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ELECTROKINETIC BEHAVIOR OF INSIDE-OUT VESICLES FROM HUMAN RED CELL MEMBRANES

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The electrokinetic behavior of red cell membrane vesicles of normal (ROV) and inverted (IOV) sidedness has been characterized using the laser Doppler technique of electrophoretic light scattering (ELS). At neutral pH ROV have a (approx. 25%) higher electrophoretic mobility than IOV and the two peaks can be resolved in the ELS spectrum to provide a quantitative estimate of the IOV/ROV ratio which is consistent with the ratio determined by assay of the activity of acetylcholinesterase. The ROV peak coincides with the mobility of fresh red blood cells and of resealed ghosts. Neuraminidase treatment reduces the ROV mobility by a factor of 2.6, while the IOV peak is reduced only slightly (<5%). Treatment with trypsin results in a single narrow ELS peak at about 60% of the mobility of ROV. Treatment of IOV with phospholipase C leaves the electrophoretic mobility unaltered, whereas treatment with phospholipase D increases their mode mobility by 22%. The mobility titration curve of IOV from pH 2 to pH 10 reveals three distinct inflection points which may be assigned to chemical groups on the cytoplasmic surface of the red cell membrane.

Introduction

The components of the membrane of human red blood cells are arranged asymmetrically such that the two surfaces of the membrane differ in their chemical composition. Integral proteins span the membrane with different portions of the molecules exposed at the two membrane surfaces [1,2]. Furthermore the peripheral proteins of the membrane are confined for the most part to the cytoplasmic surface, and the carbohydrates (as both glycoproteins and glycolipids) are only external [2]. Finally the phospholipids are also asymmetrically arranged: the ones in the external surface are mainly choline phosphatides (phosphatidylcholine and sphingomyelin); the inwardly facing lipids are mainly aminolipids (phosphatidylethanolamine and phosphatidylserine) [3,4].

The earliest study of the chemical nature of the external surface of the membrane of red cells was the measurement of the electrophoretic mobility of intact cells [5,6]. At physiological pH and ionic strength the outer surface of the membrane is negatively charged. Among numerous studies of these properties of human red cells, one of the most complete was that of Haydon and Seaman [7], in which it was shown that the charge is due to constituents of the membrane (not adsorbed ions), and that the negatively charged groups are mainly

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Abbreviations: Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; ELS, electrophoretic light scattering; IOV, inside-out vesicles; ROV, right-side-out vesicles; EGTA, ethyleneglycol bis-(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid; DTNB, 5,5'-dithiobis-(2-nitrobenzoic acid); *E*, electric field strength.

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the amino sugar *N*-acetylneuraminic acid (60%) and carboxyl groups of proteins (40%).

The cytoplasmic surface of the membrane has been inaccessible to studies of its electrical charge and the responsible chemical groups. Inside-out vesicles (IOV) prepared from the cell membranes now afford the possibility of studying these properties of the cytoplasmic membrane surface. There was a preliminary indication that it is negatively charged, but with a lower charge density than the external surface [8]. We have investigated the electrokinetic behavior of IOV using laser electrophoretic light scattering (ELS). We have confirmed the net negative charge of the membrane's cytoplasmic surface. By examining the effects of chemical probes on the mobility of the vesicles, we have been able to identify tentatively the groups responsible for its electrical charge. A preliminary report of some of these results has been published [9].

Materials and Methods

Preparation of inside-out vesicles

Blood from healthy human donors was drawn into heparinized syringes. The red cells were washed at 4°C three times by centrifugation, aspiration, and resuspension in an isotonic solution containing choline chloride (150 mM), Tris-HCl (10 mM), pH 7.4. Inside-out vesicles (IOV) were prepared by the method of Steck [1] as modified by Mercer and Dunham [10]. Unless otherwise noted, all solutions were ice cold and made using glass distilled water containing Tris-EGTA (0.1 mM), pH 7.0. The washed red cells were lysed in 40 vol. of ice-cold Tris-HCl (5 mM), pH 8.0. After 10 min on ice the solution was centrifuged and the pellet was suspended in 10 vol. of distilled H_2O. After 10 min, 20 vol. of Tris-HCl (5 mM), pH 8.0 were added and the suspension was centrifuged. Suspension and centrifugation were repeated and the pellet was suspended in 25 vol. of 0.5 mM Tris-HCl, pH 8.0. After 1 h on ice, the suspension was centrifuged and the ghosts in the pellet were homogenized by subjecting them to a shear stress, exerted by passage five times through a 1 inch 27 gauge needle on a 5 ml syringe. 2 ml of the suspension were placed onto a 3 ml Dextran T-70 shelf (4.46%, w/v, in 0.5 mM Tris-HCl, pH 8.0) and centrifuged for 40 min. The vesicles at the interface were removed and washed by centrifugation, successively, in 10, 20, and 50 mM Tris-glycylglycine, 0.1 mM $MgCl_2$, pH 7.4. The vesicles were then suspended in a solution containing NaCl (20 mM), KCl (5 mM), Tris-glycylglycine (2.5 mM), $MgCl_2$ (1 mM), pH 7.4, 52 mosmol/kg. This solution was used in all experiments as a suspension medium for the vesicles. IOV were used up to 5 days after they were prepared.

