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M.S. Hutchinson
(Ph.D. Thesis)

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ELECTROPHORETIC AND BIOCHEMICAL STUDIES OF ERYTHROCYTE MEMBRANE STRUCTURE

Majorie Smit Hutchinson

PhD Thesis

(Submitted in partial satisfaction of the requirements for the degree of Doctor of Philosophy in Biophysics, University of California, Berkeley)

December, 1984

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Electrophoretic and Biochemical Studies of Erythrocyte Membrane Structure

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Abstract

The chemical composition of the outer surface of the human erythrocyte was studied by combining electrical-surface-charge measurements (microelectrophoresis) with selective chemical modification procedures that alter various classes of ionogenic groups known to reside within the membrane. The chemical procedures that were used included: (i) the removal by enzymatic methods of sialic acid residues from membrane saccharides; (ii) removal of the choline and phosphorylcholine moieties present in the polar headgroups of membrane phospholipids; (iii) the alteration of surface charge by various charge-blocking agents.

In order to stabilize the cells against hemolysis caused by several of the chemical modification procedures, the erythrocytes were fixed with glutaraldehyde under various conditions. In this manner fixation conditions were arrived at that left unaltered the electrophoretic mobility at all ionic strengths and pH's.

The particular enzymes employed were neuraminidase, phospholipase C from *B. cereus* and from *C. perfringens*, and phospholipase D from peanut and from *S. chromofuscus*. Chemical assays were performed to determine the amount of sialic acid, phosphate, or choline removed by the various treatments. The neuraminidase results strongly suggest that the native cell has a non-zero surface conductance, which is lost in the neuraminidase-treated cell.

The phospholipase results suggest that membrane-component rearrangement occurs after removal of choline moieties from membrane phospholipids, with the remaining phosphatidic acid probably flipping to the inner surface of the membrane.

Taken together, the results of this dissertation provide new information and insights on membrane structure, stability, and biophysical responses. These include the location of the phospholipids, the physical extent and conductance of the outer carbohydrate-and-protein layer, the role of sialic acid, the ability of the membrane components to rearrange, and the nature of the fixation process.

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CHAPTER 1

Introduction

1.1. Membrane models

Modern membrane-model theory began with the lipid bilayer concepts which arose from the experiments of Gorter and Grendel (1925), in which lipids when spread in a Langmuir trough were found to cover an area approximately twice the estimated area of the cell surface. Although some of these estimates were later shown to be incorrect, these experiments led to the model proposed by Danielli and Davson (Danielli and Davson, 1935). In this model the membrane was thought to be composed of a lipid bilayer in which the hydrophobic chains of the lipids interacted in a water free environment, while the polar head-groups were oriented towards the more hydrophilic environment of the inner and outer membrane surfaces.

At that time the membrane proteins were thought to form layers covering the inner and outer surfaces. Belief that this model was largely correct was strengthened by the unit-membrane appearance seen in electron micrographs (Robertson, 1963). Further data, however, cast doubt on the complete correctness of this theory. Isotopic labeling experiments indicated that many proteins spanned the membrane, and that others were bonded to hydrophobic portions of lipids (Richardson, et al., 1963; Singer and Nicolson, 1972). In addition, freeze-fracture studies showed globular units of about 7.5nm diameter which appeared to be present in the interior (Branton, 1966; Pinto da Silva and Branton, 1970) Finally, infrared spectroscopy (Maddy and Malcolm, 1965), optical rotary dispersion (ORD) and circular dichroism (CD) experiments (Wallach and Zahler, 1966; Lenard and Singer,1966) showed that there was no detectable protein in beta form, as should be the case if the protein was in sheets on the outside

of the membrane. To have protein in globular form would require a membrane larger than the 7.5 to 9.0nm generally seen (Singer and Nicolson, 1972).

The modern membrane model, now usually called the fluid mosaic model, answered these objections (Lenard and Singer, 1966; Singer and Nicolson, 1972). This model still incorporates the lipid bilayer concept but places the protein throughout the membrane. One class of proteins, the "integral" proteins, can only be removed by drastic treatments such as by detergents or organic solvents. Once removed, they are found to be hydrophobically bound to membrane lipids, or to be insoluble in neutral aqueous buffers. The ease of extraction of lipids, either from membranes or membrane lipoprotein, indicates that the lipid-protein binding is not covalent. Proteins in another class, the "peripheral" proteins, are easily removed by increases in ionic strength or by chelating agents. This suggests that they are weakly bound to the inner and outer surfaces, perhaps by ionic bonds. The carbohydrates, which include sialic acid, hexose and hexosamines, are attached to both lipid and protein moieties at the outer surface.

Lipid organization into some form other than a bilayer has also been suggested. Lipids and proteins might form conglomerates, with the lipid hydrocarbon chains inserting into the hydrophobic portions of the protein, forming large subunits that would then interact with each other to constitute the membrane (Vanderkooi and Green, 1970). In this model, the polar head groups would not line up to the extent that they would in the bilayer. Another possible model is one in which many of the lipids would be in micelles, each of which contained large numbers of lipid molecules that interact with the proteins only at the outer edge of the micelle.

1.2. Evidence for membrane bilayer structure

The existing evidence indicates that at least part of the membrane lipid is in a bilayer arrangement. In the myelin membrane, the lipids have been shown, conclusively, to be in a bilayer arrangement, but the myelin membrane has a much higher proportion of lipid than do

most membranes. For the erythrocyte, the evidence is strong but not overwhelming. Freeze-fracture experiments in which the membrane splits down the center are taken to be good evidence for a bilayer (Branton, 1966; Pinto da Silva and Branton, 1970). Spin-label studies have shown that lipids rapidly diffuse in the plane of the membrane, (Kornberg and McConnell, 1971a), while they diffuse only very slowly across the membrane (Kornberg and McConnell, 1971b). Some studies on model membrane systems indicate that there might be two pools of lipids, one easily diffusible in the plane of the membrane, presumably within the bilayer, while the other is "bound" lipid, probably hydrophobically bound to protein (Jost, et al., 1973). Wallach and Zahler (1966), with ORD studies on plasma membranes of Ehrlich acites carcinoma, and Lenard and Singer (1966) on human erythrocytes, showed that protein conformation was consistent with the interaction between the proteins and lipids being hydrophobic in nature. Wilkins et al. (1971) performed an x-ray diffraction analysis of erythrocyte membrane fragments, and found a repeat distance of 4.5nm which they interpreted as the thickness of the lipid bilayer.

If there is strong evidence that the membrane lipids are at least partially organized into a bilayer structure, the extent of this has not been proven. Electron micrographs are made on dehydrated and embedded membrane samples, and it is well known that removal of water from lipid dispersions does cause the lipid to form bilayers, whether or not they were previously so organized. Further, Lenard and Singer (1968) showed that the fixatives used in the preparation of electron micrographs, led to altered conformation of the membrane proteins. Also, the use of techniques such as fluorescent probes and spin labels requires the insertion into the membrane of foreign molecules which could be perturbing the membrane and changing its characteristics (Op den Kamp, 1979).

1.3. Membrane asymmetry

Several studies indicate that the erythrocyte membrane is asymmetric with respect to the lipids and proteins. (Bretscher, 1972 and 1973; Gordesky and Marinetti, 1973; Zwaal and Roelofsen, 1976). The principle lipids of the erythrocyte membrane, and the points of action of the various enzymes, are shown in figure 1.1. Zwaal and Roelofsen (1976), by means of phospholipase studies, have determined that in the human erythrocyte, 80% of the phosphatidylethanolamine (PE), and all of the phosphatidylserine (PS) appear to be at the *inner* surface, while the phosphatidylcholine (PC), the sphingomyelin, and up to 20% of the PE, are accessible only at the *outer* surface of the membrane. These conclusions come from analysis of the results of combined enzyme actions on intact cells and ghosts. Bretscher (1972) in experiments in which he reacted the free amino group of PE with formyl-^{35}S-methonyl (sulphone) methyl phosphate also came to the conclusion that the membrane was asymmetrical in its lipid components. Finally, evidence for protein asymmetry has been found in studies on the availability of protein to proteases and to labels, in intact cells and in ghosts (Steck, 1974). From this asymmetry it is evident that the membrane molecular structure is other than random in nature.

1.4. Location of phospholipid components

It is interesting to examine the location of the membrane phospholipids, and the possibility that they might be masked by proteins. The membrane is made up of about 52% protein, 40% lipid and 8% carbohydrate by weight, and, as seen in electron micrograph sections, is approximately 7.5nm in thickness (Steck, 1974). If such a membrane includes of a lipid bilayer of 4.5nm thickness, as seen by Wilkins et al. (1971), then the remaining proteins and carbohydrates must take up no more than about 3.0nm. If we start with the simplest assumption for the distribution of the protein and carbohydrate, and allocate 50% to each

Figure 1.1

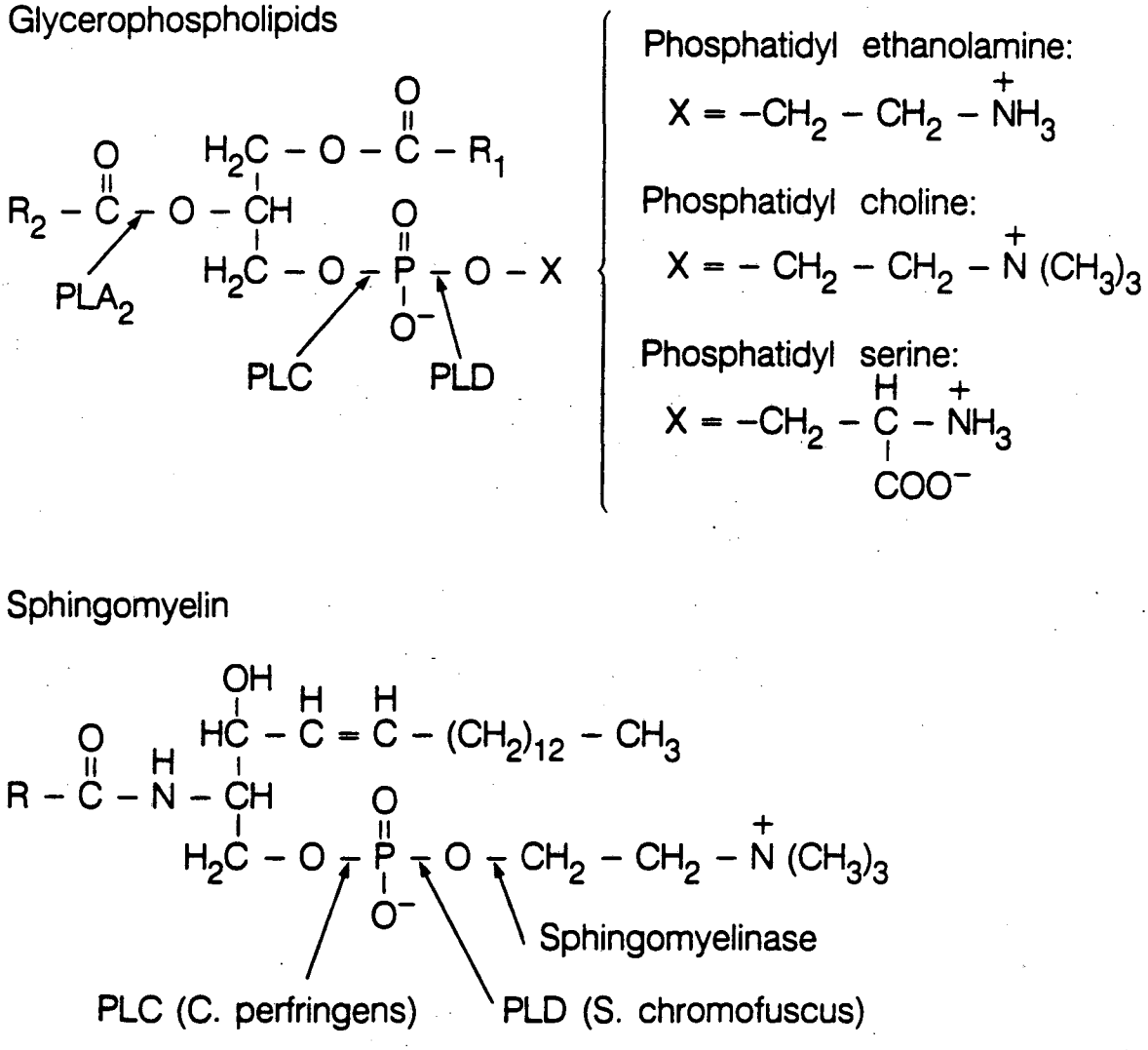


Figure 1.1. The principle lipids of the erythrocyte membrane showing the points of attack of the various phospholipases (Modified from Zwaal and Roelofsen, 1976). R, R_1, R_2 are fatty acyl chains.

surface, we would expect that the phospholipid head groups could be as far into the membrane as 1.5nm. However, the outer layer is apparently less than 1.5nm thick, since according to Rothman and Lenard (1977), and Steck (1974), labeling studies have shown that the majority of proteins (all but two of the major ones) are exposed only at the inner surface. (The remaining two major proteins are glycoproteins that are exposed at both surfaces). Thus, a reasonable assumption would be that much less than half of the protein is at the outer surface, and that the lipid head groups should, therefore, be less than about 1nm from the surface. Therefore, if indeed, the phospholipids are that close to the cell surface, it should be possible to detect them electrophoretically.

It is also possible that the phospholipids might be masked by the carbohydrates. However, using data from several sources, Tenforde (1970) estimated that the maximum surface area on the human erythrocyte covered by the carbohydrate is only 38% of the cell surface. Finean et al. (1971) estimated (from shrinkage of the membrane after PLC treatment) that 70% of the erythrocyte surface is occupied by lipid, which presumably would be largely phospholipid. Therefore, it is unlikely that the carbohydrates, alone, could effectively mask the phospholipids.

1.5. Role of the present work — experimental strategies

If the bilayer hypothesis is correct, the charge on the lipids should be detectable by the microelectrophoresis technique, when the electrophoretic mobility is measured in a solution of sufficiently low ionic strength. At low ionic strength, the charge-screening decreases due to the lower concentration of counterions. Therefore, charges which lie deeper in the membrane will contribute to the electrical potential (so called ζ-potential) effective at the surface of shear (Tenforde, 1970). The quantitative theoretical measure of this depth, measured inward from the surface of shear, is the Debye length (ℓ_D). That is, a charged particle must be within one

Debye length of the surface of shear for it to contribute to the potential at the surface of shear, and hence for it to be detected electrophoretically. At 25°C, the Debye length is given approximately by the relationship $\ell_D = 0.305/\sqrt{\Gamma/2}$ (nm), where $\Gamma/2$ is the ionic strength. An ionic strength of 0.0029 corresponds to a Debye length of 5.7nm. This is much deeper than would be needed to detect charges at the expected locations of the polar head groups of the membrane lipids as described above.

The strategy of the present experiments is to alter the membrane with enzyme treatments, and also to apply charge-neutralizing agents, particularly free-amine-combining agents, and to then measure the mobility of the treated cells at several different ionic strengths and pH values. In this way it may be determined whether there is a change in the membrane surface charge caused by the treatments, and, if so, at approximately what depth within the membrane such a change occurs. By measuring the mobility at several pH values, it is possible to determine the pK of the dissociating groups and, therefore, to infer specific charge groups that might be responsible for the electrophoretic surface-charge density. The particular enzymes used are neuraminidase and several phospholipases.

Phospholipases: **phospholipase C** (PLC) from *B. cereus,* removes the phosphocholine groups from phosphatidylcholine; PLC from *C. perfringens* removes the phospholipid head group from PC, PE and sphingomyelin. **phospholipase D** (PLD) from peanut or *Streptomyces chromofuscus,* removes only the positively-charged choline group, leaving the negatively-charged phosphate still in the membrane. As in the case of the PLC, the action of the two PLD enzymes is slightly different. The peanut PLD removes the choline head groups of PC and the ethanolamine group of PE but does not effect sphingomyelin. By contrast, the *S. chromofuscus* PLD enzyme acts on the PC and PE as above, but removes choline from sphingomyelin as well. Figure 1.1 shows the points of attack of each of the above enzymes.

Before treating the cells with any of the enzymes, except for *S. chromofuscus* PLD, the cells were necessarily fixed (with glutaraldehyde) to prevent hemolysis and cell destruction. Although, glutaraldehyde was assumed to play a passive role, it was necessary to investigate whether it, in itself, led to electrophoretic mobility changes. A similar point could be raised as to possible irreversible effects of low ionic strength acting on the membrane. It was therefore also necessary to investigate the possibility that the low-ionic-strength solutions, themselves, caused a permanent change in the cell surface charge (mobility).

Neuraminidase from *Vibrio cholerae,* removes neuraminic acid (sialic acid) from the terminal position of cell surface oligosaccharides. The rationale for the series of neuraminidase experiments is that sialic acid is the main cause of the net negative surface charge of the membrane. Thus, after removal of the sialic acid, the depth of the remaining charges could be estimated, by using the low-ionic-strength technique. By using neuraminidase in conjunction with the phospholipases, additional kinds of information can be gleaned. The effect of neuraminidase on the portion of the electrophoretic mobility that is due to the phospholipids might be quite different, depending upon how the sialic acid might interact with other membrane components.

Some possible effects of the removal of the sialic acid, either by itself, or in conjunction with phospholipase action are:

(i) Removal might *change the location of the surface of shear,* bringing the phospholipids closer to the outer surface where their charge would have a greater effect. Although, from the calculations described in section 1.4 this appears unlikely, it cannot be entirely excluded.

(ii) Without affecting the surface of shear or access to the phospholipids, removal of the phospholipid head groups might lead to *rearrangement of the sialic-acid-bearing molecules,* causing a change in the membrane charge distribution. Removal of the sialic acid would

eliminate this source of confusion for the electrophoretic results.

(iii) Sialic acid might *stearically mask the phospholipids* so that the PLC and PLD enzymes could not reach the substrate. Removal of the sialic acid, then, could lead to a significant increase in the amount of substrate removed by the enzymes, and hence to a change in the surface charge detected electrophoretically.

(iv) Sialic acid removal might have *no effect* on the charge-altering action, if any, of the phospholipases.

* * *

In sum, experiments following the above-indicated lines should lead to new insights about the location of the lipid head groups in the membrane, the location of the sialic acid moieties, the existence of electrophoretically detectable amino groups in the outer surface of the membrane, and the charge-interaction between the various molecular species in the membrane. Furthermore, such results might also provide new information about the location and extent of the lipid bilayer. For example, if the choline removal by PLD could be detected electrophoretically, the extent of the surface-charge change, and the Debye length at which the charge-change were detected, could provide new estimates of the extent and depth of the bilayer. In addition, since rearrangement within the membrane might lead to a charge change, this provides further opportunity to learn about the interactions of the membrane molecules with each other. And finally, because some experiments can be performed on both fixed and unfixed erythrocytes, this provides an opportunity to learn more about the fixation process itself, and the condition of the fixed cell.

CHAPTER 2

Methodology and Materials

2.1. Cell Electrophoresis

2.1.1. Basic relationships

The surfaces of living cells contain charge-bearing molecules that cause the cell to move when an electric field is applied. The velocity of the cells can be used to determine the electrophoretic mobility,

$$\mu \equiv v/\vec{E} \qquad \text{eqn. 1}$$

where v is the velocity (μm/sec) and $\vec{E}$ is the electric field strength (V/cm).

The relationship between the electrophoretic mobility and the surface charge density has been derived and is discussed in Tenforde (1970), Overbeek and Lijklema (1959) and Brinton and Lauffer (1959). Henry (1931) arrived at a relationship for spherical particles between μ and the zeta potential (ζ) where ζ is the electric potential at the surface of shear. The surface of shear is the imaginary surface surrounding the cell within which all ions and bound water of hydration move with the cell when the electric field is applied. Henry's relationship is

$$\mu = \zeta\epsilon f(\kappa a)/4\pi\eta \qquad \text{eqn. 2}$$

where ϵ is the dielectric constant, κ is the Debye-Hückel constant, a is the radius of the sphere, and η is the viscosity of the medium. The function $f(\kappa a)$ exhibits a complicated dependence on ionic strength and has been plotted by Henry. Smoluchowski (1903) had earlier derived the

equation $\mu=\epsilon\zeta/4\pi\eta$. When a is much larger than $1/\kappa$, as is the case of intact cells, $f(\kappa a)= 1$ and Henry's equation reduces to Smoluchowski's equation. When a is much less than $1/\kappa$, $f(\kappa a)$ approaches 2/3. A further assumption implicit in the derivation of these equations is that the particle is non-conducting.

Henry's equation for a conducting sphere is the following.

$$\mu= (\zeta\epsilon f(\kappa a)/6\pi\eta) (3\Lambda_o/(2\Lambda_o+\Lambda_i)) \qquad \text{eqn. 3}$$

where Λ_o and Λ_i are the conductivity of the external solution and the cell respectively. When Λ_i is zero, as is the case for a nonconducting sphere, this equation simplifies to eqn. 2.

The zeta potential can be related to the surface charge density σ using the Gouy-Chapman equation. This approximation treats the membrane as a planar surface with fixed charges, and it treats the counter-charge as a space charge extending out into the medium. The derivation makes use of the Debye-Hückel approximation (for which the potential, ψ, in the double layer can be approximated by $\nabla^2\psi = \kappa^2\psi$) which is valid for an impenetrable surface when the electrostatic potential, ψ is much less than kT/z_ie, where k is the Boltzmann constant, T is the temperature, z_i is the charge per ion, and e is the electronic charge. When the Gouy-Chapman approximation is carried out for a solution of singly charged ions, the surface charge, σ, can be expressed as

$$\sigma = (\epsilon kT\kappa/2\pi e) \sinh (e\zeta/2kT) \qquad \text{eqn. 4}$$

When the further approximation is made for the case where $\zeta<25$mV at room temperature, σ can be expressed as:

$$\sigma=\mu\eta\kappa = \mu\eta/\ell_D \qquad \text{eqn. 5}$$

where $\ell_D = 1/\kappa$ is known as the Debye length, and is considered to be the thickness of the ionic

double layer. For erythrocytes at room temperature, ζ is about 13mV. In equation 5, σ is in units of C m^{-2}, μ in μm s^{-1}/V cm^{-1}, η in poise, and ℓ_D in nm.

Haydon (1964) discussed the errors involved in these calculations and concluded that most of the errors are probably small with the possible exception of the error that occurs if the membrane is penetrable to charged species. He calculated that if the membrane were penetrable the true charge density could be as much as twice that of the calculated charge density.

2.1.2. Low ionic strength effects

The Debye-Hückel constant, κ, and its reciprocal, ℓ_D, are functions of the ionic strength of the medium:

$$\ell_D = (1000\epsilon kT/8\pi Ne^2\, \Gamma/2)^{1/2} \qquad \text{eqn. 6}$$

which at 25°C is approximately $0.305/(\Gamma/2)^{1/2}$ nm

where N is Avogadro's number and $\Gamma/2$ is the ionic strength $\equiv \frac{1}{2}\Sigma c_i z_i^2$. As the ionic strength decreases, the Debye length (i.e. double layer thickness) increases; fewer membrane surface charges are masked by counterions, and charges deeper in the membrane are able to contribute to the potential at the surface of shear, and hence affect the electrophoretic mobility.

2.1.3. Details of apparatus and techniques

Cell electrophoresis was performed using the modified Zeiss Cytopherometer described in Tenforde et al. (1973) employing a rectangular chamber in a lateral position. The electrodes consisted of two zinc posts immersed in saturated $ZnSO_4$ solutions. The electrodes were attached to a polarity-reversing switch placed in series with a constant-current power supply.

Velocity measurements were made by measuring the time to traverse a known distance between two grid lines. Each set of measurements was made on an average of at least 10 cells; measurements were made at the front and back stationary levels, and each cell was measured with both polarities of the applied field to cancel the effect of fluid drift. The stationary levels were determined by methods described in the manufacturer's manual (Carl Zeiss, Oberkochen/Wuertt, West Germany). The microscope was focused on the front and back walls, and the displacement of the microscope's ocular stage required to focus on the two walls was related to the chamber depth, t, using the manufacturer's tables. The positions of the front and back stationary levels were calculated by multiplying the chamber depth by 0.2024 and 0.7976, respectively (Bull, 1971). The chamber depth was approximately 700μm.

The electric field strength, $\vec{E}$, was calculated from the relation,

$$\vec{E} = I\rho/ht \qquad \text{eqn. 7}$$

where I is the current in amperes, ρ is the specific resistance of the chamber in Ω-cm, h is the chamber height (1.40cm), and t is the chamber depth in cm. The specific resistance, ρ, was determined by measuring the conductivity of the medium with a Leeds and Northrup conductivity meter. An experimental curve of solution resistance (R) vs. specific resistance was used to determine the cell constant, ξ, where $\xi = R/\rho$. (See Jones and Bradshaw, 1933)

The velocity measurements were then averaged and divided by the electric field strength to obtain the electrophoretic mobility, according to eqn. 1.

2.1.4. Electrophoresis buffers

Mobility measurements at physiological ionic strength were made in a buffered saline solution that was 0.145M in NaCl and 0.0003M in $NaHCO_3$, hereafter referred to as Standard Buffer. Low-ionic-strength buffers were prepared at several dilutions as shown in table 2-1.

TABLE 2-1

Composition of low-ionic strength buffers made up to a total volume of 200 ml using 0.3mM Na HCO_3

Debye Length (nm)	Ionic Strength	mosm/Kg NaCl[(a)]	mosm/Kg Sucrose	1.45 M NaCl[(b)] (ml)	Sucrose (g)
0.46	0.435	813	0	(4.751 g)[(c)]	0
1.13	0.0725	135.5	135.5	9.3	9.27
1.38	0.0483	90.3	180.7	6.2	12.37
1.78	0.0290	54.2	217	3.7	14.86
2.19	0.0193	36.1	235	2.48	16.09
2.52	0.0145	27.1	243.9	1.86	16.70
3.09	0.00967	18.1	253	1.20	17.32
3.58	0.00725	13.6	251.4	0.90	17.62
4.74	0.00414	7.7	263.3	0.48	18.03
5.64	0.00290	5.4	265.6	0.33	18.18

(a) - The osmolarity of Standard Buffer was measured to be 271 mosm/Kg. The osmolarity of the low ionic strength NaCl solutions were calculated from the dilution used.

(b) - The required mM NaCl was calculated from the mosm/Kg NaCl. For ionic strengths below 0.01, the contribution from the 0.3 mM NaCl has been subtracted.

(c) - This, the only non-low ionic strength buffer, was made by adding solid NaCl to the 0.3 mM $NaHCO_3$.

Sucrose was added to restore the tonicity to that of the Standard Buffer. Low-ionic-strength media were prepared by diluting 1.45M NaCl with 0.0003M $NaHCO_3$ to the proper concentration and adding sucrose to bring the tonicity to a nominal 270 mosm.

Low-ionic-strength samples were prepared for electrophoresis in the following manner. Three or four drops of cell suspension were transferred to a 35ml centrifuge tube, the tube was filled to 30ml with the low-ionic-strength solution, then centrifuged at 1100 × g for native cells or 810 × g for GA-fixed cells. The supernatant was removed by vacuum aspiration, the tube was refilled to the 30ml mark with the correct solution. The pH was corrected to pH 7.2±0.2 with an HCl or NaOH solution of the corresponding ionic strength.

2.1.5. Viscosity adjustments

Viscosity adjustments for the calculation of the surface charge density from the measured electrophoretic mobility (eqn. 5), were made using the data in figure 2.1. These data represent viscosity measurements made for buffer solutions of several ionic strengths containing sufficient sucrose to give isotonic conditions, using an Ostwald 80–100 sec viscometer (VWR-Scientific Co.) to measure the viscosity, determined from the time required for the solution to move a fixed distance under the influence of gravity. A glass pycnometer (constructed at Lawrence Berkeley Lab) was used to determine the density of the sample (ρ) relative to that of deionized water (ρ_o) by weighing a sample of both. The relative viscosity (η_r), was then calculated from the relationship (Bull, 1971)

$$\eta_r = (\eta/\eta_o)_{T=25°C} = (\rho t)/(\rho_o t_o)_{T=25°C}, \qquad \text{eqn. 8}$$

where t and η are the outflow time and viscosity, respectively, of the electrophoretic buffer solution and t_o and η_o are the corresponding quantities determined for deionized water at the same temperature. Whenever it was necessary to compare mobilities at different ionic

Figure 2.1

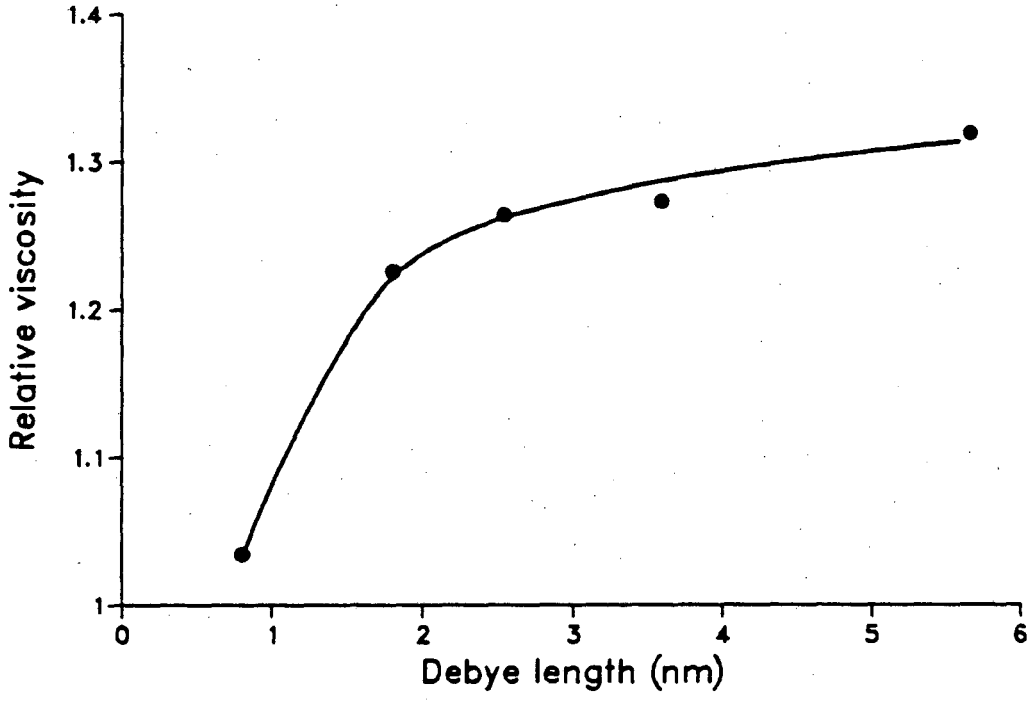


Figure 2.1. Relative viscosity vs. Debye length for the buffered saline solutions (containing added sucrose), which were used for the electrophoretic mobility measurements. Measurements were taken as described in the text.

strengths, viscosity adjustments were also made to the mobilities (without necessarily proceeding with the full surface-charge-density calculations).

2.1.6. Statistical methods

When several experiments of the same type were carried out, the data were averaged, and the errors were calculated as the standard error of the mean. Tests of significance of differences between two populations were made using Student's t-test (Spatz and Johnston, 1981).

2.2. Cell preparation techniques

2.2.1. Native cell suspensions

Expired human blood was obtained from the Alameda-Contra Costa blood bank. Fresh rat blood samples were drawn from the vena cava of anesthetized WAG/Rij rats (bred at the Lawrence Berkeley Laboratory). All blood samples were washed in approximately 15 volumes of Standard Buffer (0.145M NaCl and 0.0003M $NaHCO_3$). The cells were spun at 1100 × g for three minutes, the buffy coat was removed by aspiration, and the cells were washed three additional times, spinning at 1100 × g each time. They were then suspended as a "cell stock suspension" in an equal volume of Standard Buffer and kept at in an ice bath until needed. Cell samples were used the same day, usually within four hours after preparation.

2.2.2. Ghost preparation

2.2.2.1. Historical background

The effects of variations in the osmolality and the pH of phosphate buffer on the integrity of erythrocyte ghosts were studied by Dodge, et al. (1963). They concluded that 20

mosm and pH 7.4 were optimum conditions for performing erythrocyte lysis while minimizing the loss of membrane components. Bramely et al. (1971) did studies on the effects of variations in osmolality, using bicarbonate buffers containing no divalent cations. They tested for hemoglobin and non-heme protein retention, and also tested membrane function using measurements of phosphatase activity, cholinesterase activity, and ATPase activity. They found that the most drastic effects on membrane integrity and function occurred below 20 mosm, while hemoglobin retention increased above 40 mosm.

Johnson (1975) showed that the hemoglobin retained after ghost preparation was probably trapped inside the cell during resealing. In order to minimize the possible effect of hemoglobin adsorbed at the outer membrane surface on electrophoretic mobility measurements, the 20 mosm buffer was used for hemolysis in the experiments reported here.

Several researchers tested the effects of Ca^{++} and Mg^{++} on ghost preparation. Johnson and Kirkwood (1978) and Bramely and Coleman (1972) found that Ca^{++} or Mg^{++} ions restore the impermeability of the membrane, and prevents loss of resealing ability.

Bodeman and Passow (1972) studied the effects of temperature on the resealing process and showed that no resealing takes place at 0°C but does at higher temperatures.

2.2.2.2. Present experiments

In the experiments reported here, the 20 mosm phosphate buffer of Dodge et al. (1963) was supplemented with 1mM $MgCl_2$ to minimize the loss of membrane components. The cells were collected and washed in Standard Buffer. Twenty five ml of the hemolysing solution was added to 3ml stock cell suspension. The cells were spun at 13,000 × g for 10 minutes and washed 3 times in a 20mM phosphate solution containing 1mM $MgCl_2$. Care was taken to remove the red pellet at the bottom of the tube since this has been shown to contain protease

activity (Fairbanks, et al., 1971). The cell membranes were then resuspended in 30ml of Standard Buffer and allowed to reseal for 10 minutes at room temperature. They were then centrifuged once again at 13,000 × g, suspended 50% (v/v) in Standard Buffer, and retained in an ice bath until used.

2.2.3. Glutaraldehyde fixation

A glutaraldehyde concentration of 2.5% (v/v) was initially used for fixation. The 2.5% fixation method was carried out in Sorensens buffer (1 vol. 0.067M KH_2PO_4 + 4 vol. 0.067M $Na_2HPO_4 \cdot 7H_2O$, pH 7.4). Eight percent glutaraldehyde (Polysciences, Inc., Warrington, Pa.) was diluted with Sorensens buffer to 5% (w/v). Cells diluted 1:20 (v/v) with Sorensens buffer were mixed with an equal volume of 5% glutaraldehyde and incubated at room temperature for 10 minutes, and then spun at 1100 × g for 3 minutes and washed similarly three times in Standard Buffer.

However, Mel and Yee (1975) showed that a 2.5% concentration of glutaraldehyde produces changes in cell size and shape. These changes can be prevented by using a lower concentration of glutaraldehyde in a hyperosmolal solution. Based on their findings the following method was used for most of the work reported here. Eight percent glutaraldehyde (Polysciences, Inc., Warrington, Pa.) was diluted with Dulbecco's phosphate-buffered saline (PBS from Grand Island Biological, Grand Island, NY) to a 0.25% (v/v) concentration. The osmolality was increased to 355 mosm by the addition of NaCl. Five ml of stock cell suspension (at a concentration of approximately 5×10^9 cells/ml) were added dropwise with mixing to 35ml glutaraldehyde, and allowed to sit undisturbed for 30 minutes. The sample was centrifuged at 810 × g, the supernatant discarded, and the cells washed once in PBS and once in Standard Buffer at 810 × g.

2.3. Agglutination techniques

Concanavalin-A (lectin) from Jack Bean (Sigma Chemical Co., St. Louis, Mo.), and wheat germ agglutinin (B grade; Calbiochem-Behring Corp, La Jolla, Ca.) were both prepared at a 1 mg/ml concentration in buffer solutions of the desired ionic strength. Erythrocytes were counted in a hemocytometer, and diluted in a pH 7.4 solution at the desired ionic strength to 10^8 cells/ml (Schnebli and Bächi, 1975). Equal volumes of cell suspensions and lectin solution were transferred to a compartment of a 3040 Microtest II, flat bottom tissue culture plate (Falcon, Oxnard, Ca.). The plates were shaken for one minute on a rotary shaker and then left undisturbed for one hour. Agglutination was observed at 40× magnification and scored as (-), (+_), (+), (++) or (+++).

2.4. Assay Methods

2.4.1. Phosphate assay

Two phosphate assay methods were used, as described in Dittmer and Wells (1969). The method of Fiske and Subbarow was used in early measurements. The Bartlett modification of the Fiske and Subbarow method was employed in later measurements since it could detect smaller amounts of phosphate.

2.4.1.1. Fiske and Subbarow method

The reducing agent was prepared using 30g sodium bisulfite, 6g sodium sulfite and 0.5g 1,2,4-aminonaphthol sulfonic acid in 250 ml deionized water. This solution was kept in the dark for three hours and was then filtered into an amber bottle and kept refrigerated until used, but never longer than 8 weeks.

Samples were transferred to a round bottom tube, the liquid was evaporated over a

boiling water bath, 0.8 ml perchloric acid was added, the tubes were closed with a marble and the samples heated over a heating mantle for 20 minutes or until clear. Standards were prepared containing 0.1, 0.5 and 1.0 μmoles KH_2PO_4 (Mallinckrodt, St. Louis) in 0.1ml H_2O. Perchloric acid was added but standards were not heated, following Dittmer and Wells (1969).

To both standards and samples, 6 ml of water was added, followed by 0.5 ml of a 5% ammonium molybdate solution in water. The tubes were shaken on a vortex mixer. Then 0.4 ml reducing agent was added, plus water to bring the final solution to 10ml. After thorough mixing, the solutions were allowed to stand for one hour at room temperature. The optical density was read at 660nm on a Gilford spectrophotometer (Gilford Instrument Laboratories, Inc, Oberlin, Oh).

2.4.1.2. Bartlett Assay

The reducing agent prepared as described above was diluted 1:12 in water. The samples were digested in 0.4ml of 70–72% perchloric acid as in the Fiske and Subbarow assay. The ammonium molybdate was prepared using 1.1g ammonium molybdate, 3.5ml reagent grade 10N sulfuric acid, plus water to yield a final volume of 250ml.

After digestion, the samples were cooled, and 2.4ml ammonium molybdate solution was added. The samples were heated in a boiling water bath for 10 minutes, cooled, and the absorbance was read at 830nm on the Gilford spectrophotometer using a red filter.

2.4.2. Choline Assay

The choline assay used was the reineckate method described in Dittmer and Wells (1969), as modified by Argoudeles and Tobias (1975) to use acetonitrile as the solvent. Standards containing 0.15, 0.2, 0.3, 0.4 μmoles choline iodide were prepared and adjusted to 3ml with water. Both samples and standards were cooled, brought to pH 10.5 ± 0.5 with

concentrated NaOH, and the volume adjusted to 6ml with water. The solutions were filtered through Whatman #1 filter paper into round-bottom centrifuge tubes. The paper and the interior of the tubes were washed with three 0.5ml aliquots of water, and the washings combined with the samples which were placed in an ice bath. A solution of 1ml of 5% ammonium reineckate ($[(NH_3)_2Cr(SCN)_4]NH_4H_2O$) in methanol was added, and the samples were kept on ice in the dark for two hours. They were then centrifuged at 4°C at 3000 × g in a Beckman J6B centrifuge for 10 minutes, and the supernatant was removed by vacuum aspiration. The residue was next washed two or three times with 1ml ice-cold propanol, rinsing the sides of the tubes and mixing well, followed by centrifugation in the cold at 3000 × g. The precipitate was suspended in 3ml acetonitrile (photrex reagent, J.T.Baker, Phillipsburg, N.J.) and the optical density was measured at 312nm using uv illumination on a Gilford spectrophotometer.

