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SIALIC ACID, ELECTROPHORETIC MOBILITY AND TRANSMEMBRANE POTENTIALS OF THE *AMPHIUMA* RED CELL

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SUMMARY

Red cells from the giant salamander *Amphiuma means* are shown to contain sialic acid. The amount removed by the action of neuraminidase is equal to that released by acid hydrolysis, indicating that all of the sialic acid is present on the outer surface of the plasma membrane. These cells have a negative electrophoretic mobility and 100 % enzymatic removal of sialic acid results in a 40 % reduction in the mobility, suggesting that either a fraction of the sialic acid carboxyl groups are unavailable to the action of external electric fields, or other negatively charged groups contribute to the surface charge. A further reduction in mobility of normal and sialic acid-free cells is caused by an increased extracellular calcium concentration. The negative groups affected by calcium are most likely to be phosphate groups, since the isoelectric point of the cells is found to lie between the pK values for $H_2PO_4^-$ groups and the carboxyl groups of sialic acid. Membrane potentials of single cells, from which 80 % or more of the total sialic acid had been removed, were identical to those measured in normal cells, confirming that sialic acid plays little, if any, direct role in the maintenance of membrane potentials and ionic permeabilities.

INTRODUCTION

The electrokinetic properties of various cell types have been investigated and the results of these studies interpreted in an effort to obtain further information concerning the structure and function of the cell surface. A number of review articles dealing with both the theoretical and the experimental aspects of electrokinetic phenomena have been written (see, for example, refs 1 and 2). Electrophoretic mobility reflects the electric charges located at the surface of shear. These charges are not necessarily identical with those located on the outer surface of the plasma membrane. Since the direct measurement of surface charge of intact whole cells is as yet not possible, one is forced either to assume that these two surfaces are electrically identical, as appears to be the case under given assumptions [3], or to examine the relationship between the electrokinetic behavior of the cell and the corresponding electrophysiological properties of the membrane. Gomulkeiwicz [4] and Weiss and

Levinson [5] have investigated the effect on ion fluxes of reduction in electrophoretic mobility resulting from removal of peripheral sialic acid. Their results suggest that removal of sialic acid with changes in mobility of up to 40 % produced no significant change in anion permeability and only a small change in cation permeability. It is conceivable that a more direct measure of the interaction between the electrical properties of the plasma membrane and the surface of shear would be to observe the transmembrane potential under conditions of altered electrokinetics. Redmann et al. [6] examined the relation between transmembrane potential and the zeta potential in Fl-cells, leucocytes, and ovarian cells. They found that, under certain conditions, changes in the two potentials were related. The majority of cell types are, however, unfortunately unsuited for such studies because of the physical difficulties associated with membrane potential measurements in single isolated cells. The red cell of the giant salamander *Amphiuma means*, shown in Fig. 1, has a surface area of $\approx$ 5000 μm^2 and a volume of $\approx$ 13.000 μm^3 [7] as compared to the human red cell with corresponding values of $\approx$ 90μm^2 and $\approx$ 160 μm^3 [8]. The relatively large size of the *Amphiuma* red cell makes it possible to measure membrane potentials in these cells [9]. Thus it is feasible to measure both electrophoretic mobility and