Right-side-out vesicles (ROV) were prepared in an identical fashion except that the solution for the second incubation on ice and for homogenization (0.5 mM Tris-HCl, pH 8.0) also contained 1 mM $MgCl_2$. ROV were not stable beyond the day they were prepared (see below).

Enzymatic treatments of vesicles

Neuraminidase: incubation with 5 units/ml of enzyme (Type V, Sigma) at 37°C for 60 min.

Trypsin: incubation with 0.2 mg/ml of enzyme (Type I, Sigma) at 37°C for 45 min. The trypsin was tested for purity by polyacrylamide gel electrophoresis using a method described previously [10]. More than 95% of the protein was in a single, discrete band with remainder of low molecular weight (< 20000) in a diffuse zone.

The trypsin was tested for contamination with glycosidase using *p*-nitrophenylglucoside as a substrate. Incubations were carried out at 37°C with trypsin at 0.2 mg/ml and *p*-nitrophenylglycoside at concentrations from 10 to 100 μM. In 1 h no *p*-nitrophenol was released (measured by absorbance at 410 nm), indicating no contamination with glycosidase.

Phospholipase C and D: incubation with 2 units/ml of enzyme (Sigma; phospholipase C, type V; phospholipase D, type II) at 37°C for 60 min.

The vesicles were at 0.1 mg protein per ml suspension. The enzymatic treatments were terminated by cooling to 0°C and then diluting at least 10-fold in the usual suspension medium.

Assay for sidedness of vesicles

The ratio of inside-out to right-side-out vesicles (IOV/ROV) was determined from the activity of acetylcholinesterase (which is restricted to the outer surface of the membrane [11]) as described by Steck [1]. The enzyme assay uses DTNB to follow

the appearance of free thio groups during the hydrolysis of the substrate, acetylthiocholine. Vesicles in buffer with and without Triton X-100 (which disperses the membranes) were added to a solution containing DTNB and acetylthiocholine chloride. The reaction is followed with a recording spectrophotometer; an increase in absorbance at 412 nm corresponds to acetylcholinesterase activity. The ratio, IOV/ROV, is given by: A_{tr}/A_{in}, where A_{tr} is the acetylcholinesterase activity of the vesicles disrupted with triton and A_{in} is the activity of the intact vesicles. All preparations of IOV had an IOV/ROV ratio between 3.5 and 4.6.

Preparation of resealed ghosts

These were made by the method of Bodemann and Hoffman [12]. Briefly, fresh, washed red cells were lysed at 0°C in 10 vol. of a solution containing $MgCl_2$ (4 mM) and Tris-HCl (2 mM), pH 7.4. After 5 min at 0°C, isotonicity was restored by addition of KCl. This mixture was incubated 1 h at 37°C in order to reseal the ghosts, which were then collected and washed by centrifugation. The majority of ghosts produced by this technique (60–75%) have a cation permeability similar to that of untreated red cells [12]. Electrophoretic mobility of the ghosts was determined the same day they were prepared.

When comparing electrophoretic mobilities of the resealed ghosts and intact cells to that of vesicles, it was desirable to suspend the ghosts and cells in a medium of the same ionic strength as that used for IOV, but which was also isotonic to the cells. Accordingly, the solution contained NaCl (25 mM), Hepes (2.5 mM), pH 7.4, and sufficient sorbitol to raise the osmolality to 270 mosmol/kg.