Several precautions were necessary for this assay to be successful. All glassware was washed in an acid bath, the solutions were kept cold and were protected from light as much as possible, the vacuum aspiration was done with great care to prevent the removal of any precipitate, and the samples were well mixed before each centrifugal wash.

2.4.3. Sialic acid assay

Sialic acid was assayed using the method of Warren (1959). The following solutions were prepared.

A. Sodium periodate (meta), 0.2M in 9M phosphoric acid.

B. Sodium arsenite, 10% in 0.5M sodium sulfate-0.1N H_2SO_4.

C. Thiobarbituric acid, 0.6% in 0.5M sodium sulfate.

A standard containing 0.04μmoles of neuraminic acid in 0.4ml water was prepared. A 0.4ml aliquot of each sample was transferred to a 12ml centrifuge tube with 0.2ml of solution A. The

tube was shaken and allowed to stand 20 minutes at room temperature. One ml of solution B was added and the tube was shaken until the yellow-brown color disappeared. Three ml of solution C was then added, the tube capped with a glass bead, and boiled vigorously in boiling water for 15 minutes. The tube was then placed in cold water for 5 minutes, and 2ml was transferred to a tube containing 2ml of cyclohexanone. The tube was shaken twice, then centrifuged at 1100 × g for three minutes. The upper layer was removed and the optical density measured on a Gilford spectrophotometer at 549nm and 532nm in visible light with no filter. The sialic acid was calculated using the relationship (Warren, 1959):

$$\mu\text{moles sialic acid} = 0.09\times(OD)_{549} - 0.033\times(OD)_{532}$$

2.4.4. Lowry protein assay

The method used is that of Lowry, et al., 1951. Three solutions were prepared.

A. 2% Na_2CO_3 in 0.10N NaOH.

B. 0.5% $CuSO_4.5H_2O$ in 1% Na-K-Tartrate.

C. 50ml A + 1ml B (used the same day).

Standards containing from 25μg to 250μg protein in 0.5ml saline were prepared from purified bovine serum albumin (Pentex, Miles-Lab, Inc., Kankakee, Il). Half an ml of each sample was mixed well with 4ml of solution C and allowed to stand at room temperature for at least 10 minutes. Half an ml of Folin-Ciocaleau phenol reagent (Uni-tech, Sun Valley, Ca, diluted 1:1 with water just before using) was added, mixed very quickly (1 or 2 seconds), and allowed to stand for 30 minutes. The optical densities were then read at 600nm on a spectrophotometer (Bausch and Lomb, Rochester, NY).

2.4.5. Azocoll analysis for proteolytic enzymes

Azocoll analysis (Calbiochem-Behring) for contaminating proteolytic enzymes (Seaman

et al. 1967; Tenforde, 1970) was carried out on several of the phospholipase enzymes. This procedure involves a colorimetric measurement of the amount of dye released by a proteolytic enzyme from an insoluble collagen-azo dye complex. The sample is incubated at 37°C for 15 minutes, the insoluble substrate is removed by filtration (Swinnex #13 filter), and the optical density of the supernatant is read at 580nm with a Bausch and Lomb spectrophotometer. As a standard, the amount of dye solubilized by 0.1mg/ml trypsin (highly purified porcine pancreas trypsin from Sigma Chemical, St. Louis), pH 7.4, was determined. The activity of the sample is reported as the ratio of its optical density to that of the standard, in "equivalent trypsin units".

Some measurements of the proteolytic activity were also carried out in the presence of 1mM Di-isopropyl-fluoro-phosphate (DFP) from Aldrich (St. Louis, Mo.) and with 1mM phenyl methyl sulfonyl fluoride (PMSF) from Calbiochem-Behring, La Jolla, Ca., both of which inhibit proteolytic enzyme activity of serine proteases. The former is an extreme poison and must be handled with care. Both inhibitors were a gift of Herb Moise and Junko Hosoda of Lawrence Berkeley Lab.

2.5. Enzyme treatments

2.5.1. Neuraminidase treatment

Neuraminidase from *Vibrio cholerae,* (Calbiochem-Behring Corp., La Jolla, Ca.) was mixed with 1% $CaCl_2$ (w/v) in Standard Buffer in a concentration of 62 units/ml, where 1 unit is the amount of the enzyme required to release 1 μg of N-acetylneuraminic acid from human α_1-acid glycoprotein in 15 minutes at 37°C. The pH was adjusted with 0.145M NaOH to fall within the range of 7.0 to 7.2. Inactivated neuraminidase for the controls was prepared by heating in a boiling water bath for 5 minutes.

Cells and neuraminidase at a concentration of 62 units neuraminidase per 10^{10} cells

were incubated at 37°C for 30 minutes. The samples were spun down and the supernatant was saved for later sialic acid determination. The sample was then washed two times in Standard Buffer (method modified from Tenforde, 1970).

2.5.2. Phospholipases

2.5.2.1. Phospholipase-C treatment

Phospholipase-C (PLC) from *Bacilus cereus* (50 units/mg solid) and *Clostridium perfringens* (210 units/mg protein, 7% protein) were obtained from Sigma Chemical Co. (St. Louis, Mo.). PLC from both *B. cereus* or *C. perfringens* sources removed phosphocholine from glutaraldehyde-fixed cells. (Neither enzyme could be used on unfixed cells without some hemolysis). Both require calcium ions for full activity. The *C. perfringens* enzyme from Sigma Chemical was chromatographically purified by the manufacturer. Another *C. perfringens* enzyme from Calbiochem-Behring (La Jolla, Ca.) was not highly purified. Table 2-2 contains results of testing these enzymes for proteolytic activity using the azocoll method.

TABLE 2-2

Proteolytic activity of PLC enzymes using the Azocoll method.

	% activity relative to 0.1mg/ml trypsin[a]	
Enzyme tested	Enzyme alone	Enzyme with PMSF[b]
PLC (*B. cereus*) (Sigma)	1.4	
PLC (*C. perfringens*) (Calbiochem-Behring)	39.	36.
PLC (*C. perfringens*) (Sigma) chromatographically purified	1.7	

(a) - Highly purfied trypsin from Porcine Pancreas (Sigma Chem, St. Louis)

(b) - Phenyl methyl sulfonyl fluoride (1mM)

For treatment with *B. cereus* PLC, glutaraldehyde fixed erythrocytes were suspended at a concentration of 2.5×10^9 cells/ml in Standard Buffer containing 1mM $CaCl_2$ and 10 units of *B. cereus* PLC. (One unit liberates 1.0 μmole of water-soluble organic phosphorous from soybean lecithin per minute at 37°C, pH 7.3). Half of the cell suspension was made 4mM in o-phenanthroline which acts as an inhibitor of PLC activity (Ottolenghi and Bowman, 1970). The cells were incubated at 37°C for 10 minutes, and then spun at 810 × g. The supernatant was saved for later phosphate assay. The cells were washed three times in Standard Buffer at 810 × g.

For treatment with *C. perfringens* PLC, glutaraldehyde-fixed cell suspensions at 2.5×10^9 cells/ml were suspended in an equal volume of Standard Buffer containing 1.0mM $CaCl_2$ and 15 units of the enzyme (1 unit releases 1 μmole of water-soluble organic phosphorus from L-α-phosphatidyl choline per minute at 37°C, pH 7.3). The control was made 4mM with o-phenanthroline which acts as an inhibitor of PLC. The cells were incubated at 37°C for 15 minutes, centrifuged at 810 × g, and washed twice in Standard Buffer.

2.5.2.2. Phospholipase-D treatment

Phospholipase-D (PLD) from several sources was used in these investigations. PLD from peanut was obtained from Sigma Chemical Co. (St. Louis, Mo) and from Calbiochem-Behring (La Jolla, Ca). Since this enzyme lysed unfixed cells it was used only on glutaraldehyde-fixed cells. PLD from *Streptomyces chromofuscus* was obtained from Dr. Milton Friedman and Dr. Nejat Duzgenes of the University of California, San Francisco and from Dr. Shigeyuki Imamura of the Toyo Jozo Co., LTD. (Mifuku 632, Ohito-Cho, Tagata-Gun, Shizuoka, 410-23, Japan). The enzyme from Dr. Imamura, a very highly purified enzyme, was received and kept suspended in 25% sucrose-10mM tris-HCl buffer at pH 8.0. This enzyme could be used on the intact cell without extensive hemolysis. Only the highly purified enzyme

could be used on erythrocytes. A less highly purified enzyme, also received from Dr. Imamura, caused hemolysis of the unfixed erythrocytes, and was only slightly active on GA-fixed erythrocytes.

The proteolytic activities of the peanut enzyme and a cabbage PLD from Sigma Chemical Co. (catalog number P-7758) were assayed using the azocoll method described in section 2.4.5. The results, shown in table 2-3, show very little, if any, proteolytic activity of any of these enzymes. (Since only a few attempts were made to use the cabbage enzyme at an early stage in the experiments these results are not included in the discussion).

TABLE 2-3

Proteolytic activity of PLD enzymes using the Azocoll method.

	% activity of 0.1mg/ml trypsin	
Enzyme	Enzyme Alone	Enzyme with DFP
PLD (cabbage-Sigma)	1	—
PLD (peanut - Sigma)	3	2
PLD (peanut - Calbiochem-Behring)	1.2	1

2.5.2.2.1. Peanut PLD

The peanut enzyme was assayed by Sigma at 100 units/mg where one unit will liberate 1 μmole choline per hour at 30°C, pH 5.6. To verify the activity, Sigma's assay method (see Sigma Chemical reference) was used to assay the activity by measuring the release of ^{14}C-choline from L-α-phosphatidyl [N-methyl-^{14}C] choline, dipalmitoyl (Specific activity 25μCi/ml, from Amersham Corp, Arlington Heights, Il). The radioactive choline was quantitated in a scintillation counter after extracting the choline from the 1ml reaction mixture with 4 volumes

of chloroform/methanol (2:1). A 0.5ml aliquot of the upper methanol-water phase was removed, and added to 10ml Aquasol scintillation fluid (New England Nuclear, Boston, Ma.). Reineckate-choline assays (see section 2.4.2) were done in parallel using the same reaction conditions (0.1M acetate, 2.5mM sodium lauryl sulfate, 50mM $CaCl_2$) on a substrate of 0.05ml lecithin (5μmoles/ml) with 0.05mg PLD/sample in a total volume of 1ml.

Assays gave results consistent with those of Sigma Chemical with activities of from 65 to 109 units/mg. A time study of enzyme activity using both the radioactivity assay on lecithin substrate and the reineckate-choline assay on cells (table 2-4) showed that under these reaction conditions, the reaction was almost complete after 90 minutes incubation.

TABLE 2-4

PLD (peanut) reaction time study

Reaction time (min)	μmoles released from 1 μmole lecithin	μmoles released from 10^{10} cells
30	0.20	—
60	0.50	0.42
90	0.87	0.53
120	0.90	0.55

The following method was used to treat the cells with the peanut enzyme. Cells fixed with glutaraldehyde (0.25% v/v) to stabilize them against hemolysis, were washed once in a 0.1M acetate solution. Two and one half ml cells (50% v/v in the reaction solution) were added to 1.3ml of the reaction mixture (0.1M acetate, 2.5mM sodium lauryl sulfate and 50mM calcium chloride) containing 1mg PLD (100 units/sample). The control sample was incubated in the same reaction mixture but without the PLD. All samples were incubated with shaking at 30°C for 90 minutes, the samples were spun at 810 × g, the supernatant was

removed and mixed with 4 volumes chloroform/methanol (2:1). The phases were separated by centrifugation and the upper methanol/water phase was removed and saved for later assay of choline. The cells were washed twice in Standard Buffer and kept on ice until used for electrophoresis. Unless otherwise noted, cells were used the same day.

2.5.2.2.2. S. chromofuscus PLD

The treatment with the *S. chromofuscus* enzyme was carried out on unfixed cells for most experiments, and on 0.25% glutaraldehyde-fixed cells to prevent hemolysis at extreme values of pH during the measurements of electrophoretic mobility vs. pH. Early attempts to incubate the cells in weakly-buffered standard saline as in Fujii and Tamura (1979) were unsuccessful, probably because of the difficulty in keeping the pH at a constant value. The method reported in the authors' later paper (Tamura and Fujii, 1981) using tris-HCl saline was more successful in removing choline from the cells. The cells were washed once in a tris-HCl saline solution made 5mM in $CaCl_2$ and 0.25mM in $MgCl_2$, pH 7.4. PLD (0.25 ml, 170 U/ml) in 5ml of the tris solution was mixed with 0.5 ml stock cell suspension. The reaction mixture contained about 40 units of the PLD enzyme. The control contained no PLD. The mixture was incubated with gentle shaking at 37°C for 2 hours, then spun down at 1100 × g, and the supernatant was treated as above with chloroform/methanol (2:1) and saved for later choline assay.

2.6. Lipid extraction

Lipid extraction in chloroform/methanol was performed using the method described in Nelson (1967). One ml of glutaraldehyde-fixed erythrocytes was added to 17ml methanol while stirring with a magnetic stirrer. 34ml chloroform was added and stirring continued for 5 minutes. The samples were centrifuged at 1100 × g, and the extract filtered through fast filter paper (Whatman #41) and saved for later assay.

Lipid extraction with ethanol was performed as in Tenforde (1969). Erythrocytes were collected and washed in Phosphate-buffered saline (Grand Island Biological Company, Grand Island, N.Y.), fixed in 0.25% glutaraldehyde, and suspended in 10 volumes of 100% ethanol for 40 minutes at room temperature, then spun at 810 × g. The supernatant was retained for later assay. The cells were washed three times with Standard Buffer. An attempt was made to break up the cell clumps by either shaking on a vortex mixer or expulsion through a #25 gauge (5/8″) needle. The latter method was slightly more effective but neither was truly successful.

2.7. Charge blocking agents

In several experiments, charge blocking agents were used to determine if positive groups were contributing to the surface charge of the cell membrane.

2.7.1. 1,5-difluoro-2,4-dinitrobenzene

1,5-difluoro-2,4-dinitrobenzene (DFNB) from J.T.Baker, Phillipsburg, N.J., was prepared at a concentration of 2.8mM by first mixing 2 mg DFNB with 20ml methanol, and then diluting a 0.57ml aliquot to 100ml in Standard Buffer as described in Berg et al. (1965). One ml of cells (1:1 in Standard Buffer) was added to 20ml DFNB solution and incubated at room temperature with occasional stirring. A control in Standard Buffer was similarly incubated. After 30 minutes, the suspensions were spun at 1100 × g, and then washed three times in Standard Buffer at 1100 × g.

2.7.2. P-toluenesulfonyl chloride

P-toluenesulfonyl chloride (tosyl chloride, J.T.Baker, Phillipsburg, N.J.) was used as an amino group blocking agent. The compound was difficult to solubilize and caused slight hemolysis of unfixed cells. The method used was based on that described in Seaman and Heard (1960). Five volumes of tosyl chloride (1 mg/ml) in Standard Buffer was mixed on a

magnetic stirrer for 30 minutes, and then mixed with 1 volume stock cell suspension and incubated 30 minutes at 37°C. Controls were similarly incubated in Standard Buffer. Samples were washed three times in Standard Buffer at 810 × g.

2.7.3. Acetaldehyde

The acetaldehyde treatment method was that of Haydon and Seaman (1962). Twenty volumes of 1% acetaldehyde (w:v) in 50% 0.145M saline, 50% Sorensens phosphate buffer (0.0667M, pH 7.4) was added to a 50% cell suspension (v:v). After incubating at room temperature for 30 minutes, the suspension was centrifuged at 810 × g and washed once in Standard Buffer. (Some earlier acetaldehyde treatments were done in Standard Buffer rather than the more strongly buffered Sorensens buffer.)

CHAPTER 3

Effects of ionic strength, pH, and cell treatment

3.1. Effects of ionic strength on cell agglutinability

Because low ionic strengths were used in many of my experiments, it was important to investigate certain possible specific effects of low ionic strength on the membrane. It is possible that the low-ionic-strength medium might cause a rearrangement of the membrane structure, or the elution of components from the membrane surface. To investigate such effects on the carbohydrate-containing components of the membrane surface, several experiments were conducted to examine the effect of agglutinins (which are known to combine with the carbohydrates) on human erythrocyte membranes at low ionic strengths. Changes in membrane carbohydrate concentrations or orientations would be expected to lead to a change in agglutinability of the erythrocytes.

The agglutinins (or lectins) used were concanavalin A (ConA), wheat germ agglutinin (WGA), and soy bean agglutinin (SBA). These three agglutinins bind to different structures on the membrane. ConA binds to nonreducing terminal D-mannose, D-glucose or D-fructofuranosyl units, WGA binds saccharides containing N-acetyl-glucosamine (D-GlcNAc) residues, while soybean agglutinin binds to N-acetyl-galactosamine (D-GalNAc). Trypsinized cells are known to be agglutinated by several lectins. As a test of the activity of the lectin, trypsinized cells were treated with agglutinins and tested for agglutinability in a 0.145M NaCl solution, and all of the lectins used caused agglutination of trypsinized cells. Cells in the absence of lectin showed no agglutination under any conditions.

Non-trypsinized cells were tested at an ionic strength of 0.145 and at lower ionic

strengths. ConA agglutinated the native erythrocytes at all ionic strengths, with a slight decrease in agglutinability at low ionic strengths (see table 3-1). This was evidently not due to any permanent change in the cell surface as was shown by resuspending cells in 0.145M buffer after keeping them in a low-ionic-strength solution for one hour. All were as strongly agglutinated by ConA as was the 0.145M NaCl control. Other workers found that ConA does not agglutinate freshly drawn erythrocytes but Singer and Morrison (1976) determined that it does agglutinate ATP-depleted cells.

Cells fixed in 2.5% glutaraldehyde were also treated with ConA. The results were similar to those of the unfixed cells, except for a larger decrease in agglutinability at the lowest ionic strength (Debye length = 5.7nm). The decrease in agglutination at low ionic strength might be a result of the reduced accessibility of specific groups by the lectin caused by the increased unshielded charge at low ionic strength. The additional reduction upon fixation might be due to the rigid structure of the fixed membrane.

Wheat germ agglutinin led to larger effects at the low ionic strengths, with no agglutination at all occurring at the lowest ionic strength (table 3-1). To test whether an irreversible denaturing of the WGA itself might be occurring at low ionic strength, some of the WGA in the low ionic strength medium was brought back up to high ionic strength by adding NaCl, and then tested for its ability to agglutinate cells. It induced near-normal agglutination under these conditions. Cells were also tested for any permanent (irreversible) effects of the low-ionic-strength exposure, by resuspending them in Standard Buffer and retesting for agglutination. All showed at least normal agglutination. Thus, it appears that either the WGA or the structures containing D-GlcNAc are affected by low ionic strength media, but that the effects are reversible.

Soy bean agglutinin did not agglutinate the normal red cells to any significant degree.

TABLE 3-1

Agglutination of human erythrocytes. Agglutination was tested with Conconavalin A and wheat germ agglutinin[a]. Control experiments on cells without lectin were carried out for all samples and showed no agglutination (results not shown).

		ConA[b] (0.5mg/ml)		WGA[c] (0.5mg/ml)	
Ionic Strength	Dil-ution	Native[d] Erythrocytes	GA-fixed[e] Erythrocytes	Native[d] Erythrocytes	GA-fixed[e] Erythrocytes
0.145M	1/1	++	++	++	++
0.029	1/5	+	++	+	+
0.0145	1/10	++	++	+-	+-
0.00725	1/20	+	+	+-	+-
0.00290	1/50	+	-	-	-

(a) - ConA and WGA from Boehringer Mannheim Biochemicals, Indianapolis, Indiana. Soybean agglutinin from Miles-Yeda Ltd., Israel, at a concentration of 200 μg/ml showed no agglutination with this technique.

(b) - Average results of four experiments.

(c) - Results of one experiment

(d) - As a reversibility test, cells at all low ionic strengths were resuspended in 0.145M Standard Buffer and tested for agglutinability; they were scored ++ with ConA, and +++ with WGA.

(e) - Cells were fixed with 2.5% glutaraldehyde.

SBA did, however, agglutinate trypsinized cells.

3.2. Comparisons of unfixed and fixed erythrocytes, and ghosts

3.2.1. Variation of mobility with ionic strength and pH.

Furchgott and Ponder (1941) did important early studies in the electrophoretic properties of erythrocytes at varying ionic strengths. They measured the mobility of unfixed erythrocytes as a function of both pH and ionic strength, and showed that at neutral pH, the electrophoretic mobility increased approximately linearly with Debye length, until the ionic strength fell below 0.02 (Debye length $\approx$ 2 nm).

Most of the definitive electrophoretic work on the erythrocyte using low-ionic-strength solutions has been by Seaman and his associates. Heard and Seaman (1960) determined the effects of pH- and ionic-strength-variations on the untreated erythrocyte. Isotonicity was maintained at low ionic strength by addition of sorbitol. Measurements were also taken without sorbitol, and these gave identical results, when corrected for the change in viscosity. These authors showed that the mobility increased as the ionic strength decreased, indicating that more negative charge was contributing to the mobility at the lower ionic strength. However, the calculated negative surface charge decreased (see chapter 2, eqn. 5), as the ionic strength decreased. This calculation indicated, then, that the increase in mobility was not due to new negative charges on the membrane, but rather to greater unmasking of charges, as a result of fewer counterions at the lower ionic strengths. And, in fact, the calculations would indicate that perhaps fewer negative charges were actually on the membrane at the lower ionic strengths, or that positively charged groups were contributing to the mobility in low-ionic-strength media.

These authors found that there was a pH range within which the cells remained stable,

(i.e. where reversible behavior occurred), and that this range became increasingly narrow as the ionic strength decreased. They determined mobility-pH curves at a series of ionic strengths, within the ranges for which the cells were stable at each ionic strength. Their conclusion was that no simple amino groups could be present in the outermost 1 to 2 nm of surface, since the mobility at each ionic strength was essentially constant, from pH 5.5 to 10.0. A further support for Heard and Seaman's conclusion came from their observation that there was no difference in the mobility of cells in aqueous solutions of chloride, bromide, iodide or thiocyanate ions at any ionic strength. If there were either non-ionic binding or ionic binding to positive groups, the degree of binding should depend upon the radius of hydration of the ion and should be different for different ions. (Some slight differences were seen for iodide ions.)

Figure 3.1 presents my electrophoretic data for one experiment on unfixed human red cells. Erythrocytes were suspended in solutions of low ionic strength made isotonic by the addition of sucrose. The electrophoretic mobility was measured and corrected for the increased viscosity of the sucrose solution using the data in figure 2.1. Also plotted is the surface charge density (figure 3.2), calculated using the equation $\sigma = \mu\eta/\ell_D$, (Chapter 2, eqn. 5).

Examination of figures 3.1 and 3.2 show that the results are similar to those of Furchgott and Ponder (1941), and Heard and Seaman (1960). Note that as the Debye length increases, the mobility also increases. However, as in the case of Heard and Seaman's data, this mobility increase is evidently not due to a true increase in membrane surface charge, as can be seen from figure 3.2. The calculated surface charge decreases as the Debye length increases, when the effect of the decreased screening of charge groups (at the lower ionic strength) is taken into effect by the Debye-Hückel calculations. Possible charge groups that could contribute to such a positive charge are the positive amino groups of protein side chains (lysine, arginine and histidine), the positive choline of PC or sphingomyelin, or the positive amino group of PE.

Figure 3.1

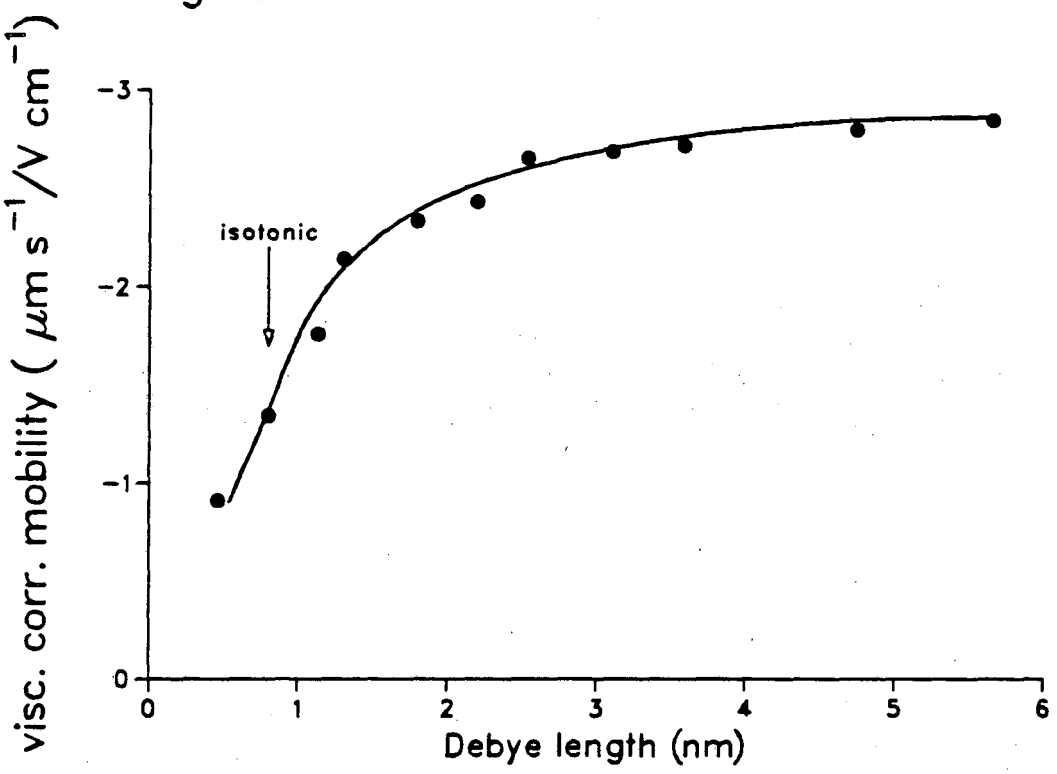


Figure 3.1. Viscosity-corrected electrophoretic mobility vs. Debye length for unfixed human erythrocytes. The cells were collected and washed, and the low ionic strength (high Debye length) suspensions were prepared, as described in section 2.1.4. Measurements were made at pH 7.2 ± 0.2; each mobility is the average for 20 measurements, on a total of 10 cells, 5 at each of the two stationary layers, with measurements in each direction for each cell. Viscosity corrections have been made using the data from figure 2.1. The arrow indicates the Debye length corresponding to isotonicity.

Figure 3.2

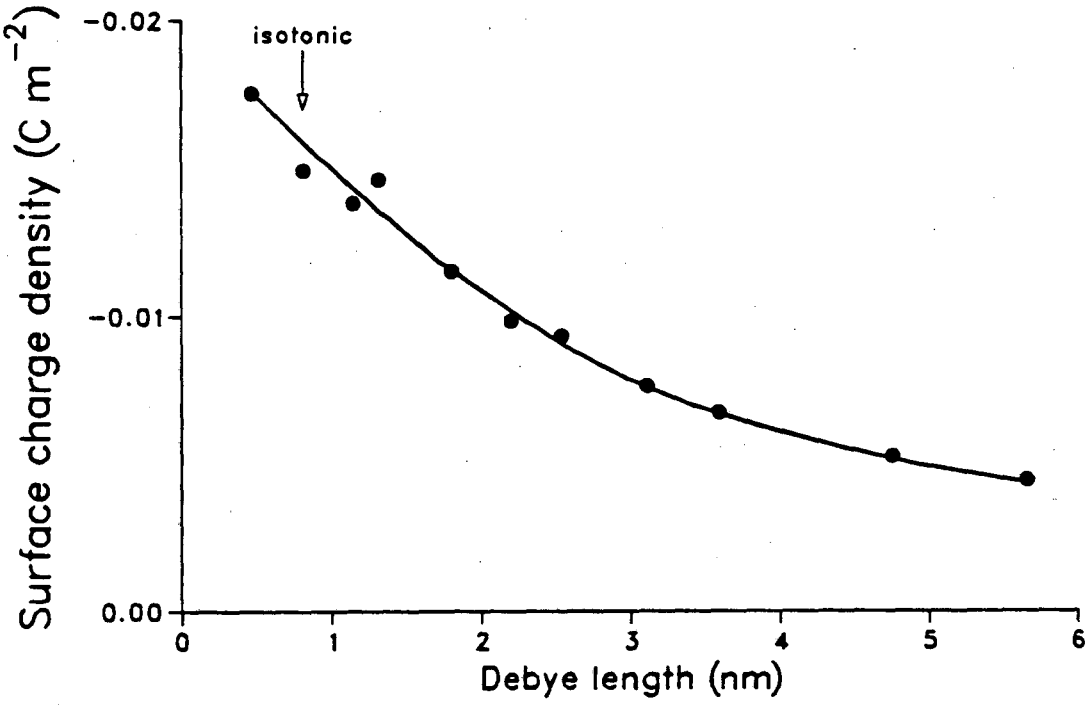


Figure 3.2. Surface charge density vs. Debye length for unfixed human erythrocytes. The surface charge density, σ, was calculated from the data of figure 3.1 using the relation, $\sigma=\mu\eta/\ell_D$, where μ is the electrophoretic mobility in μm s^{-1}/V cm^{-1}, η is the relative viscosity of the medium in poise, and $\ell_D \equiv 1/\kappa$ is the Debye length in nm. The arrow indicates the Debye length corresponding to isotonicity.

To further test for the presence of amino groups at the surface, native human erythrocytes were treated with tosyl chloride and DFNB (as described in chapter 2). Tosyl chloride reacts with positive amines. DFNB reacts with amino, sulfhydryl, tyrosyl, or histidyl groups, forming cross-links between groups if they are separated by about 0.5nm (Berg, et al., 1965). The results, shown in figure 3.3 (tosyl chloride) and figure 3.4 (DFNB), confirm Heard and Seaman's findings that no positive amino groups are available to these probes, at least to a depth of 5.7nm below the hydrodynamic surface of shear.

The presence of the other above mentioned positive groups will be discussed later.

3.2.2. Aldehyde fixation.

Several aldehydes have been used for fixation including glutaraldehyde (GA), acetaldehyde, and formaldehyde. Tenforde (1970) summarized the action of these fixatives. Briefly, glutaraldehyde reacts only with positive amino bases, forming a five-carbon bridge between neighboring groups. Evidence is that the amino groups of phosphatidyl serine, phosphatidyl ethanolamine, and lysine residues all can participate in the crosslinking reaction. The amino groups are thought to be converted to secondary amines, rather than Schiff bases, with the reaction product remaining stable at extremely acidic pHs. The probable reaction is shown in figure 3.5.

Acetaldehyde and formaldehyde are believed to combine with the positive amino groups of protein side chains, and with the amino group of phosphatidyl ethanolamine, but they apparently do not form cross bridges. Acetaldehyde, unlike glutaraldehyde, also combines with the guanidinium base of arginine. The reaction of acetaldehyde forms a Schiff base (figure 3.6), which again effectively neutralizes the positive charge, but the product may not be stable at low pH.

Figure 3.3

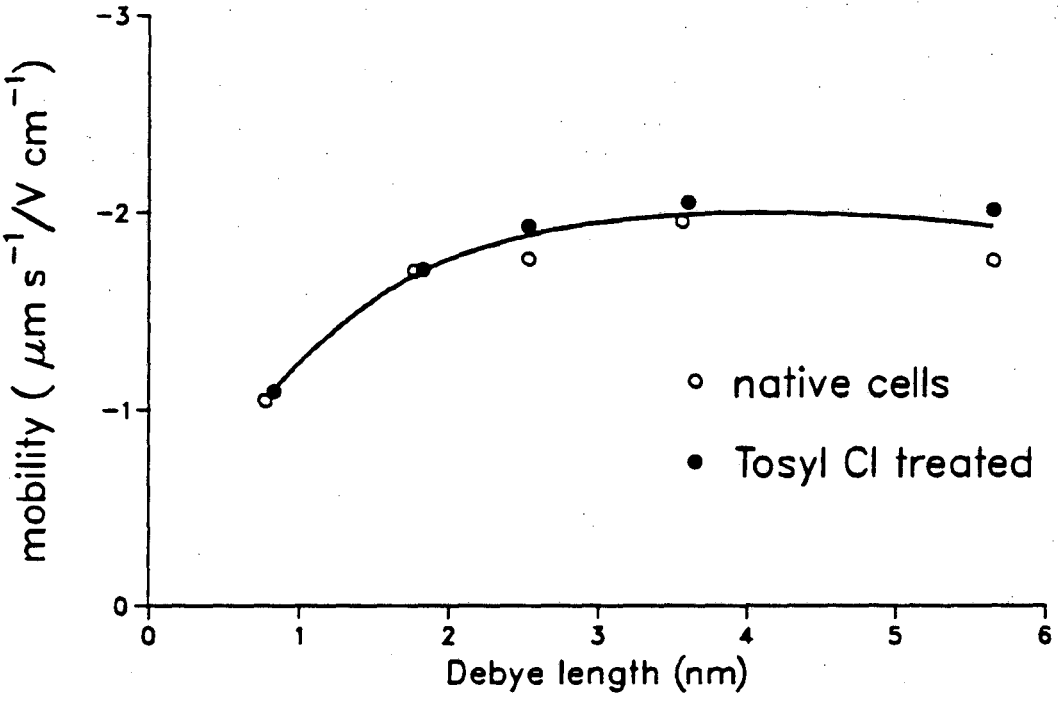


Figure 3.3. Electrophoretic mobility as a function of Debye length for native- (o) and tosyl-chloride- (•) treated human erythrocytes. For the tosyl chloride treatment, five volumes of tosyl chloride (1mg/ml) in Standard Buffer were mixed with 1 volume stock cell suspension for 30 minutes at 37°C.

Figure 3.4

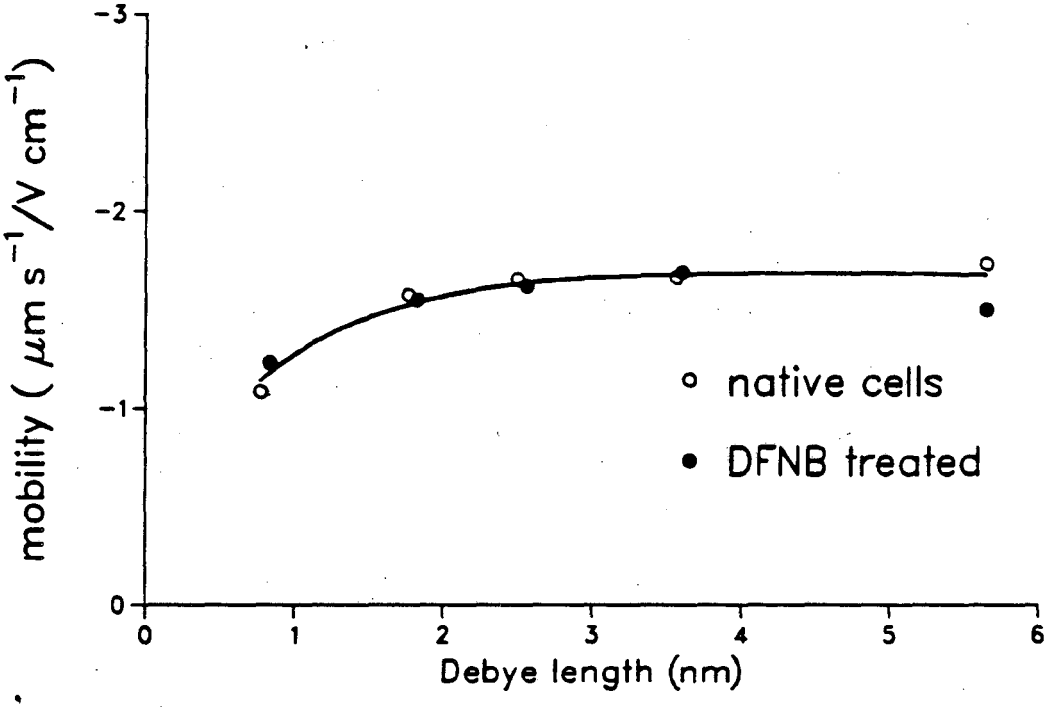


Figure 3.4. Electrophoretic mobility as a function of Debye length for native- (o) and DFNB- (•) treated-human erythrocytes. For the DFNB treatment, 20 volumes 2.8mM DFNB in Standard Buffer, were added to 1 volume stock cell suspension, and incubated for 30 minutes at room temperature.

Figure 3.5

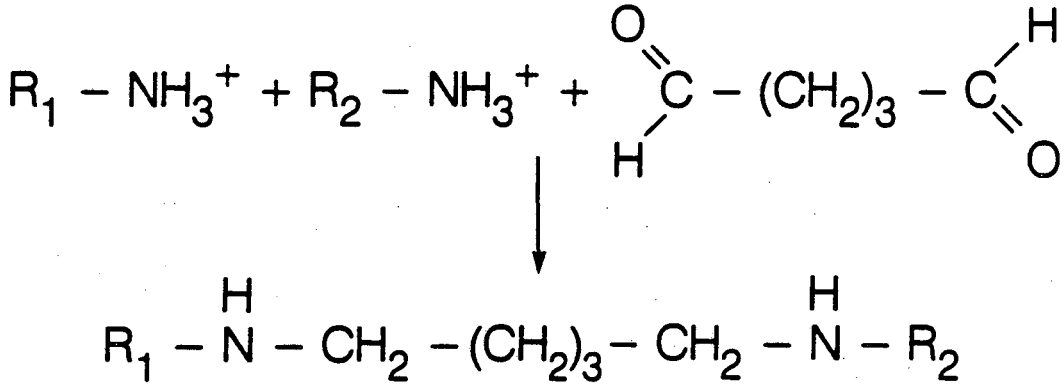
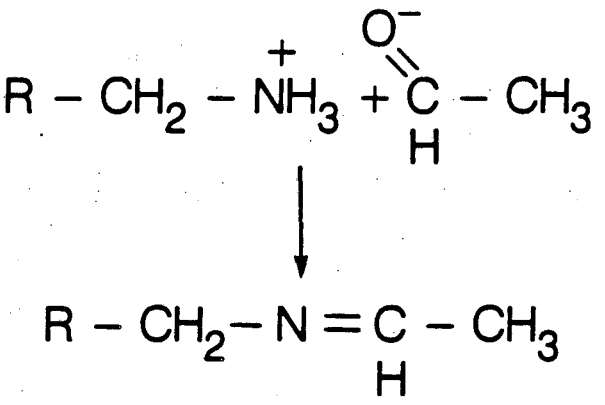


Figure 3.5. The presumed reaction of glutaraldehyde with positive amino groups. The primary amines of the reactants are protonated at pH 7.4, while the secondary amines of the reaction products are not.