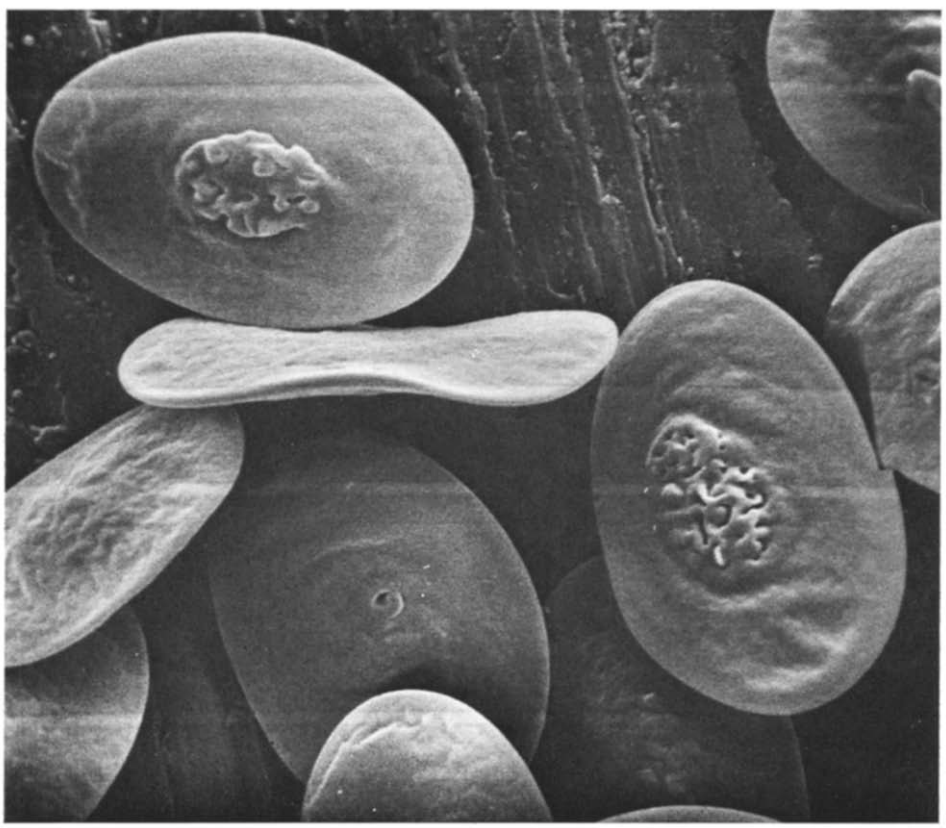

Fig. 1. Scanning electron micrograph of *Amphiuma* red cells. The cells have a characteristic disc shape and are nucleated. Magnification $\times$ 1050.

the transmembrane potentials in single cells of this type. The present study was undertaken in order to characterize the electrokinetic properties of Amphiuma red cells as well as their relationship to transmembrane potentials and cation permeability.

MATERIALS AND METHODS

Red cells from the giant salamander *Amphiuma means* were used in the present study. Blood samples were obtained by cardiac puncture as reported by Lassen et al. [9] and immediately washed twice in 10 vols of cold, stirred Ringer's solution containing 118 mM Na^+, 2.5 mM K^+, 124 mM Cl^-, 1.8 mM Ca^{2+}, and 10 mM morpholino-propane sulphonic acid buffer (Sigma Chemical Co.). All Ringer's solutions used for collecting and storing cells were maintained at pH 7.2 and in addition contained 0.1 % albumin in order to avoid hemolysis. Albumin was omitted from solutions used for electrophoretic mobility measurements.

Sialic acid content

Hydrolytic release of sialic acid was carried out in 0.025 M H_2SO_4 at 80 °C for 1 h. After cooling, the solution was neutralized with an equal volume of 0.05 M Na_2HPO_4 and centrifuged. Under this relatively mild hydrolysis, the sialic acid is considered to be fairly stable [10]. The clear supernatant was freeze dried in a Hetosicc Type CD 3 freeze dryer (Heto, Denmark) and sialic acid determinations were made on the reconstituted concentrated supernatant samples using the Warren method [11]. Absorption at 549 nm was measured on a Pye Unicam SP 8000 recording spectrophotometer.

For examination of the enzymatic release of sialic acid, cells which had been collected as described above were immediately washed twice in Ringer's, pH 6.4, and adjusted to a hematocrit of 5 %. Cells were incubated at 27 °C with neuraminidase (Type VI, chromatographically purified, Sigma Chemical Co., U.S.A.), and aliquots removed at appropriate time intervals. After centrifugation the supernatant samples were freeze dried and stored for sialic acid determinations while the cells were washed and resuspended in normal Ringer's, pH 7.2, for further studies. The amount of neuraminidase used was sufficient to release the total membrane sialic acid within 30–60 min of incubation. Under these conditions hemolysis was negligible.