Electrophoretic light scattering

The principles and methods of electrophoretic light scattering (ELS) have been described previously [13–15]. The spectrum of Doppler shifts of laser light scattered from particles which are migrating in an electric field is measured to determine the electrophoretic velocities. One important feature of the measurements obtained by this method is that they represent charge densities of the particles, and are independent of the size of the particles. For these experiments a thermostatically controlled scattering chamber [14] was fitted with a pair of Ag/AgCl electrodes and maintained at 22°C by a circulating water bath. The samples were illuminated with light from a He-Ne laser (Spectra-Physics 125A), and the applied electric field was provided by means of a constant current power supply (Electronic Measurements, C612). Values of applied current ranged from 2 to 5 mA, corresponding to electric fields of 28 to 70 V/cm. The scattering angle θ_s, was 36.7° throughout this study. The conductivities of each solution, required for the calculation of the field strengths, were determined with a conductivity meter (Radiometer, Model CDM-3). The Doppler signal was analyzed and averaged by a real-time spectrum analyzer (Hewlett-Packard Model 3582A). Typically 64 averages, requiring a total time of 16 min, were performed for each spectrum. All of the ELS spectra shown are representative of results from at least four separate experiments.

Results

We have performed two kinds of experiments: The first kind was undertaken to provide a basis for distinguishing between IOV and ROV by differences in their electrophoretic mobilities. In the second we examined the effects of enzyme treatments and of varying pH on the mobility of IOV as approaches to identifying the chemical groups

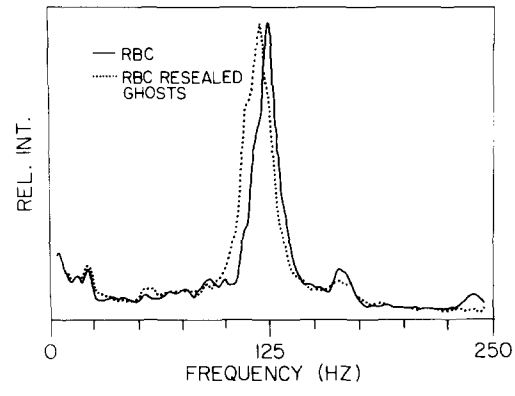

Fig. 1. ELS spectra of intact red cells (RBC) and resealed red cell ghosts. Relative scattered light intensity (Rel. int.) is plotted against Doppler shift frequency (Hz) (The same units are used in Figs. 2–6). Cells and ghosts were suspended at 10^5/ml in the buffered solution given in Materials and Methods. $E = 38$ V/cm.

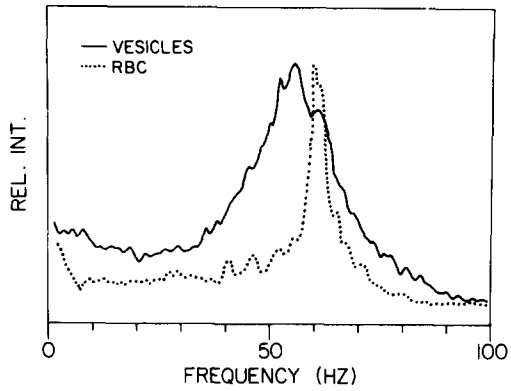

Fig. 2. ELS spectra of intact red cells (RBC) and vesicles with IOV/ROV ratio=4.0. Cells and vesicles were suspended in the media specified in Materials and Methods. The vesicles in this experiment (and in all subsequent ones) were suspended at 1 μg membrane protein per ml. The cells were at 10^5/ml. $E=31$ V/cm.

responsible for the electrical charge of the cytoplasmic membrane surface.

Electrophoretic mobility of intact cells, resealed ghosts, and vesicles

We first examined whether the trauma of osmotic lysis to the membranes had any significant effect on electrophoretic mobility. Fig. 1 shows the ELS spectra of both fresh, washed intact red cells and of resealed red cell ghosts. It is clear from these spectra that there is little or no difference in the electrophoretic mobilities and thus little effect on the cell surface charge density caused by lysis.

Fig. 2 shows superimposed ELS spectra of a preparation of membrane vesicles and of intact red cells. (The shift for the intact cells was lower than in Fig. 1 since the ionic strength of the medium for suspending the cells was reduced when they were compared with vesicles (see Materials and Methods).) The spectrum for the vesicles in Fig. 2 exhibits a bimodal distribution, indicating the presence of two distinct electrophoretic species. The mobility of the higher of the two coincides with that of intact cells. The IOV/ROV ratio determined by the cholinesterase assay was 4.1. From a series of similar experiments, we concluded that the relative proportions of the ELS peaks as a measure of IOV/ROV ratio were consistent with the estimates from analyses of cholinesterase. Therefore we can tentatively assign the higher mobility vesicles as ROV and the lower mobility ones as IOV.