Figure 3.6



XBL 8411-8938

Figure 3.6. The presumed reaction of acetaldehyde with positive amino groups with the formation of a Schiff base.

Heard and Seaman (1961) also measured the electrophoretic properties of human erythrocytes using cells that had been fixed in formaldehyde and acetaldehyde. They found that in the range of pH where the unfixed cells were stable, the fixed cells had the same electrophoretic mobilities as the unfixed cells, and that in the "metastable regions" (regions where the mobilities varied in time but could be restored to the initial values by washing and resuspending the cells in Standard Buffer), the fixed cells with added hemolysate gave the same results as the initial values of the unfixed cells. This indicated that the main effect of the instability at the pH extremes was the alteration in mobility due to hemoglobin leakage and adsorption. The aldehyde-fixed cells could not be taken below pH 2.7 without irreversible effects occurring. (Mel et al. (1973) were able to show that GA-fixed (rat) cells could be taken to low pH without irreversible effects; see below).

Heard and Seaman determined from the mobility-pH measurements that hydrogen ion dissociation occurs over a pH range of 2.5 to 4.0 for all ionic strengths tested, and they reported that an isoelectric point exists at about pH 2.5 for acetaldehyde-fixed cells. The aldehyde would be expected to combine with free amines. Since the aldehyde-treated cells and the native cells had the same mobility in the pH range of 6 to 10, this was further evidence that no amino groups were contributing to the mobility. (One caveat in the interpretation of these results is the work of Bishop and Richards (1968), who reacted glutaraldehyde with alpha-lactoglobulin lysine residues. They found that although glutaraldehyde reacts with primary amines, it has a minimal effect on pK; reducing it by at most 1 or 2 pK units. There have apparently been no similar studies using acetaldehyde).

Vassar et al. (1972) examined the effects of acetaldehyde, formaldehyde and glutaraldehyde (1.65%) on human erythrocytes. They determined that both acetaldehyde- and formaldehyde-fixed cells showed great leakage of hemoglobin whereas glutaraldehyde-fixed cells showed no leakage of hemoglobin. Their electrophoresis results, unlike Heard and Seaman's,

showed that the mobility of the glutaraldehyde-fixed cells increased by 10% at pH 7. They also determined an isoelectric point of 1.5 with the 1.65% glutaraldehyde-fixed cells, using cells that were examined from 1 hour to several days after glutaraldehyde fixation.

Mel, et al. (1973) examined some of the discrepancies in low pH measurements using an independent technique (stable-flow free boundary electrophoresis on rat erythrocytes) and showed that the report by Haydon and Seaman (1967) that erythrocytes do not have a positive mobility at low pH, was incorrect. They attributed variations in the electrophoretic mobility to operational factors causing changes in electro-osmotic flow profiles due to increased adhesion between cells and the glass chamber walls. Such adhesion has been observed for aldehyde-fixed cells at low pH. They gave a reasonable explanation for the greater distortion seen with the cylindrical chambers used by most experimenters, as opposed to the rectangular chamber used in their experiments (and the present experiments).

The effect of glutaraldehyde at certain concentrations on the charge properties of neuronal membranes has been demonstrated by Burry and Wood (1979) using electron micrographs of cationized-ferritin-treated cultured neurons. They determined that glutaraldehyde in high concentrations greatly changed the membrane surface charge of these cells. However, neuronal membranes differ in composition from erythrocyte membranes.

Because of the great variety of results reported in the literature regarding the effects of different concentrations of glutaraldehyde, it was necessary to repeat some of these experiments, using both a high (2.5%) and a low (0.25%) concentration of glutaraldehyde. Mel and Yee (1975) have shown that low concentrations of glutaraldehyde (0.05 to 0.25%), coupled with a specified higher salt content of the (hyperosmolar) medium, left the cells unchanged in size and form. The data described below show that higher concentrations of glutaraldehyde do in fact lead to a change in membrane electrophoretic properties, but that this change is not seen in

preparations treated at the lower (0.25%) fixative concentration.

As described in Chapter 2, human erythrocytes were fixed with 2.5% or 0.25% glutaraldehyde while rat erythrocytes (in earlier experiments) were fixed with 2.5% glutaraldehyde. The results of electrophoretic mobility measurements, as a function of Debye length, for the 2.5% GA-fixed human cells are shown in table 3-2 and figure 3.7, and for the rat cells in table 3-3. The results for the 0.25% GA-fixed human cells are shown in table 3-4 and in figures 3.8 and 3.9. The tables display both the mobilities and the calculated surface charges. Table 3-4 shows the surface charge calculations for a single experiment in greater detail. Figure 3.8 (raw mobilities) and figure 3.9 (viscosity corrected) display average data from a number of experiments.

The data show that a change in negative mobility occurs after fixation with 2.5% glutaraldehyde, and is especially pronounced at the lower ionic strengths. The human GA-fixed cells had significant (as determined by Student t-test) changes in mobility, which ranged from a 13% increase in Standard Buffer to a 17% decrease at 0.0029M (Debye length = 5.7nm). The rat erythrocyte data showed qualitatively similar results, but the Student t-test indicated that the differences were not significant.

For the 0.25% GA-fixed human cells, no significant difference is observed between the fixed and unfixed cell mobilities over the range of ionic strengths used. Thus the apparent effects of glutaraldehyde on electrophoretic mobility measurements are apparently the result of fixation at high GA concentration. With the lower concentration of glutaraldehyde in a 355mosm solution, the effect disappears.

Figures 3.10 (Standard Buffer) and 3.11 (low ionic strength medium, Debye length = 2.53nm) display the mobility vs. pH data for human erythrocytes. An isoelectric point of pH 2.75 was found for these cells, which is to be compared with Heard and Seaman's pH value of

TABLE 3-2

Electrophoretic properties of native and 2.5% glutaraldehyde-fixed human erythrocytes. The mobility and surface charge density are reported for several ionic strengths. The errors are standard errors of the mean.

Debye Length (nm)	Dilution	Electrophoretic mobility (μ)[a] ($\mu m\ s^{-1}/V\ cm^{-1}$)				Surface charge density ($10^{-3}\ C\ m^{-2}$)	
		Native[b] Erythrocyte	GA-fixed[c] Erythrocyte	% change	p Value[d]	Native Erythrocyte	GA-fixed Erythrocyte
0.80	1/1	1.17±0.02	1.32±0.03	12.8	<0.001	13.1±0.2	14.8±0.3
1.79	1/5	2.17±0.04	2.03±0.05	−6.5	0.02<p<0.03	10.9±0.2	10.2±0.3
2.53	1/10	2.58±0.06	2.31±0.06	−10.5	<0.01	9.1±0.2	8.2±0.2
3.57	1/20	2.86±0.09	2.43±0.06	−15.0	<0.01	7.2±0.2	6.1±0.2
5.65	1/50	3.02±0.11	2.50±0.07	−17.0	<0.01	4.8±0.2	4.0±0.1

(a) - Mobilities include a viscosity correction.

(b) - Each datum represents 340 measurements carried out in 17 separate experiments.

(c) - Each datum represents 180 measurements carried out in 9 separate experiments.

(d) - Calculated from Student's t-test.

Figure 3.7

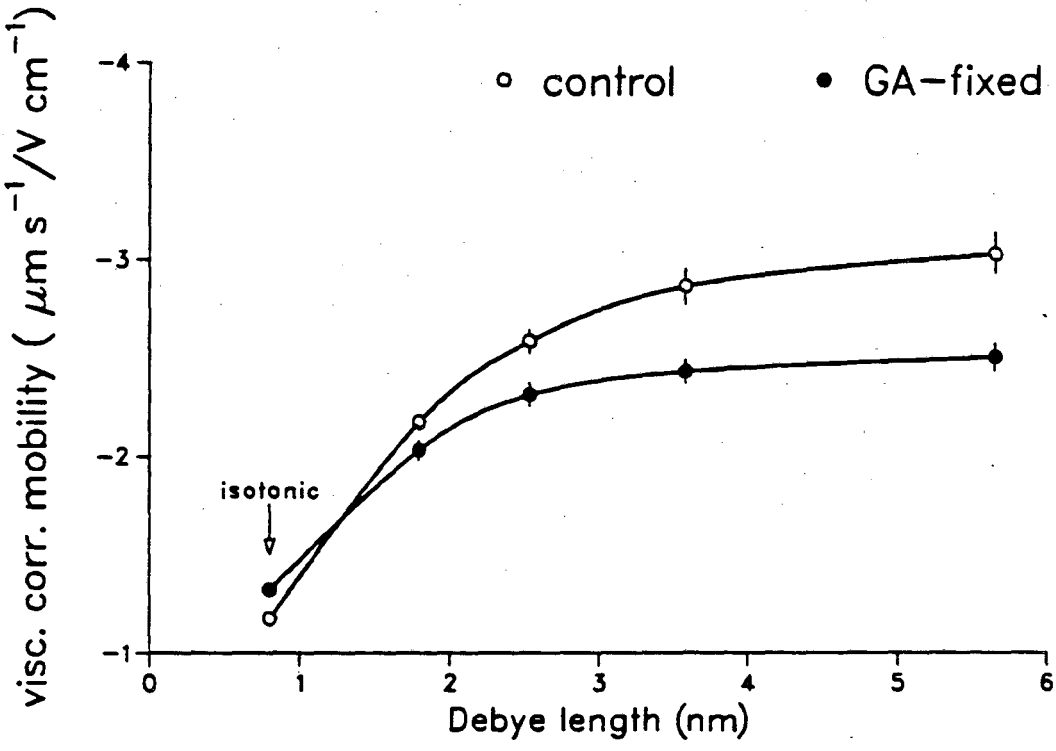


Figure 3.7. Viscosity-corrected electrophoretic mobility as a function of Debye length for native- (o) and 2.5% GA-fixed- (•) human erythrocytes. Data from table 3-2.

TABLE 3-3

Electrophoretic properties of native and 2.5% glutaraldehyde-fixed rat erythrocytes. The mobility and surface charge density are reported for several ionic strengths. The errors are standard errors of the mean.

Debye Length (nm)	Dilution	Electrophoretic mobility (μ)[a] ($\mu m\ s^{-1}/V\ cm^{-1}$) Native Erythrocyte		GA-fixed Erythrocyte		% change	p Value[b]	Surface charge density ($10^{-3}\ C\ m^{-2}$) Native Erythrocyte	GA-fixed Erythrocyte
0.80	1/1	1.37±0.03	(20)[c]	1.47±0.04	(16)[c]	7.3	0.05	15.3±0.3	16.4±0.4
1.79	1/5	2.66±0.11	(6)	2.50±0.22	(6)	−6.0	>0.5	13.3±0.6	12.5±1.1
2.53	1/10	3.22±0.06[d]	(5)	2.82±0.19[e]	(7)	−12.4	>0.1	11.4±0.2	10.0±0.7
3.57	1/20	3.94±0.14	(13)	3.40±0.24	(7)	−13.7	>0.05	9.9±0.4	8.5±0.6
5.65	1/50	4.20±0.17[d]	(15)	3.52±0.27[e]	(7)	−16.2	0.02<p<0.03	6.6±0.3	5.6±0.4

(a) - Mobilities include a viscosity correction.

(b) - Calculated from Student's t-test.

(c) - Numbers in parentheses represent the number of separate experiments, each of which represent 20 measurements.

(d) - When samples were resuspended in standard buffer, the mobility was 1.38 for the 1/10 and 1.42 for the 1/50 sample.

(e) - When samples were resuspended in standard buffer, the mobility was 1.35 for the 1/10 and 1.27 for the 1/50 sample.

TABLE 3-4

Electrophoretic mobility and surface charge density as a function of Debye length for 0.25% glutaraldehyde-fixed human erythrocytes.

Debye Length (nm)	Dilution	Uncorrected mobility (μ) ($\mu m\ s^{-1}/V\ cm^{-1}$)	Viscosity adjustment factor	Viscosity corrected mobility	Surface charge density (σ) ($10^{-3}\ C\ m^{-2}$)
0.46	3/1	0.88	1.03	0.91	17.5
0.80	1/1	1.30	1.03	1.34	14.9
1.13	1/2	1.54	1.14	1.76	13.8
1.30	1/3	1.78	1.20	2.14	14.6
1.79	1/5	1.90	1.23	2.33	11.5
2.19	1/7.5	1.94	1.25	2.43	9.8
2.53	1/10	2.10	1.26	2.65	9.3
3.10	1/15	2.11	1.27	2.68	7.6
3.58	1/20	2.11	1.29	2.71	6.7
4.74	1/35	2.13	1.31	2.79	5.2
5.67	1/50	2.15	1.32	2.84	4.4

(a) - $(\sigma) = \mu\eta/\ell_D$, where ℓ_D is the Debye length and η, the viscosity, is the viscosity correction factor times the viscosity of water (0.008904 poise).

Figure 3.8

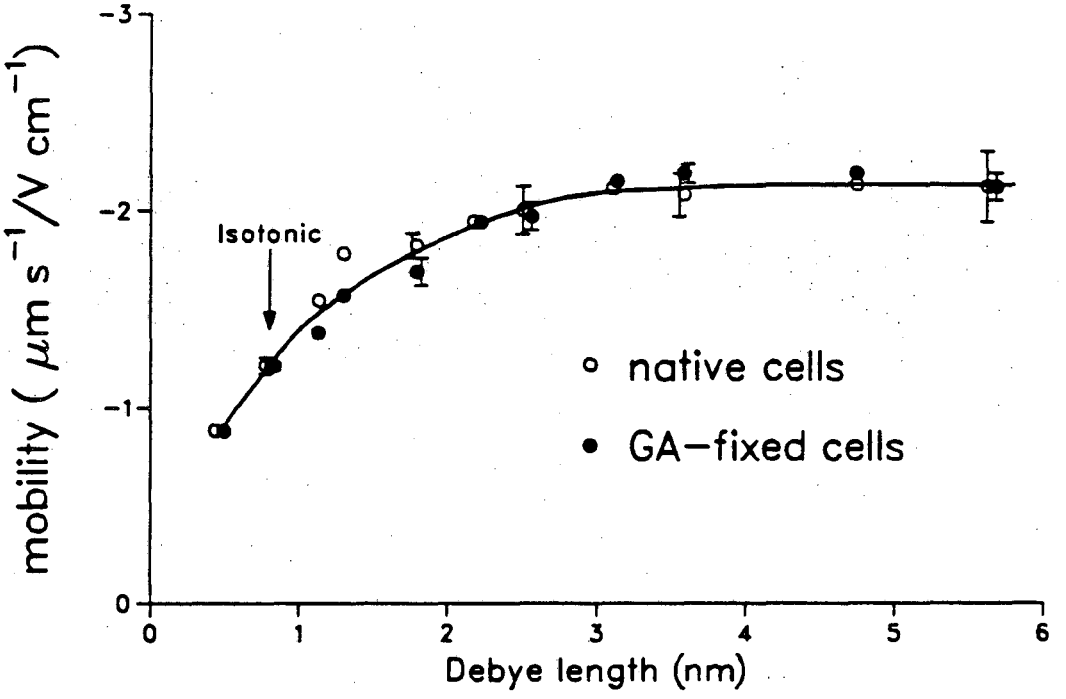


Figure 3.8. Electrophoretic mobility as a function of Debye length for native- (o) and 0.25% glutaraldehyde- (GA) fixed (•) human erythrocytes. Fixation was accomplished by incubating the cells for 30 minutes at a GA concentration of 0.25% at room temperature. Mobility was measured in Standard Buffer at pH 7.2±0.2. Error bars are shown for points measured in more than one experiment, and represent ±1 standard error.

Figure 3.9

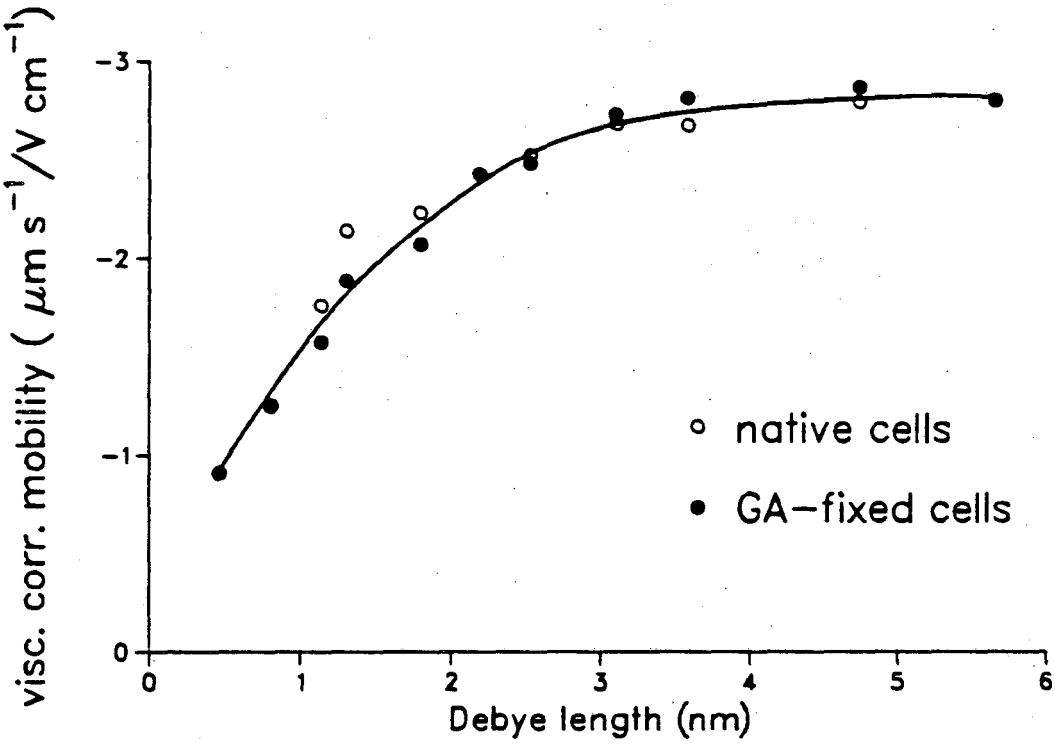


Figure 3.9. The data of figure 3.8 after making the viscosity correction.

Figure 3.10

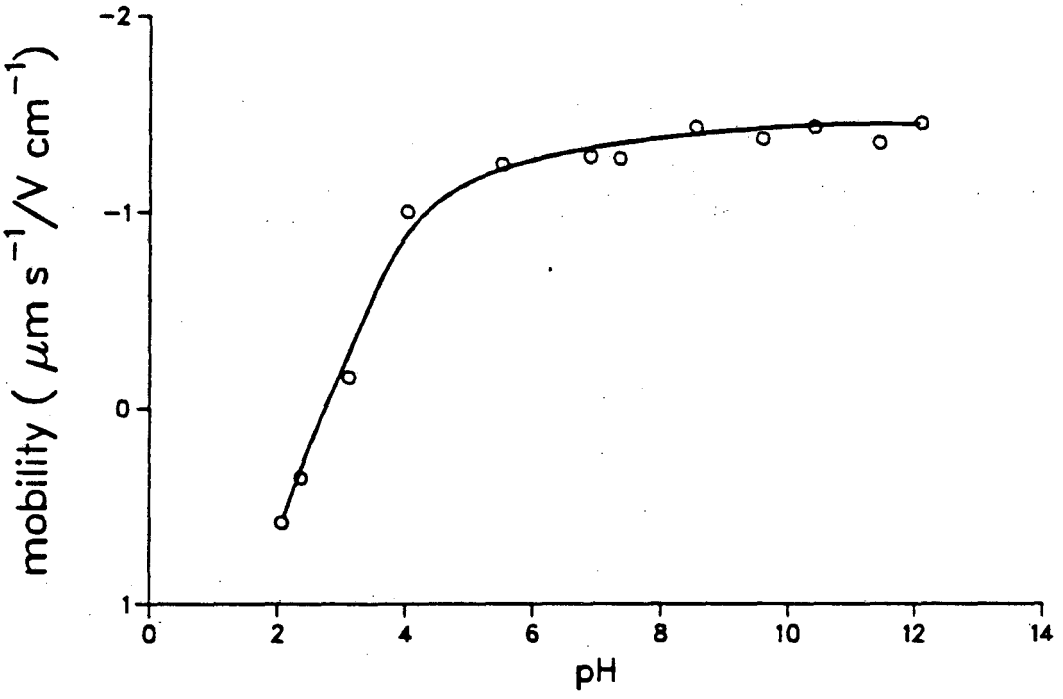


Figure 3.10. Electrophoretic mobility of human erythrocytes fixed in 0.25% GA, measured in Standard Buffer (Debye length = 0.8nm) as a function of pH. The mobilities have not been corrected for the effects of viscosity. The pH adjustments were made using 0.145M NaOH and HCl.

Figure 3.11

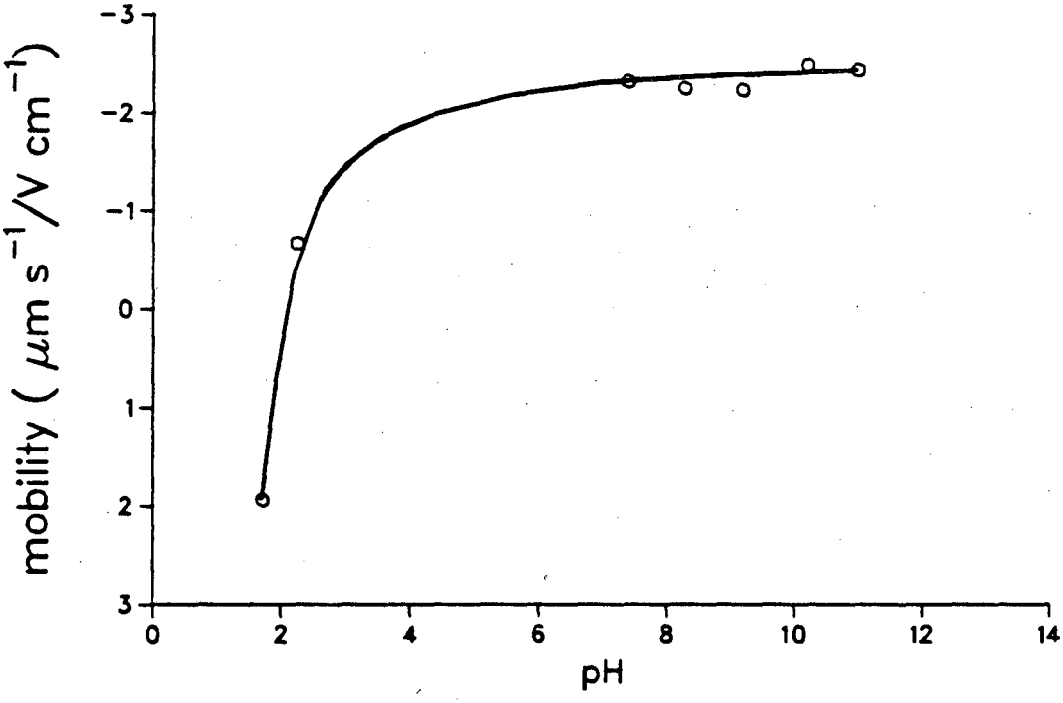


Figure 3.11. Electrophoretic mobility of human erythrocytes fixed in 0.25% GA, measured in low ionic strength buffer (Debye length = 2.53nm) as a function of pH. The suspension medium was 0.0145M buffer made isotonic by the addition of sucrose. The pH adjustments were made with 0.0145M NaOH or HCl.

2.5 for acetaldehyde-fixed cells.

It should be pointed out that the lack of any effect of glutaraldehyde on the mobility is further evidence that the increased positive charge at low pH and low ionic strength is not due to positive amino groups, at least not amino groups which are accessible to glutaraldehyde.

Table 3-5 contains isoelectric point estimates from several experiments. Much of this data is from control curves measured for other experiments. Those experiments will be described in later sections, but the isoelectric point data is compiled here for completeness. In those cases where a range of pH is given, the data was not complete enough to determine an accurate isoelectric point. Also included in table 3-5 are isoelectric points for neuraminidase-treated cells. These data will be described in section 3.3. The figure numbers from which the data were derived are given in the table. The data show that the isoelectric point varies with increasing Debye length, first increasing slightly and then decreasing.

In summary, the first few figures in this section indicate that the high concentrations of glutaraldehyde used in many studies change the surface properties of the cells, while a lower (0.25%) concentration in a hypertonic solution leaves the membrane surface charge properties unchanged.

A few words on acetaldehyde use in these studies is appropriate here. Both acetaldehyde and glutaraldehyde neutralize positive amino groups, but acetaldehyde also reacts with guanidinium bases. In order to detect any positive groups that may have been uncovered by other cell treatments, post-treatment with acetaldehyde was therefore used.

3.2.3. Lipid extraction

Seaman and Cook, 1965, treated acetaldehyde-fixed cells with ethanol, methanol, ether and chloroform-methanol, and measured the mobilities in 0.145M NaCl. They detected no

TABLE 3-5

Isoelectric points for GA-fixed and neuraminidase-treated human erythrocytes.[a]

Debye length (nm)	Dilution	Figure number	Isoelectric point	
			GA-fixed	GA-fixed, neuraminidase-treated
0.80	1/1	3.10	2.75	
		4.14	2.7	
		4.23	2.7	
		4.24	2.7	
		4.19		3.2
1.79	1/5	3.19		3.5
		4.10	3.2	
2.19	1/7.5	4.11	3.3	
		4.25	2.9	
2.53	1/10	3.11	2.1	
		3.20		2.5–3.3[b]

(a) - All cells were fixed with 0.25% glutaraldehyde. Neuraminidase treated cells were incubated for 30 minutes at 37°C in a Standard Buffer solution containing 120 units neuraminidase *(V. cholerae)* per 10^{10} cells.

(b) - The data were not complete enough to make an accurate estimate.

Note: Heard and Seaman (1961) measured an isoelectric point of 2.5 for acetaldehyde and formaldehyde fixed human erythrocytes at Debye length 0.8nm.

differences in the mobilities of the extracted cells relative to the controls. Thus, they concluded that cholesterol and phospholipids must play very little or no role in the mobility measured at an ionic strength of 0.145. However, they did not measure the mobilities at low ionic strengths.

In my experiments, several studies were performed at low ionic strength to determine the effect of lipid extraction on the electrophoretic mobility of GA-fixed cells. These studies were difficult to perform because the cells formed large clumps which caused extraneous convective currents in the measuring chamber. Generally, the low-ionic-strength samples clumped less than did the higher-ionic-strength samples. For electrophoresis it was helpful to break up some of these clumps by ejecting the cell suspensions through a #25 needle. This was more effective than shaking the suspensions in a vortex mixer, but neither was truly effective.

The mobility vs. Debye length curve for chloroform/methanol-extracted samples is shown in figure 3.12. The phosphate assay data for this experiment determined that 3.48 μmoles phosphate were removed per 10^{10} cells treated. Steck, et al. (1971) estimated the total phospholipid for ghost membranes to be 3.84 μmoles per 10^{10} cells. The error bars show the larger-than-usual standard errors of the extracted-cell measurements. Although no difference is seen at the high ionic strength, a decrease in mobility is seen at low ionic strengths.

The mobility vs. Debye length results for ethanol-extracted cells are shown in figure 3.13 for one experiment and in figure 3.14 and in table 3-6 for the average of several experiments. The data in figure 3.14 are expressed as fractions of their corresponding control values, so as better to compare results of different experiments. These data also show little, if any, variation at high ionic strength, in agreement with the Seaman and Cook (1965) experiments. A slight decrease in mobility of the extracted sample is seen at medium ionic strengths (Debye length of 1.5 to 2.5). This would suggest that the negative groups of the phospholipids may have a greater effect on the mobilities at medium ionic strengths than do the positive groups of the

Figure 3.12

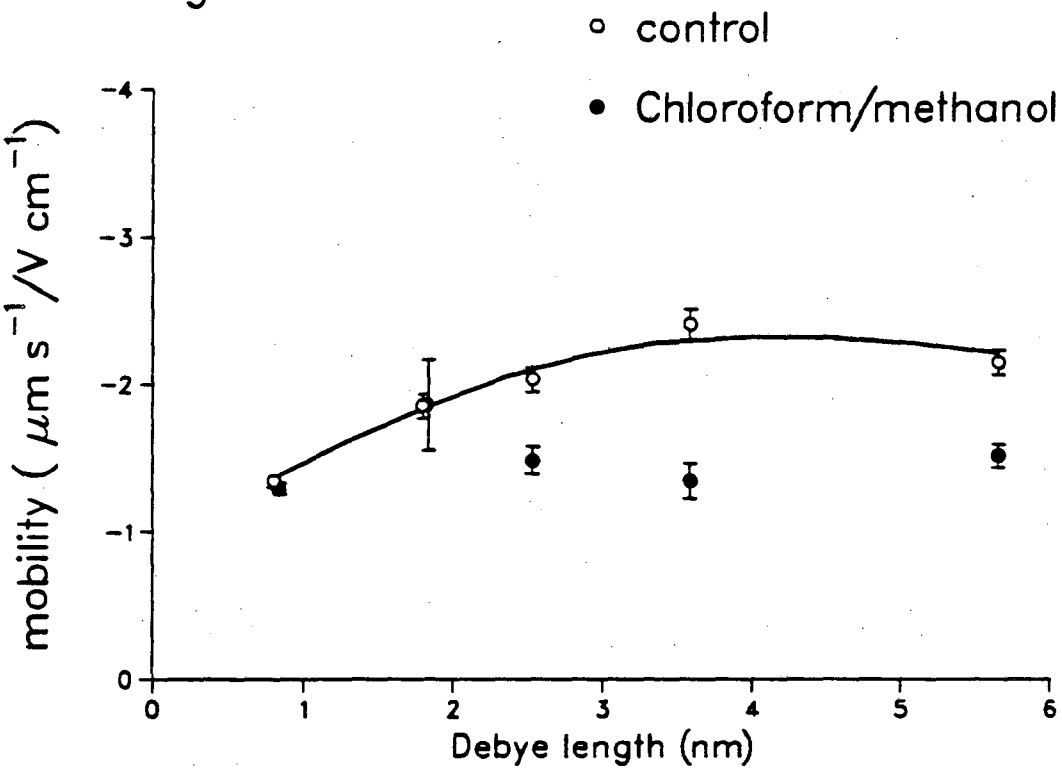


Figure 3.12. Uncorrected electrophoretic mobility of chloroform/methanol- extracted (•), GA-fixed, human erythrocytes as a function of Debye length (see text). The controls (o) were not treated with chloroform/methanol. The error bars indicate ± 1 standard error.

Figure 3.13

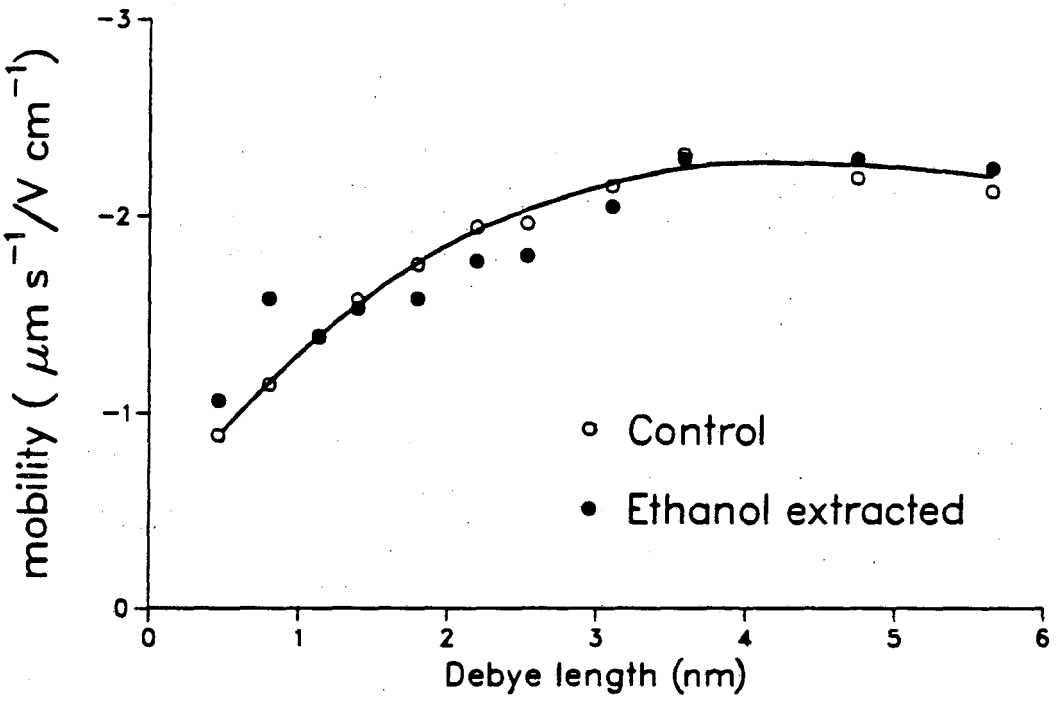


Figure 3.13. Uncorrected electrophoretic mobility of ethanol-extracted (•) and non-extracted (o), GA-fixed, human erythrocytes as a function of Debye length (the mobilities measured in a single experiment).

Figure 3.14

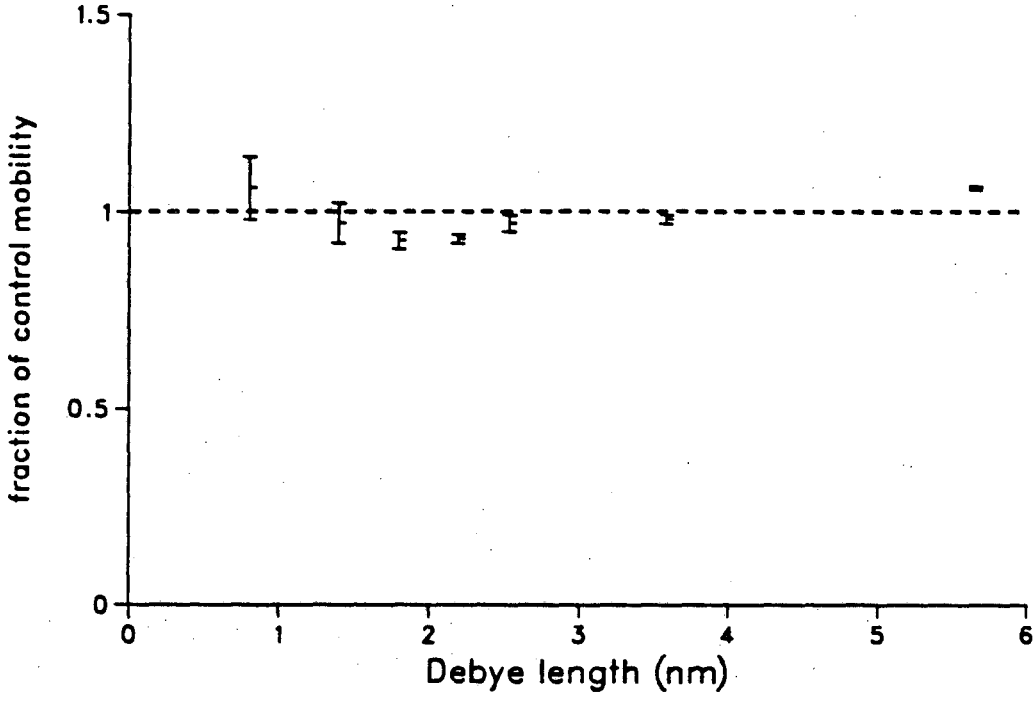


Figure 3.14. Electrophoretic mobility of ethanol-extracted, GA-fixed, human erythrocytes as a function of Debye length (averages for two to four experiments). The ordinate is expressed as a fractional mobility, compared to the control mobility at the same ionic strength. See table 3-6 for details.

TABLE 3-6

Glutaraldehyde fixed human erythrocytes treated with ethanol.[a] Electrophoretic mobility as a function of debye length. Expressed as fraction of the control.

Debye length (nm)	Dilution	Fraction of control	Number of experiments[b]
0.46	3/1	1.20	1
0.80	1/1	1.06 ± 0.08[c]	4
1.13	1/2	1.00	1
1.39	1/3	0.97 ± 0.05	3
1.79	1/5	0.93 ± 0.02	4
2.19	1/7.5	0.93 ± 0.01	3
2.53	1/10	0.97 ± 0.02	4
3.10	1/15	0.95	1
3.58	1/20	0.98 ± 0.01	3
4.74	1/35	1.05	1
5.65	1/50	1.06 ± 0.003	2

(a) - Washed cells were fixed in 0.25% glutaraldehyde for 45 minutes, and then suspended in 10 volumes 100% ethanol for 30–40 minutes at room temperature. Controls were similarly treated but suspended in standard buffer.

(b) - Each experimental result was based on an average of twenty measurements.

(c) - Errors are standard errors for the average of the several means.

phospholipids.

A phosphate assay using the Fiske and Subbarow method was performed on some of the ethanol extracts to document the removal of phospholipid. Table 3-7 shows some representative assay data for four separate experiments on GA-fixed cells, with each sample normalized to a 50% (v/v) packed cell fraction. If we assume a rough approximation of 5×10^9 cells/ml, the average of the four experiments (0.37μmoles/ml), corresponds to 0.74μmoles/10^{10} cells or 19% of the total phosphate found by Steck, et al. (1971). Gigg and Payne (1969) and Roozemond (1969) showed that neither PE nor PS can be extracted with organic solvents after GA-fixation.

TABLE 3-7

Phosphate assay for glutaraldehyde-fixed human erythrocytes treated with ethanol.

Expt. No.	μmoles Phosphate per ml cell suspension		
	Control	Ethanol extract	Difference
1		0.22	0.22
2	0.27	0.56	0.30
3	0.16	0.93	0.77
4	0.16	0.36	0.20
Mean			0.37

The cells were treated with 100% ethanol as described in Table 3-6.

Studies were also performed comparing phosphate removal in unfixed cells to that of GA-fixed cells. The fixed cells released approximately half the phosphate of the unfixed cells (0.409 μmoles/ml vs. 0.853 μmoles/ml for the unfixed). Because the unfixed cells formed a sticky brown residue at the bottom of the tube, they could not be used for electrophoresis. The percent ethanol used in the extraction was also significant to the assay results. When 50% ethanol was used on fixed cells, rather than the usual 100%, only about half the phosphate was

released (0.215 μmoles/ml vs. 0.409 μmoles/ml)

In summary, the mobilities after lipid extraction were difficult to measure because of the increased aggregation of the cells. However, it appears that some decrease in mobility does occur at low ionic strengths. From these data, the most probable location of the lipid head groups is at a depth of between 2 and 3 nm beneath the surface of shear.

Another possible explanation for the decreased mobility is an increased conductivity of the membrane after lipid removal. As can be seen in equation 3 of chapter 2, if the membrane became conductive, the mobility could decrease by a factor of 2, even with no surface charge change. Ghosh and Bull (1963) showed, in experiments on glass beads, that the effect of surface conductance is negligible at ionic strengths greater than 0.01 but that it becomes important at lower ionic strengths. However, complete particle conductance can have an effect on the mobility, even at the higher ionic strengths. This was demonstrated by Einolf and Carstensen (1967) on bacterial cells. Peros (1981), using resistive pulse spectroscopy, showed that the membrane became transparent to an electric field when all lipids were removed with Triton X-100.