In order to ascertain the primary type of sialic acid present in *Amphiuma* red cell membranes, a sample of supernatant from enzyme-treated cells was deproteinized with perchloric acid to remove any traces of hemoglobin and desalted by elution with water from a 1×50 column packed with Sephadex G-10. Column samples containing the major fraction of sialic acid were combined, freeze dried, and analyzed by thin-layer chromatography on cellulose phosphate plates using ethanol/H_2O/NH_3 (80:20:1) as the solvent. Plates were developed using a spray described by Aminoff [12].

Electrophoretic mobility

Because of the large size of these cells and their rapid sedimentation, a specially designed chamber was used for electrophoretic mobility measurements. Fig. 2 illustrates the general features of the experimental setup. The main electrophoretic chamber consists of a vertically oriented rectangular cell with an internal cross-section measuring 1×20 mm, and a length of 24 mm.

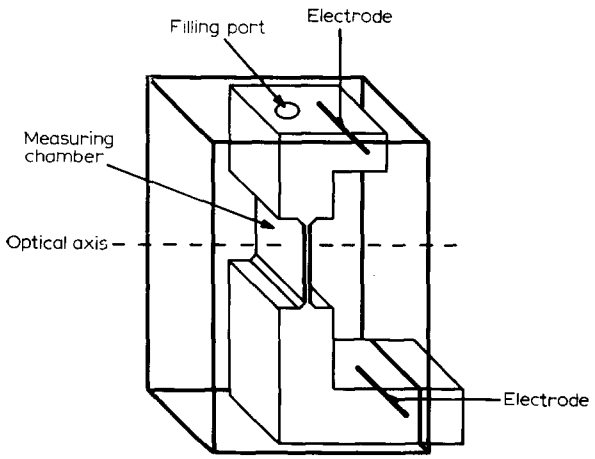

Fig. 2. Schematic diagram of electrophoretic measuring chamber. External water-circulating chamber for thermostatic control is not shown.

Motion of the cells was followed with an externally located Zeiss stereo microscope (400×) containing an ocular micrometer. The microscope was focused at the stationary plane to avoid electroosmotic effects and only cells which were in focus throughout the period of measurement were used in the calculation of mobility. Each cell was observed under both positive and negative polarity with sufficient time between changes in polarity to insure that velocity during the measurement period was constant. The temperature of solutions in the measuring chamber was thermostatically maintained at 25 °C. Viscosity (Ostwald viscometer) and conductance (Conductivity Meter, Radiometer, Copenhagen) of the suspending solutions were also measured at 25 °C.

A constant-current generator (Shandon Southern Instruments Ltd., England) was used to provide the necessary constant electric field strength. The data from each run, including the relevant parameters such as migration distance, current, electric field and ionic strength, were stored on magnetic tape for subsequent treatment.

Potential measurements

Membrane potentials of the *Amphiuma* red cells were measured by means of KCl-filled microelectrodes and the data handled via an A/D converter and PDP 8/e digital computer. Details of the measurement technique and data handling have been described by Lassen et al. [9, 13] and Bengtson and Lassen (in preparation).

Ficoll gradient

Aliquots of a 20 % by weight concentration of Ficoll 400 (Pharmacia, Sweden) in Ringer's solution were diluted with Ringer's solution to give a range of concentrations from 5–20 %. 500 µl of each concentration was layered into a glass column measuring 1 cm in diameter to produce a discontinuous gradient in increments of 2.5 %. 250 µl of a suspension of fresh *Amphiuma* red cells in Ringer's solution (5 %

hematocrit) were deposited on top of the gradient column and the distribution of cells observed after 40 min when a clear separation had taken place.

RESULTS

The sialic acid content of red blood cells from *Amphiuma means* as determined by acid hydrolysis was found to be 22.8 μg/ml packed cells. The enzymatic release of sialic acid by neuraminidase follows normal enzyme kinetics as shown in Fig. 3. Furthermore, the maximum amount of sialic acid per ml packed cells released by enzymatic digestion is equal to that obtained by acid hydrolysis.