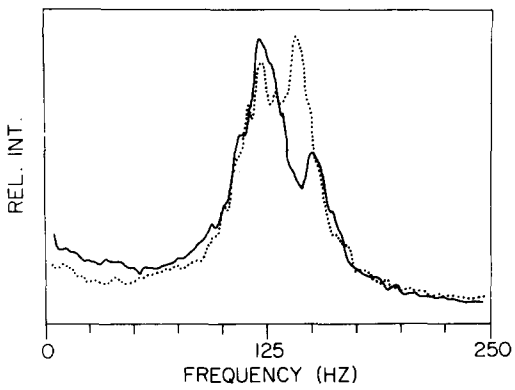

Fig. 3. ELS spectra of two preparations of vesicles made to be predominantly IOV (———) or ROV (·····). The methods of their preparation are given in Materials and Methods. $E=60$ V/cm.

Mobility of right-side-out and inside-out vesicles

In an attempt to confirm the identification of IOV and ROV in Fig. 2, we obtained ELS spectra for two preparations of vesicles, one made without added Mg^{2+} to maximize the fraction of IOV, and the other prepared with Mg^{2+} added, to increase the fraction of ROV (see Materials and Methods). The results are shown in Fig. 3. The solid trace indicates the spectrum of a preparation made to contain mostly IOV (the IOV/ROV ratio was 4.3); the dotted trace is a spectrum of a preparation with mostly ROV. Obviously the preparation with the higher fraction of ROV also had the higher proportion of higher mobility vesicles. These results confirm the interpretation of the results in Fig. 2, and also illustrate that the two methods for making vesicles do indeed result in different IOV/ROV ratios.

Instability of right-side-out vesicles

The inside-out vesicles (IOV) were stable for up to one week, as judged from measurements of both mobility and cholinesterase activity. ROV were not stable in ROV-enriched preparations. During storage at 4°C for a week the ELS spectra of ROV-rich preparations slowly and spontaneously converted to the IOV/ROV ratio of 4, which is characteristic of standard IOV preparations. Steck

[1] earlier mentioned that after incubation for 2 h at room temperature the percentage of IOV increased. This apparent spontaneous conversion of ROV to IOV is provocative, but without explanation.

Treatment with neuraminidase

Most of the negative charge on the external surface of red cells is due to the amino sugar *N*-acetylneuraminic acid, and this compound is absent from the cytoplasmic surface [7,16]. Therefore, the treatment of a suspension of vesicles with neuraminidase, which cleaves the amino sugar groups from the membrane surface, should reduce the mobility of ROV but should not affect the IOV. The results of an experiment designed to test this prediction are shown in Fig. 4. The electrophoretic mobility of the higher mobility, less numerous vesicles, was reduced at least 50%, confirming again the designation of these vesicles as ROV. The extent of the reduction is consistent with earlier estimates of the extent of the contribution of *N*-acetylneuraminic acid to the total surface charge density. The mobility of the (initially) slower fraction of vesicles was only slightly affected by neuraminidase, confirming their assignment as IOV. The 5% or so decrease seen in mobility of IOV may be attributable to residual protease contamination of the neuraminidase; similar experiments using more highly purified neuraminidase have been inconclusive. It is interesting to note that the ratios of the intensities of the two peaks and the linewidths of the two peaks are unaffected by neuraminidase treatment, which we take as evidence that the integrity of the two species was conserved.

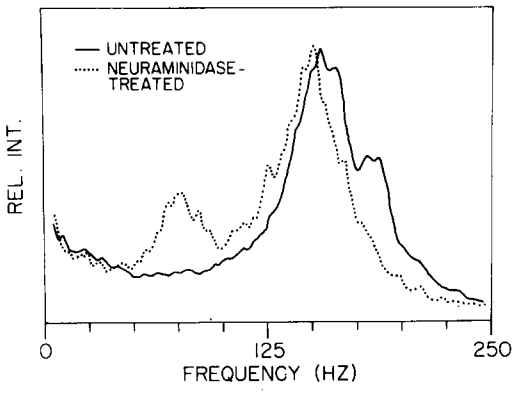

Fig. 4. ELS spectrum of vesicles (IOV/ROV≈4) treated with neuraminidase (·····) compared with the spectrum of untreated vesicles (———) from the same preparation. The procedure for the enzymatic treatment is given in Materials and Methods. $E = 67$ V/cm.