3.2.4. Resealed ghosts

Ghosts have been used extensively in experiments to study the difference between the inner and outer surfaces of the membrane. An implicit assumption has been that the membrane has not been drastically modified by the lysis and subsequent resealing of the cells. Cabantchik, et al. (1975) detected no difference between ghosts and native cells in the response to surface probes such as 4,4'-diisothiocyano-2,2'stilbene disulfonic acid (DIDS) and pyridoxal phosphate as seen after sodium dodecylsulfate/acrylamide gel electrophoresis. Staros, et al. (1974), also using the gel separation technique, did detect a difference in the labeling by N-(4-azido-2-nitrophenyl)-2-ethane sulfonate (NAP-taurine).

Furchgott and Ponder (1941) first measured the electrophoretic mobility of ghost membranes. Their measurements, at an ionic strength of 0.172, were made on ghost fragments which were produced by hemolysis in distilled water. Their results showed variability of mobility with time, but, when measurements were made immediately, the mobilities were the same as those of native cells.

Table 3-8 and figure 3.15 contains my mobility vs. Debye length data for human erythrocyte ghosts. For these measurements the ghosts were formed at 20 mosm, as described in the Chapter 2; the ghosts were then resuspended in Standard Buffer to allow for resealing, before transferring them to the measuring solution. The results show that there is no significant difference between the mobilities of the intact cells and the ghosts.

Table 3-9 contains the mobility vs ionic strength data for rat erythrocyte ghosts. Again, there is no significant difference between the mobilities of the intact cells and the ghosts except at the lowest ionic strength (Debye length = 3.6nm) where a weakly significant p-value of 0.014 indicated the 11% decrease in mobility may be a real effect.

3.3. Neuraminidase treatment

Most of the negative surface charge density of human erythrocytes has been shown to be due to the negative-carboxyl groups of N-acetyl-neuraminic acid (sialic acid). The pK of sialic acid has been determined by Svennerholn (1956) to be about 2.6.

A number of researchers have measured electrophoretic properties of neuraminidase-treated erythrocytes. Cook et al. (1961) measured the mobility of cells treated with what was then called "recepter destroying enzyme" (RDE) and measured the pH mobility curve for unfixed cells between pH's 3.0 and 9.5. They determined by direct chemical analysis that twice as much sialic acid was removed from the cell surface than could be accounted for by the

TABLE 3-8

Electrophoretic properties of native human erythrocytes and ghosts. The mobilities and surface charge densities are reported for several ionic strengths. The errors are standard errors of the mean.

Debye Length (nm)	Dilution	Electrophoretic mobility (μ) ($\mu m\ s^{-1}/V\ cm^{-1}$)[a]		p Value[d]	Surface charge density ($10^{-3}\ C\ m^{-2}$)	
		Native Erythrocyte[b]	Resealed Ghost[c]		Native Erythrocyte	Resealed Ghost
0.80	1/1	1.17±0.02	1.17±0.05	>0.5	13.1±0.2	13.1±0.6
1.79	1/5	2.17±0.04	2.21±0.09	>0.5	10.9±0.2	11.0±0.5
2.53	1/10	2.58±0.06	2.52±0.13	>0.5	9.1±0.2	8.9±0.5
3.57	1/20	2.86±0.09	2.98±0.11	>0.4	7.2±0.2	7.5±0.3
5.65	1/50	3.02±0.11	3.06±0.21	>0.5	4.8±0.2	4.8±0.3

(a) - Mobilities include a viscosity correction.

(b) - Each datum represents 340 measurements carried out in 17 separate experiments.

(c) - Each datum represents 140 measurements carried out in 7 separate experiments.

(d) - Calculated from Student's t-test.

Figure 3.15

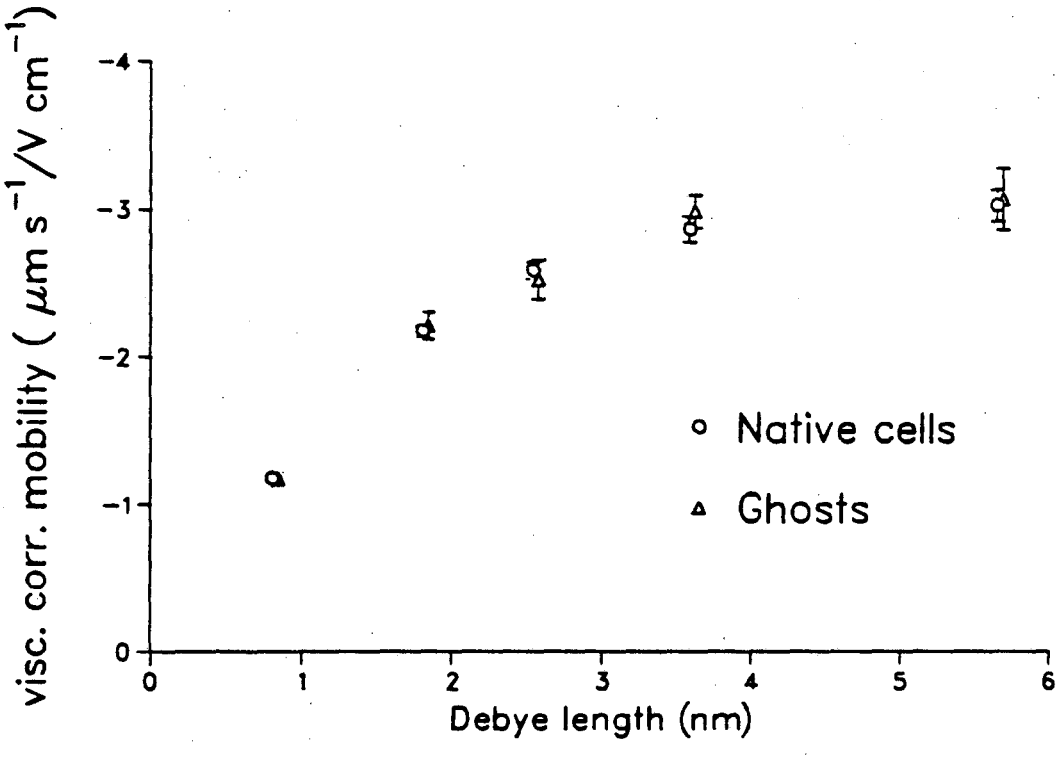


Figure 3.15. Viscosity-corrected electrophoretic mobility as a function of Debye length for resealed human erythrocyte ghosts (Δ). Controls (o) were mobility measurements made on non-hemolysed cells of the same blood sample. Data from table 3-8.

TABLE 3-9

Electrophoretic properties of native rat erythrocytes and ghosts. The mobility and surface charge density are reported for several ionic strengths. The errors are standard errors of the mean.

Debye Length (nm)	Dilution	Electrophoretic mobility (μ)[a] ($\mu m\ s^{-1}/V\ cm^{-1}$)						Surface charge density ($10^{-3}\ C\ m^{-2}$)	
		Native Erythrocyte[b]		Resealed Ghost		% change	p Value[c]	Native Erythrocyte	Resealed Ghost
0.80	1/1	1.37±0.03	(20)[b]	1.35±0.05	(12)[b]	−1.5	>0.7	15.3±0.3	15.1±0.6
1.79	1/5	2.66±0.11	(6)	2.44±0.09	(7)	−8.3	>0.1	13.3±0.6	12.2±0.5
2.53	1/10	3.22±0.06	(5)	3.12±0.11	(8)	−3.1	>0.5	11.4±0.2	11.0±0.4
3.57	1/20	3.94±0.14	(13)	3.49±0.08	(14)	−11.4	0.014	9.9±0.4	8.7±0.2
5.65	1/50	4.20±0.17	(15)	4.28±0.22[d]	(18)	1.9	>0.7	6.6±0.3	6.8±0.3

(a) - Mobilities include a viscosity correction.

(b) - Numbers in parentheses represent the number of separate experiments, each of which represent 20 measurements.

(c) - Calculated from Student's t-test.

(d) - When samples were resuspended in 0.145M standard buffer, the mobility was 1.35 for the 1/10 sample and 1.27 for the 1/50 sample.

decrease in mobility from 1.08 to 0.38 μmoles $s^{-1}/V\ cm^{-1}$. They repeated the measurements on cells that were fixed for 20 days with acetaldehyde. The acetaldehyde-fixed, neuraminidase-treated cells showed a slight increase in mobility over those of the unfixed cells.

Eyler et al. (1962) examined neuraminidase-treated, native cells from several species of animals, including humans. In an ionic strength 0.072 phosphate buffer, the mobility-pH curve could be measured between pH's 3 and 9. The isoelectric point was estimated for the untreated cells to be approximately 2.8. The isoelectric point for the neuraminidase-treated cell increased to 5.5.

Haydon and Seaman (1967) fixed human cells in acetaldehyde first, and subsequently reacted them with neuraminidase. They found no positive branch to the mobility-pH curve under those circumstances. However, as stated above in the section on aldehydes, Mel et al. (1973) showed that this lack of a positive branch is due to operational factors in the apparatus used and is not a property of the cells.

Vassar, et al. (1972), using a cylindrical chamber, measured the pH vs. mobility in Standard Buffer for neuraminidase-treated cells that were postfixed with glutaraldehyde. They found an isoelectric point of about 3.25 for the neuraminidase-treated vs. 1.5 for the non-neuraminidase-treated cells.

In an attempt to study the residual charge at low ionic strength, I treated both unfixed and glutaraldehyde-fixed human erythrocytes with neuraminidase. The sialic acid assay data are shown in table 3-10. No difference was seen between unfixed and fixed cells for either the assay data or the mobility measurements. A representative ionic strength vs. mobility curve on unfixed cells is shown in figure 3.16, along with data from a control sample. Averages from results of 4 to 8 experiments (GA-fixed cells), at 5 different ionic strengths, are tabulated in table 3-11. The mobilities are plotted in figure 3.17, and the corresponding calculated surface

TABLE 3-10

Sialic acid removed from neuraminidase-treated, glutaraldehyde-fixed and unfixed human erythrocytes.[a]

	$\mu g/10^{10}$ cells	$\mu moles/10^{10}$ cells
Experiment I:		
Unfixed erythrocytes	116	0.37
GA-fixed erythrocytes	131	0.42
Experiment II:		
Unfixed erythrocytes	186	0.60
GA-fixed erythrocytes	103	0.33
Experiment III:		
GA-fixed erythrocytes	129	0.42
Mean ± S.E.	133 ± 12.7	0.43 ± 0.04
Cook et al., 1961 (RDE)	42.8–108.1	
Eylar et al., 1962 (RDE)		0.4

(a) - Cells were collected, washed, fixed in 0.25% glutaraldehyde, and incubated for 30 minutes at 37°C in Standard Buffer solution containing 120 units neuraminidase *(V. cholerae)* per 10^{10} cells.

Lowry protein assay of the extract found no detectable protein removed with the sialic acid.

Figure 3.16

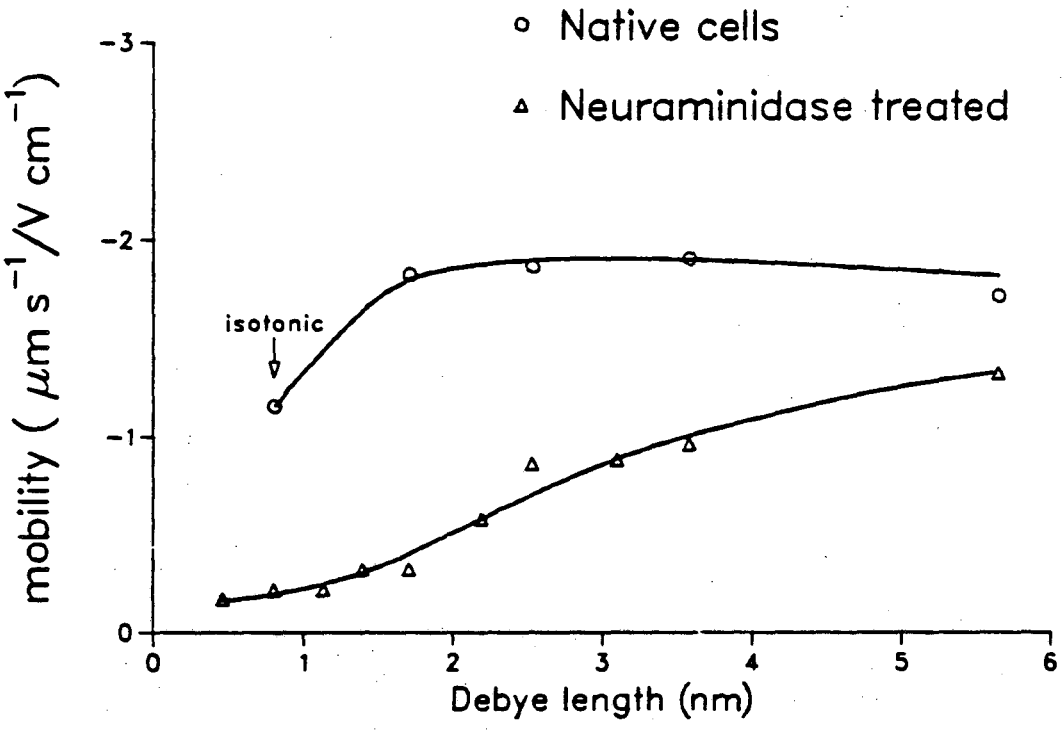


Figure 3.16. The effects of neuraminidase *(V. cholerae)* on the (uncorrected) electrophoretic mobility of unfixed human erythrocytes as a function of Debye length. The treated cells (Δ) were incubated for 30 minutes at 37°C in Standard Buffer solution containing 120 units neuraminidase/10^{10} cells. Control cells (o) were similarly incubated in a solution containing neuraminidase that had been inactivated by heating in a boiling water bath for 5 minutes.

TABLE 3-11

Electrophoretic mobility and surface charge density as a function of ionic strength for neuraminidase treated glutaraldehyde fixed human erythrocytes.[a]

Debye length (nm)	Dilution	Uncorrected mobility	Number of experiments[b]	Surface charge density(σ)[c] (10^{-3} C m^{-2})
0.80	1/1	0.26 ± 0.06[d]	6	3.01 ± 0.64
1.79	1/5	0.55 ± 0.06	8	3.35 ± 0.38
2.19	1/7.5	0.75 ± 0.07	4	3.81 ± 0.35
2.53	1/10	0.87 ± 0.05	8	3.88 ± 0.22
3.58	1/20	1.01 ± 0.04	4	3.24 ± 0.13

(a) - Cells were collected, washed, fixed in 0.25% glutaraldehyde, and incubated for 30 minutes at 37°C in a standard buffer solution containing 120 units neuraminidase *(V. cholerae)* per 10^{10} cells.

(b) - Each experimental result was based on an average of 20 individual measurements.

(c) - $\sigma = \mu\eta/\ell_D$ where η=viscosity and ℓ_D=Debye length.

(d) - Errors are standard errors of the mean.

Figure 3.17

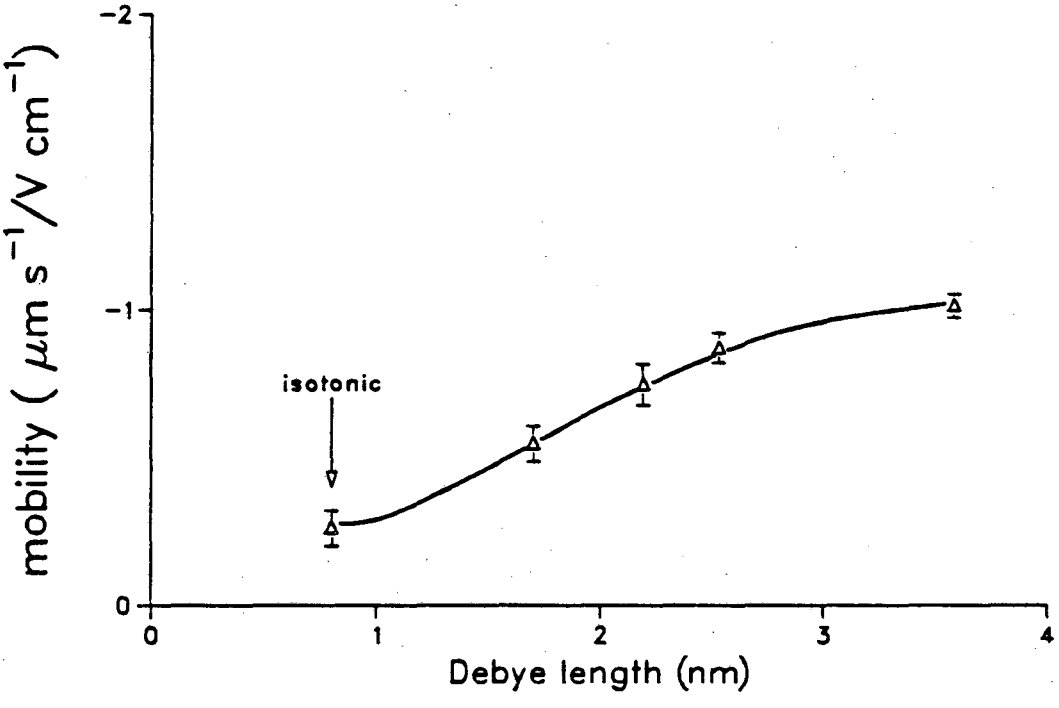


Figure 3.17. The effects of neuraminidase on the (uncorrected) electrophoretic mobility of GA-fixed human erythrocytes as a function of Debye length. Erythrocytes were collected, washed and fixed in 0.25% glutaraldehyde, then treated with neuraminidase (120 units/10^{10} cells). The error bars depict the standard errors of the mean for the several experiments. (Data from table 3-11).

charge densities are plotted in figure 3.18 (the bottom curve). The surface charge density curve for the untreated cells from figure 3.2 is repeated in figure 3.18, for ease of comparison (top curve). Reversibility data is tabulated in table 3-12.

The curves show that although the mobility at physiological ionic strength falls to a very low value, it rises to and remains at a much higher value at the low ionic strengths. There is also a possible inflection point around Debye length 1.7 to 2.5nm where some increase in negative surface charge is seen. The relative constancy of the surface-charge-density plot indicates that the higher mobility at lower ionic strength is mostly due to the decreased masking of charges at the low ionic strength caused by a reduction in the number of counter ions.

PH vs. mobility curves after neuraminidase treatment of GA-fixed cells were measured at 1/5th and at 1/10th physiological ionic strength (Debye lengths 1.79 and 2.53nm). The results given in figures 3.19 and 3.20 show that the isoelectric point at these ionic strengths occurs at pH 3.5 for the 1.79nm sample and between 2.5 and 3.3 for the 2.53nm sample. When these isoelectric points are compared with control values measured in other experiments, a slight increase in isoelectric point is seen at low ionic strength (see table 3-5) for the neuraminidase-treated sample.

Because there was the possibility that the added sucrose might be affecting the results, a few additional points were measured with no sucrose present in the solutions. When these points are compared with the viscosity-corrected mobilities of the sucrose-containing solutions, no significant variation is found.

Neuraminidase-treated, GA-fixed cells were also treated with DFNB to ascertain if any positive groups were available after neuraminidase treatment. As discussed previously in section 3.2, positive amino groups would be expected to react with the DFNB such that the treated cells would have a higher (negative) mobility than the untreated cells. Examination of

Figure 3.18

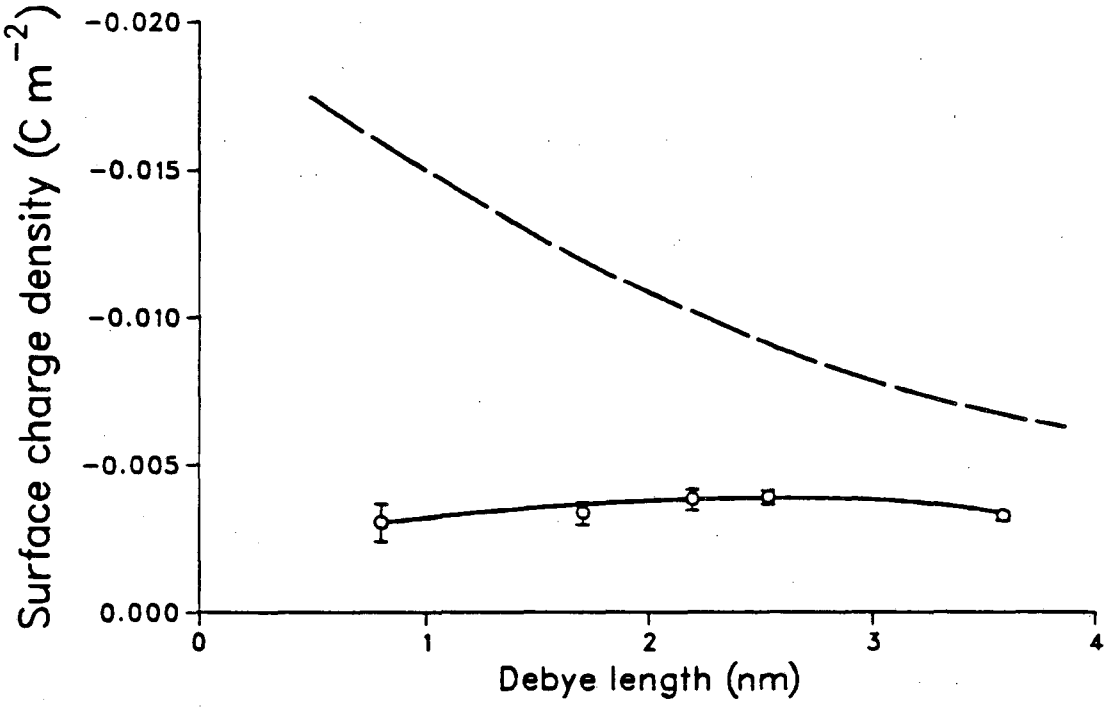


Figure 3.18. Surface charge density as a function of Debye length for neuraminidase-treated, GA-fixed human erythrocytes (o). (Data from table 3-11). The upper dashed curve (shown for comparison) is the relevant portion of the native cell surface charge from figure 3.2.

TABLE 3-12

Electrophoretic reversibility as a function of Debye length for neuraminidase treated glutaraldehyde fixed human erythrocytes.[a]

Debye length (nm)	Dilution	Control		Neuraminidase treated	
		Fraction of Standard Buffer Value	Number of Experiments[b]	Fraction of Standard Buffer Value	Number of Experiments[b]
1.79	1/5			1.01	1
2.19	1/7.5	1.05	1	1.42 ± 0.078[c]	2
2.53	1/10	1.03 ± 0.03	3	1.08 ± 0.12	4
3.58	1/20	.98	1	1.41 ± 0.07	3

(a) - Cells were collected, washed, fixed in 0.25% glutaraldehyde, and incubated for 30 minutes at 37°C in a standard buffer solution containing 120 units neuraminidase *(V. cholerae)* per 10^{10} cells.

(b) - Each experimental result was based on an average of 20 individual measurements.

(c) - Errors are standard errors of the mean.

Figure 3.19

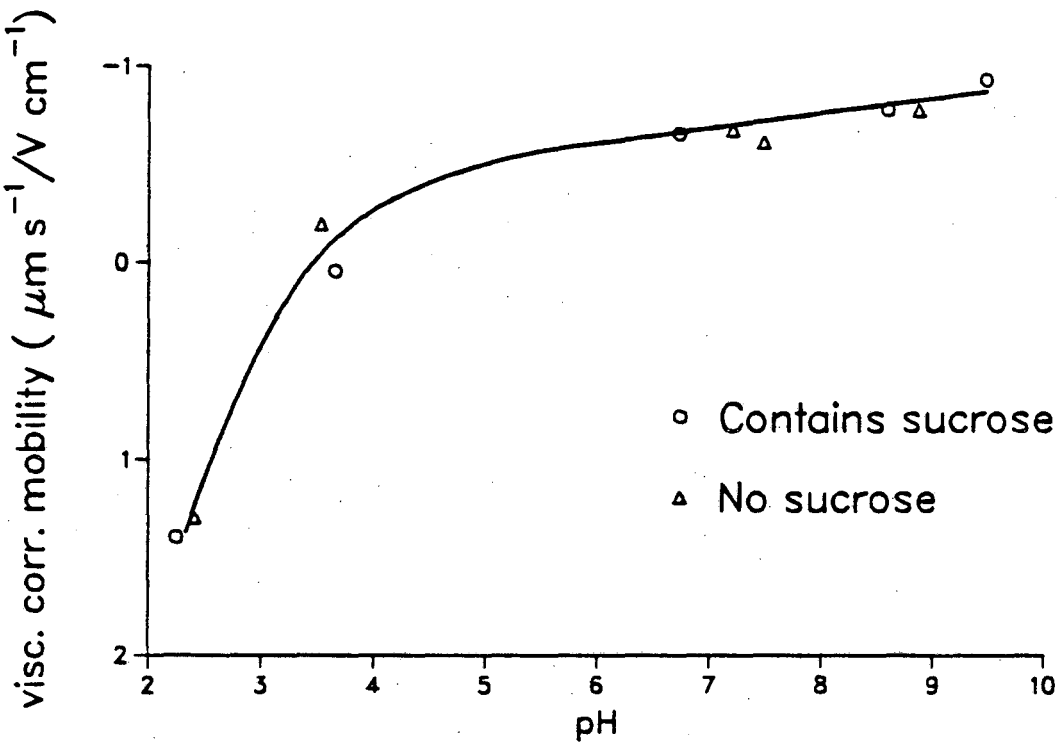


Figure 3.19. Viscosity-corrected electrophoretic mobility of GA-fixed, neuraminidase-treated human erythrocytes as a function of pH: Effects of low ionic strength (Debye length = 1.79nm), and of sucrose. Human erythrocytes were collected, washed, fixed with 0.25% glutaraldehyde, and treated with neuraminidase (120 units/10^{10} cells). The pH adjustments were made with 0.029M NaOH or HCl. Some mobility measurements (o) were made in 0.029M saline buffers with sucrose added to bring the osmolality to 0.145M. Other measurements (Δ) were made in buffers with no added sucrose.

Figure 3.20

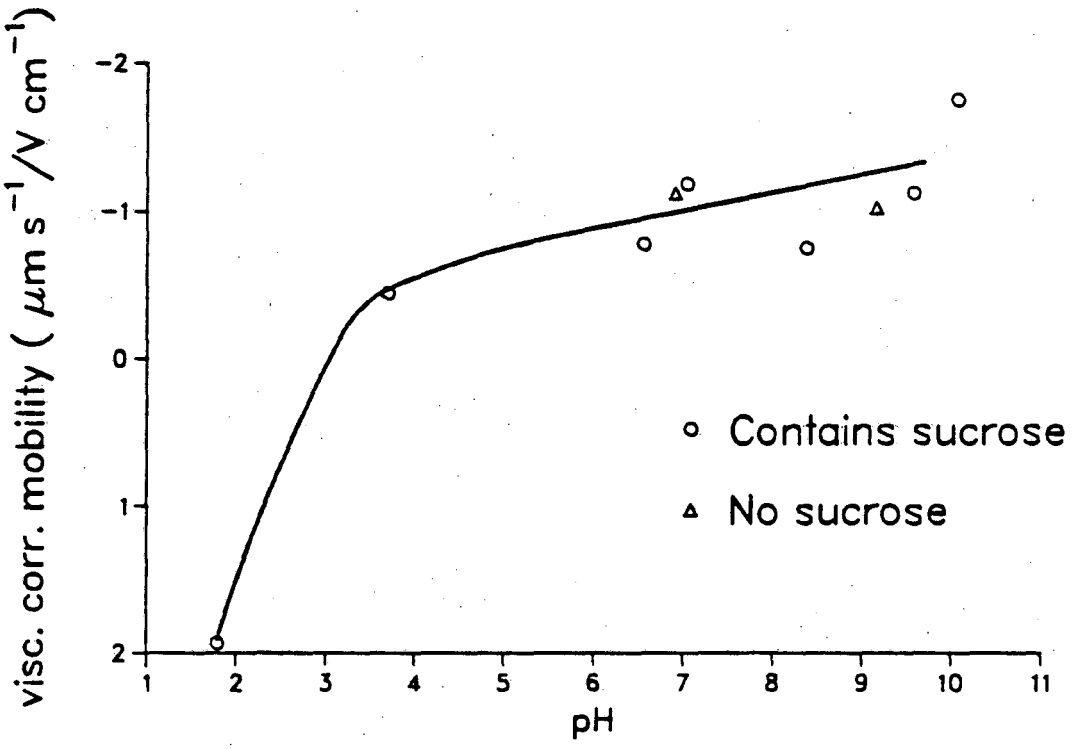


Figure 3.20. Viscosity-corrected electrophoretic mobility of GA-fixed, neuraminidase-treated human erythrocytes as a function of pH: Effects of low ionic strength (Debye length = 2.53nm) and of sucrose. Human erythrocytes were collected, washed, fixed in 0.25% GA and treated with neuraminidase (120 units/10^{10} cells). The pH corrections were made with 0.0145M NaOH or HCl. Most mobility measurements (o) were made in 0.0145M saline with sucrose added to bring the osmolality to 0.145M. A few measurements (Δ) were made in buffers with no added sucrose.

figure 3.21 shows that there is no evidence of this occurring. However, in a separate experiment reported in section 4.2.1, neuraminidase-treated, GA-fixed erythrocytes were treated with acetaldehyde. This treatment did slightly increase the (negative) mobility of the cells.

What can we say about the effects of neuraminidase on the erythrocyte? Compare the two curves in figure 3.18. If we ignore for the moment the slight increase in charge in the bottom curve, the remaining charge density after sialic acid removal is constant over the entire Debye length range, indicating that all of this negative charge is within 0.8nm of the surface of shear. On the other hand, examination of the upper curve shows a continual decrease in negative charge as the Debye length increases with, apparently, increasing positive charges becoming uncovered at the greater Debye lengths. Could it be possible that neuraminidase is removing all this positive charge, down to a depth of 5.7nm, leaving only some negative charge groups within 0.8nm of the surface of shear? This seems unlikely.

However, an alternative explanation exists. The neuraminidase may be changing the location of the surface of shear. Further, the decreased surface charge seen for the native cell (upper curve) at higher Debye lengths, may not be a true charge decrease, but rather, an artifact caused by the high conductivity within a sparsely populated outer surface containing mostly sialic acid. Once sialic acid is removed, the surface of shear moves toward the "true" non-conductive cell surface and the remaining surface charges are mostly within 0.8nm of that surface. The results with DFNB indicate that the residual surface charge after sialic acid removal does not contain amino, sulfhydryl, tyrosyl or histidyl groups that are accessible to the probe. However, the acetaldehyde results (section 4.2.1) indicate that either an amino group or a guanidinium base may be uncovered by the action of neuraminidase.

Figure 3.21

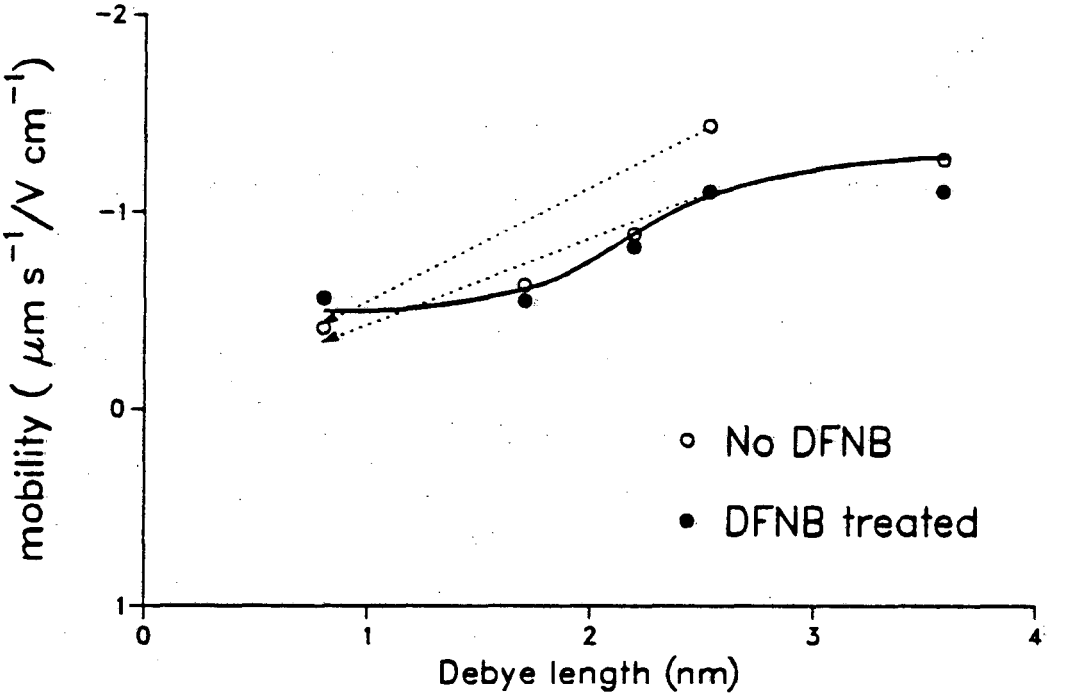


Figure 3.21. Effects of 1,5-difluoro-2,4-dinitrobenzene (DFNB) on the (uncorrected) electrophoretic mobility of neuraminidase-treated, GA-fixed, human erythrocytes as a function of Debye length. Cells were collected, washed, fixed with 0.25% GA, treated with neuraminidase (120 units/10^{10} cells), and then incubated for 30 minutes at room temperature in Standard Buffer that was made 2.8mM in DFNB (•). A control (o) was similarly treated but incubated in Standard Buffer without DFNB. The arrow heads of the dotted lines depict the reversibility measurements, made by resuspending the low ionic strength samples (2.53nm points) in Standard Buffer.

3.4. Summary

The principle results of this chapter may be summarized as follows. Low-ionic-strength buffers have an effect on the agglutinability of erythrocytes but the results are reversible. Therefore, no permanent effect occurs on the charge moieties responsible for agglutination. Low-ionic-strength buffers cause an increase in the (negative) electrophoretic mobility of human erythrocytes, and a corresponding decrease in the surface charge density. Treatment of the cells with tosyl chloride, DFNB, and glutaraldehyde demonstrated that this decrease in mobility is probably not due to amino, sulfhydryl, tyrosyl, or histidyl groups.

Glutaraldehyde in high concentrations (2.5%, w:v) affects the cell mobility, but lower concentrations (0.25%) in a hypertonic buffer, do not affect the mobility at any ionic strength tested.

Removal of the bulk of the lipid by treating the GA-fixed cells with chloroform/methanol caused a large decrease in the cell's mobility in low-ionic-strength buffers. However, it is not possible to separate the effect of the increased electrical conductivity of the cell from the effect of the change in surface charge.

Extraction of a portion of the lipids with ethanol led to a slight decrease in mobility in the range of Debye lengths from 1.5 to 2.5nm.

Resealed ghosts from both human and rat erythrocytes, made by the method of Dodge et al. (1963) but with added Mg^{++}, had the same mobility at all ionic strengths as did the native erythrocytes.

Neuraminidase treatment removes most of the negative charge of the membrane but a small residual negative charge remains. This residual charge could be from the carboxyl groups of proteins or phosphorous-containing groups of the phospholipids. At low ionic strength this

charge does not change appreciably (except for a slight increase in the Debye length range of 2 to 3nm), suggesting that all but a small portion of the surface charge remaining after neuraminidase treatment occurs within 0.8nm of the surface of shear. The data strongly suggests that the major effect of sialic acid removal is to move the surface of shear closer to the cell membrane, and that the decreasing surface charge as a function of increasing Debye length seen in the native cell is an artifact of the high conductivity of the more sparsely populated outer section of the membrane surface.

The isoelectric point of the neuraminidase-treated cells increased slightly over that of the untreated cells.

CHAPTER 4

Effects of phospholipases

The cell membrane bilayer structure has been shown to be asymmetric with respect to the lipid (Gordesky and Marinetti, 1973; Bretscher, 1973; Zwaal and Roelofsen, 1976). The inner layer contains most of the phosphatidylethanolamine (PE) and phosphatidylserine (PS) while the outside layer contains phosphatidylcholine (PC), sphingomyelin, and possibly as much as 20% of the total PE (Zwaal and Roelofsen, 1976).

There is some evidence that the phosphate groups of the outer membrane layer may be accessible to chemical probes, and hence would be expected to contribute to the membrane surface charge. Burry and Wood (1979) used the electron microscope to detect the markers, anionized ferritin and cationized ferritin, in lipid vesicles. They determined that the phosphates of PC vesicles did react in a limited manner with cationized ferritin, indicating that the phosphate group is partially accessible. However, when free glutaraldehyde was present in the reaction mixture, PC no longer reacted with cationized ferritin. Burry and Wood (1979) further found that PE vesicles also react with cationized ferritin. That the choline head group is in an aqueous environment was demonstrated by Chapman (1968) with NMR studies. Phillips et al. (1972) believe from x-ray data that the choline head group of PC is perpendicular to the cell surface while the head group of PE is parallel to the membrane surface. The PC result supports the similar conclusions of Shah and Schulman (1967) derived from Ca^{+2} binding studies on lecithin bilayers. The choline head group is not rigid but can bend to expose phosphate groups (Burry and Wood, 1979).

4.1. Phospholipase-C treatment

Two phospholipase-C (PLC) enzymes were used to remove phosphocholine groups from the membrane of human erythrocytes: PLC from *Bacilus cereus* and PLC from *Clostridium perfringens*. The *B. cereus* enzyme has been shown to attack the phosphocholine of lecithin but not that of sphingomyelin; however, in the purified form it is inactive against the intact cell membrane (Zwaal and Roelofsen, 1976), at least under the conditions of their experiments. On the other hand, the *C. perfringens* enzyme attacks phosphocholine of both lecithin and sphingomyelin. It also hydrolyzes PE at a very slow rate, but does not hydrolyze PS. This form of the enzyme is believed to be active on the erythrocyte membrane, causing hemolysis of the native cell (Zwaal and Roelofsen, 1976).

4.1.1. Bacilus cereus PLC

As stated above, Zwaal and Roelofsen (1976) found the *B. cereus* enzyme to be inactive on the native erythrocyte. This was also true even after pretreatment of the cells with pronase, trypsin or neuraminidase. However, it has been shown to be active on erythrocytes after sphingomyelinase treatment (Colley, et al., 1973), and after ATP depletion (Frish, et al., 1973). My results obtained with this commercial version of the enzyme, on glutaraldehyde-fixed erythrocytes, and using expired blood from a blood bank, do show activity. The phosphate assay indicated an average of 0.69 μmoles phosphate were removed per 10^{10} cells (table 4-1). The mobility vs. ionic strength data after treatment with the *B. cereus* PLC are also given in table 4-1, and plotted in figures 4.1 (average of two experiments), 4.2 and 4.3 (the individual experiments). In these experiments the controls contained either o-phenanthroline (which acts an inhibitor) with PLC, or in some cases, contained no PLC in the reaction mixture. No mobility differences were observed for these two treatments. Although these measurements show no significant effect of this commercial grade enzyme on the electrophoretic mobility of

TABLE 4-1

Electrophoretic properties of fixed human erythrocytes treated with PLC *(Bacilus cereus)*.