Thin-layer chromatography of membrane sialic acid showed a single spot with an R_F value identical to that of reference *N*-acetylneuraminic acid.

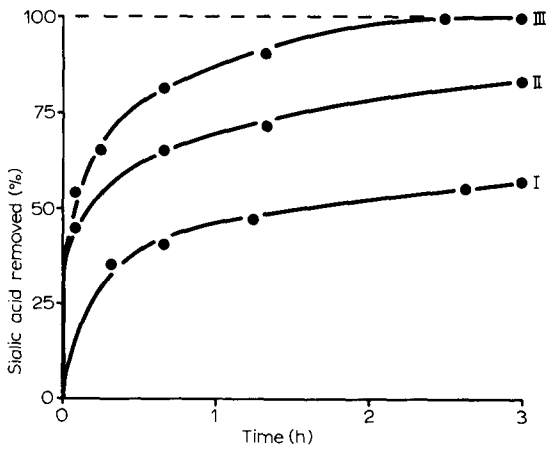

Fig. 3. Enzymatic release of sialic acid from *Amphiuma* red cells. The percent sialic acid removed is plotted as a function of time for various quantities of neuraminidase. (I) $9 \cdot 10^{-3}$ units; (II) $27 \cdot 10^{-3}$ units; (III) $43 \cdot 10^{-3}$ units. The dotted line indicates the amount removed by acid hydrolysis, taken to be 100 %.

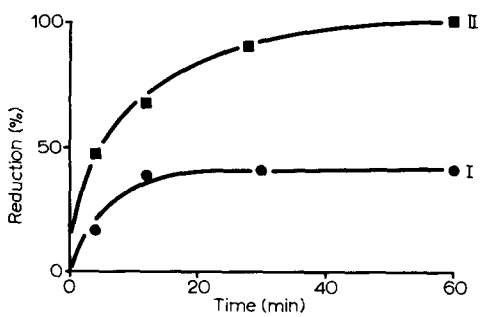

Fig. 4. Effect of reduced membrane sialic acid on *Amphiuma* red cell electrophoretic mobility. The percent reduction in mobility (I) and sialic acid (II) for the same cell batch are plotted versus incubation time in neuraminidase.

Electrophoretic mobility of *Amphiuma* red cells calculated from velocity measurements in normal Ringer's, pH 7.2, ionic strength 0.126 M at 25 °C is -2.81 ± 0.06 $\mu m \cdot cm \cdot V^{-1} \cdot s^{-1}$. This is an average value based on mobility measurements for 272 cells. All the cells measured showed a negative mobility reflecting the net negative charge on the cell surface under these conditions. The removal of membrane sialic acid results in a reduction in negative mobility as seen in Fig. 4. Loss of 100 % of surface sialic acid results in an electrophoretic mobility of -1.67 ± 0.15 $\mu m \cdot cm \cdot V^{-1} \cdot s^{-1}$. This value represents a reduction in negative mobility of 40 %.

The effect of calcium on the electrokinetic behavior of *Amphiuma* red cells was investigated by measuring the electrophoretic mobility of cells in a Ringer's solution containing 15 mM Ca^{2+}. The mobility of both control cells and sialic acid-free cells was 11–16 % less negative than mobilities measured in normal Ringer's containing 1.8 mM Ca^{2+}.

In order to determine the isoelectric point (p*I*) for normal *Amphiuma* red cells, a study of electrophoretic mobility as a function of pH was carried out. The results are shown in Fig. 5. Electrophoretic mobility was measured for pH values ranging from 3.0 to 8.0 and extrapolation of the pH vs mobility curve gives a p*I* of 2.3.

The average membrane potential in normal Ringer's solution (1.8 mM Ca^{2+}) for cells which had lost more than 80 % of their sialic acid was −17.3 mV. This is essentially unchanged from the average membrane potential of control cells, which was −16.3 mV. In order to measure the effect of increased extracellular calcium concentrations, cells were rapidly transferred to Ringer's solution containing 15 mM Ca^{2+} and membrane potentials measured during the first 10 min after suspension. The same characteristic distribution of hyperpolarized membrane potentials was seen in both the control cells and cells from which a major fraction of sialic acid had been removed. The potential distributions for control cells in high-calcium Ringer's are identical to those observed by Lassen et al. (in preparation).