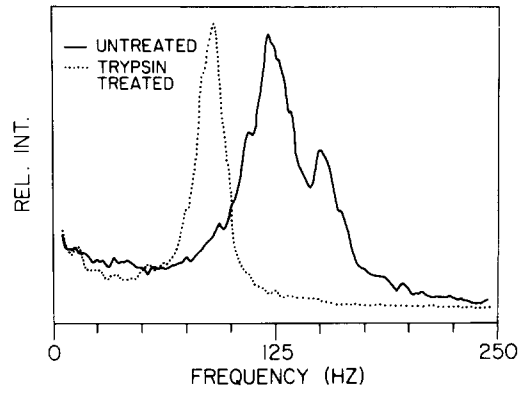

Fig. 5. ELS spectrum of vesicles (IOV/ROV = 4.0) treated with trypsin (·····) compared with the spectrum of untreated vesicles (———) given in Materials and Methods. $E = 60$ V/cm.

Treatment with trypsin

Trypsin cleaves exposed membrane proteins at basic amino acid residues and reduces the electrophoretic mobility of intact red cells, thereby inducing aggregation [6]. If ROV respond in the same way, the effects would be manifest as a decrease in both the Doppler shift and the width of the ROV peak in the ELS spectrum. The ELS spectra of a sample of untreated and trypsin-treated vesicles from a preparation with an IOV/ROV ratio of 4.0 are shown in Fig. 5. The spectra show that the mobilities of both IOV and ROV have been reduced and that the result is a single peak at lower electrophoretic mobility. The collapse of the bimodal spectrum into a single peak indicates either that the mobilities have coincidentally been reduced to a single value or, more likely, that the two species have interaggregated. Two mechanisms for aggregation can be distinguished. Enzymatic cleavage of peptide bonds on the external surfaces of the respective vesicles may increase their propensity to aggregate, or, alternatively, trypsin may act as the binding site to bridge the aggregating vesicles. We view the latter possibility as being less likely, and we cite as evidence the fact

that trypsin, even at much higher concentrations (10 mg/ml), does not cause aggregation of intact cells. The reduction in mobility of both ROV and IOV is probably a consequence of cleavage of portions of proteins containing carboxyl groups.

Treatment with phospholipases

Of the various cell membrane components, the contribution of phospholipids to membrane surface charge have been the least explicitly established. Of the four major phospholipids in the red cell membrane, three (phosphatidylcholine, phosphatidylethanolamine and sphingomyelin) are uncharged zwitterions and the third (phosphatidylserine) bears a negative charge at pH 7. The external surface contains mainly phosphatidylcholine and sphingomyelin, while phosphatidylserine and phosphatidylethanolamine are the primary lipid components of the cytoplasmic surface.

To determine the extent of the contribution by phospholipids to charge density of the membrane surface, we have made ELS measurements on vesicles treated with two phospholipases: phospholipase C, which hydrolyzes the P-O bond between the phosphate and glycerol moieties, and phospholipase D, which hydrolyzes the other P-O bond, leaving an exposed phosphatidic acid. Phospholipase C can be expected to have no effect (at pH 7) on the net charge of phosphatidylethanolamine or phosphatidylcholine (removing both the phosphate and the amino groups), while the charge of phosphatidylserine would become more positive (removal of two negative charges and one positive charge). Conversely, phospholipase D would have no effect on the charge of phosphatidylserine (removal of the zwitterionic serine), but would increase by one the negative charge on phosphatidylethanolamine and phosphatidylcholine (removal of an amino group).

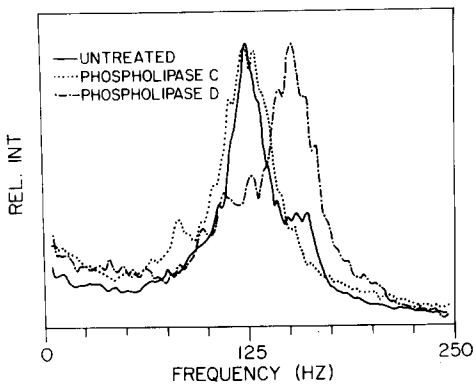

Fig. 6. ELS spectra of vesicles (IOV/ROV=4.0) treated with phospholipase C (·····), phospholipase D (·-·-·), and untreated vesicles (———). The methods of treatment are given in the text. $E=60$ V/cm.