Debye length (nm)	Dilution	Uncorrected mobility [a] ($\mu m\ s^{-1} / V\ cm^{-1}$) Control[b]	Uncorrected mobility [a] ($\mu m\ s^{-1} / V\ cm^{-1}$) PLC treated[c]	Number of experiments[d]
0.80	1/1	1.00 ± 0.06[e]	0.92 ± 0.025[e]	2
1.79	1/5	1.82 ± 0.30	1.69 ± 0.28	2
2.19	1/7.5	1.98 ± 0.23	1.74 ± 0.20	2
2.53	1/10	1.93 ± 0.23	1.80 ± 0.12	2
3.58	1/20	1.94	2.03	1
μmoles phosphate / 10^{10} cells			0.69 ± 0.11	7

(a) - All cells were fixed in 0.25% glutaraldehyde prior to phospholipase treatment.

(b) - Control cells were incubated either with PLC plus o-phenanthroline, or with neither of these in the reaction mixture. No mobility differences were observed for the two treatments.

(c) - PLC treatment consisted of 10 minute incubation at 37°C in Standard Buffer with 10 units PLC, 5×10^9 cells, and 0.5mM $CaCl_2$.

(d) - Each experimental result was based on an average of 20 individual measurements.

(e) - Errors are standard errors of the mean.

Figure 4.1

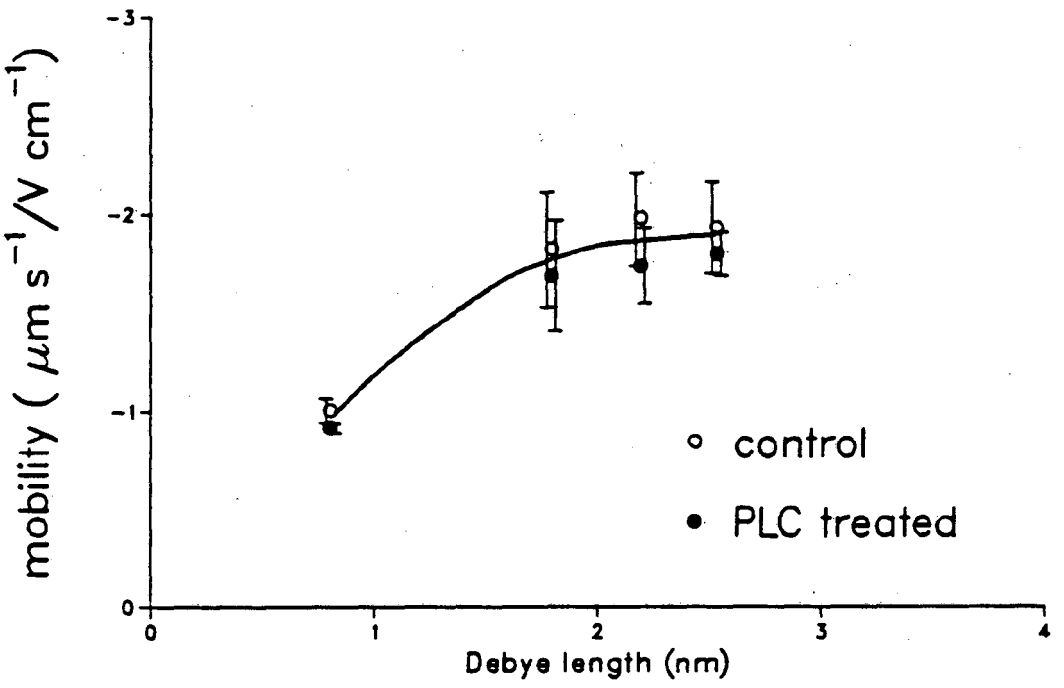


Figure 4.1. Uncorrected electrophoretic mobility of PLC *(B. cereus)*- treated (•) and untreated (o), GA-fixed, human erythrocytes as a function of Debye length. Data are the averages for two experiments (plotted separately in figures 4.2 and 4.3), from table 4-1. Error bars are the standard errors of the mean.

Figure 4.2

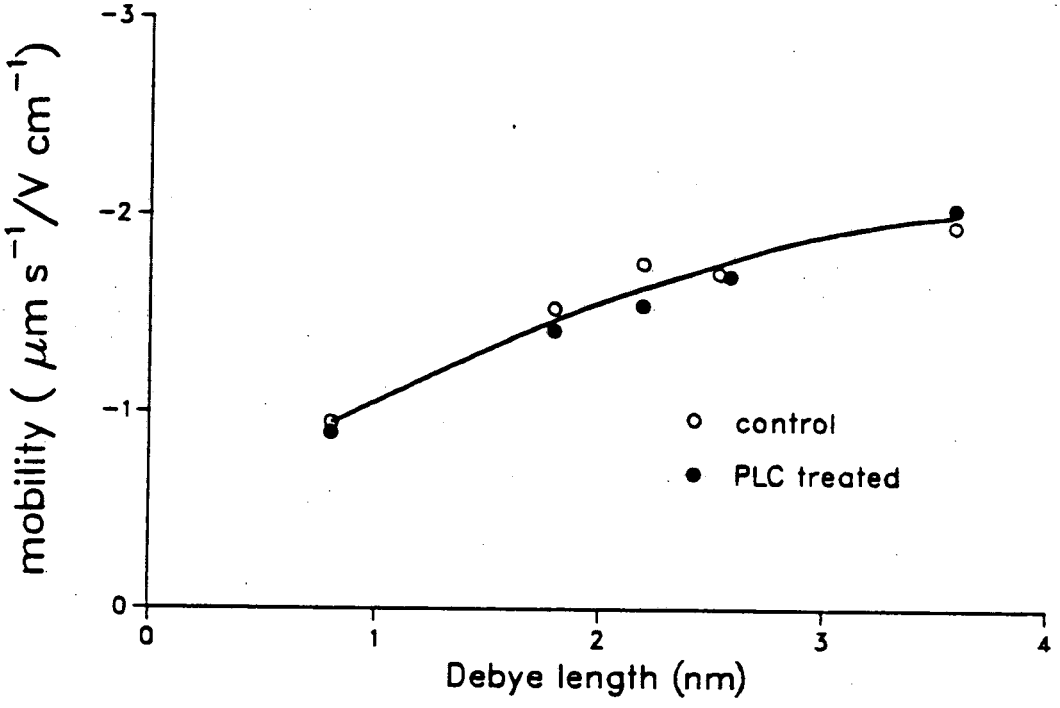


Figure 4.2. Uncorrected electrophoretic mobility of PLC *(B. cereus)*- treated (•) and untreated (o), GA-fixed, human erythrocytes as a function of Debye length. Data are from the first experiment included in the average results plotted in figure 4.1 (and table 4-1).

Figure 4.3

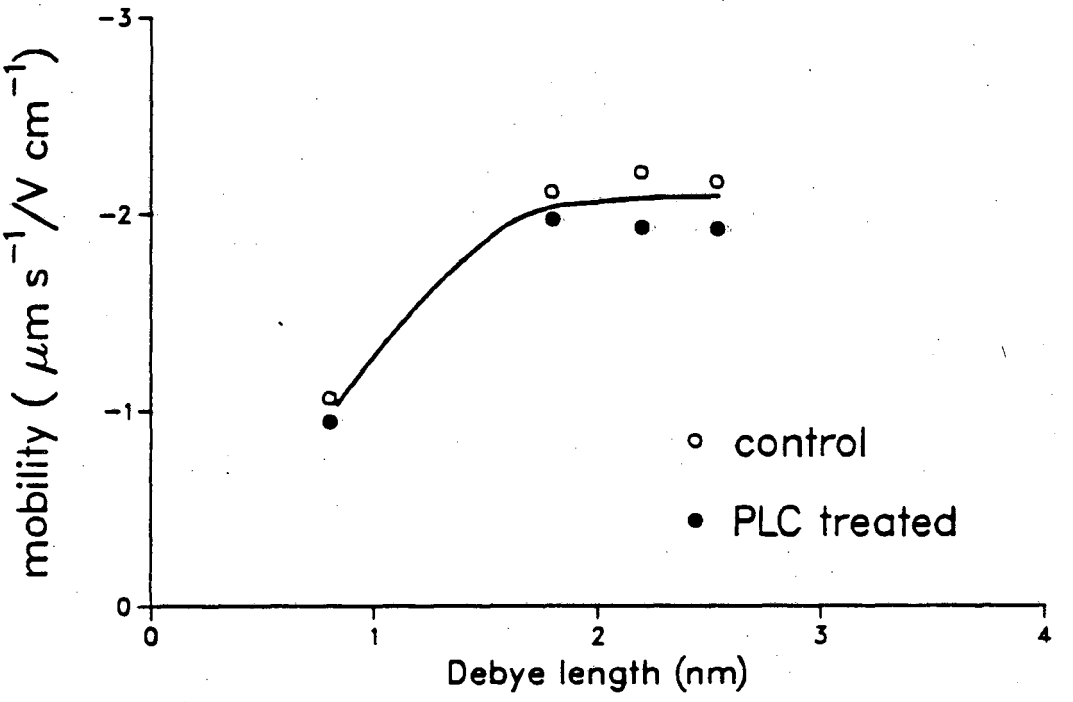


Figure 4.3. Uncorrected electrophoretic mobility of PLC *(B. cereus)*- treated (•) and untreated (o), GA-fixed, human erythrocytes as a function of Debye length. Data are from the second of the two experiments included in the average results of figure 4.1 (and table 4-1).

the cells, it is suggestive that once again a slight decrease is seen in the range 1.5 to 2.5nm, as with ethanol extraction.

Neuraminidase-treated, GA-fixed cells were also reacted with *B. cereus* PLC. The results of these experiments are shown in figure 4.4. In these experiments the controls contained PLC that was inhibited with o-phenanthroline. The PLC removed 1.2μmoles phosphocholine per 10^{10} cells, which is a larger amount than was removed in any of the non-neuraminidase-treated samples No effect of the PLC on the mobility is seen. (The apparent positive shift for the low-Debye-length control probably is a result of chamber drift for this, the first measurement of the day; Numerous other results, eg. figures 3.16 and 3.17, show no such effect.)

Thus the results of the *B. cereus* enzyme (less purified) indicated very little effect, if any, of the PLC on the electrophoretic mobilities except for the slight decrease of the mobility in the Debye length range of 1.5 to 2.5nm. Since other workers with highly purified enzymes did not find the activity that I did, it is possible that the contaminants in the commercial enzyme are partially responsible for the activity. Another explanation is that the age of the cells or the fixation procedure might have modified the membrane and allowed the enzyme to better interact with the substrate. Because of these considerations, I repeated some of these experiments with a more highly purified enzyme from another source.

4.1.2. Clostridium perfringens PLC

Highly purified PLC from *C. perfringens* was used on GA-fixed cells. The phosphate assay indicated that approximately 0.79 micromoles of phosphate were removed per 10^{10} cells (Table 4-2). Data on the electrophoretic mobility vs. Debye length for these cells are tabulated in table 4-3, and plotted in figures 4.5 and 4.6. The former shows the uncorrected data, the latter the data corrected for the viscosity of the sucrose in the low ionic strength solutions. The PLC-treated cells appear to have a somewhat less negative mobility than the untreated cells,

Figure 4.4

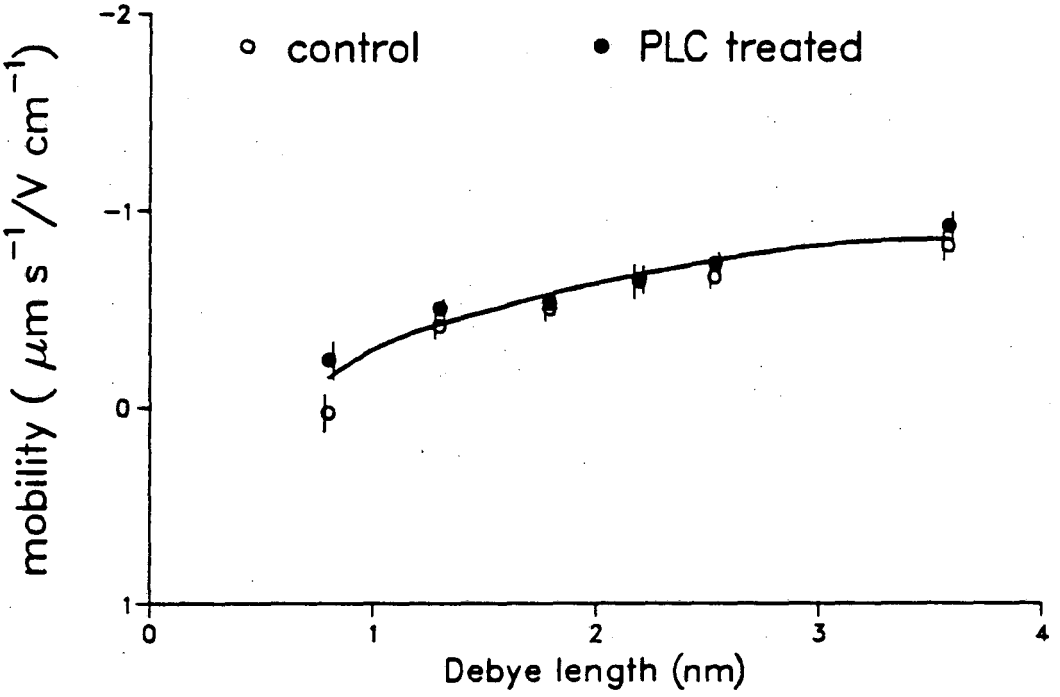


Figure 4.4. The effect of PLC *(B. cereus)* on neuraminidase-treated, GA-fixed, human erythrocytes: uncorrected electrophoretic mobility as a function of Debye length. Neuraminidase treatment was performed on 0.25% GA-fixed cells using *V. cholerae* neuraminidase (60 units/ml in Standard Buffer, 1% $CaCl_2$, pH 7.1 ± 0.1) for 5 minutes at 37°C. PLC treatment (•) consisted of 10-minute incubation at 37°C in Standard Buffer with 10 units PLC, 0.5mM $CaCl_2$, and 5×10^9 cells. Controls (o) were treated the same way except for the addition of 4mM o-phenanthroline. The phosphate assay indicated that 1.2 μmoles phosphate was removed per 10^{10} cells. Error bars represent ± 1 standard error for each average.

TABLE 4-2

Phosphate removed by treatment with phospholipase-C from *C. perfringens.*

Micromoles phosphate[a]

Expt. No.	Per 2 ml cells		Difference: PLC-control	
	Control[b]	PLC treated	Per 2 ml cells	Per 10^{10} cells
1	0.66	1.80	1.14	0.95
2	0.46	1.25	0.79	0.86[c]
3	1.02	1.47	0.45	0.49[c]
4	1.02	1.93	0.91	0.99[c]
5	1.40	2.30	0.90	0.98[c]
6	0.88	1.47	0.59	0.64[c]
7	1.07	1.76	0.69	0.75[c]
8	1.13	1.87	0.74	0.69
Mean				0.79 ± 0.06[d]

(a) The assay used was that of Fiske and Subbarow.

(b) The "control" samples contained PLC that was inactivated by the addition of o-phenanthroline (4mM).

(c) An average cell count of 0.922×10^{10} cells per 2ml sample was used for these experiments. This was the average from 5 experiments in which cell counts were made (1.38×10^{10}, 1.2×10^{10}, 1.08×10^{10}, 2.7×10^{9}, and 6.8×10^{9}).

(d) The error is the standard error of the mean.

TABLE 4-3

Electrophoretic properties of fixed human erythrocytes treated with PLC *(C. perfringens)*.

		Uncorrected mobility [a] ($\mu m\ s^{-1}$ / $V\ cm^{-1}$)		
Debye length (nm)	Dilution	Control[b]	PLC treated[c]	Number of experiments[d]
0.80	1/1	1.16 ± 0.04 [e]	1.13 ± 0.02	5
1.39	1/3	1.53 ± 0.06	1.41 ± 0.07	3
1.79	1/5	1.65 ± 0.03	1.56 ± 0.03	5
2.19	1/7.5	1.78 ± 0.05	1.64 ± 0.05	5
2.53	1/10	1.93 ± 0.08	1.79 ± 0.11	5
3.58	1/20	1.93	1.65	1
μmoles phosphate / 10^{10} cells			0.79 ± 0.06	8

(a) - All cells were fixed in 0.25% glutaraldehyde prior to PLC treatment.

(b) - Control cells were incubated either with PLC plus o-phenanthroline, or with neither of these in the reaction mixture. No mobility differences were observed for the two treatments.

(c) - PLC treatment consisted of 10-minute incubation at 37°C in Standard Buffer with 10 units PLC, 5×10^9 cells, and 0.5mM $CaCl_2$.

(d) - Each experimental result was based on an average of 20 individual measurements.

(e) - Errors are standard errors of the mean.

Figure 4.5

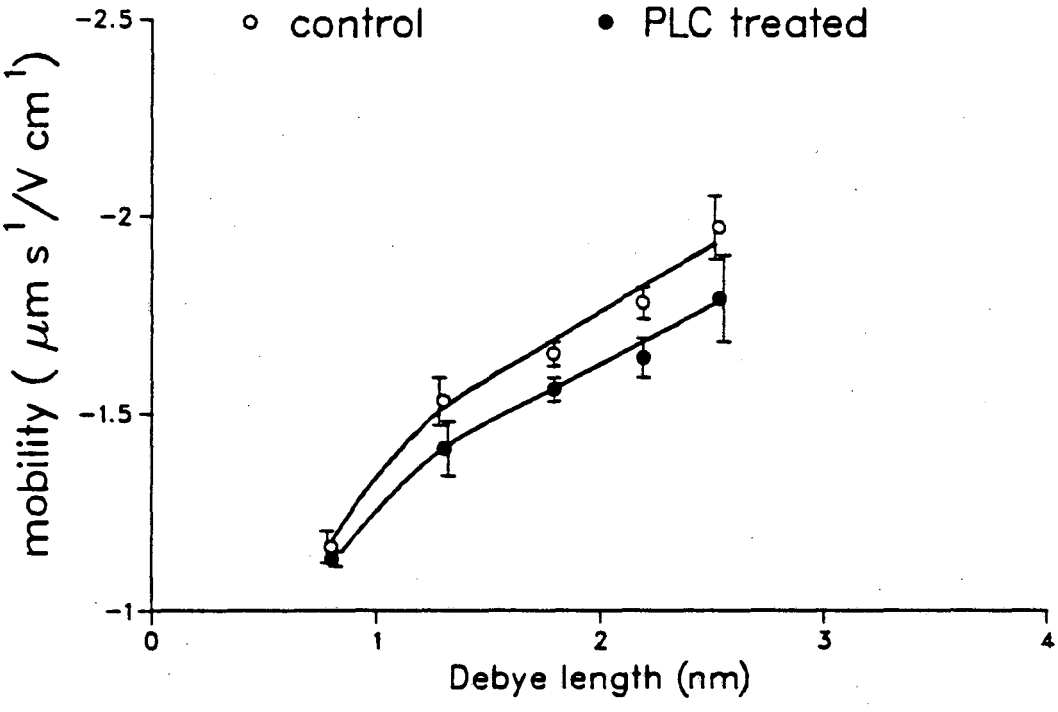


Figure 4.5. Uncorrected electrophoretic mobility of PLC *(C. perfringens)*- treated, GA-fixed, human erythrocytes (•) as a function of Debye length. The controls (o) were similarly treated but without PLC. Error bars are standard errors of the mean. Data from table 4-3. See figure 4.7 for reversibility data.

Figure 4.6

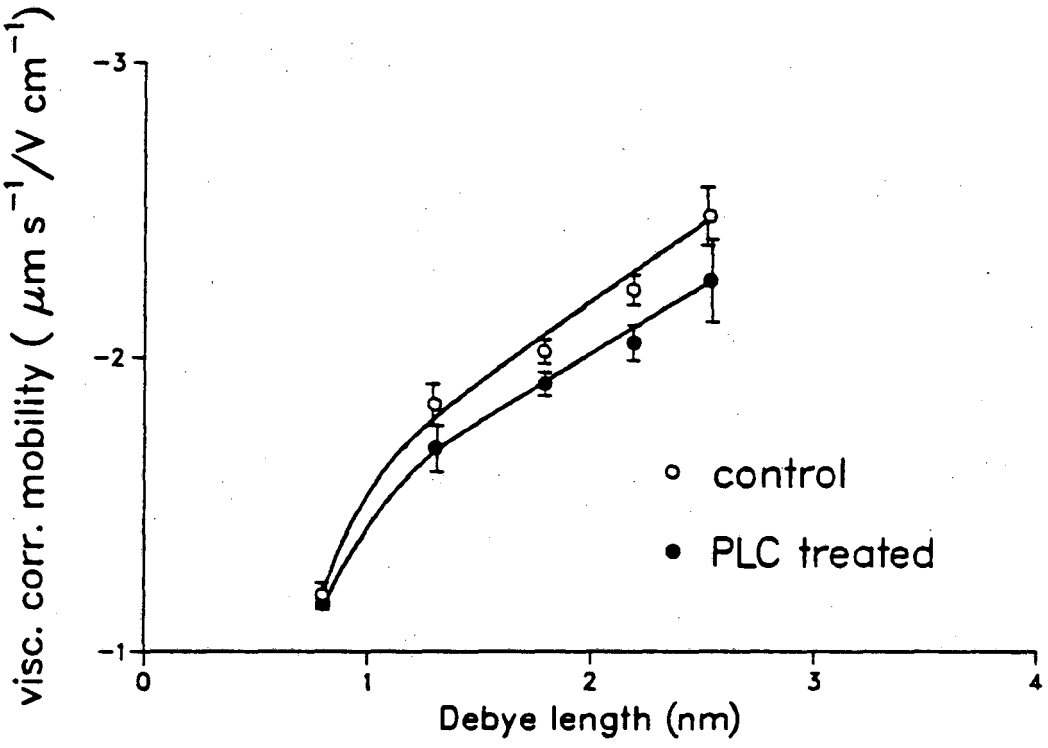


Figure 4.6. The data of the figure 4.5 but after making the viscosity correction. See figure 4.7 for reversibility data.

especially between Debye lengths 1.5 and 2.5nm, but the significance of each individual difference is marginal ($P>.05$ as determined by the Student t test). However, the Wilcoxon matched pairs signed rank test, performed on all the data at all low ionic strengths, shows that the data from the two treatments differ with a level of significance of $<.01$. Interestingly, this Debye-length range (1.5 to 2.5) where the difference is most significant is the same range in which a decrease in mobility was seen after treatment with PLC from *B. cereus*, (section 4.1.1) and with ethanol treatment (section 3.2.3).

The reversibility measurements for the *C. perfringens* treated cells are shown in figure 4.7. These data were obtained by resuspending each low-ionic-strength sample in Standard Buffer, measuring the electrophoretic mobility and expressing the result as a fraction of the mobility of the original-Standard Buffer sample. These data represent the average values from several experiments: 2 experiments at Debye length 1.39, 3 at Debye lengths 1.79 and 2.19, and 4 at Debye length 2.53. The results show that the cells pass the reversibility test reasonably well. Therefore, the low-ionic-strength buffers have not, themselves, caused a permanent change in the electrophoretic properties of either the control- or the PLC-treated cells.

Postfixing PLC-treated cells with acetaldehyde produced little effect on the mobilities (table 4-4, figures 4.8 and 4.9), although a slight increase is seen in one of the experiments in the range of Debye lengths in which the decrease in mobility was seen in figures 4.5 and 4.6. However, this increase is within the measurement errors of the technique. Therefore, I conclude that little, if any, of the mobility increase is due to the uncovering of free amines by the enzyme action.

The pH vs. mobility measurements at low ionic strength showed little effect of the PLC treatment: figure 4.10 (Debye length=1.79nm) and figure 4.11 (Debye length=2.19nm). The mobility values for the PLC-treated cells were generally lower than for control cells over the pH

Figure 4.7

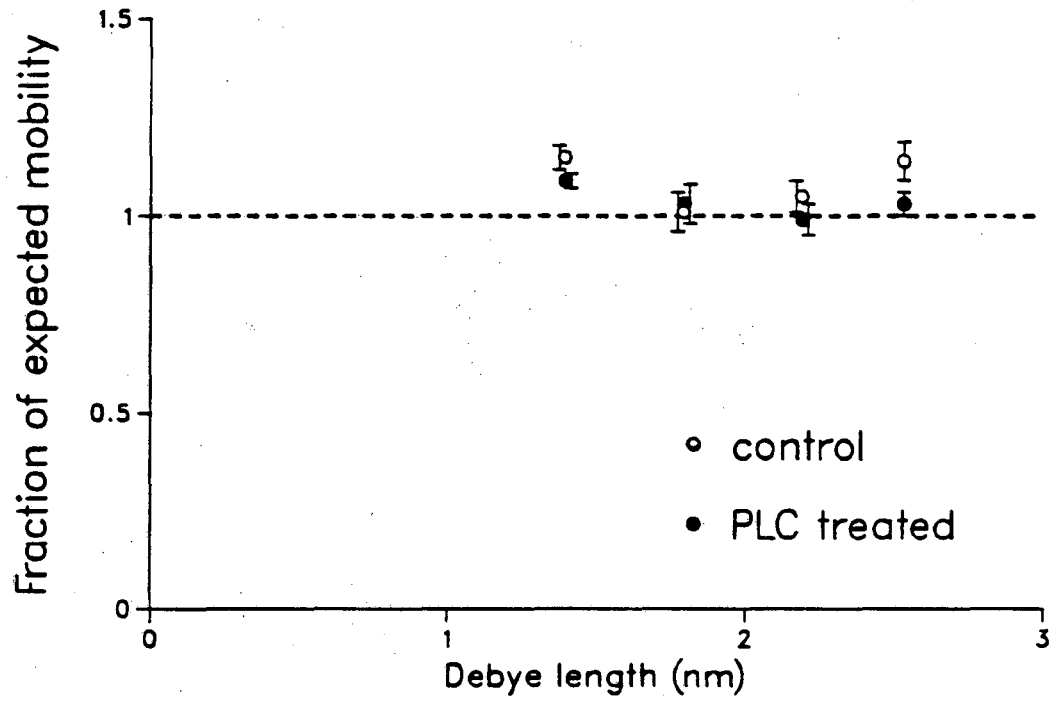


Figure 4.7. Reversibility data for the data presented in figures 4.5 and 4.6. After making the mobility measurements at the indicated ionic strengths, the samples were centrifuged and resuspended in Standard Buffer and the mobility was again measured. The data are presented for both the PLC-treated (•) and controls (o), as the fraction of the mobility of the original Standard-Buffer sample. The number of experiments (shown in parentheses) for each Debye length was: 1.39(2), 1.79(3), 2.19(3), 2.53(4). Error bars are the standard error of the mean.

TABLE 4-4

Effects of acetaldehyde fixation on PLC (*C. perfringens*)-treated human erythrocytes that were prefixed with 0.25% glutaraldehyde.

			Uncorrected negative electrophoretic mobility[a] ($\mu m\ s^{-1}$ / $V\ cm^{-1}$)			
			No PLC[b]		PLC	
Debye length (nm)	Dilution	Expt. #[c]	No acetaldehyde	Postfixed with acetaldehyde	No acetaldehyde	Postfixed with acetaldehyde
0.80	1/1	1	1.10	1.15	1.05	1.14
		2	1.56	1.66	1.62	1.53
1.79	1/5	1	1.84	1.77	1.77	1.78
		2	2.43	2.43	2.23	2.32
2.19	1/7.5	1	2.00	2.08	1.83	1.89
		2	2.45	2.52	2.28	2.41
Micromoles phosphate[d]			0.88		1.47	

(a) - Each value is the average from 20 measurements on 10 cells.

(b) - The "No PLC" samples contained PLC that was inactivated by the addition of o-phenanthroline (4mM).

(c) - The two experiments contain data collected on different days. The second experiment shows unusually high mobility values due to an equipment calibration problem. It is presented here for horizontal comparison only.

(d) - Phosphate assay is from experiment 1 only.

Figure 4.8

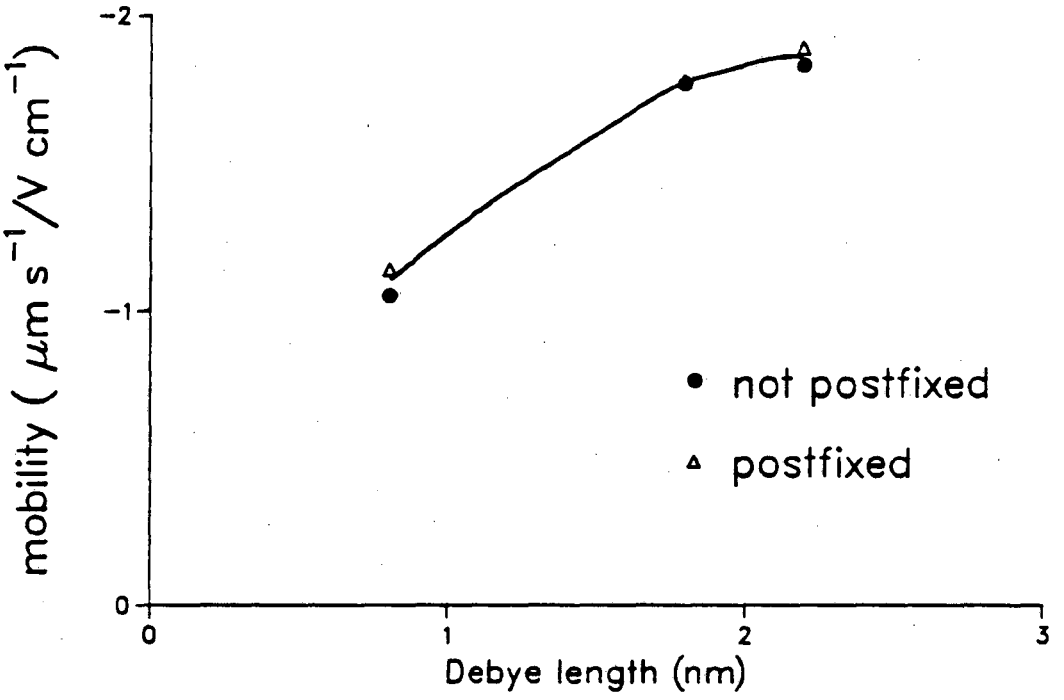


Figure 4.8. The effects of post-fixation with acetaldehyde on the electrophoretic mobilities of PLC *(C. perfringens)*- treated, GA-fixed, human erythrocytes as a function of Debye length. The data are from the first experiment of each of the three data sets in table 4-4. All cells were collected, washed, fixed with 0.25% glutaraldehyde, treated with PLC. The postfixed cells (Δ) were then incubated in 0.5% (w/v) acetaldehyde plus Standard Buffer for 10 minutes at room temperature. The non-postfixed cells (•) were similarly incubated in Standard Buffer.

Figure 4.9

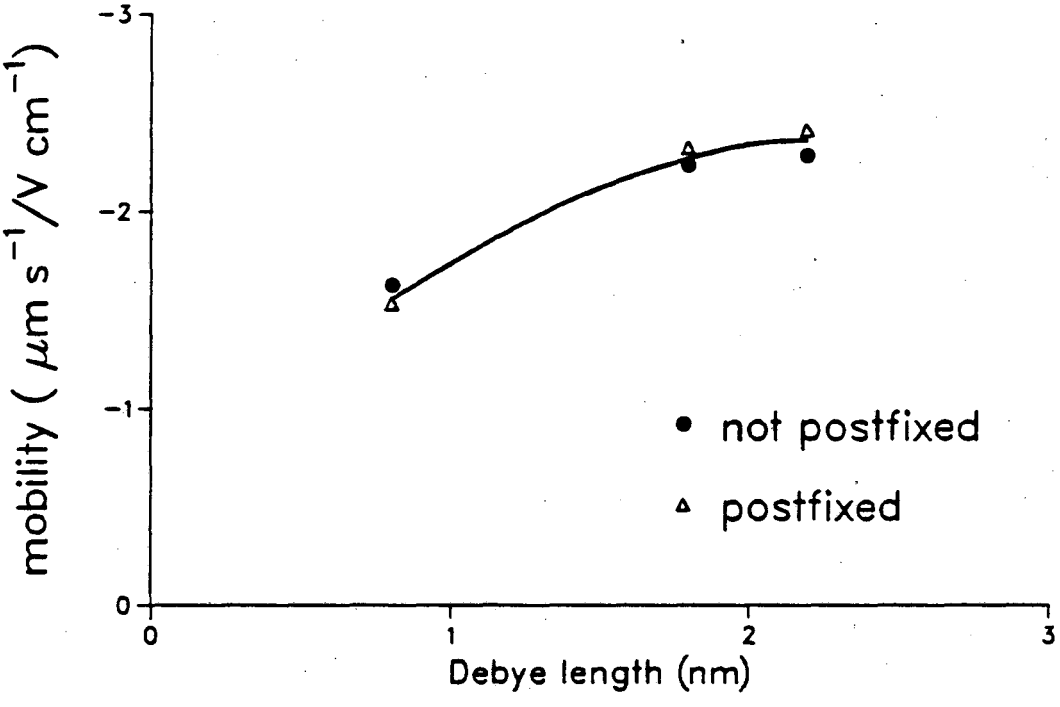


Figure 4.9. A repeat of the experiment in figure 4.8, data taken from the second experiment of each data set in table 4-4. The mobilities here were unusually high due to an equipment-calibration problem, but are presented here for internal comparisons.

Figure 4.10

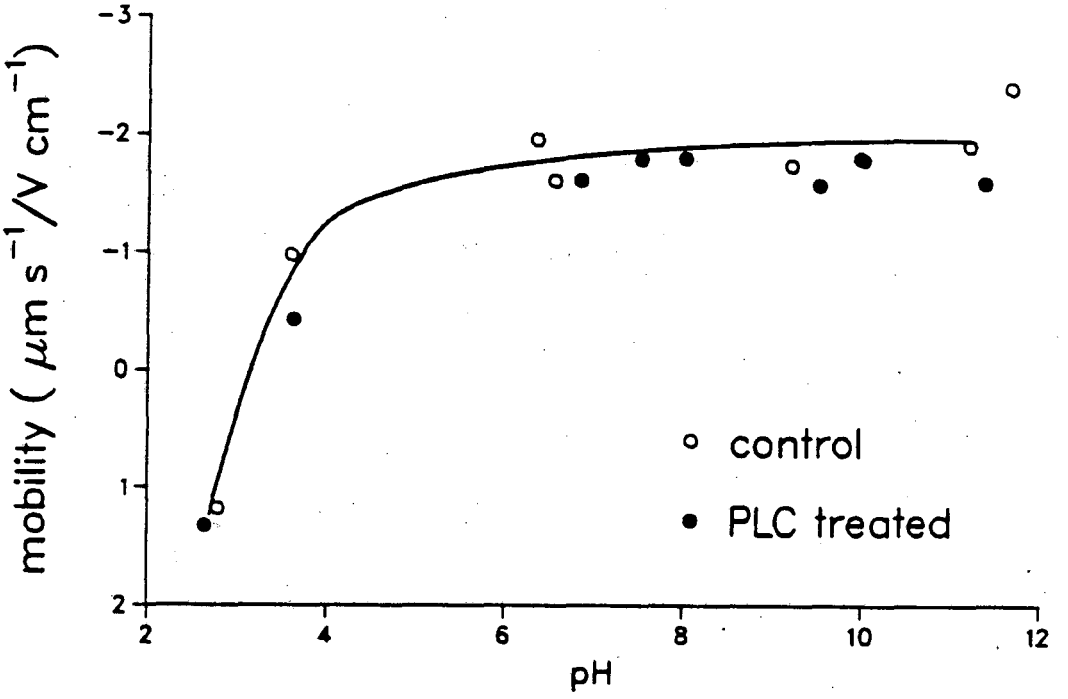


Figure 4.10. Uncorrected electrophoretic mobility of PLC *(C. perfringens)*- treated (•) and untreated (o), GA-fixed, human erythrocytes, measured in low ionic strength buffer (Debye length = 1.79nm) as a function of pH. The suspension medium was 0.029M buffer made isotonic by the addition of sucrose. The pH adjustments were made with 0.029M NaOH or HCl.

Figure 4.11

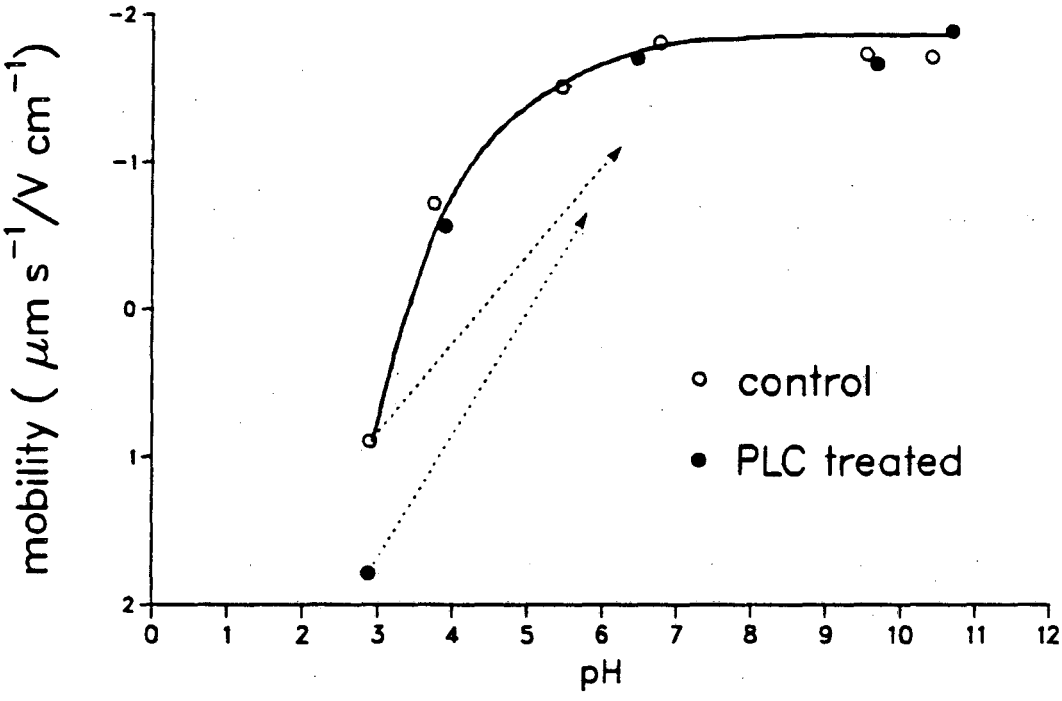


Figure 4.11. Uncorrected electrophoretic mobility of PLC *(C. perfringens)*- treated (•) and untreated (o), GA-fixed, human erythrocytes, measured in low ionic strength buffer (Debye length = 2.19nm) as a function of pH. The suspension medium was 0.019M buffer made isotonic by the addition of sucrose. The pH adjustments were made with 0.019M NaOH or HCl . The arrow ends of the dotted lines depict reversibility measurements, made after raising the pH of the lowest pH sample, then remeasuring the mobilities. The measurements demonstrate that the cells were unstable at the lowest pH.

range 2 to 12, but the differences between the two samples were very small. However, at a Debye length of 2.19nm a difference is noted at the lowest pH, but the PLC-treated cells, and to some extent the controls, were electrically unstable at this pH and ionic strength, even though fixed with glutaraldehyde. This behavior is reflected in figure 4.11 by the lack of reversibility of the low pH sample. The phosphate assay showed that the enzyme was active on these cells; removing approximately 1.5μmoles phosphate for each sample.

