, it appears that some sialic acid molecules are located at the surface of shear and contribute more strongly to the zeta potential than others more internal. It would be expected that the more surface-prominent sialic acid would be more accessible to the action of neuraminidase. Thus the per cent change in charge would always exceed the per cent loss of sialic acid. A simple illustration can be made by assuming half of the sialic acid is located 11 Å ($1/\kappa$ is about 11 Å for ionic strength 0.072) within the cell surface (Model 2a of Haydon). Calculation with Equation 3 of Haydon reveals that this internal layer of sialic acid, assuming normal counter ion distribution, would increase the value of σ by about 50% over the value given by the outer layer expressed by Equations 1 and 2. Thus removal by enzyme of the outer layer of sialic acid (50% of the total) would decrease the calculated σ by 66%. Obviously the precise distribution of the sialic acid molecules is not known, but from our knowledge of glycoprotein structure (36), more accurate models can be designed. It is certainly unlikely that every sialic acid molecule would be located in precisely the same position relative to the surface of shear.

The low pK of the sialic acid carboxyl group near 2.6 is compatible with the low isoelectric point of erythrocytes which is quoted (4, 5) from 1.7 to 2.3. The electrophoretic behavior of the erythrocyte surface appears to parallel that of the α_1 -glycoprotein. The latter (31) has a high negative charge at pH 6 and a low isoelectric point near 2.7 which is mainly due to 16 sialic acid carboxyl groups. On neuraminidase treatment¹ the isoelectric point is raised to pH 5.0. Similarly the isoelectric point of the erythrocytes shown in Fig. 1 is raised to values near pH 5. In contrast to mobility-pH curves of proteins, however, the erythrocytes curves are conspicuous (except for the chicken) for their relatively small response in the pH range 3 to 5, where β , γ -carboxyl groups titrate. Also the virtual absence of ϵ -amino groups is denoted by the minor effect of formaldehyde on the mobility of human erythrocytes in the pH range 7 to 9. Neuraminidase treatment does not appear to affect any groups that express themselves in the pH range 3.5 to 9 since the slopes of the curves in Fig. 1 for a given species are essentially the same.

Trypsin is known to reduce erythrocyte mobility by approximately 25% (37). Direct efforts by Seaman and Heard (38) to locate phosphate liberated by trypsin were unsuccessful. Recently it has been found that trypsin releases a glycopeptide that contains sialic acid from human erythrocytes (39, 40). Klenk and Uhlenbruck (41) have obtained a "sialomuroid" from bovine and human erythrocytes by phenol or papain treatment which interacts strongly with virus.

The over-all results offer strong evidence for glycoprotein at the surface of the erythrocyte. The negative surface charge in all cases was not completely accounted for by sialic acid and leaves room for minor contributions from β , γ -carboxyl groups (Fig. 2), possibly phosphate groups, and also cation desorption on nonionogenic areas (42). The availability of the total cellular sialic acid to neuraminidase offers for the first time specific chemical evidence for a unique structure at the outer surface of the plasma membrane (43). The presence of sialic acid in the membrane of all erythrocytes so far examined, in white cells, and Ehrlich ascites carcinoma cells (44) suggests it as a general

constituent of cell surfaces. It has also been demonstrated in the erythrocytes of the lamprey eel (45).

It is well established (5) for human erythrocytes that the charge density shows little variation over a wide range of ionic strength, from 0.172 to approximately 0.01. For a given pH and ionic strength the mobility is invariant in many types of buffers (3, 27) including phosphate, chloride, thiocyanate, iodide, Tris, and others. It was concluded from these data that the surface charge is due mainly to fixed anionic components of the plasma membrane rather than anion adsorption. The concept of fixed surface charge has been emphasized in many interpretations (1, 4-6) in terms of phospholipid phosphate. The location of phosphate groups at the periphery of the plasma membrane has not been demonstrated, however. Undoubtedly this choice has been influenced by the large proportion of phospholipid in the erythrocyte stroma. The likely candidates among these lipids are not lecithin, phosphatidylethanolamine, or sphingomyelin, but rather phosphatidylserine, monophosphoinositide, and phosphatidic acid which have a surfeit of negative charge. In Table VI these lipids are compared quantitatively with sialic acid for each species. It is evident that sialic acid is the only component in sufficient quantity to consider seriously. In order to detect any general relationship among the species, the sialic acid and phosphatidylserine per μ^2 were compared with mobility as shown in Fig. 4. The roughly linear relationship between the sialic acid surface density and mobility illustrates the consistent position of sialic acid carboxyl groups in the general make-up of the erythrocyte surface. The phosphatidylserine, on the other hand, shows a scatter of points which indicates a relationship opposite to that anticipated.

