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MICROELECTROPHORETIC STUDIES ON THE SURFACE CHEMISTRY OF ERYTHROCYTES

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I. Introduction

II. The Microelectrophoretic Method

A. Theoretical Aspects

1. The Relationship of Electrophoretic Mobility to the Zeta Potential of a Charged Surface

2. Calculation of Surface Charge Density from the Zeta Potential

B. Experimental Procedures

III. Studies on the Surface Chemistry of Erythrocytes at Physiological Ionic Strength

A. The Dependence of Surface Charge Properties on Ionic Strength

B. The Role of Sialic Acid in Determining Electrophoretic Mobility; Surface Carbohydrates

C. Chemical Modification Coupled with Microelectrophoresis

1. Stabilization of Erythrocyte Membranes Using Aldehydes and Osmium Tetroxide; Mobility-pH Relations for Human and Rat Erythrocytes

2. Studies on Anion Adsorption as a Possible Charging Mechanism

3. Susceptibility of Surface Groups to Methylation and Reaction with a Water Soluble Carbodiimide

4. Evidence Against Dissociating Groups Above Neutral pH

5. Studies on Possible Phospholipid Involvement in Erythrocyte Surface Charge Properties

6. Mobility-pH Characteristics of Neuraminidase-Treated Erythrocytes

D. Summary: The Ionic Character of the Erythrocyte Membrane Surface at Physiological Ionic Strength

I. Introduction

In the fifty years following its development by Northrup and Kunitz (83, 96, 97), the microscope method of electrophoresis has been used to obtain considerable information on the surface charge properties of living cell membranes (1, 5, 138). The microelectrophoretic method has been employed, for example, to characterize the surface charge of cells in normal and pathological states. During the past decade, microelectrophoresis has also proved to be a powerful technique for directly investigating the character of ionic moieties at the outer surface of intact cells. This has been accomplished through the quantitation of changes in surface charge resulting from specific chemical alteration of the cell surface, e.g. through the action of enzymes. In this manner, ionic groups responsible for the membrane surface charge may be identified, the most notable success to date being the discovery of sialic acid at the surface of a wide variety of cells.

The primary objective of this paper is to discuss the surface structure of erythrocytes as deduced from electrophoretic studies on chemically modified cells. An attempt has been made to present experimental results that are directly related to the identification of charged groups at the red cell surface. A discussion of the microelectrophoretic method has also been included, with a particular view towards presenting the theory of colloid electrophoresis as it applies to intact cells in physiological media.

It is clear from the preceding paragraphs that the term "cell surface" is being used here in a highly restricted sense, namely, as the "electrokinetic

cell surface." This is a purely operational description based upon ionic components of the plasma membrane that are detectable by electrophoretic means. Alternative operational descriptions could be given in terms of functional properties localized at the outer membrane surface (e.g. cell antigens and intercellular contact phenomena). However, from this viewpoint the cell surface is extraordinarily complicated. As a means of demonstrating this complexity, it is worthwhile to discuss briefly several properties of erythrocytes that are known to be associated with the outer membrane surface.

The functional components that are best understood at a molecular level are the blood group antigens. It has been well established that these are oligosaccharides linked to membrane protein and lipid (29, 135). In addition to neutral sugars, many of the oligosaccharide chains contain sialic acid, the carboxyl group of which is a major contributor to the erythrocyte surface charge. The surface mucoids have also been demonstrated to function as receptors for influenza and other strains of virus (130).

Several transport functions have been shown to depend upon molecular components at the erythrocyte surface. Perhaps the best known is the sodium-potassium ionic pump. This active transport system is susceptible to inhibition by low concentrations of cardiac glycosides, a group of steroids that act directly upon allosteric transport sites at the outer surface of the cell membrane (7, 71, 142). A set of sulfhydryl groups at the outer surface of erythrocytes has been demonstrated by Vansteveninck *et al.* (133) to be essential for the transport of glucose. Stein (125) has proposed a model of facilitated glycerol transport in human erythrocytes

in which the formation of glycerol dimers is mediated by protein components at the outer membrane surface. He hypothesizes that dimers would be essentially free of bound water, and could thereby penetrate lipoidal regions of the membrane more readily than single molecules of glycerol.

One of the enzymes present in the erythrocyte membrane has been shown to be located at the outer surface. Using a lead stain in the presence of thiolacetic acid, Shinagawa and Ogura (122) have demonstrated that acetylcholinesterase is primarily associated with the outermost layer of the membrane. It has also been observed that membrane-bound acetylcholinesterase can be inhibited by reacting erythrocytes with trypsin (70), an enzyme that is known to effect the release of surface components (119).

Although by no means complete, the list of properties given in the preceding paragraphs serves to demonstrate the large variety of cellular functions associated with the erythrocyte surface. In many instances, the corresponding macromolecular components have not been isolated. The chemical characterization of these functional entities, and their correlation with surface properties determined by electrophoretic methods, remains as one of the major unsolved problems in membrane biology.

by Henry (68), and contains the following assumptions: (i) The particle suspension is sufficiently dilute that no electrical or hydrodynamic interactions occur between neighboring particles. (ii) The solution conductivity, dielectric constant, and viscosity have the same values within the double layer as in the bulk medium surrounding the particle. (iii) The applied field obeys Ohm's law, and can be taken as superimposed on the field associated with the electrical double layer. (iv) The hydrodynamic equations for the motion of a viscous fluid hold in the double layer, and the electrophoretic motion is sufficiently slow that inertia terms may be neglected.