The results with the PLC enzymes were somewhat inconsistent between experiments. Both PLC enzymes caused a slight decrease in (negative) electrophoretic mobility between Debye lengths of approximately 1.5 and 2.5nm. This is the same range of Debye lengths where a decrease was seen after ethanol treatment (Section 3.2.3). However, for the two samples for which the pH mobility curves were measured, less effect of the PLC was seen. Also, when neuraminidase-treated cells were reacted with PLC, no effect was seen.

From the results of several experiments with the two PLC enzymes, and particularly the combined results of the several experiments shown in figures 4.5 and 4.6, I conclude that there is a real effect of the phosphocholines on the electrophoretic mobility. However, the effect is very small and I must conclude that any effect of PLC on the charge properties of the membrane is close to the limits of precision of the measurements (i.e. that any contribution to the membrane surface charge from the phosphocholine is very small)

4.2. Phospholipase-D treatment

As indicated previously, phospholipase-D (PLD) removes choline but not the phosphate from phospholipids (see figure 1.1). If the positive choline is removed, and if the phospholipid head groups are contributing to the surface charge, the negative electrophoretic mobility should increase after treatment. Treatment of human erythrocytes was carried out with PLD enzymes from two different sources. The PLD from peanut was used only on glutaraldehyde-fixed cells,

since it hemolysed the unfixed cells. By contrast, PLD from *S. chromofuscus* could be used on unfixed cells without excessive hemolysis. The peanut enzyme hydrolyses PC, PE, and PS, but not sphingomyelin, (Heller, 1978). The *S. chromofuscus* enzyme hydrolyzes all of these plus sphingomyelin as well (Imamura and Horiuti, 1978).

Several workers have reported different positive actions on red cells with various PLD treatments. Fujii and Tamura (1979) induced invagination in human erythrocytes using *S. chromofuscus* PLD. Treatment was in 0.9% NaCl with 5mM $CaCl_2$. After 2 hours of incubation only about 60% of the PC remained in the cell. No change occurred in PE, PS, or sphingomyelin content. The phosphatidic acid content increased, but by less than the amount needed to make up for the decreased PC. With cabbage enzyme, they found no effect on cell shape. This is consistent with Zwaal et al. (1975), who reported that cabbage PLD is inactive on the human erythrocyte.

Muller, et al. (1981) used conditions of ATP depletion, and examined the mobility of the lipid vesicles formed by the blebs lost from the membrane. They found an increase in the mobility of these vesicles when the cells had previously been treated with PLD from cabbage. This increase was preserved after neuraminidase treatment.

Zwaal and Roelofsen (1976) state that PLD produces an accumulation of phosphatidic acid in the membrane, accompanied by an increase in negative charge. However, no supporting data were given.

In my experiments, both of the enzymes used were shown to remove choline as determined by the choline reineckate assay, but the *S. chromofuscus* enzyme removed larger amounts: an average of 1.11 ± 0.17 μmoles/10^{10} cells for the *S. chromofuscus* enzyme, as compared to 0.50 ± 0.07 μmoles/10^{10} cells for the peanut enzyme (discussed with tables 4-6 and 4-9, below).

4.2.1. Peanut PLD

The properties of the peanut-PLD enzyme have been extensively studied by Heller et al. (1975). This enzyme has a very narrow pH range for maximum activity with a peak about pH 5.6. In my experiments this pH was maintained with an acetate buffer. A very high Ca^{+2} level, of at least 50mM, is also required. A detergent such as lauryl sulfate serves as an activator of the reaction. In order to determine the effects of these additional chemical conditions on erythrocytes, the electrophoretic mobilities of cells incubated in this solution were compared with those for cells incubated for the same length of time in Standard Buffer. The results, shown in table 4-5, indicate little effect of these conditions on the mobilities of the erythrocytes. Since each individual experiment includes its own controls, which are treated with identical conditions except for the absence of PLD, slight differences will not affect the comparisons of the PLD-treated and the controls.

The choline assay data are given in table 4-6. They indicate that on the average 0.50 ± 0.07 μmoles choline were removed from 10^{10} cells, when incubated with the peanut-PLD for 90 minutes. Assays for phosphate and sialic acid were also performed to determine if either of these moieties was lost from the membrane as a result of treatment with the PLD enzyme. Examination of the data in table 4-7, shows that this was not the case (i.e. in no case is the result for the test sample greater than that for the (sum of the) controls). Assays for protein also showed that no detectable protein was removed by the peanut enzyme. (In a 0.5ml aliquot taken from the supernatant after treatment, 80μg protein was recovered from the enzyme-treated cell sample, 75μg from the PLD control, and 15μg from the untreated-cell control).

Measurements on PLD-(peanut)-treated, GA-fixed cells.

The results of measurements of mobility vs Debye length after treatment with peanut PLD are given in table 4-8 and figure 4.12. The same data are plotted slightly differently in

TABLE 4-5

The effects of incubation in acetate, lauryl sulfate and calcium chloride on the electrophoretic mobility of GA-fixed human erythrocytes.

		Uncorrected Electrophoretic Mobility ($\mu m\ s^{-1}$ / $V\ cm^{-1}$)		
Debye length (nm)	Dilution	Control[a]	Test cells[b]	number of experiments[c]
0.80	1/1	1.19 ± 0.03[d]	1.11 ± 0.03[d]	3
2.53	1/10	2.06 ± 0.08	1.97 ± 0.06	3

(a) - Control cells were incubated in Standard Buffer.

(b) - Test cells were prepared by incubating 0.25%-GA-fixed human erythrocytes for 10 to 30 minutes at 30°C in a solution containing 0.1M acetate, 2.5mM sodium lauryl sulfate and 50mM $CaCl_2$, at pH 5.6. The control cells were incubated in Standard Buffer.

(c) - Each experimental result was based on an average of twenty individual measurements.

(d) - Errors are standard errors of the mean.

TABLE 4-6

Choline removed by treatment with peanut PLD.

	μmoles choline per 10^{10} cells			
	Controls		Test sample	
Expt. No.	Cells, no PLD[a]	No cells, PLD[b]	Cells with PLD[c]	Difference[d]
1	0	—	0.53	0.53
2	0.04	—	0.76	0.72
3	0.13	—	0.40	0.27
4	—	0.29	0.93	0.64
5	0	0	0.31	0.31
6	0	0	0.51	0.51
Mean				0.50 ± 0.07[e]

(a) - Control cells were treated in the same manner as the test sample but without PLD.

(b) - These controls, containing PLD but no cells, were treated in the same manner as the test sample.

(c) - Test cells were incubated for 90 minutes at 30°C, with shaking, in a solution containing PLD (33 U/ml), 0.1M sodium acetate, 2.5mM lauryl sulfate, and 50mM $CaCl_2$. The cells were centrifuged, the supernatant was removed and mixed with 4 volumes chloroform/methanol (2:1). The upper layer was saved in the freezer for the assay.

(d) - Difference between the test-sample result and the sum of the control values.

(e) - The error is the standard error of the mean.

TABLE 4-7

Phosphate and sialic acid assays[a] after treatment of GA-fixed human erythrocytes with PLD (peanut)[b].

Assay	μmoles per 10^{10} cells		
	Controls		Test sample
	Cells, no PLD[b]	No cells, PLD[b]	Cells with PLD[b]
Phosphate	0.180	0.096	0.216
Sialic acid			
Predigested[c]	0.042	0.006	0.033
Not predigested	0.030	0.026	0.033

(a) - The phosphate assay was that of Bartlett, the sialic acid assay that of Warren.

(b) - PLD treatment and controls as in Table 4-6.

(c) - Predigested in 0.1N H_2SO_4 at 80°C for 1 hour.

TABLE 4-8

Viscosity corrected electrophoretic mobility as a function of Debye length for Ga-fixed human erythrocytes treated with PLD (peanut).

		Electrophoretic mobility ($\mu m\ s^{-1}/Vcm^{-1}$)			
		Control[a]		PLD treated[a]	
Debye length	Dilution	Mobility	Number of experiments[c]	Mobility	Number of experiments[a]
0.8	1/1	1.03 ± 0.05[d]	4	1.03 ± 0.02[d]	4
1.79	1/5	1.94	1	1.89	1
2.19	1/7.5	2.86	1	2.51	1
2.53	1/10	2.96 ± 0	2	2.78 ± 0.04	2
3.57	1/20	3.25 ± 0.15	3	2.84 ± 0.25	3

(a) - Fixed cells were incubated in PLD (100 units/2.5ml of 50% cell suspension (v:v)) for 90 minutes at 30°C in a buffer containing 0.1M acetate, 5mM sodium lauryl sulfate and 50mM $CaCl_2$. Controls were similarly incubated but without PLD.

(b) - Viscosity corrections were made using the data in Figure 2.1.

(c) - Each experimental result was based on an average of twenty individual measurements.

(d) - Errors are standard errors of the mean.

Figure 4.12

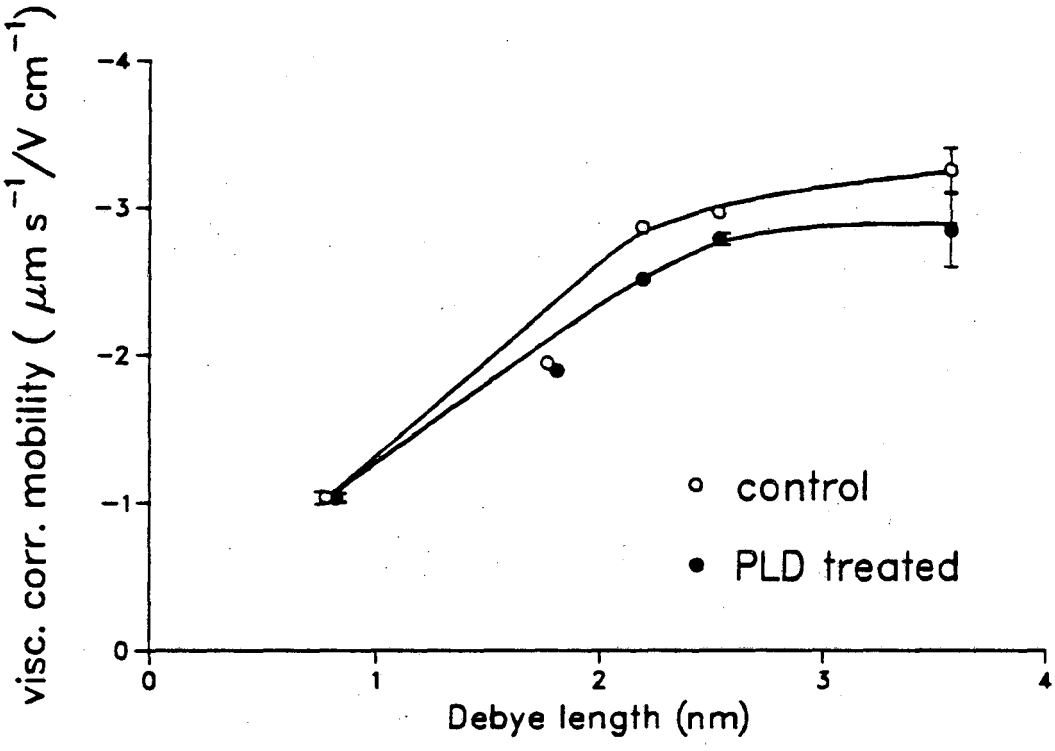


Figure 4.12. Viscosity-corrected electrophoretic mobility of PLD (peanut)-treated (•) and untreated (o), GA-fixed, human erythrocytes as a function of Debye length. Data from table 4-8. Error bars, representing standard errors of the mean, are presented for those data which are averages of more than one experiment.

figure 4.13, as the fraction of the control mobilities after PLD treatment. Error bars representing the standard errors of the mean are shown for those points that are based on more than one measurement. The curves indicate a slight decrease in the (negative) mobility at the lower ionic strengths after PLD treatment. If the only effect on the surface charge were that due to the removal of the positive choline, an *increase* rather than a decrease would be expected.

Figure 4.14 shows the results of measuring electrophoretic mobility vs. pH, in Standard Buffer, for the (peanut) PLD-treated cells. Care was taken to measure points for both curves at the same pH, as nearly as possible. In all of these data below pH 7, the mobility value of the PLD-treated cells is lower than that of the untreated cells. The isoelectric point increased from about 2.75 for the untreated to 3.0 for the PLD-treated cells.

In order to test for positive amino groups, that might contribute to the mobility after PLD treatment, control and PLD-treated cells were postfixed with acetaldehyde. The results are shown in figure 4.15. From a comparison of figure 4.15 with figure 4.14, I conclude that acetaldehyde has little or no effect on the decreased negative mobilities of the PLD-treated cells and hence that amino groups are not responsible for the measured effect.

In preparing the analysis for this section, another possible basis for the "positive shift" came to mind, namely that Ca^{+2} in the enzyme-treatment medium might be exerting a specific effect (i.e. reacting with the phosphatidic acid after the choline removal), even though the cells were washed twice in Ca^{+2} free buffer following incubation with the enzyme solution. I was able to test this idea with a single experiment, at several different ionic strengths, by placing the cells in a 10mM EDTA solution (which would presumably remove the Ca^{+2}) and measuring the electrophoretic mobility. The result was that in some cases the mobility of the EDTA-treated cells increased slightly, but not systematically, and not up to the values for the non-

Figure 4.13

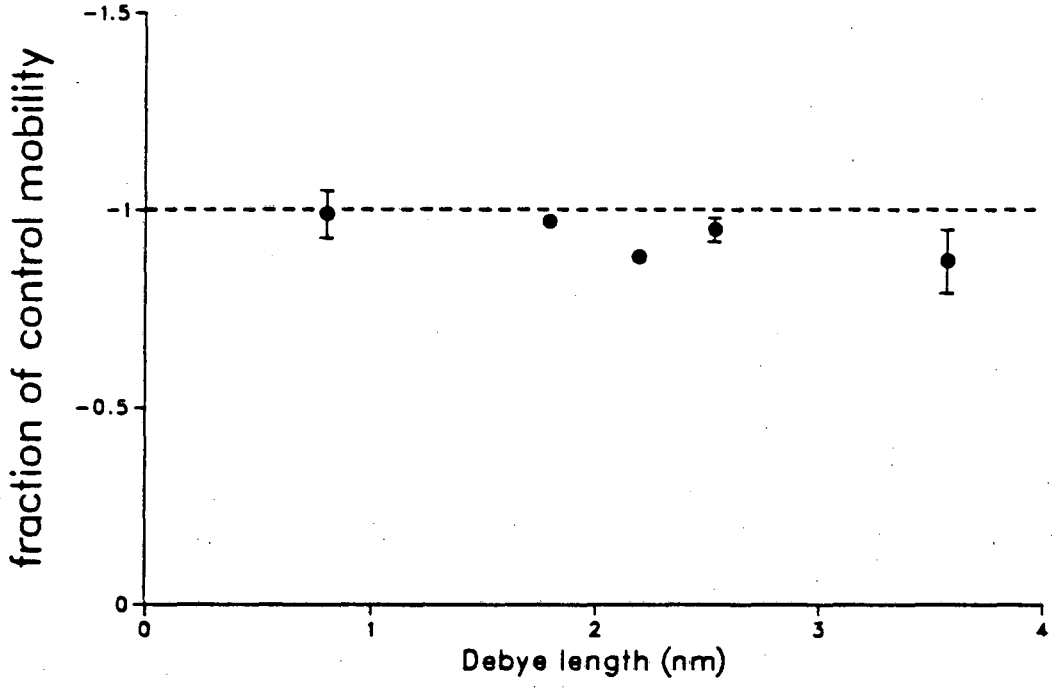


Figure 4.13. The data of figure 4.12 plotted with the ordinate expressed as the fraction of the control mobility.

Figure 4.14

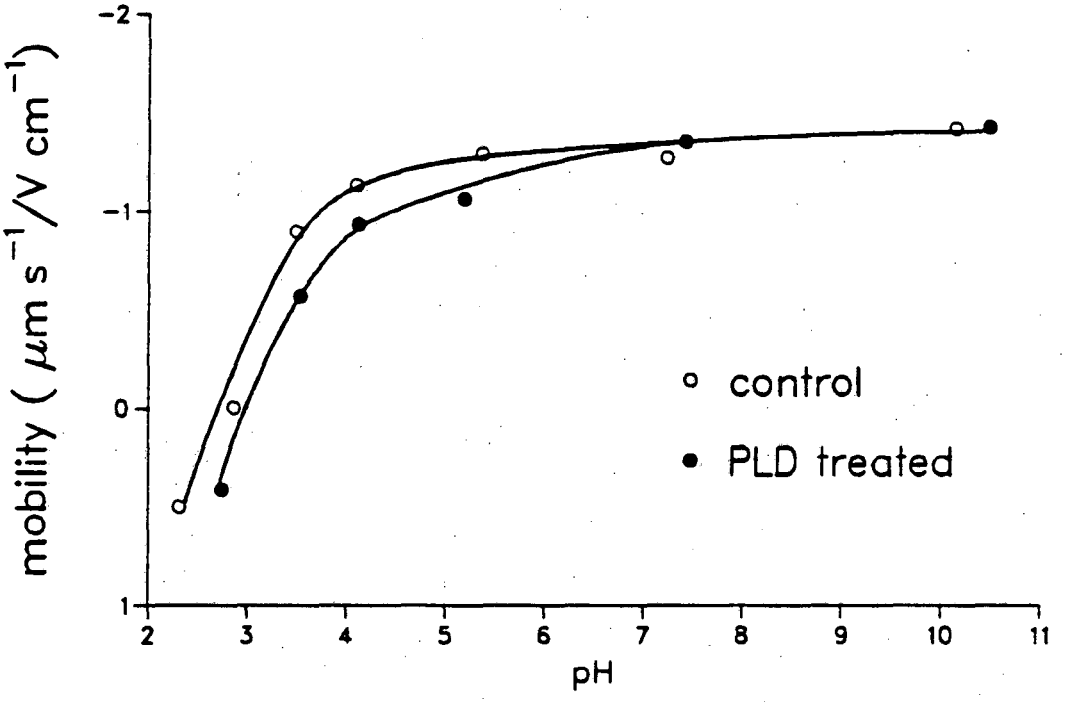


Figure 4.14. Uncorrected electrophoretic mobility in Standard Buffer of PLD (peanut)-treated (•) and untreated (o), GA-fixed, human erythrocytes as a function of pH.

Figure 4.15

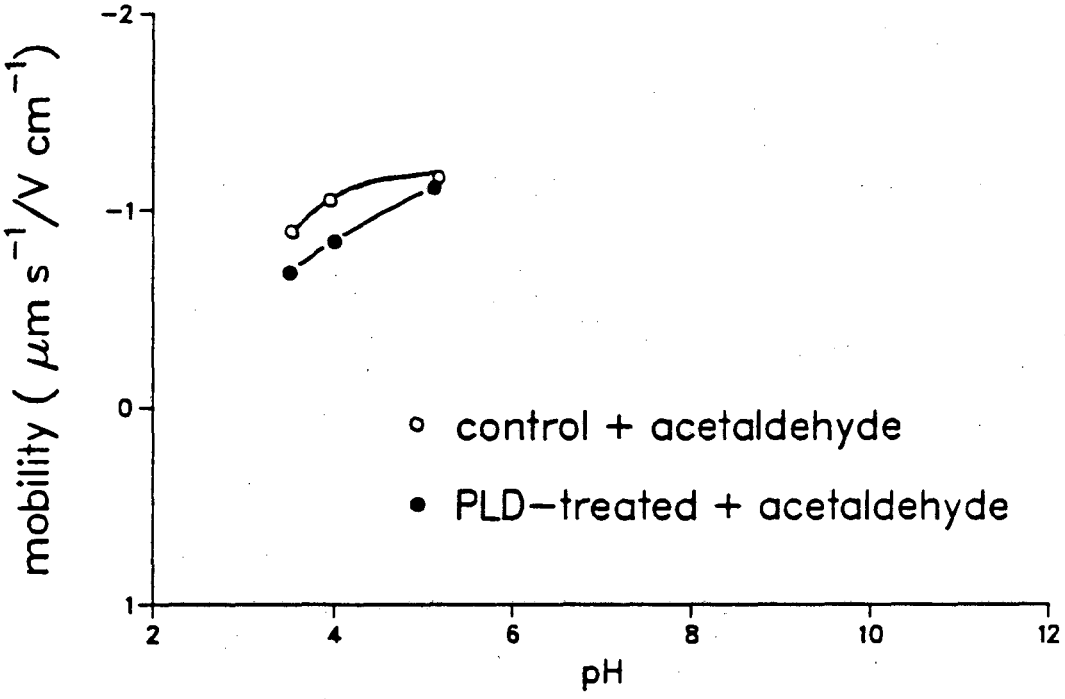


Figure 4.15. The effect of post-fixing with acetaldehyde on the uncorrected electrophoretic mobility of PLD (peanut)-treated (•) and untreated (o), GA-fixed, human erythrocytes at low pH. All cells were incubated in 1% acetaldehyde in Standard Buffer for 30 minutes at room temperature.

PLD-treated cells. Another argument against Ca^{+2} playing a significant role comes from the pH-mobility experiments. If Ca^{+2} were binding to the phosphorus group, it would be expected that at low pH, the Ca^{+2} would be displaced by singly-charged protons, and therefore, that the mobility difference between the two curves would decrease. Examination of the pH vs. mobility curve plotted in figure 4.14 shows that, in fact, the opposite effect is seen.

Measurements on PLD (peanut)-treated, neuraminidase-treated, GA-fixed cells.

The peanut-PLD treatment was also applied to neuraminidase-treated, GA-fixed cells in order to test whether the presence of sialic acid affected either the action of the enzyme or the positive-shift experimental result. The amount of choline removed from the treated cells, in one experiment, was 0.21 μmoles per 10^{10} cells, compared to 0.28 μmoles per 10^{10} from the non-neuraminidase-treated, GA-fixed cells. Therefore, based on this (one) experiment, removal of sialic acid does not allow an increased action of the PLD enzyme. Figures 4.16 (mobility) and 4.17 (surface charge density) display the average values (for 2 to 4 experiments) as a function of Debye length. In every case, the PLD-treated cells had a *higher* (negative) mobility than did the untreated. Reversibility measurements (back to Standard Buffer) were also conducted for one of the experiments contributing to the average mobility in figure 4.16, for each of the two highest Debye lengths; reversibility was found to be almost perfect. Though in the non-neuraminidase-treated cells, choline removal did not lead to an increase in the (negative) mobility (the expectation which originally motivated the PLD experiments), such an increase is now seen once the sialic acid has been removed. However, the mobility increase after PLD treatment is much less than would be expected if there were a one-to-one correspondence with the amount of choline removed. Some rough calculations demonstrate this point. A 0.001 C/m^2 charge-change corresponds to 8.7×10^5 charges/cell, assuming $1.4 \times 10^{-10} m^2$/cell as the cell surface area. The removal of 0.21 μmoles choline per 10^{10} cells translates to 1.26×10^7 charges per cell, a figure more than an order of magnitude larger than

Figure 4.16

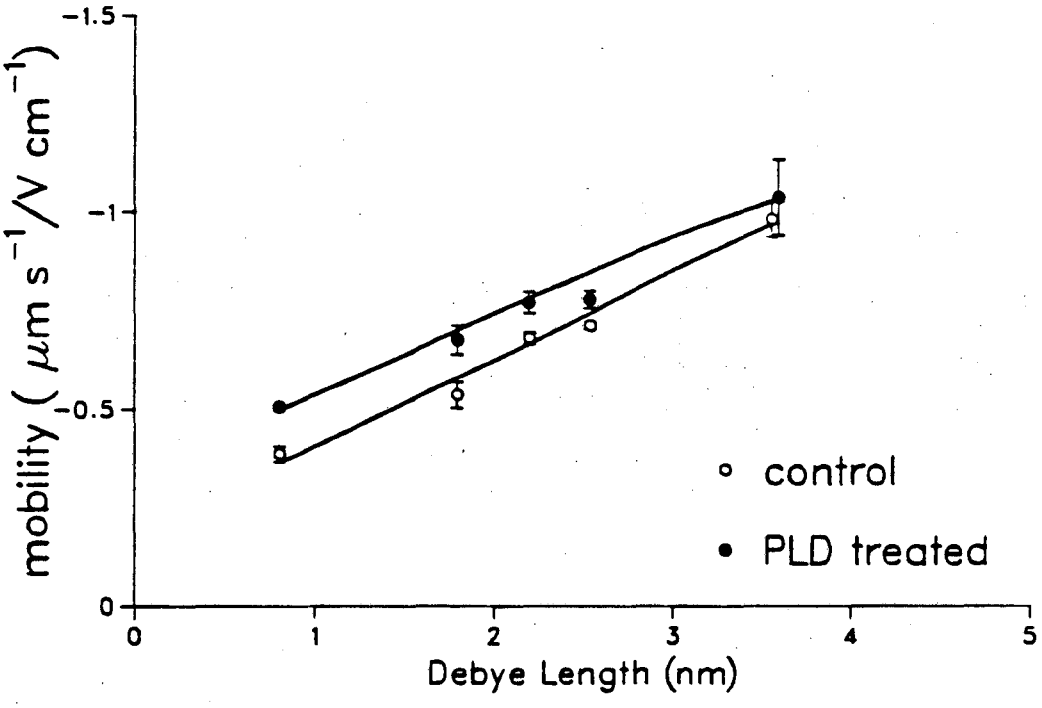


Figure 4.16. The effect of PLD (peanut) on neuraminidase-treated, GA-fixed, human erythrocytes: uncorrected electrophoretic mobility as a function of Debye length. Neuraminidase treatment was performed on 0.25% GA-fixed cells using *V. cholerae* neuraminidase (60 units/ml in Standard Buffer, 1% $CaCl_2$, pH 7.1 ± 0.1), at 37°C for 5 minutes. For the PLD treatment (•), cells were incubated in peanut PLD (100 units/2.5ml of 50% cell suspension, v/v) for 90 minutes at 30°C in a buffer containing 0.1M acetate, 5mM sodium lauryl sulfate and 50mM $CaCl_2$. Controls (o) were similarly incubated but without PLD.

Figure 4.17

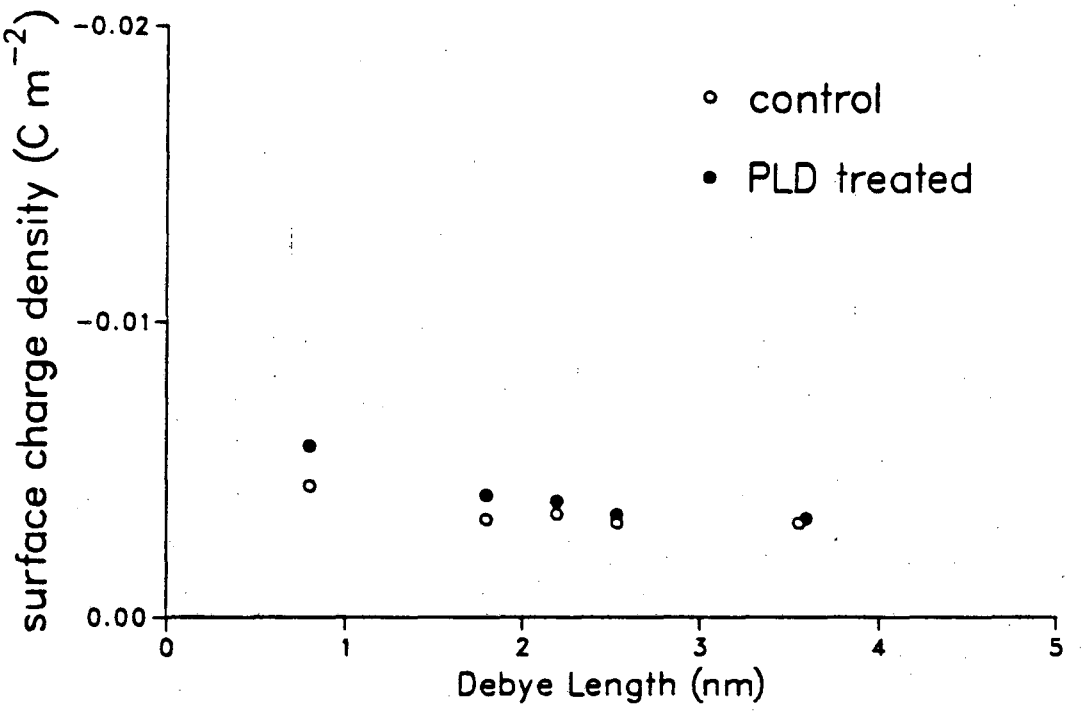


Figure 4.17. The surface charge calculations made from the data of figure 4.16.

the observed surface-charge change.

Might amino groups be exerting some influence on the electrophoretic mobility once the sialic acid is removed? To test this idea, a few mobility measurements were made on acetaldehyde-post-treated cells (figure 4.18). The mobilities of the PLD-treated cells were unchanged. The mobilities of the controls, however, came up to those of the PLD-treated cells. The presence of free amines after neuraminidase treatment is consistent with the results of Cook et al. (1961), who also saw an increase in (negative) mobility after acetaldehyde treatment of neuraminidase-treated cells. What is not at all clear, however, is why this increase should bring the mobility up to almost exactly that of the PLD-treated cells. It is possible that these amines are associated through ionic bonds to the choline head groups, and the amino-containing moieties are lost from the membrane when the choline groups are removed enzymatically. Alternatively, the membrane surface components may rearrange after the removal of choline groups in such a way the the amines are no longer electrophoretically detectable. A rearrangement of membrane lipids may also explain the absence of a contribution to the negative surface charge by the phosphatidic acid residues that remain at the cell surface following removal of choline groups by PLD.

Another possible explanation for the increased mobility after PLD, on neuraminidase-treated cells, is that the removal of sialic acid allows the collapse of the remaining surface components, which might, thereby, increase the surface charge density. To test this hypothesis, microhematocrit measurements were performed in collaboration with Dr. Gary Richieri. In no case was the size of the neuraminidase-treated cells less than that of the non-neuraminidase-treated cells, thus ruling out this explanation. Much more work is required to explain the increased mobility after PLD-treatment of neuraminidase-treated cells.

Several mobility measurements were also made on the above cell samples at high and

Figure 4.18

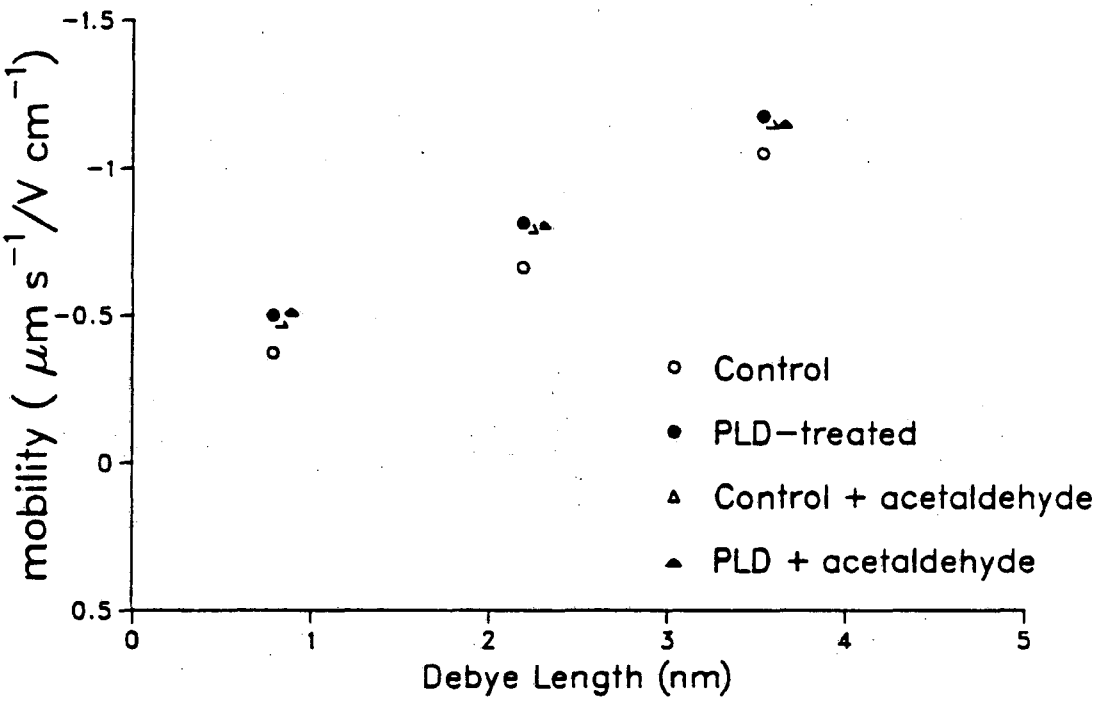


Figure 4.18. The effect of acetaldehyde on the cells from one of the experiments of figure 4.16. Triangles represent cells postfixed with acetaldehyde. Twenty volumes of acetaldehyde (1%, w/v, in a 50%, v/v, mixture of Sorensens Buffer and Standard Buffer), were added to 1 volume of a 50% cell suspension in Standard Buffer, and incubated at room temperature for 30 minutes.

low pH values (figure 4.19). At high pH (pH 10), the cells retained the mobility separation between the control and the PLD-treated cells. Furthermore, when reversibility measurements were performed, the mobilities were reversible when resuspended in a pH 7.4 (Standard Buffer) solution. At-lower-than-normal pH values (pH 4 to 4.5) the PLD-treated cells displayed highly irreversible mobilities (the mobilities increased sharply when remeasured at about pH 7, as shown in figure 4.19). At pH 3 and below, the PLD-treated cells became very unstable. The large asterisks in figure 4.19 show the mobility measurements that were made on a few cells in the short time before all the cells lysed.

Several plausible explanations exist for this array of results with neuraminidase-treated, GA-fixed cells that were post-treated with PLD. *First,* rearrangement of membrane components may occur after PLD treatment. This could result from the flipping of some of the (negative) phosphatidic acid molecules to the inner surface, and/or from the flipping of positively-charged lipids or proteins from the inner to the outer surface of the membrane. An additional consequence of the rearrangement could be the neutralization of some of the phosphatidic acid moieties by positive amines in proteins. This latter appears unlikely, however, considering that acetaldehyde failed to increase the (negative) mobility of the PLD-treated cells. A *second* explanation is that phosphatidic acid may be lost from the membrane. No phosphate was detected in the supernatant from PLD-treated cells, but no test was made for phosphate after PLD treatment of neuraminidase-treated cells. A *third* explanation which cannot be ruled out by the available data is the following. The increase in mobility after PLD treatment might be due to a loss from the membrane of the positive groups that are uncovered by the neuraminidase treatment. That these positive groups are electrophoretically detectable was shown by the acetaldehyde experiments. As previously suggested, if these positive groups were held in place by ionic bonds to the choline groups, choline removal could release them as well. The failure to detect a contribution to the negative surface charge by the phosphatidic

Figure 4.19

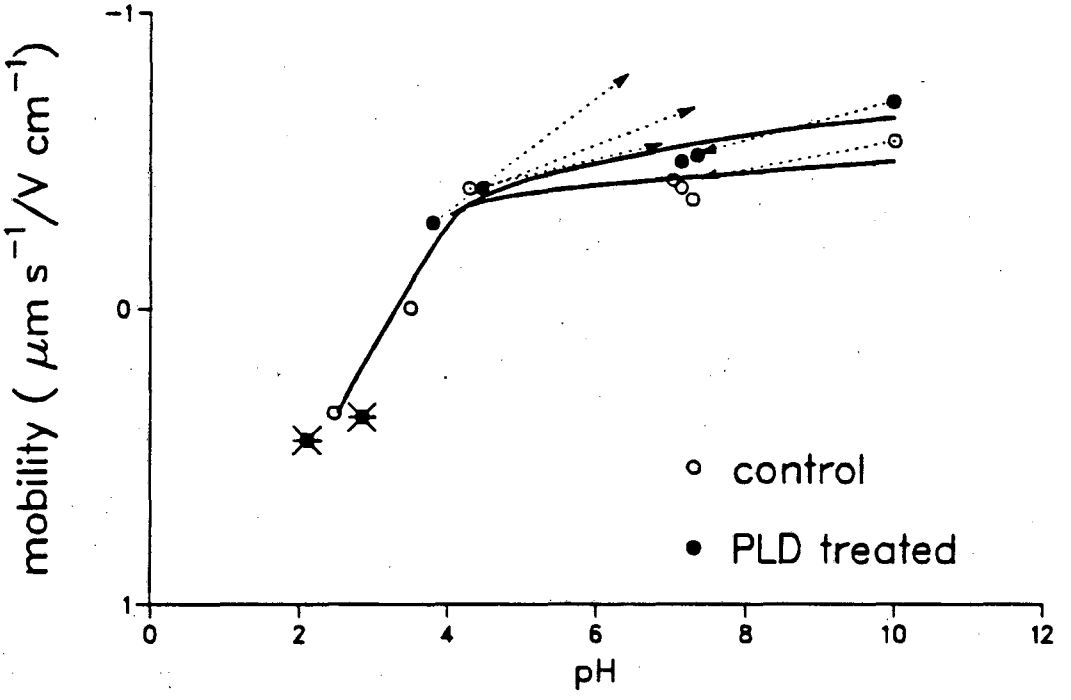


Figure 4.19. Uncorrected electrophoretic mobility in Standard Buffer of PLD (peanut)-treated, neuraminidase-treated, GA-fixed human erythrocytes as a function of pH. Enzyme treatments were as in figure 4.16. The large asterisks represent cells that disintegrated before more-than-a-few mobility measurements could be made. The arrow heads of the dotted lines depict reversibility measurements. To study reversibility of the surface charge, after determining the mobility of cells at a given pH, the cells were resuspended in Standard Buffer, and the pH and the mobility measured again.

acid residues that are present after enzymatic removal of the choline groups suggests that the phosphate groups are rearranged within the membrane following PLD treatment, possibly by "flipping" to the inner membrane surface.

The instability of the cells in the low-pH solutions, once the sialic acid has been removed as well as the choline, demonstrates that the resulting membrane is held in a somewhat precarious balance. The excess of protons in the low pH solutions may upset the charge balance, leading to cell destruction. Thus the sialic acid moieties appear to be playing a role of their own, along with the protein and lipids, in insuring membrane stability.

Summary of PLD (peanut) results.

In summary, PLD (peanut) lowered the (negative) mobility of non-neuraminidase-treated, GA-fixed erythrocytes, when an *increase* would be expected if the only effect of the PLD on the membrane were the removal of the positive choline. Associated with this charge change, the isoelectric point of the treated cells increased by about 0.25 pH units. The excess positive charge after PLD treatment was not due to amino groups. In contrast, the peanut PLD did increase the mobility of *neuraminidase-treated*, GA-fixed cells, although less than would be expected from an analysis of the choline removed. In addition it produced cells that were unstable at low pH but not at high pH. Acetaldehyde had no effect on the PLD-treated cells, but increased the control-cell mobility to that of the PLD-treated cells.