ELS spectra of phospholipase-treated vesicles are shown in Fig. 6. Treatment with phospholipase D produced a spectrum with a significant increase in the mode mobility of the IOV, the expected effect consequent to the action of the enzyme on phosphatidylethanolamine, a lipid of the cytoplasmic membrane surface. (It is not clear if phospholipase D also increased the mobility of the ROV.) Therefore phosphatidylethanolamine contributes significantly to the charge of the cytoplasmic membrane surface.

Treatment with phospholipase C had no effect on the mobility of the IOV. The low mobility shoulder was not reproducible among different preparations of vesicles. The failure of phospholipase C to reduce the mobility of IOV is difficult to interpret. It may mean that phosphatidylserine does not contribute significantly to surface charge, or that the enzyme does not have access to its target P-O bond in this phospholipid.

Dependence of mobility on pH

Most of the ionizable surface groups on the red cell membrane have pK values within an experimentally accessible pH range. The study of the pH dependence of the electrophoretic mobility of red blood cells has been a classical method for investigating these surface groups [6]. One of the most detailed electrophoretic titration curves of red blood cells was obtained by Uzgiris and Kaplan [17] using a laser Doppler technique. Like earlier workers, however, they were unable to obtain stable results at extremes of pH, presumably because of damage to the cells. The range of pH in which the cells, and their mobility, were stable was subsequently extended by fixation of the cells with aldehydes [18]. It is probable that fixation affected the titration curves, but the nature and extent of the effects are inaccessible.

We have found that membrane vesicles have a much wider range of electrokinetic stability than

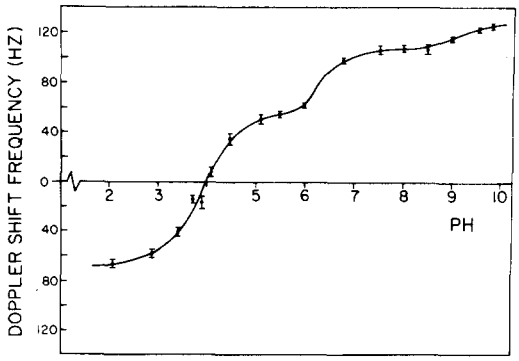

Fig. 7. Electrophoretic mobilities of a preparation of IOV (IOV/ROV=4.1) measured at various pH values. The points were taken from the height of the IOV peak of the individual spectra. Shown are means of at least three determinations±S.E. The ordinate is Doppler shift frequency, which can be converted to electrophoretic mobility in the usual units (10^{-4} cm^2/V per s) by multiplying by 0.013.

intact cells, as pH is varied, and thus (though the reason for this is not clear) are an excellent model system for studying membrane titration behavior. We have succeeded in measuring the titration curve for electrophoretic mobility of IOV, and the data are shown in Fig. 7. The buffer was that described in the Materials and Methods, except that citric acid was substituted for glycylglycine from pH 2 to pH 7. Titration was carried out by adding dilute HCl or NaOH to the vesicle suspension. Three distinct inflection points, at pH 4, 6.3, and 9.2, can be seen in this curve. The isoelectric point is about 4.0, close to the isoelectric point of the external membrane surface, estimated for intact cells [17–19]. The reversal of mobility below pH 4 shows the presence of positive charges, possibly amino groups and adsorbed cations. The titrating groups with a pK of 4.0 are probably carboxyl groups of proteins and maybe also of phosphatidylserine. The other inflections, at pH 6.3 and 9.2, are probably attributable to phosphate groups and amino groups, respectively.

Discussion

We have been able to distinguish inside-out from right-side-out vesicles in terms of their electrophoretic mobility in ELS spectra. This distinction was based upon three criteria: (1) the identity of the mobility of the smaller, more rapid fraction of vesicles with the mobility of both intact cells and resealed ghosts; (2) the consequence of treatment with neuraminidase in lowering the mobility of the smaller more rapid fraction of vesicles, and not affecting the mobility of the larger fraction designated IOV by criterion one; (3) the similarity of estimates throughout the range of IOV/ROV ratios from cholinesterase activity and relative sizes of the two peaks of mobility in the ELS spectra.