Samples of *Amphiuma* blood placed on a discontinuous ficoll gradient showed a persistent tendency to separate into three distinct groups. Cells from each group were measured for average size and electrophoretic mobility. The pooled results of a number of such separations, as well as the relative distribution of cells in the three groups, is shown in Table I.

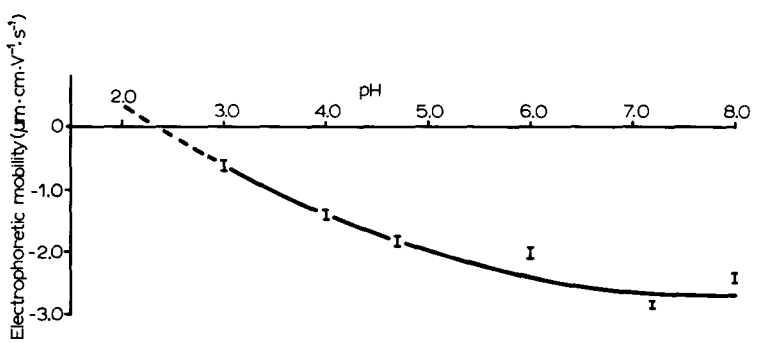

Fig. 5. Effect of pH on *Amphiuma* red cell electrophoretic mobility. The low pH end of the curve is extrapolated (– – – –) to give p*I*. Error bars indicate ±1 S.D.

TABLE I

DISTRIBUTION OF *AMPHIUMA* RED CELLS ON A DISCONTINUOUS FICOLL GRADIENT

Ficoll concentration	10 %	12.5 %	15 %
Fraction of sample	≈ 10 %	≈ 60 %	≈ 30 %
Average size (major and minor axis in μm)	61 × 37 (44 cells)	65 × 38 (37 cells)	66 × 39 (23 cells)
Electrophoretic mobility in $\mu m \cdot cm \cdot V^{-1} \cdot s^{-1}$	−2.95	−2.80	−2.58

DISCUSSION

Sialic acid which is a common component of glycoproteins present in cellular membranes is a substrate for the enzymatic cleavage of the *O*-glycosidic bond by neuraminidase. Since neuraminidase does not penetrate the plasma membrane, sialic acid removed by the enzyme is present on the outer surface. On the other hand, the sialic acid content measured by hydrolysis could represent sialic acid present in membranes other than the plasma membrane. This has particular significance in the case of *Amphiuma* red cells which contain mitochondria and are nucleated. The presence of sialic acid on these inner membranes as well as enzymatically unavailable sialic acid molecules on the plasma membrane would be expected to lead to differences in sialic acid content measured enzymatically as compared to values obtained from hydrolysis. The fact that both methods give identical values for the sialic acid content of *Amphiuma* red cells indicates that essentially all of the sialic acid is present on the outer surface of the plasma membrane and is available to enzymatic release. Furthermore, the results of thin-layer chromatography indicate that *N*-acetylneuraminic acid is the main type of sialic acid present in the *Amphiuma* red cell.

In a survey of p*I* values for various cell types Sherbert et al. [14] suggest that these values reflect the overall p*K* of the cell surface, which is determined by the groups present on the surface. The p*I* value for *Amphiuma* red cells as determined by extrapolation of the electrophoretic mobility vs pH curve is 2.3 and lies between the expected pK_a values for phosphate and sialic acid carboxyl groups. This finding is consistent with the presence of sialic acid (pK_a 2.6) on the membrane and furthermore suggests the possibility that phosphate groups also are present and contribute to the net negative charge. For example, membrane phosphoglycerides have in general a negatively charged phosphate group and have a pK_a of 1–2 at pH 7.0. It should be noted that the values for mobility in the pH dependence curve (Fig. 5) have not been corrected for the presence of Ca^{2+} in the normal Ringer's solution. As discussed below, calcium has a titrating effect on negative groups contributing to the electrokinetic properties of these cells. However, based on our observations of the effect of 15 mM Ca^{2+}, the presence of 1.8 mM Ca^{2+} would be expected to reduce the observed mobilities and extrapolated value for p*I* only slightly.