4.2.2. Streptomyces chromofuscus PLD

The highly purified *S. chromofuscus* enzyme was active on the *unfixed* cell and did not usually cause hemolysis of the cell. Therefore, the use of this enzyme provides us an unique opportunity to compare the effects of the PLD on the fixed cell with those on the unfixed cell. The assay of the choline removed by this enzyme is shown in table 4-9, and demonstrates that

TABLE 4-9

Choline removed by treatment with phospholipase-D from *S. chromofuscus*

Expt. No.	μmoles choline / 10^{10} cells
	Test cells with PLD[a]
1	0.73[b]
2	1.48[b]
	1.78[b]
3	0.81[b]
	0.65[c]
4	1.2[c]
Mean	1.11 ± 0.17[d]

(a) - Test cells were treated with 85 U PLD/ml of 50% cell suspension containing 5 mM $CaCl_2$, tris-HCl buffered saline and incubated for 2 hours at 37°C with shaking. The cells were centrifuged, the supernatant was removed and mixed with 4 volumes chloroform/methanol (2:1). The upper layer was stored at −20°C prior to the assay. Control samples containing either no PLD or no cells were similarly treated and in all cases they yielded negligible quantities of choline (not included).

(b) - The cells used for the PLD treatment were washed with the tris buffer but were unfixed.

(c) - The cells used for the PLD treatment were first fixed in 0.25% glutaraldehyde, then washed in tris buffer.

(d) - Error is the standard error of the mean.

a considerable quantity of choline was removed (about twice that removed by the peanut PLD, 1.11 ± 0.17 μmoles/10^{10} cells). Steck et al. (1971) determined that a total of 3.84 μmoles phospholipid is contained in 10^{10} cells. This assumes a value of 5.7×10^{-10} mg of protein/ghost as determined by Fairbanks et al. (1971). No consistent difference was observed in the amount of choline removed from GA-fixed, compared to unfixed cells.

Tenforde (1970) calculated the number of molecules per cell of each type of phospholipid using the data of Ways and Hanahan (1964). He calculated that the numbers of PC, PE, PS, and sphingomyelin, respectively, were 10.7×10^7, 9.3×10^7, 5.4×10^7, and 8.6×10^7. In μmoles/10^{10} cells, this corresponds to 1.78, 1.55, 0.9 and 1.43 respectively. These numbers add up to more than the total determined by Steck. However, if we use these as a rough estimate of the true values, and assume that choline is removed by the PLD only from PC as was found by Fuji and Tamura (1979), it appears that about 60% of the choline was removed by the enzyme. (By comparison, Fuji and Jamura (1979) found about 40% was removed). The difference between the actions of the peanut (section 4.2.1) and the *S. chromofuscus* enzymes may be due to the removal of a larger proportion of the choline of the PC molecules by the latter enzyme. An alternative explanation, that the difference is due to removal of the sphingomyelin cholines by the *S. chromofuscus* enzyme appears unlikely in view of the results of Fuji and Tamura (1979).

To obtain further information on other possible effects of this enzyme, sizing experiments were conducted following treatment with the *S. chromofuscus* enzyme, as shown in table 4-10. Resistive pulse spectroscopy (RPS) was kindly performed on these GA-fixed cells by Dr. Gary Richieri of Donner Laboratory, UC, Berkeley. (See Akeson and Mel (1983) for a discussion of the method). The results showed a decrease of 16 to 18% in the cell size. Microhematocrits performed on the same cell samples showed a 12.5% decrease.

TABLE 4-10

Resistive Pulse Spectroscopy sizing and hematocrit on GA-fixed human erythrocytes after treatment with PLD *(S. chromofuscus)*

Current μamp	Uncorrected, normalized peak channel (proportional to cell volume)		
	No PLD	PLD	% Decrease
100	30.94	25.88	16%
200	31.35	26.12	17
400	32.00	26.35	18
800	32.46	26.69	17
1600	33.37	28.17	16
Hematocrit	32%	28%	12.5%
Cells/ml	1.5×10^9	1.47×10^9	
Choline removed	$1.2\mu moles/10^{10}$ cells		

The results of table 4-10 can be summarized as follows. (i) A size decrease after *S. chromofuscus* PLD treatment averaging 16.8% is measured by RPS after PLD treatment. (ii) This is not inconsistent with the results from the independent microhematocrit test showing a decrease of 12.5%. (iii) No greater relative size reduction is seen in the high-current RPS than in the low-current RPS. This indicates that PLD treatment has not led to any electrical "leakiness" of the cell membrane. This result is to be contrasted with a related type of experiment whereby the (kinetic) removal of lipids from the GA-fixed red cell membrane, by action of low concentrations of Triton X-100, did lead to a complete electrical transparency of the membrane (Peros, 1981).

Mobility measurements for PLD-(S. chromofuscus)-treated cells.

Figure 4.20 presents the mobility as a function of Debye length for unfixed human erythrocytes treated with the *S. chromofuscus* PLD. The data are the means for the three individual experiments tabulated in table 4-11. The curve shows that there is *no effect of this enzyme on the mobility of unfixed cells* over the entire ionic strength range used. Results of reversibility measurements for this system are given in figure 4.21. These data were collected by using samples at the indicated ionic strengths, resuspending them in Standard Buffer, and remeasuring the electrophoretic mobility. The results are plotted as the fraction of the original Standard-Buffer mobility. Within the accuracy of the measurements, the cellular mobilities at low ionic strength are reversible for the unfixed cells.

A different result for the mobility vs. Debye length is obtained for PLD-treated, *GA-fixed* erythrocytes, figure 4.22 and table 4-12. Here, the (negative) mobilities of the PLD-treated cells are clearly *lower* than those of the untreated cells.

Figures 4.23 (combined data) and 4.24 (single experiment) show results of electrophoretic mobility vs. pH in Standard Buffer for the PLD-treated, GA-fixed erythrocytes. These plots

Figure 4.20

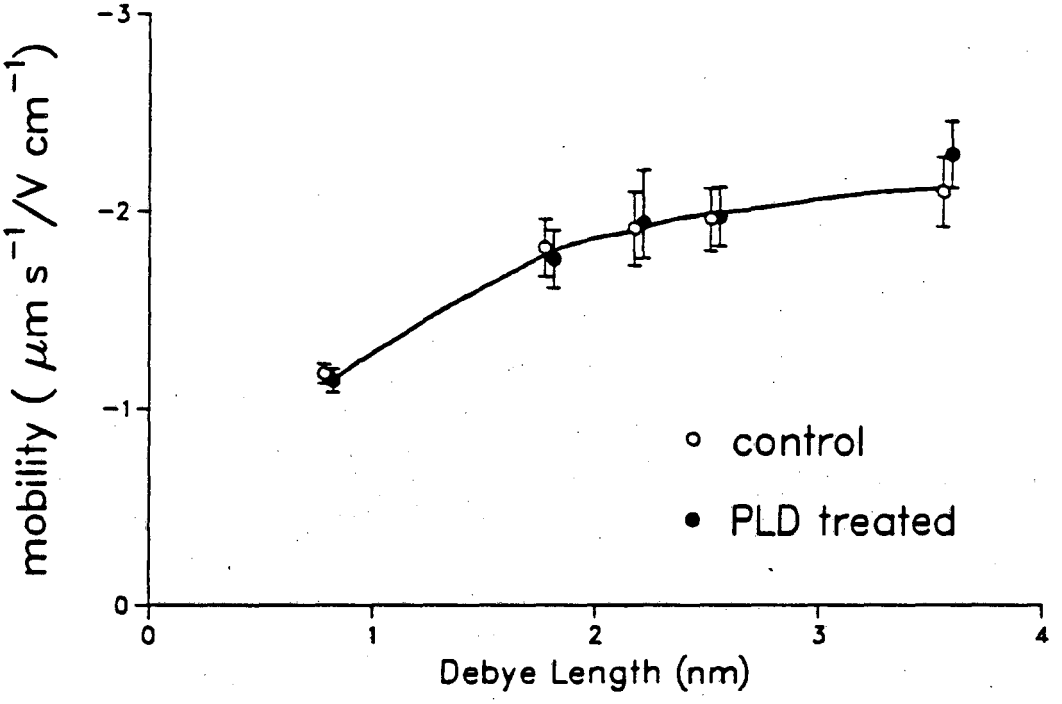


Figure 4.20. Uncorrected electrophoretic mobility of PLD *(S. chromofuscus)*- treated (•) and untreated, unfixed, human erythrocytes as a function of Debye length. For the PLD treatment, cells were washed with tris-HCl saline, pH 7.4, and an aliquot of cells was incubated with 80 units PLD (in tris-HCl-saline, 5mM $CaCl_2$) per ml cells, for two hours at 37°C with constant shaking. The data are the averages shown in table 4-11. Error bars are standard errors of the mean.

TABLE 4-11

Effects of PLD *(S. chromofuscus)* on the electrophoretic mobilities of native human erythrocytes at several Debye lengths.

		Uncorrected negative electrophoretic mobilities ($\mu m\ s^{-1}$ / $V\ cm^{-1}$)			
		No PLD		PLD	
Debye length (nm)	Dilution	Expt. No.	Individual expts.	mean	Individual expts.	mean
0.80	1/1	I.	1.10±0.13 (20)[a]		1.07±0.11 (20)[a]	
		II.	1.15±0.14 (24)	1.17±0.05[b]	1.09±0.11 (20)	1.14±0.06[b]
		III.	1.28±0.12 (24)	S.D. 0.13 (11%)[c]	1.29±0.13 (30)	S.D. 0.12 (11%)[c]
1.79	1/5	I.	1.48±0.17 (20)		1.53±0.25 (20)	
		II.	1.92±0.23 (20)	1.81±0.15	1.66±0.25 (20)	1.76±0.15
		III.	2.07±0.29 (30)	S.D. 0.23 (13%)	2.11±0.31 (38)	S.D. 0.25 (14%)
2.19	1/7.5	I.	1.69±0.25 (20)		1.69±0.25 (20)	
		II.	1.70±0.17 (20)	1.91±0.19	1.77±0.14 (20)	1.94±0.18
		III.	2.36±0.27 (20)	S.D. 0.23 (12%)	2.38±0.29 (24)	S.D. 0.23 (12%)
2.53	1/10	I.	1.75±0.31 (20)		1.80±0.20 (20)	
		II.	1.81±0.16 (20)	1.96±0.16	1.82±0.18 (20)	1.97±0.15
		III.	2.34±0.34 (20)	S.D. 0.27 (14%)	2.34±0.36 (26)	S.D. 0.25 (13%)
3.57	1/20	I.	1.87±0.21 (20)		2.07±0.28 (20)	
		II.	—	2.10±0.18	—	2.29±0.17
		III.	2.35±0.31 (34)	S.D. 0.26 (12%)	2.53±0.47 (40)	S.D. 0.38 (16%)

(a) - Errors are standard deviations and the numbers in parentheses represent the number of mobility measurements.

(b) - Errors are standard errors of the mean.

(c) - S.D. is the mean of the standard deviations. The numbers in parentheses represent the standard deviation as the percentage of the mean.

Figure 4.21

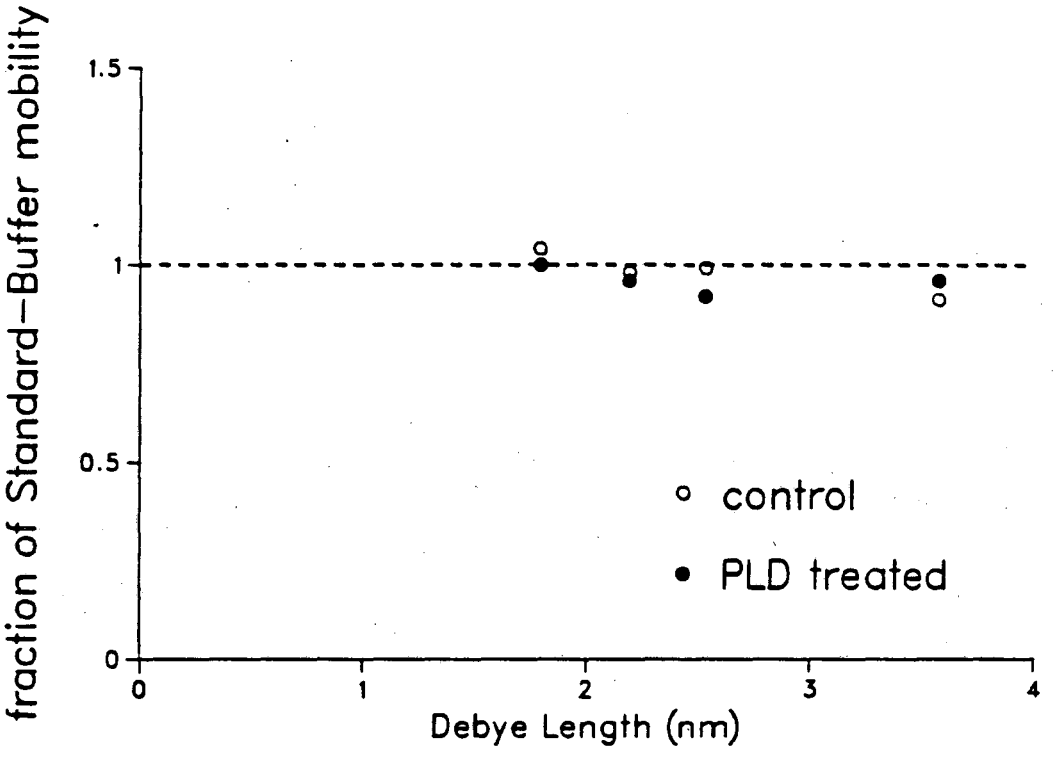


Figure 4.21. Reversibility measurements for some of the data in figure 4.20. After making the mobility measurements at the indicated ionic strengths, the samples were centrifuged and resuspended in Standard Buffer, and the mobility was again measured. The data are presented for both the PLD-treated cells (•) and the control cells (o), as the fraction of the mobility of the original Standard Buffer sample.

Figure 4.22

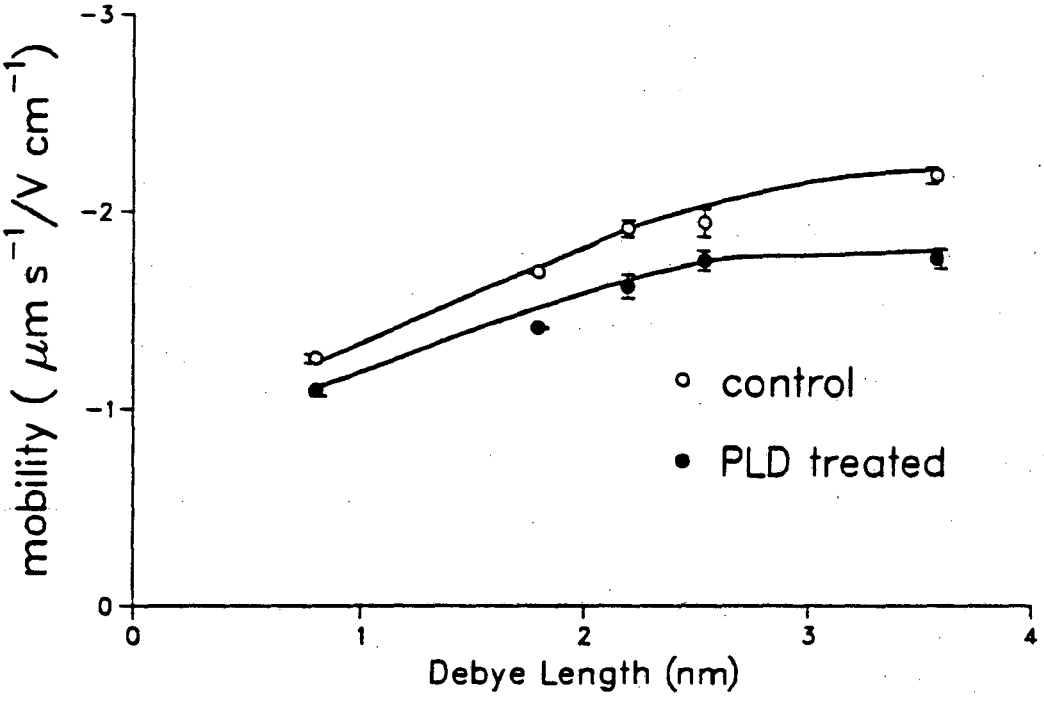


Figure 4.22. Uncorrected electrophoretic mobility of PLD *(S. chromofuscus)*- treated, GA-fixed, human erythrocytes as a function of Debye length. PLD treatment was as described in figure 4.20, but on 0.25% GA-fixed erythrocytes. The data are the averages shown in table 4-12. The error bars are the standard errors of the mean.

TABLE 4-12

Effects of PLD *(S. chromofuscus)* on the electrophoretic mobilities of GA-fixed, human erythrocytes at several Debye lengths.

		Uncorrected negative electrophoretic mobilities[a] ($\mu m\ s^{-1}$ / $V\ cm^{-1}$)			
		No PLD		PLD	
Debye length (nm)	Dilution	Mobility	Number of expts.	Mobility	Number of expts.
0.80	1/1	1.25 ± 0.02[b]	6	1.09 ± 0.03	5
1.79	1/5	1.69 ± 0.003	2	1.41 ± 0.003	2
2.19	1/7.5	1.91 ± 0.04	9	1.62 ± 0.06	10
2.53	1/10	1.94 ± 0.07	4	1.75 ± 0.05	4
3.57	1/20	2.18 ± 0.04	3	1.76 ± 0.05	3

(a) - GA-fixed human erythrocytes were mixed with PLD at a concentration of 80 units/ml 50% cell suspension (1:1 in tris-buffered saline, 5mMCaCl$_2$ and 0.25mM MgCl$_2$). The samples were incubated with gentle shaking for 2 hours at 37°C. Controls were similarly treated but without PLD.

(b) - Errors are standard errors of the mean.

Figure 4.23

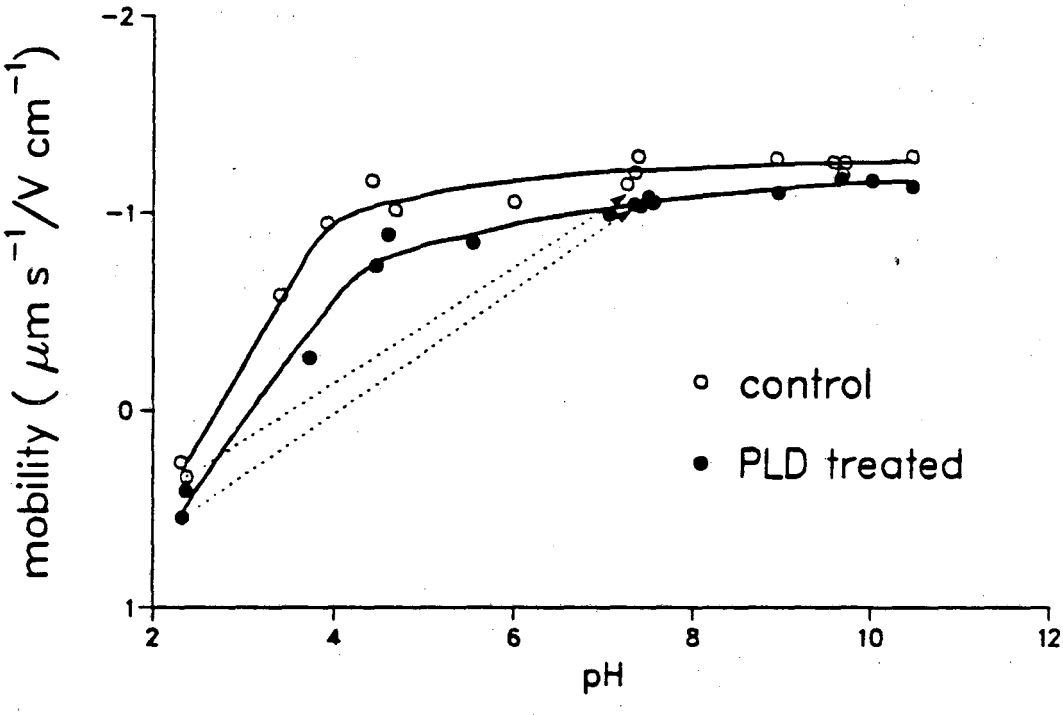


Figure 4.23. Uncorrected electrophoretic mobility in Standard Buffer of PLD *(S. chromofuscus)*- treated, GA-fixed, human erythrocytes as a function of pH. PLD treatment as in figure 4.20. Data from two experiments are shown. One of these experiments is shown in figure 4.24. The arrow heads of the dotted lines depict the reversibility measurements, made by readjusting the pH with 0.145M NaOH.

Figure 4.24

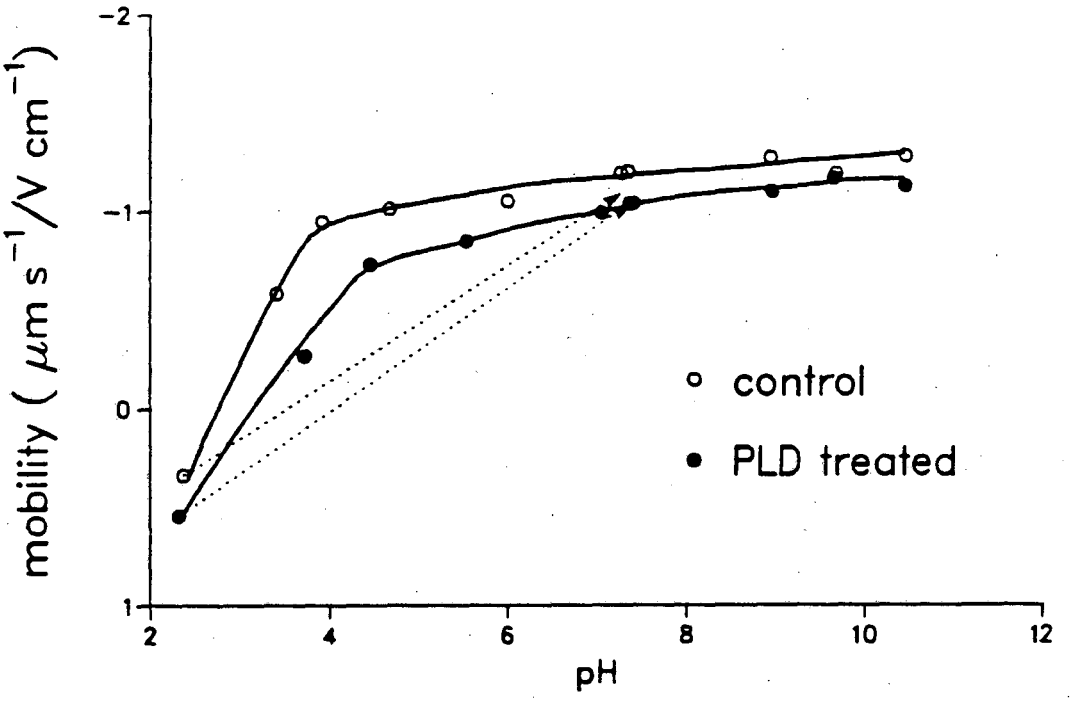


Figure 4.24. Uncorrected electrophoretic mobility in Standard Buffer of PLD *(S. chromofuscus)*- treated, GA-fixed, human erythrocytes as a function of pH. PLD treatment as in the previous figures. One of the experiments plotted in the previous figure. The arrow heads of the dotted lines depict two of the reversibility measurements (those at the lowest pH for both the control and the PLD-treated samples). For reversibility tests, after making the mobility measurements at the indicated pH's, the pH of the samples were adjusted back to neutral pH by the addition of 0.145M NaOH or HCl and the mobility was again measured. The following are the reversibility values expressed as % of the expected mobility: Control samples: pH 2.37, 94%; pH 10.45, 103%. PLD samples: pH 2.32, 101%; pH 4.45, 96%; pH 5.53, 106%; pH 10.45, 102%.

show a small but definite decrease in mobility for the PLD-treated samples at all pH values, coupled with an increase in the isoelectric point, from about pH 2.7 to pH 3.2. Figure 4.25 shows a similar plot for an experiment at a Debye length of 2.19nm. The mobility decrease is even more apparent at this ionic strength. Here the isoelectric point shift is from pH 2.8 in the untreated to 3.4 in the PLD-treated sample.

Thus, for the GA-fixed cells, highly significant decreases in negative mobility are seen, when an increase would be expected if the only effect were associated with the PLD acting to remove positively charged choline moieties. A similar, though smaller, decrease was seen with the PLD (peanut) enzyme as discussed above. These results, in the face of no changes in the electrophoretic mobility for unfixed cells, require an explanation.

Possible explanations for the decreased mobility following PLD treatment.

What are some of the possible explanations for these results? First, the more positive mobility for the PLD-treated, GA-fixed cells could reflect *an actual change in surface charge.* This could arise by either the loss of negative groups such as the carboxyl group of sialic acid, or by the uncovering of positive groups such as amines, or by the interaction of Ca^{+2} ions with the phosphatidic acid that remains after choline treatment. Why would this mobility change not be seen for the unfixed cells? Their membrane components being less constrained, they would possibly be able to rearrange their surface structure after treatment, for example by losing components or by pushing charged groups to the interior of the membrane.

To determine whether there were sialic-acid-containing membrane components being removed along with the choline during PLD treatment, the supernatant following PLD treatment (GA-fixed cells) was assayed for sialic acid. The results, shown in table 4-13, indicate that a negligible amount of sialic acid was lost from the cell membrane.

Figure 4.25

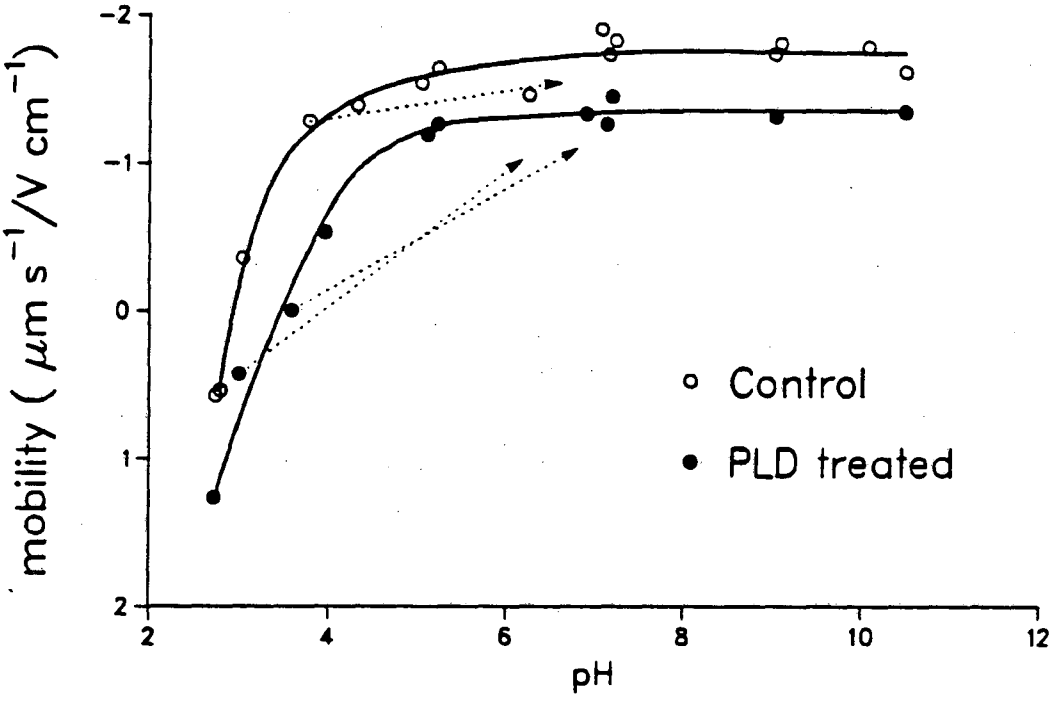


Figure 4.25. Uncorrected electrophoretic mobility at low ionic strength (Debye length = 2.19nm) of PLD *(S. chromofuscus)*- treated, GA-fixed, human erythrocytes as a function of pH. PLD treatment as in figure 4.20. The arrow heads of the dotted lines show reversibility measurements made by restoring the pH to a value of 7.0±0.5, then spinning down, and resuspending in the low ionic strength medium.

TABLE 4-13

Sialic acid assay of Ga-fixed human erythrocytes after treatment with PLD *(S. chromofuscus)*

μmoles sialic acid / 10^{10} cells		
Controls		PLD test sample
Cells, no PLD	No cells, PLD	Cells with PLD
0.001	0.003	0.003

The assay was that of Warren, 1959. A standard containing 0.04 μmoles neuraminic acid yielded 0.038μmoles.

If new positive charges were being uncovered by the treatment, it is likely that this charge would be an amine. Since acetaldehyde reacts with amines the (negative) electrophoretic mobility should increase after acetaldehyde treatment. Table 4-14 gives the results of several experiments in which the mobility was measured at Debye length 2.19nm for acetaldehyde-treated erythrocytes. The results show no increase in mobility following acetaldehyde treatment either with or without PLD pretreatment. In these several experiments, there was either no effect of acetaldehyde, or a slight decrease in mobility.

Figure 4.26a displays mobility as a function of pH, at Debye length 2.19nm, with and without PLD treatment. Figure 4.26b show the effects of acetaldehyde on the mobility of the PLD-treated sample. Examination of the figure indicates that, once again, no uncovering of positive groups induced by PLD treatment can be electrophoretically detected.

Although the possibility that the Ca^{+2} ions were interacting with the phosphatidic acid was not explicitly tested with the *S. chromofuscus* PLD, arguments against this explanation

TABLE 4-14

Effects of acetaldehyde on the electrophoretic mobility of PLD *(S. chromofuscus)* treated, GA-fixed, human erythrocytes measured at Debye length 2.19 nm[a]

	Uncorrected negative electrophoretic mobilities[b] ($\mu m\ s^{-1}$ / $V\ cm^{-1}$)			
	No acetaldehyde		With acetaldehyde	
Expt.	Control	PLD	Control	PLD
1	2.02 ± 0.26[c]	1.83 ± 0.30[c]	2.04 ± 0.29[c]	1.77 ± 0.38[c]
2	2.10 ± 0.30	1.78 ± 0.31	1.82 ± 0.46	1.52 ± 0.51
	—	—	1.86 ± 0.30	—
3	2.03 ± 0.37	1.80 ± 0.39	1.87 ± 0.23	1.81 ± 0.50
	1.99 ± 0.30	1.80 ± 0.45	—	1.76 ± 0.34
mean[e]	2.03 ± 0.02	1.80 ± 0.01	1.90 ± 0.04	1.71 ± 0.06
mean S.D.[d)(e)]	0.31 ± 0.02	0.36 ± 0.03	0.32 ± 0.04	0.43 ± 0.07

(a) - GA-fixed human erythrocytes were mixed with PLD at a concentration of 80 units/ml 50% cell suspension (1:1 in tris-buffered saline, 5mM$CaCl_2$ and 0.25mM $MgCl_2$). The samples were incubated with gentle shaking for 2 hours at 37°C. Controls were similarly treated but without PLD. Half of each sample was then incubated for 30 min at room temperature with 1% (w/v) acetaldehyde in Sorensen's phosphate buffered saline, pH 7.4. The other half (controls) was incubated in Sorensen's buffered saline alone.

(b) - Each mobility is the mean for at least 20 measurements.

(c) - Errors are standard deviations.

(d) - S.D. values are the mean of the Standard Deviation values reported above. Errors are the standard error of the mean.

(e) - All blood was obtained from the blood bank. Expt 1 used fresh blood "presumed normal from a therapeutic phlebotomy." Mobilities were measured the day after cell treatment. Expt 2 used the same blood sample but measurements were made two and three days after cell treatment. Expt 3 used expired "normal" blood and measurements were made the day of cell treatment.

Figure 4.26 A

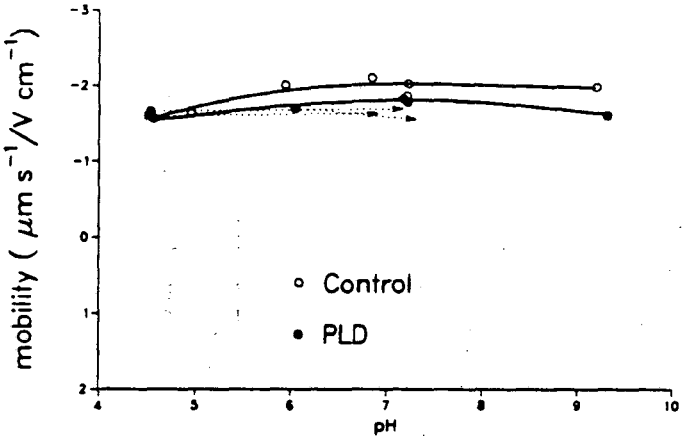


Figure 4.26 B

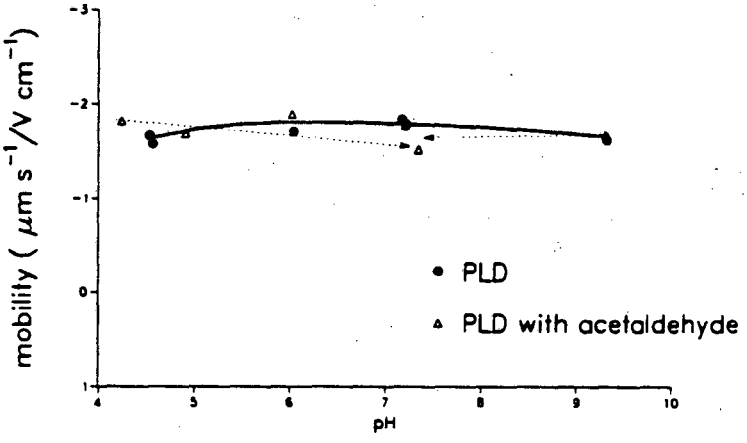


Figure 4.26. Effect of acetaldehyde on the electrophoretic mobility of PLD (*S. chromofuscus*)-treated erythrocytes measured in low ionic strength (Debye length = 2.19nm) and at several pH values. PLD treatment *(S. chromofuscus)* was as in figure 4.20. For acetaldehyde treatment, twenty volumes of acetaldehyde (1%, w/v in a 50%, v/v, mixture of Sorensen's buffer and Standard Buffer), was added to 1 volume of a 50% cell suspension in Standard Buffer, and incubated at room temperature for 30 minutes. Figure 4.26A shows the control- (o) and the PLD-(•) treated cells for this sample. Figure 4.26B shows the PLD treated cells with (Δ) and without acetaldehyde (•). The arrow head of the dotted lines in each case depict reversibility measurements. After making the mobility measurements at the indicated pH's, the pH of the samples were adjusted by the addition of 0.145M NaOH or HCl and the mobility was again measured.

were presented in conjunction with the peanut PLD results.

A second possible explanation is that the *GA action might have introduced a "hidden" chemical artifact,* i.e. a chemical change that is not seen electrophoretically until the choline has been removed by the enzyme. Recall that a higher concentration of GA (2.5%) was observed to lead to an artifactual mobility reduction, and similar high-concentration, GA-induced alterations have also been observed in other systems (Peros, 1981). It was for this reason that the lower concentration of 0.25% GA was adopted for the present experiments. There is even slight evidence of such a "hidden" effect even for GA concentrations of 0.25% (Peros, 1981). If this were the case, the surface rearrangement explanation for the lack of a PLD-induced mobility drop for unfixed cells would not be the relevant one.

A rather different role might conceivably be ascribed to the glutaraldehyde: *introduction of mechanical tension into the rigid membrane, which may be relieved, with shrinkage, upon removal of membrane components by PLD.* This amounts to a membrane rearrangement for the *fixed* PLD-treated cell that could conceivably cause *hitherto-hidden-positive charges* to become seen electrophoretically. What evidence can be cited for this hypothesis? (i) GA-fixation kinetics indicate that it is during the course of cell-shrinkage that the cells become "frozen" during fixation (Yee and Mel, 1978). (ii)RPS measurements plus the single hematocrit measurement both indicate a shrinkage of the fixed cells (~12–16%) following the PLD treatment. (iii) When fixed cells were completely delipidized, using Triton X-100 in the Peros (1981) experiments, the results of the sizing data taken at high- and low-field strengths were consistent with an actual physical shrinkage being one component of the effect. (iv) The instability of the PLD-(peanut)-treated, neuraminidase-treated, GA-fixed cells discussed in section 4.1 are consistent with the tension hypothesis. An argument against this mechanism of revealing positive charges, is the lack of effect on the mobility of PLD-treated, GA-fixed cells when acetaldehyde post-treatment is used to block positively-charged amine groups at the cell

surface.

A fourth possibility for the decrease in mobility after PLD treatment is *a change in internal conductivity within the membrane of the cell.* The RPS experiments described above ruled out the possibility of the membranes becoming transparent to the electric field, but they did not rule out a moderate increase in conductance within the surface of the membrane. Henry's equation for the electrophoretic mobility of a sphere having a conducting surface is

$$\mu = [\zeta\epsilon f(\kappa a)/6\pi\eta][3\Lambda_o/(2\Lambda_o+\Lambda_i)],$$

(chapter 2, eqn 3, where Λ_o and Λ_i are the external and the in-membrane conductances, respectively). Thus, if the electrical conductivity of the membrane increases, the mobility would be expected to decrease. A difference in mobilities of PLD-treated and control cells was observed only for GA-fixed erythrocytes. It is possible that the GA-fixation, in preventing membrane rearrangement after removal of membrane components by PLD treatment, opens a less-encumbered, and hence lower-electrical-resistance pathway, leading to an increased in-membrane conductance.

A final possibility to consider is that the decrease in mobility following PLD treatment is *an artifact of the measuring technique.* The PLD-treated cells tend to form slightly more clumps than do the untreated (or the PLC-treated cells), and thus they may adhere to the sides of the measuring chamber and modify its electrical characteristics.

Attempts were made to minimize this possible effect by cleaning the chamber with bleach between samples (followed by thorough rinsing) and by always measuring control and PLD-treated cells alternately to prevent any long-term change in chamber characteristics from affecting the difference in mobility between controls and treated cells.

If any such effects were taking place during the course of a single sample measurement, it

would be reasonable to expect that the mobility would change with time, as more cells attached themselves to the chamber walls. In figure 4.27 is shown a series of measurements taken over time at the front stationary layer of the chamber. No time effect is seen in these plots.

4.3. Summary

Four phospholipase enzymes were used, and all were active on the membrane of GA-fixed erythrocytes (i.e. they removed either phosphocholine or choline). The two PLC enzymes removed nearly the same amount of phosphocholine per 10^{10} cells (0.69 for the *B. cereus* enzyme and 0.79 for the *C. perfringens* enzyme). The two PLD enzymes removed quite different quantities of choline (0.50 for the peanut enzyme compared to 1.11 for the S. chromofuscus enzyme).

The PLC enzymes caused a very slight but apparently real decrease in mobility, and the difference was greatest in the Debye-length range of 1.5 to 2.5nm. This effect was not noted on all cell preparations, and was not seen after neuraminidase treatment.

The PLD enzymes caused a definite decrease in mobility for GA-fixed cells, at all low ionic strengths, and the effect was much greater with the *S. chromofuscus* enzyme than with the peanut enzyme. However, when unfixed cells rather than GA-fixed cells were treated with the *S. chromofuscus* enzyme, no mobility effect was noted. Furthermore, when the peanut PLD was used on neuraminidase-treated cells, a slight increase in mobility was observed.