There is a net negative charge of the IOV, and therefore of the cytoplasmic surface of the red cell's membrane. Most of the charge, about 2/3, is attributable to carboxyl groups. The remainder of the negative charges are phosphates of the phospholipid molecules. There are also positive charges, demonstrable below the isoelectric point, which are probably amino groups. The tentative identification of the three types of groups is based in large part on the titration curve (Fig. 7). In addition the increase in mobility caused by treatment with phospholipase D (which cleaves the zwitterion to leave an anion) suggests a contribution of the amino group of ethanolamine. The carboxyl groups are probably on both proteins and phosphatidylserine. Phospholipase C did not reduce mobility as anticipated if it were cleaving serine phosphate. However, lack of access of the enzyme to its target P-O bond does not rule out contribution to the charge by serine's carboxyl group.

The method used for preparing IOV results in removal of most of the peripheral proteins from the cytoplasmic membrane surface. For example, up to 70% of spectrin and actin are removed [10]. In preliminary experiments we found that further removal of peripheral protein (specifically, complete removal of band 6, glyceraldehyde-3-phosphate dehydrogenase) by elution with 0.15 M NaCl had no effect on ELS spectra. But the results do not permit assessment of the contribution of peripheral proteins to the charge of the membrane. It has been shown that at pH 5 or lower the intramembranous particles of red cell membranes aggregate [20,21], and that the distribution in the membrane of the particles is probably controlled by spectrin and other peripheral proteins [21–23]. It follows that the aggregation occurs as the isoelectric point of spectrin is reached. This phenomenon might have a second order effect on the charge of vesicles at low pH, but not a primary

effect. Furthermore, as mentioned above, our method of preparing IOV results in loss of much of the spectrin [10].

We observed, as stated above, that our IOV were stable, as judged by electrophoretic mobility, for up to a week. Membrane enzymes with proteolytic activity have been observed which cleave, for example, band 3 protein [24]. We can conclude only that this proteolysis, if it takes place in our preparations, is sufficiently slow and with sufficiently subtle consequences, that net charge is unaffected.

Attention directly toward the function of electrical charges on the external surface of membranes has been focused largely on interactions with chemical messengers and with other cells. Clearly these kinds of interaction cannot be a function of the electrical charge at the cytoplasmic surface. A striking feature of the structure of membranes is the asymmetric arrangement of the constituent molecules. The maintenance of the asymmetry, that is the minimized probability of reorientation of the molecules between the two surfaces of the membrane, is likely a consequence of their charges. Since the internal domain of membranes is hydrophobic, maintenance of asymmetry is assured by the charged groups on the portions of the molecules which protrude into the internal or external aqueous phases.

Inside-out vesicles have lower amounts of peripheral proteins than intact cells. The interesting suggestion has recently been made that maturation of circulating reticulocytes occurs in part by endocytosis of portions of the membrane free of spectrin, the major peripheral protein [25]. Thus the properties (mechanical and chemical) of spectrin-free portions of the membrane are important determinants of the developmental process. Since IOV have a low spectrin content, and are formed experimentally by invagination, their properties are relevant to the maturation of reticulocytes. It remains to be seen if electrical charge of the cytoplasmic surface is related.

Another role of spectrin has been proposed, that of maintenance of the asymmetric distribution of phospholipids in red cell membranes. After treatment of red cells with SH-oxidizing agents, availability to cleavage was observed of phosphatidylserine and phosphatidylethanolamine, phospholipids predominantly of the cytoplasmic surface of the membrane bilayer [26]. In another study the distribution of phospholipids appeared to be less asymmetric in sickled red cells (both irreversibly and reversibly sickled cells) than in normal ones, and the distribution appeared to become asymmetric again when reversibly sickled cells were reoxygenated [27]. In both of these studies apparent loss of asymmetry was ascribed to an alteration in the interaction of cytoskeletal proteins, particularly spectrin, with the membrane, which occurs during sickling and with oxidation.

However it has been shown conclusively that IOV (made by the same method employed in the present study) have the same asymmetric distribution of phospholipids as intact cells [28], even though IOV made by this procedure have relatively much less spectrin than do intact cells [10]. Perhaps the apparent loss of asymmetry sometimes observed [26,27] reflects increased accessibility to externally applied lipases of the phospholipids at the cytoplasmic membrane surface (cf. Ref. 29).

Acknowledgements

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