Forrester [15] found that BHK 21 cells showed a 10–20 % reduced mobility when electrophoresed in buffer containing 10 mM Ca^{2+}. This is consistent with the binding of Ca^{2+} by surface phosphate groups and a resultant reduction in net negative surface charge. The electrophoretic mobility of Amphiuma red cells in Ringer's solution containing 15 mM calcium is reduced from -2.81 ± 0.06 to

-2.37 ± 0.06 $\mu m \cdot cm \cdot V^{-1} \cdot s^{-1}$, a reduction of $\approx 16\%$. The increase in ionic strength due to the increase in calcium concentration in the buffer can have only a minor effect in the ionic strength region (0.126–0.137) involved [16]. The observed reduction in mobility may thus be due to the specific interaction between calcium and surface negative charges. Cells from which the available sialic acid groups had been enzymatically removed showed a comparable reduction in negative electrophoretic mobility when transferred to Ringer's solution containing 15 mM Ca^{2+}. Therefore it is reasonable to conclude that the effect is due to an interaction between Ca^{2+} and negative groups other than sialic acid present on the cell surface. It is interesting that there is not a comparable calcium-induced reduction in the negative mobility in the case of human erythrocytes, where the total net negative surface charge is assumed to be generated by sialic acid residues [15].

The fraction of sialic acid removed enzymatically as a function of time, and the associated reduction in electrophoretic mobility for the same cell population, seen in Fig. 4, indicates that, as in other cell types, there is a direct correlation between the loss of sialic acid and change in mobility. Removal of 100 % of the sialic acid results in an electrophoretic mobility of -1.67 $\mu m \cdot cm \cdot V^{-1} \cdot s^{-1}$ which represents a 40 % reduction. In their study of sialic acid and erythrocyte surface charge, Eyler et al. [10] found that the reduction in electrophoretic mobility for erythrocytes from a number of animals was less than that expected from the number of sialic acid molecules removed. They concluded that the difference represented a surface roughness which effectively shields a portion of the sialic acid carboxyl groups from electrophoretic interaction. For example, Seaman and Cook [17] pointed out that, in 0.145 M NaCl, carboxyl groups lying 20–30 Å within the slip plane will make no significant contribution to the zeta potential. The reduction in mobility of *Amphiuma* red cells resulting from the removal of sialic acid indicates a similar restriction in the amount of enzymatically available sialic acid carboxyl groups which contribute

TABLE II

DATA SUMMARY

Sialic acid content	22.8 μg/ml cells $6.0 \cdot 10^8$ molecules/cell* $1.2 \cdot 10^{13}$ molecules/cm²**	
Spacing between sialic acid molecules (average)	$\approx$ 30 Å	
Electrophoretic mobility (pH 7.2, 25 °C, $\Gamma/2 = 0.126$ M)	-2.81 ± 0.06 $\mu m \cdot cm \cdot V^{-1} \cdot s^{-1}$	
Electrophoretic mobility after 100 % removal of sialic acid	-1.67 ± 0.15 $\mu m \cdot cm \cdot V^{-1} \cdot s^{-1}$	
Surface charge characteristics	Based on	
	Sialic acid	Electrophoretic mobility
σ esu/cm²	$5.8 \cdot 10^3$	$6.7 \cdot 10^3$
Charges/cell	$6.0 \cdot 10^8$	$7.0 \cdot 10^8$
Charges removed/cell (enzymatically)	$6.0 \cdot 10^8$	$2.8 \cdot 10^8$
Fraction of surface charge due to sialic acid	87 %	40 %

* Based on an average volume of $1.3 \cdot 10^4$ μm^3.