Several possible explanations for these results have been considered. The unmasking of electrophoretically detectable amino groups as an explanation for the mobility decreases has been ruled out by acetaldehyde post-treatment after the PLC and PLD treatments. Similarly, the loss of membrane charges associated with sialic-acid residues, lipid phosphates, and membrane protein following PLD treatment has been ruled out, at least for the numerous

Figure 4.27 A

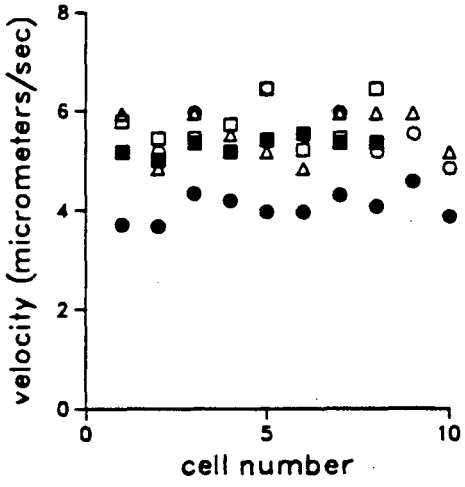


Figure 4.27 B

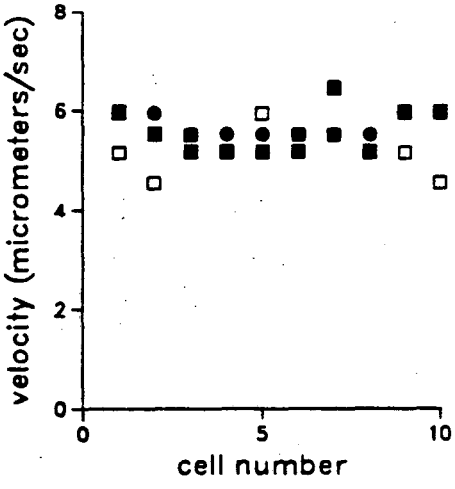


Figure 4.27 C

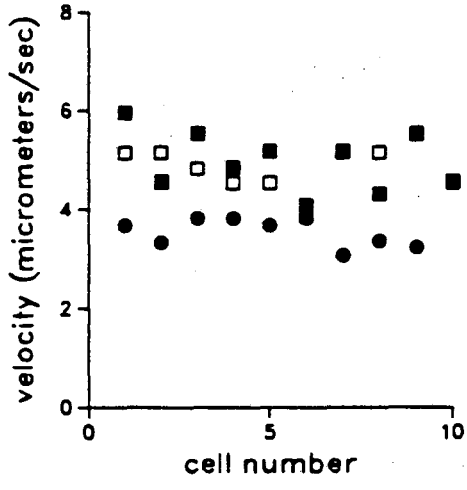


Figure 4.27 D

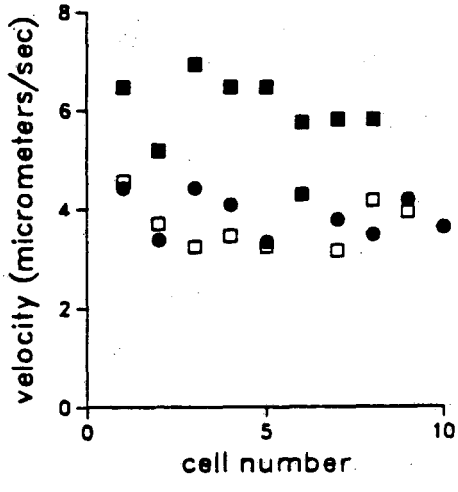


Figure 4.27. Effect of experimental measurement time on electrophoretic *velocity* measurements at various ionic strengths and currents. Velocity measurements on representative single cells over time are shown for; A - control cells, B - PLD-treated cells, C - control cells postfixed with acetaldehyde, and D - PLD-treated cells postfixed with acetaldehyde. The label "cell number" refers to the sequence in time for each successive measurement, for each experimental series. Approximately 5 to 10 minutes is required to measure 10 cells. The different symbols depict different experimental sequences.

conditions for which chemical assays were carried out.

For the mobility decrease after PLD treatment of GA-fixed cells, five potential explanations were evaluated. These alternatives will be considered in more detail in the next chapter.

CHAPTER 5

Summary and Conclusions

5.1. Overview

The bulk of this work has involved treatment of native and GA-fixed human erythrocytes with phospholipases and neuraminidase followed by measurement of the electrophoretic mobility of the cells in solutions of several low ionic strength and pH values.

Treatments which leave the cell mobility unaltered.

The experiments described in the preceding chapters have shown that several chemical and physical treatments of human erythrocytes leave their electrophoretic properties unaltered, over a range of ionic strengths and over a broad pH range. These treatments include:

(i) For native red cells, low ionic strength buffers, by themselves, cause no irreversible membrane changes that are detectable with agglutinins or by electrophoretic mobility measurements.

(ii) Fixation, alone, of both human and rat erythrocytes, in 355 mosm, 0.25% (w/v) glutaraldehyde, leaves the surface charge properties unchanged; this is not true for 2.5% glutaraldehyde.

(iii) Hemolysis in 20 mosm saline (containing 1mM Mg^{++}), and subsequent resealing in Standard Buffer, leaves the electrophoretic mobility unchanged over the entire ionic strength range tested (Debye length 0.8 to 5.7nm).

(iv) Phospholipase D *(S. chromofuscus)* treatment of *unfixed* erythrocytes leads to no change at all in the electrophoretic mobility of the cells, except possibly at a Debye length of 3.5nm.

Treatments which alter the electrophoretic mobility of the cells.

On the other hand, several other treatments of the cell do cause modification of the electrophoretic properties of human erythrocytes:

(i) Fixation in high concentrations of glutaraldehyde (2.5%) leads to an increase in electrophoretic mobility at physiological ionic strength and a decrease in mobility at low ionic strengths.

(ii) Neuraminidase treatment of either native or GA-fixed cells decreases electrophoretic mobility at all ionic strengths, although all the mobilities increase with increasing Debye length. The calculated surface charge densities show disparate behavior: For the untreated cells, the negative charge density *decreases* significantly with increasing Debye length, while for the neuraminidase-treated cells, charge density remains nearly constant (with a slight increase between Debye lengths 1.7 and 2.5nm).

(iii) Ethanol extraction of membrane lipids from GA-fixed erythrocytes leads to a slight decrease in mobility in the Debye length range of 1.5 to 2.5nm.

(iv) Chloroform-methanol extraction of membrane lipids from GA-fixed erythrocytes leads to a major decrease in mobility at Debye lengths >2nm.

(v) Phospholipase C *(B. cereus* and *C. perfringens)* treatment of GA-fixed human erythrocytes leads to a slight decrease in mobility in the Debye length range 1.5 to 2.5nm.

(vi) Phospholipase D *(S. chromofuscus)* treatment of *GA-fixed* cells causes a decrease in mobility. PLD from a different source (peanut) also leads to a mobility decrease, albeit a smaller one.

(vii) PLD (peanut) treatment of neuraminidase-treated, GA-fixed cells causes a small increase in negative surface charge density and leads to cells that are unstable at low pH.

What kind of cell-membrane structure can fit all of these observations? Consider first the effects of neuraminidase. In Chapter 1, several possible effects, in conjunction with

phospholipase action, were postulated.

(i) Removal of sialic acid might change the location of the surface of shear, bringing the phospholipids closer to the outer surface (so that their charge contribution could then be "seen" electrophoretically.

(ii) Removal of sialic acid might lead to rearrangement of the membrane components, thereby changing the location of phospholipid polar groups.

(iii) The removal of sialic acid might lead to an increase in the amount of substrate available to the phospholipases.

(iv) Sialic acid removal might have no effect on the action of phospholipases.

What can we now say about those possible effects?

Neuraminidase: changes in the location of the surface of shear or membrane rearrangement.

(i) Did the neuraminidase treatment change the location of the surface of shear? The discussion in section 3.3 indicates there is a high probability for this to be the case. The two curves in figure 3.18 demonstrate the very different behaviors of the non-neuraminidase-treated cells, when compared to the neuraminidase-treated. This is best explained if we assume that the surface of shear of the untreated cells is located a considerable distance from the true nonconductive cell surface, while that of the neuraminidase-treated cells is very close to that surface. The alternative explanation, that neuraminidase removes both negative and positive moieties down to a depth of 5.7nm appears unlikely, particularly in view of the (estimated) membrane thickness of only 7.5nm and the specificity of the enzyme's action for negatively charged neuraminic acid residues.

(ii) What about rearrangement of membrane components as a consequence of the removal of sialic acid? This also appears unlikely as an explanation for the full effect, since this

hypothesis would require that all charges, originally at a depth of 0.8 to 5.7nm within the membrane, be moved out to the outermost 0.8nm. My results do not preclude smaller molecular rearrangements following neuraminidase treatment, but do exclude this possibility as the principal explanation for the full observed surface charge density variations.

PLC plus neuraminidase plus GA: Surface conductance and membrane rearrangement.

(iii) The phosphate assay after PLC *(B. cereus)* treatment of neuraminidase-treated, GA-fixed human erythrocytes indicated that 1.2μmoles of phosphate were removed per 10^{10} cells, while the average removed from untreated GA-fixed cells was 0.79 ± 0.17. This experiment does suggest that more phosphocholine was indeed removed from the neuraminidase-treated cells. However, a single experiment performed with peanut PLD on neuraminidase-treated, GA-fixed cells indicated a slight decrease occurred in the amount of choline removed. It may be that the negative sialic acids aid the action of the peanut PLD, but hinder the action of the *B. cereus* PLC.

(iv) Since removal of sialic acid did affect the action of the PLC, as stated above, the possibility of "no effect" must now be discarded.

What else can we conclude from the PLC results? Phospholipase C *(B. cereus)* acting on neuraminidase-treated, GA-fixed erythrocytes removed significant quantities of phosphocholine, but had no effect on the mobility of the cells at any ionic strength. Bearing in mind Henry's equation for the mobility of a conducting sphere (Chapter 2, eqn 3), it is apparent that removal of phosphocholine does not cause an increased surface conductance of the cells. The decrease in mobility of a conducting sphere, in low ionic strength solutions was demonstrated by Ghosh and Bull (1963). Thus, the lack of such an effect after PLC treatment is at least mildly surprising since the bi-products of PLC treatment are lost from the cell, and hence might be expected to leave gaps in the membrane. If the membrane is able to close up gaps left by lost components, it must be doing so in spite of fixation. This is consistent with, but does not

require, the possibility that the GA-fixed cell is under increased surface tension as discussed in section 4.2.2.

PLC without neuraminidase.

PLC acting on the GA-fixed erythrocytes that were not treated with neuraminidase, did cause a slight decrease in mobility. This may be due, not to a loss of the phosphocholine, but rather to a simultaneous loss of a small amount of sialic acid at the same time. If this is the case, this sialic acid is located at a depth of from 1.5 to 2.5nm into the membrane. Unfortunately this sialic acid elution was not tested.

Lipid extraction: membrane conductance changes.

What about ethanol and chloroform/methanol treatments? The *ethanol* results, discussed in Chapter 3, are very similar to the PLC results, in that both lead to a small decrease in mobility in the Debye-length range 1.5 to 2.5nm. The same argument can be made, that the slight decrease in (negative) mobility is a result of a slight loss of sialic acid but again, this was not tested. (Note: since the sialic acid contributes most of the charge to the red cell membrane, it is a likely candidate for affecting charge change.)

The *chloroform/methanol* treatment which caused a large decrease in cell mobility, may best be explained in terms of an increase in conductivity across the treated-cell membrane system. The results of Peros (1981) showed unequivocally that the membranes of GA-fixed cells became transparent to an electric field after delipidization with Triton X-100. Surprisingly, in his experiments the GA-fixed cytoplasm conducted electrically almost like that of the unfixed cytoplasmic hemoglobin.

PLD: unfixed vs. GA-fixed cells.

The PLD results are puzzling. Fuji and Tamura (1979), using 2-dimensional thin layer chromatography, measured the lipid composition of the membrane following PLD *(S. chromofuscus)* treatment. They demonstrated that (negative) phosphatidic acid remains in the membrane following PLD treatment, and hence one might expect a large increase in (negative) electrophoretic mobility following treatment (removal of positive choline), at least for low ionic strengths. (The action of PLD on the PC molecule is shown in figure 1.1). Both of the PLD enzymes acting on GA-fixed erythrocytes caused a decrease in mobility at all low ionic strengths. The PLD (peanut) enzyme acting on neuraminidase-treated, GA-fixed cells led to an increase in mobility at all ionic strengths, but the increase was less than expected from the amount of choline removed. However, the (highly purified) *S. chromofuscus* enzyme acting on *unfixed* cells led to absolutely no change in mobility (except possibly at a Debye length of 3.5). The PLD (peanut) enzyme, acting on neuraminidase-treated, GA-fixed cells led to an increase in mobility at all ionic strengths, but the increase was less than that expected from the choline removed. Acetaldehyde fixation indicated that the effect on the surface charge may have been due to loss of positive amines which may have been lost from the membrane surface along with choline groups.

Let us first consider only the unfixed-cell results. A direct chemical assay indicated that choline was indeed removed. Fuji and Tamura (1979), using the same *S. chromofuscus* enzyme, determined that phosphatidic acid (in large quantities) remained in the membrane after PLD treatment, but that the amounts were not quite sufficient to make up for the decreased phosphatidyl choline. (They attributed this discrepancy to errors in recovering all the phosphatidic acid.) And yet, I observed absolutely no change in mobility (except for a non-significant increase at a Debye length of 3.5nm).

What about applying the argument to the unfixed cells that I used in section 4.2.2 to explain the PLD effects on GA-fixed cells — namely that an increase in surface conductance might occur after PLD treatment? Such a change in conductance would have to be such as to exactly equal the change in mobility due to the increased negative charges. It appears quite unlikely that this would occur at all ionic strengths.

Another possibility is that the phosphatidylcholine head group is at a depth greater than 3.5nm, and that the non-significant result for Debye length 3.5nm represents a real effect. Although my data are from only two experiments, in both cases the PLD *(S. chromofuscus)*-treated cells had a somewhat higher mobility than the untreated. The problem with this explanation is that 3.5nm is probably too far into the membrane as a location for the outer head groups of the lipid bilayer. That is, when the known components of the outer membrane are considered, there is not enough other material (proteins or carbohydrates) at the outer surface to fill such a large volume. However, we can perhaps reconsider this possibility in light of the conclusions drawn from the neuraminidase results. Since, in the untreated cell, increased membrane surface conductance may have been detected with the low-ionic-strength technique, it is apparent that the outer membrane surface may be loosely packed. The probable explanation for the constant surface charge density after the removal of the sialic acid is a collapse of the remaining outer protein and carbohydrate moieties. It is therefore likely that the molecules of protein and sialic acid taken together, do extend to a greater distance from the phospholipid head groups than would be expected if the outer components were more tightly packed.

Another explanation also seems plausible: *membrane rearrangement.* This could come about either by the phosphatidic acid flipping further into the membrane, such that its charges would not be detected electrophoretically, or by some other positive group rearranging to the vicinity of the phosphatidic acid. There is evidence that modification of membranes can

increase the "flip-flop" rate of some lipid species. Experiments by de Kruijff and Baken (1978) on dimyristoyl lecithin vesicles show that after treatment with PLD, the half time for lecithin flip-flop decreased from several days to 8 hours. In addition, in other artificial membrane systems, the presence of glycophorin increases the flip-flop rate by two orders of magnitude (de Kruijff et al., 1978). In a somewhat different kind of experiment, transfer of phospholipids between labeled sealed ghost membranes and PC liposomes, Bloj and Zilversmit (1976) found a half-time rate of 2.3h in the presence of purified phospholipid exchange protein from beef heart cytosol. Based on all these results, it would not be surprising if removal of choline by the PLD also caused increased flip-flop between the inner and outer layers.

The PLD results obtained for GA-fixed cells were discussed extensively in the previous chapter. The mobilities of the PLD-treated, GA-fixed cells were lower at all ionic strengths and pH values. The possible explanations put forth for these unexpected results were:

(i) GA-fixation might constrain the membrane from rearranging, and thus allow a real gain of positive or loss of negative charges, which became electrophoretically detectable.

(ii) GA-fixation might have introduced a "hidden" chemical artifact.

(iii) GA-fixation might have introduced mechanical tension into the membrane structure, which would serve as a driving force for a membrane rearrangement enabled by removal of membrane components by PLD.

(iv) GA-fixation might constrain the membrane from filling in gaps, causing an increase in membrane surface conductance after PLD treatment.

(v) The result may be an artifact of the experimental measuring technique.

The first explanation does not seem plausible, based on the information available. No sialic acid, phosphate, or protein moieties were lost from the membrane as a result of treatment with PLD. No positive amino groups were detected electrophoretically following acetaldehyde treatment. The negative phosphatidic acid is known to remain in the membrane following

PLD treatment (Fuji and Tamura, 1979). What about the possibility, however, that Ca^{++} from the treatment medium may have become bound to the phosphatidic acid molecule. This does not appear likely as an explanation because the decreased negative mobility after PLD treatment becomes even more pronounced in low pH solutions. It would be expected that, as the pH was lowered, the Ca^{++} would be displaced by singly charged protons, with the lessening of the observed effect. Other evidence against this possible explanation was found in the EDTA experiment. The EDTA treatment did not cause a consistent change in the mobility of the PLD-treated cells.

The second possibility cannot be ruled out, but there is no evidence that the GA effects cited in the previous chapter would carry with them such a consequence for surface charge change.

If the third explanation were to be valid, the effect of this mechanical tension would have to be to push the phosphatidic acid to the interior of the membrane (since no phosphate was detected in the supernatant after PLD treatment). If inside-out vesicles could be formed after PLD treatment, further experiments with the effects of GA on that system might throw more light on the subject.

The fourth, or surface-conductance explanation, cannot be ruled out with the information at hand.

Regarding the fifth explanation, all efforts were made to minimize this possibility, and the existing data strongly suggest that it is not a basis for the observed surface charge effects of PLD on GA-fixed cells.

The third and fourth possibility seem to describe the most probable explanations for the GA-fixed cell results.

5.2. The bilayer revisited

In Chapter 1 it was stated that one of the aims of this research was to determine the extent and the location of the lipid polar groups relative to the surface of shear. What can we say about this now? Most of the new data are not fully supportive of a very extensive bilayer, but neither do they rule out this model. The increase in surface charge density that would be expected after PLD treatment of a bilayer structure was not seen (except possibly after neuraminidase treatment). Several plausible explanations were given for this result that are not inconsistent with the bilayer hypothesis.

However, another explanation, that the lipid is not in a bilayer, or at least that the bilayer is not very extensive, is certainly viable, based on these results. If the lipid is more thoroughly mixed with the proteins, in some instances forming micelles or just miniature "local" bilayer regions, it would seem more likely that rearrangement would take place after any perturbation in the local charge properties by removal of choline. In this event, the lipids might not be as near to the outer surface as a bilayer would postulate, and the unchanged mobility that was seen after PLD treatment would then be reasonable. Alternatively, PLC or PLD treatment could lead to some rearrangement of membrane proteins that were previously held in place by the intact lipid polar groups. The decreased mobility following PLD treatment of GA-fixed cells would then have to be due to negative charges leaving the outer surface of the membrane, rather than positive charges appearing there, since acetaldehyde post-treatment gave no evidence for the presence of positive amines, at least not electrophoretically detected ones.

5.3. Conclusions

One conclusion that can be drawn, based on the experiments with neuraminidase, is that the native-cell membrane is partially electrically conductive below the surface of shear, but that removal of sialic acid exposes the non-conductive surface — possibly (although not necessarily)

the polar head groups of the lipid bilayer.

In addition it can be said that positive amino groups must exist near the outer membrane surface that can be electrophoretically detected once the sialic acid has been removed.

Another conclusion that can be drawn about the lipid head groups is that the charges of the choline and phosphorus groups are probably in close enough proximity to each other to effectively neutralize one another. This follows from the lack of any charge change after removal of the two groups following treatment of the neuraminidase-treated cell with PLC.

I believe the most probable distance of the lipid head groups from the surface of shear, based on these experiments, to be about 3.5nm. This conclusion is a preliminary finding from the experiments with the *S. chromofuscus* enzyme, in which no charge increase was seen until the mobility was measured in a 3.5nm Debye length solution. In drawing this conclusion it is assumed that the correct explanation for the lower mobility seen at the higher ionic strengths is either an increased surface conductance of the cell, or a surface-component rearrangement. However, not all of the data support the 3.5nm estimate. No mobility increase at 3.5nm was seen after treatment with PLD (peanut). (In this case, however, less choline was removed with the peanut PLD, and the total charge-change seen after the peanut enzyme treatment was smaller than it was for the *S. chromofuscus* enzyme.) The slight decrease in mobility seen after PLC treatment or ethanol treatment of GA-fixed cells in the Debye-length range of 1.5 to 2.5nm is probably best ascribed to the removal of small amounts of protein or sialic acid along with the phospholipids.

Conclusions can also be drawn about the ability of the membrane components to rearrange after molecular modification. The complete lack of any surface charge change in the *unfixed* cell following treatment with the *S. chromofuscus* PLD strongly suggests that the lipids

are able (and, in fact, are in some way required) to rearrange, to restore the original charge properties of the surface. The manner in which this rearrangement occurs is changed by GA-fixation.

Several unexpected results of GA-fixation were observed. High concentrations of glutaraldehyde led to a decrease in membrane electrophoretic mobility. (This is, however, not inconsistent with other reports of alterations to red cell membranes by such high concentrations of glutaraldehyde.) Lower concentrations, in a hypertonic solution, did not have this effect. Glutaraldehyde used in combination with PLD *(S. chromofuscus)* treatment, indicates that the effects of GA-fixation on rearrangement of membrane components are complex.

REFERENCES

Akeson, S.P. and H.C. Mel, Biochim. Biophys. Acta, *718* (1982) 201–211. Osmotic hemolysis and fragility. A new model based on membrane disruption, and a potential clinical test.

Argoudelis, C.J. and J. Tobias, Analytical biochem *64* (1975) 276–280. The determination of small quantities of choline reineckate.

Berg, C.H., J.M. Diamond and P.S. Marfey, Sci., *150,* (1965) 64–67. Erythrocyte Membrane: Chemical Modification.

Bishop, W.H. and F.M. Richards, J. Mol. Biol. *33* (1968) 415–421. Isoelectric point of a protein in the crosslinked crystalline state: β-lactoglobulin.

Bodemann, H. and H. Passow, J. Memb. Biol. *8* (1972) 1–26. Factors controlling the resealing of the membrane of human erythrocyte ghosts after hypotonic hemolysis.

Bloj, B. and D.B. Zilversmit, Biochem., *15* (1976) 1277–1283. Asymmetry and transposition rates of phosphatidylcholine in rat erythrocyte ghosts.

Bramley, T.A. and R. Coleman, Biochim. Biophys. Acta *290* (1972) 219–228. Effects of inclusions of Ca^{2+}, Mg^{2+}, EDTA or EGTA during the preparation of erythrocyte ghosts by hypotonic haemolysis.

Bramley, T.A., R. Coleman and J.B. Finean, Biochim. Biophys. Acta, *241* (1971) 752–769. Chemical, enzymological and permeability properties of human erythrocyte ghosts prepared by hypotonic lysis in media of different osmolarities.

Branton, D. Proc. Nat. Acad. Sci., *55* (1966) 1048–1056. Fracture faces of frozen membranes.

Bretscher, M.S. Nature (New Biology), *236* (1972) 11–12. Phospholipids—more about less.

Bretscher, M.S., Sci., *181* (1973) 622. Membrane structure: some general principles.

Brinton, C.C., Jr. and M.A. Lauffer in "Electrophoresis" ed. M. Bier, Academic Press, N.Y. (1959) Chapter 10, pp 427–491. The electrophoresis of viruses, bacteria, and cells, and the microscope method of electrophoresis.

Bull, H.B., "An Introduction to Physical Biochemistry" (1971) F.A. Davis Co., Philadelphia, Pa.

Burry, R.W. and J.G. Wood, J. Cell Biol., *82* (1979) 726–741. Contributions of lipids and proteins to the surface charge of membranes.

Cabantchik, Z.I., M. Balshin, W. Breuer, H. Markus and A. Rothstein, Biochim. Biophys. Acta, *382* (1975) 621–633. A comparison of intact human red blood cells and resealed and leaky ghosts with respect ot their interactions with surface labelling agents and proteolytic enzymes.

Chapman, D., V.B. Kamat, J. deGier, and S.A. Penkett, J. Mol Biol., *31* (1968) 101–114. Nuclear magnetic resonance studies of erythrocyte membranes.

Colley, C.M., R.F.A. Zwaal, B. Roelofsen and L.L.M. van Deenen Biochim. Biophys. Acta, *307* (1973) 74–82. Lytic and non-lytic degradation of phospholipids in mammalian erythrocytes by pure phospholipases.

Cook, G.M.W., D.H. Heard and G.V.F. Seaman, Nature, *191* (1961) 44–47. Sialic acids and electrokinetic charge of the human erythrocyte.

Danielli, J.F. and H. Davson, J. Cell. Comp. Physiol., *5* (1935) 495–508. A contribution to the theory of permeability of thin films.

Dittmer, J.C. and M.A. Wells, in "Methods in Enzymology", vol 14, Lipids (1969) ed. J.M. Lowenstein,, Acad. Press, N.Y., p482. Quantitative and qualitative analysis of lipids and lipid components.

Dodge, J.T., C. Mitchell and D.J. Hanahan, Arch. Biochem. Biophys., *100* (1963) 119–130. The preparation and chemical characteristics of hemoglobin-free ghosts of human erythrocytes.

Einolf, C.W. and E.L. Carstensen, Biochim. Biophys. Acta, *148* (1967) 506–516. Bacterial conductivity in the determination of surface charge by microelectrophoresis.

Eylar, E.H., M.A. Madoff, D.V. Brody and J.L. Oncley, J. Biol. Chem., *237* (1962) 1992–2000. The contribution of sialic acid to the surface charge of the erythrocyte.

Fairbanks, G., T.L. Steck and D.F.H. Wallach, Biochem., *10* (1971) 2606–2617. Electrophoretic analysis of the major polypeptides of the human erythrocyte membrane.

Finean, J.B., T.A. Bramley, R. Coleman, Nature, *229* (1971) 114. Lipid layer in cell membranes.

Frish, A., Y. Gazitt and A. Loyter, Biochim. Biophys. Acta, *291* (1973) 690–700. Metabolically controlled hemolysis of chicken erythrocytes.

Fujii, T. and A. Tamura, J. Biochem., *86* (1979) 1345–1352. Asymmetric manipulation of the membrane lipid bilayer of intact human erythrocytes with phospholipase A, C, or D induces a change in cell shape.

Furchgott, R.F. and E. Ponder, J. Gen. Physiol., *24* (1941) 447–457. Electrophoretic studies on human red blood cells.

Gazitt, Y., I. Ohad and A. Loyter, Biochim. Biophys. Acta, *382* (1975) 65–72. Changes in phospholipid susceptibility toward phospholipases induced by ATP depletion in avian and amphibian erythrocyte membranes.

Ghosh, S. and H.B. Bull, J. Colloid Sci., *18* (1963) 157–160. Electrophoresis and surface conductance.

Gigg, R. and S. Payne, Chem. Phys. Lipids, *3* (1969) 292–295. The reaction of glutaraldehyde with tissue lipids.

Gordesky, S.E. and G.V. Marinetti, Biochem. and Biophys. Res. Comm., *50* (1973) 1027–1031. The asymmetric arrangement of phospholipids in the human erythrocyte membrane.

Gorter, E. and F. Grendel, J. Exp. Med., *41* (1925) 439–443. On bimolecular layers of lipoids on the chromocytes of the blood.

Green, D.E. and J.F. Perdue, Biochem., *55* (1966) 1295–1302. Membranes as expressions of repeating units.

Haydon, D.A. and G.V.F. Seaman, Proc Royal Soc. B, *156* (1962) 533–549. An estimation of the surface ionogenic groups of the human erytrhocyte and of Escherichia coli.

Haydon, D.A. in "Recent Progress in Surface Science", *1* (1964) 94–158. eds. J.F. Danielli, K.G.A. Pankhust and A.C. Riddiford. The electrical double layer and electrokinetic phenomena.

Haydon, D.A. and G.V.F. Seaman, Archives of Biochem and Biophys, *122* (1967) 126–136. Electrophoretic studies of the ultrastructure of the human erythrocyte. I. Electrophoresis at high ionic strengths — the cell as a polyanion.

Heard, D.H. and G.V.F. Seaman J. Gen. Physiol., *43* (1960) 635–653. The influence of pH and ionic strength on the electrokinetic stability of the human erythrocyte membrane.

Heard, D.H. and G.V.F. Seaman, Biochim. Biophys. Acta, *53* (1961) 366–374. The action of lower aldehydes on the human erythrocyte.

Heller, M., N. Mozes and E. Maes, in "Methods in Enzymology", *35* Lipids, Part B (1975), ed. J.M. Lowenstein, p 226. Phospholipase D from peanut seeds.

Heller, M., in "Advances in lipid research", *16*, eds. R. Paoletti and D. Kritchevsky, Acad. Press (1978) Phospholipase D.

Henry, D.C., Proc. Roy. Soc. A133, *1* (1931) 106–129. The cataphoresis of suspended particles. Part I. — The equation of cataphoresis.

Hope, M.J. and P.R. Cullis, FEBS letters, *107* (1979) 323–326. The bilayer stability of inner monolayer lipids from the human erythrocyte.

Imamura, S. and Y. Horiuti, J. Biochem., *83* (1978) 677–680. Enzymatic determination of phospholipase D activity with choline oxidase.

Johnson, R.M., J. Memb. Biol., *22* (1975) 231–253. The kinetics of resealing of washed erythrocyte ghosts.

Johnson, R.M., and D. H. Kirkwood, Biochim. Biophys. Acta, *509* (1978) 58–66. Loss of resealing ability in erythrocyte membranes. Effect of divalent cations and spectrin release.

Jones, G. and B.C. Bradshaw, J. Amer. Chem. Soc., *55* (1933) 1780–1800. The measurement of the conductance of electrolytes. V. A redetermination of the conductance of standard potassium chloride solutions in absolute units.

Jost, P.C., O.H. Griffith, R.A. Capaldi and G. Vanderdooi, Proc. Nat. Acad. Sci. USA, *70* (1973) 480–484. Evidence for boundary lipid in membranes.

Kornberg, R.D. and H.M. McConnell, Proc. Nat. Acad. Sci. USA, *68* (1971a) 2564–2568. Lateral diffusion of phospholipids in a vesicle membrane.

Kornberg, R.D. and H.M. McConnell, Biochem., *10* (1971b) 1111–1120. Inside-outside transitions of phospholipids in vesicle membranes.

de Kruijff, B. and P. Baken, Biochim. Biophys. Acta, *507* (1978) 38–47. Rapid transbilayer movement of phospholipids induced by an asymmetrical perturbation of the bilayer.

de Kruijff, B., E.J.J. VanZoelen and L.L.M. VanDeenen, Biochim. Biophys. Acta, *509* (1978) 537–542. Glycophorin facilitates the transbilayer movement of phosphatidylcholine in vesicles.

Lenard, J. and S.J. Singer, Proc. Nat. Acad. Sci., *56* (1966) 1828–1835. Protein conformation in cell membrane preparations as studied by optical rotary dispersion and circular dichroism.

Lenard, J. and S.J. Singer, J. Cell Biol., *37* (1968) 117–121. Alteration of the conformation of proteins in red blood cell membranes and in solution by fixatives used in electron microscopy.

Lowry, O.H., N.J. Rosebrough, A.L. Farr and R.J. Randall, J. Biol. Chem., *193* (1951) 265–275. Protein measurement with the folin phenol reagent.

Maddy, A.H. and B.R. Malcolm, Sci., *150* (1965) 1616–1618. Protein conformations in the plasma membrane.

Mel, H.C., T. Tenforde and R.M. Glaeser, Archives of Biochem. and Biophys., *158* (1973) 533–538. New electrophoretic information on the surface composition of the rat erythrocyte.

Mel, H.C. and J.P. Yee, Blood Cells, *1* (1975) 391–399. Erythrocyte size and deformability studies by resistive pulse spectroscopy.

Müller, H., U. Schmidt and H.U. Lutz, Biochim. Biophys. Acta, *649* (1981) 462–470. On the mechanism of vesicle release from ATP-depleted human red blood cells.

Nelson, G.J., J. Lipid Research *8* (1967) 374–379. Composition of neutral lipids from erythrocytes of common mammals.

Nicolson, G.L., J. Cell Biol., *57* (1973) 373–387. Anionic sites of human erythrocyte membranes — effects of trypsin, phospholipase C and pH on the topography of bound positively charged colloidal particles.

Op den Kamp, J.A.F. Ann. Rev. Biochem., *48* (1979) 47–71. Lipid asymmetry in membranes.

Ottolenghi, A.C. and M.H. Bowman, J. Memb. Biol., *2* (1970) 180–191. Membrane structure: Morpholigical and chemical alterations in phospholipase-C-treated mitochondria and red cell ghosts.

Overbeek, J.Th.G. and J. Lijklema in "Electrophoresis" ed. M. Bier, Academic Press, N.Y. (1959) chap 1, pp 1–33. Electric potentials in colloidal systems.

Peros, S., Senior Honors Thesis, Dept. Biophys and Med Phys, UC, Berkeley (1981). Interactive effects of electric current; glutaraldehyde and detergent on red blood cell membrane systems.

Phillips, M.C., E.G. Finer and H. Hauser, Biochim. Biophys. Acta, *290* (1972) 397–402. Differences between conformations of lecithin and phosphatidylethanolamine polar groups and their effects on interactions of phospholipid bilayer membranes.

Pinto da Silva, P. and D. Branton, J. Cell Biol., *45* (1970) 598–605. Membrane splitting in freeze-etching. Covalently bound ferritin as a membrane marker

Richardson, S.H., H.O. Hultin, and D.E. Green, Biochem., *50* (1963) 821–827. Structural proteins of membrane systems.

Robertson, J.D., J. Cell Biol., *19* (1963) 201–221. The occurrence of a subunit pattern in the unit membranes of club endings in mauthner cell synapses in goldfish brains.

Roozemond, R.C., J. Histochem and cytochem *17* (1969) 482–486. The effect of fixation with formaldehyde and glutaraldehyde on the composition of phospholipids extractable from rat hypothalamus.

Rothman, J.E. and J. Lenard, Sci., *195* (1977) 743–753. Membrane Asymmetry.

Schnebli, H.P. and T. Bachi, Experimental Cell Research, *91* (1975) 175–183. Reactions of lectins with human erythrocytes. I. Factors governing the agglutination reaction.

Seaman, G.V.F. and D.H. Heard, J. Gen. Physiol., *44* (1960) 251,268. The surface of the washed human erythocyte as a polyanion.

Seaman, G.V.F., L.J. Jackson, and G. Uhlenbruck, Archives of Biochem and Biophys, *122* (1967) 605–613. Action of alpha-amylase preparations and some proteases on the surface mammalian erythrocytes.

Seaman, G.V.F. and G.M.W. Cook in "Cell electrophoresis", ed. E.J. Ambrose, (1965) 48–65. Modification of the electrophoretic behaviour of the erytrhocyte by chemical and enzymatic methods.

Shah, D.O. and J.H. Schulman, J. Lipid Res., *8* (1967) 227–233. The ionic structure of lecithin monolayers.

Sigma chemical Co., Phospholipase-D assay procedure. Available from Sigma Chemical Co., P.O. Box 14508, St. Louis, Mo, 63178.

Singer, J.A. and M. Morrison, Biochim. Biophys. Acta, *426* (1976) 123–131. Effect of metabolic state on agglutination of human erythocytes by concanavalin A.

Singer, S.J. and G.L. Nicolson, Sci., *175* (1972) 720–731. The fluid mosaic model of the structure of cell membranes.

Smoluchowski, M., Bull. Acad. Sci. Crucovie (1903)

Spatz, C., J.O. Johnston, "Basic Statistics, Tales of Distributions" (1981) Brooks/Cole Publishing Co., Monterey, Ca.

Staros, J.V., B.E. Haley, and F.M. Richards, J. Biol. Chem., *249* (1974) 5004–5007. Human erythrocytes and resealed ghosts. A comparison of membrane topology.

Steck, T.L., G. Fairbanks and D.F.H. Wallach, Biochem., *10* (1971) 2617–2624. Disposition of the major proteins in the isolated erythrocyte membrane. Proteolytic dissection.

Steck, T.L., J. Cell Biol., *62* (1974) 1–19. The organization of proteins in the human red blood cell membrane — a review.

Svennerholm, L., Acta Chem. Scand., *10* (1956) 694. On sialic acid in brain tissue.

Tamura, A and T. Fujii, J. Biochem. *90* (1981) 629–634. Roles of charged groups on the surface of membrane lipid bilayer of human erythrocytes in induction of shape change.

Tenforde, T., Ph.D. Thesis (1969), University of California, Berkeley. Electrophoretic and chemical studies on the rat erythrocyte membrane interface.

Tenforde, T. in "Adv. Biological and Med. Physics" *13*, ed. T. Hayes, Acad. Press (1970) 43–105. Microelectrophoretic studies on the surface chemistry of erythocytes.

Tenforde, T., E.J. Clarke, R.D. MacGregor,Jr., M.J. Streibel and P.W. Todd, Med. and Biol. Eng., *11* (1973) 236–238. A convenient microelectrophoresis assembly.

Vanderkooi, G. and D.E. Green, Proc. Nat. Acad. Sci., *66* (1970) 615–621. Biological membrane structure, I. The protein crystal model for membranes.

Vassar, P.S., J.M. Hards, D.E. Brooks, B. Hagenberger and G.V.F. Seaman, J. Cell Biol., *53* (1972) 809–818. Physicochemical effects of aldehydes on the human erytrhocyte.

Wallach, D.F.H. and P.H. Zahler, Biochem., *56* (1966) 1552–1559. Protein conformations in cellular membranes.

Walter, H., E.J. Krob, G.S. Ascher, Biochim. Biophys. Acta, *641* (1981) 202–215. Aging of erythrocytes results in altered red cell surface properties in the rat, but not in the human.

Warren, L., J. Biol Chem., *234* (1959) 1971–1975. The thiobarbituric acid assay of sialic acids.

Ways, P. and D.J. Hanahan, J. Lipid Res., *5* (1964) 318–328. Characterization and quantification of red cell lipids in normal man.

Wilkins, M.H.F., A.E. Blaurock and D.M. Engelman, Nature, new biol., *230* (1971) 72–76. Bilayer structure in membranes.

Yee, J.P. and H.C. Mel, Blood Cells *4* (1978) 485–497. Kinetics of glutaraldehyde fixation of erythrocytes: size, deformability, form, osmotic and hemolytic properties.

Zwaal, R.F. and B. Roelofsen, in " Biochem. Analysis of Membranes", ed. A.H. Maddy, Wiley and sons, N.Y. (1976) Applications of pure phospholipases in membrane studies.

Zwaal, R.F.A., B. Roelofsen, P. Comfurius and L.L.M.vanDeenen, Biochim. Biophys. Acta, *406* (1975) 83–96. Organization of phospholipids in human red cell membranes as detected by the action of various purified phospholipases.

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