The horse cells are conspicuous because they have a high mobility which is not accounted for by their sialic acid content. Furthermore, neuraminidase releases only about half of the total sialic acid of the horse erythrocyte (and stroma) in contrast to the other species shown in Table III. The data may be reconciled however by the recent report of Yamakawa, Irie, and Inanaga (48), who showed that the glycolipids extracted from

TABLE VI
Comparison of number of molecules* $\times 10^{-6}$ per cell of lipid† and sialic acid components in the erythrocyte stroma which are negatively charged at pH 6.0

Species	Sialic Acid	Phosphatidylserine	Diphosphoglyceroinositide	Phosphatidic acid
Horse	5.9	3.0	1.0	0.5
Lamb	7.6	3.3	0.9	0.2
Human	24.0	6.6	0.9	0.5
Calf	10.0	5.6	1.4	0.2
Chicken	12.9	7.5	3.0	1.5
Pig	5.6	6.0	1.2	0.3

* In order to be compatible with the theory of the bimolecular leaflet, the lipid values were divided by 2.

† Values for the lipid composition of human red blood cells were taken from Kates, Allison, and James (46). For chicken cells, the data of Kates and James (47) were used.

For other species, the values were computed from the composition of the erythrocyte stroma given by Dawson, Hemington, and Lindsay (7). The total phospholipid composition for each species was computed from the value for human cells (46) after correcting for the difference in area.

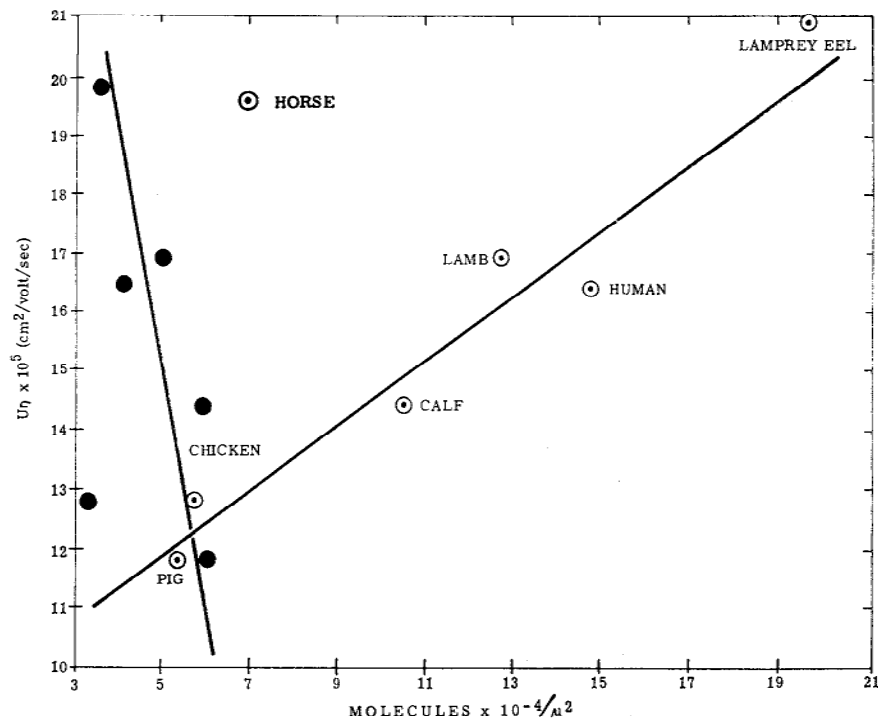


FIG. 4. The relationship between the electrophoretic mobility and the number of molecules of sialic acid (○), or phosphatidylserine (●) per μ^2 . The areas used for this calculation were taken from Table II; the sialic acid and phosphatidylserine values from Table VI. The data for the lamprey cells were taken from Reference (47).

horse and cat erythrocytes contained high contents of sialic acid (approximately 20%) whereas glycolipids from human, sheep, guinea pig, and rabbit erythrocytes contained no sialic acid. It is probable that the difference between the acid- and enzyme-released sialic acid for horse erythrocytes in Table III represents lipid-bound sialic acid that is known to be resistant to enzyme attack (49) in the case of gangliosides. Similarly the high mobility of the horse erythrocytes may be explained by sialic acid located at the surface but joined to glycolipid rather than what is probably glycoprotein for the other species. Another possibility is that the horse erythrocytes have a type of sialic acid which is itself resistant to enzymatic attack.

SUMMARY

The action of neuraminidase on erythrocytes from various species was studied. It was found that the electrophoretic mobility and surface charge were greatly reduced in each case except that of the horse. The reduction in surface charge was accompanied by the release of both *N*-glycolylneuraminic and *N*-acetylneuraminic acids. It was concluded that the carboxyl group of sialic acid is mainly responsible for the negative surface charge rather than lipid phosphate, especially phosphatidylserine. The sialic acid per μ^2 was found to relate linearly to the mobility among the species studied.

The surface of the erythrocyte was found to behave like a glycoprotein surface in the sense that it has a low isoelectric point which was raised to pH 4 to 5 on release of the sialic acid. All the erythrocyte sialic acid is located at the outer surface of the plasma membrane and is accessible to neuraminidase. Progressive release of sialic acid did not give a 1 to 1 relationship with the per cent change in charge since the latter always exceeded the per cent loss of sialic acid.

On a molecule per charge basis, the sialic acid released in each

case exceeded the surface charge computed from the mobility. The results could be reconciled by a consideration of the factors influencing the potential at the surface of shear, particularly the radius of curvature of the mobility-determining structures, and the distribution of the sialic acid molecules a few angstroms from the surface of shear. In each case the estimate of the surface charge was increased to a more appropriate value.

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