** Based on an average area of $5 \cdot 10^3$ μm^2.

to the electrokinetic behavior of the cell surface. However, the presence of a Ca^{2+} effect, discussed above, shows that there are other groups present which also contribute to the net negative surface charge. A summary of the calculated surface charge based on both sialic acid and electrophoretic mobility is presented in Table II. Assuming a single charge per sialic acid molecule, the charge density σ is $5.8 \cdot 10^3$ esu/cm^2 with a spacing between sialic acid molecules of $\approx$ 30 Å. The surface charge density as calculated from electrophoretic mobility using the relation

$$U\eta = \sigma \left(\frac{1}{K} + a_i\right)$$

where U is the electrophoretic mobility, K = Debye-Hückel function, η is the viscosity of the buffer solution, and a_i the radius of the counter ion taken to be Na^+, gives a value of $6.7 \cdot 10^3$ esu/cm^2. The above values of surface charge density are calculated based on an area of 5000 nm^2 for the *Amphiuma* red cell. The greater charge density calculated on the basis of mobility is a further indication that sialic acid is not the only source of negative charge on the surface. These findings, however, do not rule out the possibility that a portion of surface sialic acid molecules are structurally hidden from interaction with external electrical fields.

A number of investigators have studied the possible relationship between surface charge density and membrane permeability. Gomulkiewicz [4] found that permeability of the erythrocyte membrane to orthophosphate and SO_4^{2-} was unaltered after neuraminidase treatment and concluded that the negative surface charge does not control anion permeability of the erythrocyte membrane. In their study of the relationship between changes in the transmembrane and zeta potentials in single-cultured cells, Redmann et al. [6] found that with temperature changes and use of mitotic blocking agents, changes in these potentials were in the same direction. On the other hand they claim that changes in external Cl^- concentration affected the transmembrane potential but not the zeta potential. Although anion permeability appears to be unaffected by removal of surface sialic acid groups, Weiss and Levinson [5] observed a 10–17 % change in K^+ permeability in Ehrlich ascites tumor cells after treatment with neuraminidase. In addition, Gilbert and Ehrenstein [18] found that changes in the negative surface charge of squid axon membranes resulted in a change in K^+ conductance. They explained this effect by proposing that the axon is sensitive to the double-layer potential which results from the binding of Ca^{2+} by the outer surface fixed negative charge. Lassen et al. [9] have shown that the membrane potential measured in single *Amphiuma* red cells is sensitive to increased internal concentrations of Ca^{2+}. An increase in internal Ca^{2+} resulting from microelectrode puncture caused a transient increase in K^+ permeability and hyperpolarization of the membrane. In a subsequent study (in preparation) the same authors found that a transient hyperpolarization and an increase in K^+ permeability could be generated by raising the normal extracellular calcium concentration from 1.8 mM to 6 mM or more.

In order to determine whether negative surface charge in the form of sialic acid carboxyl groups plays a role in the calcium dependent K^+ permeability, membrane potentials were measured in *Amphiuma* red cells from which sialic acid had been enzymatically removed. The membrane potentials of these cells, measured in normal Ringer's (1.8 mM Ca^{2+}) as well as Ringer's containing 15 mM Ca^{2+},

were identical to membrane potentials in normal cells. Thus it must be concluded that neither the transmembrane potential nor the calcium-induced increase in K^+ permeability of these cells is affected by the removal of sialic acid carboxyl groups.

In the course of evaluating the mobilities obtained from individual runs we found a consistent tendency for a trinodal distribution pattern. This suggested the possibility that blood samples taken from the *Amphiuma* contained inhomogenous cell populations. At the same time fresh blood samples placed on a discontinuous ficoll gradient ranging from 0 to 20 % in Ringer's solution formed three distinct groups. Cells were removed from each group and their dimensions and mobility measured. The results are shown in Table I and indicate that the fractions differ in both physical dimension and electrophoretic mobility. It has been shown that mobility is independent of particle size; therefore the variations in mobility suggest that, in addition to differences in size, the fractions are characterized by differences in net negative surface charge.

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