(b) The Equations of Smoluchowski and Henry. When a suspended charged particle reaches its steady state velocity in an applied electric field, the electrical force is exactly balanced by the frictional resistance resulting from movement through a viscous medium. This is expressed by the equation:

$$\vec{F}_{elec} + \vec{F}_{mech} = 0 \qquad (1)$$

In Eq. (1), $\vec{F}_{elec}$ is the force on the suspended particle due to the net electrical field $\vec{E} = \vec{E}_Q + \vec{E}_o$, where $\vec{E}_Q$ is the field associated with the total charge carried by the particle and $\vec{E}_o$ is the applied field. In the case of intact cells, there is no evidence for a net internal charge (46), so that $\vec{E}_Q$ can be considered to arise only from the electrical double layer at the surface. $\vec{F}_{mech}$ represents the total mechanical force exerted by the viscous medium on the moving particle. Calculation of these two forces can be made by means of integrating electrical and hydrodynamic stress

tensors ($\vec{\vec{T}}_{elec}$ and $\vec{\vec{T}}_{mech}$) over the particle surface:

$$\vec{F}_{elec} = \oiint \vec{\vec{T}}_{elec} \cdot \vec{dS} \tag{2a}$$

and

$$\vec{F}_{mech} = \oiint \vec{\vec{T}}_{mech} \cdot \vec{dS} \tag{2b}$$

In the case of a rigid sphere, the mathematical calculations of $\vec{F}_{elec}$ and $\vec{F}_{mech}$ were originally presented by Henry (68) and have recently been reviewed by Glaeser (46). When the resulting expressions for the electrical and mechanical forces are substituted into Eq. (1), the following equation is obtained for the mobility (μ) of a charged sphere:

$$\mu = \frac{\varepsilon}{6\pi\eta}\left\{\zeta + a^3\left(\frac{\lambda-\lambda'}{2\lambda+\lambda'}\right)\left[3a^2\int_{\infty}^{a}\frac{1}{r^5}\frac{\partial\psi}{\partial r}dr - 2\int_{\infty}^{a}\frac{1}{r^3}\frac{\partial\psi}{\partial r}dr\right]\right\} \tag{3}$$

Here ε and η represent, respectively, the dielectric constant and viscosity in the double layer; λ is the solution conductivity and λ' the particle conductivity; r is the radial coordinate and a is the electrophoretic radius, i.e. the distance from the center of the sphere to the surface of shear. ψ is the potential associated with the electrical double layer, and is defined by the relation $\vec{E}_Q = -\nabla\psi$. The integrals in Eq. (3) can be evaluated in two special cases:

(i) If the thickness of the ionic double layer is small relative to the electrophoretic radius a, then Eq. (3) reduces to

$$\mu = \left(\frac{3\lambda}{2\lambda+\lambda'}\right)\frac{\varepsilon\zeta}{6\pi\eta} \tag{4}$$

For a non-conducting particle ($\lambda' = 0$), Eq. (4) becomes

$$\mu = \frac{\varepsilon \zeta}{4\pi\eta} \tag{5}$$

This is the equation originally derived for large non-conducting colloids by Smoluchowski (123), who made the simplifying assumption that the applied electric field lines are parallel to the particle surface at all points within the double layer.

(ii) A second condition under which the integrals of Eq. (3) can be evaluated is the special case when the zeta potential is sufficiently small that the ionic double layer can be described in the Debye-Hückel (34) approximation. Representing the valences of small ions in the double layer by z_i, the electronic charge by e, Boltzmann's constant by k, and the absolute temperature by T, the necessary condition is given by

$$\frac{z_i e \psi}{kT} \ll 1 \tag{6}$$

At room temperature, Eq. (6) requires that ψ be much less than $25/z_i$ millivolts. The Poisson-Boltzmann equation describing the potential ψ in the double layer (see Section II, A, 2, a) can then be written in a linear form (the Debye-Hückel approximation):

$$\nabla^2 \psi = \kappa^2 \psi \tag{7a}$$

where

$$\kappa^2 = \frac{4\pi e^2}{\varepsilon kT} \sum_i n_i z_i^2 \tag{7b}$$

Here n_i represents the number of ions per unit volume of the ith species in the bulk medium surrounding the charged colloid. The quantity $1/\kappa$ has the dimension of a length, and is frequently referred to as the "Debye length." For aqueous solutions at room temperature, the Debye length in angstrom units is given by

$$\frac{1}{\kappa} = \frac{3.05}{(\Gamma/2)^{1/2}} \tag{8}$$

The quantity $\Gamma/2$ is the ionic strength defined by Lewis and Randall (85):

$$\Gamma/2 = \frac{1}{2}\sum_i c_i z_i^2 \tag{9}$$

where c_i is the bulk molar concentration of the ith ionic species. Physically, the Debye length can be regarded as a rough measure of the thickness of the ionic double layer.

The solution of Eq. (7a) for a rigid charged sphere is given by:

$$\psi = \zeta\left(\frac{a}{r}\right)e^{-\kappa(r-a)} \tag{10}$$

Substituting ψ from Eq. (10) into the integrals of Eq. (3), Henry (68) obtained the following expression for the mobility of an insulating particle:

$$\mu = \frac{\varepsilon\zeta}{4\pi\eta} f(\kappa a) \tag{11}$$

The function $f(\kappa a)$ exhibits a complicated dependence upon ionic strength, and has been plotted by Henry (68) over the range $-2 < \log_{10}\kappa a < +3$. If the particle size and ionic strength satisfy the relation $\kappa a > 300$,

then to an accuracy of 1%, $f(\kappa a)$ can be set equal to unity and Eq. (11) reduces to the Smoluchowski equation. For values of $\kappa a < 0.5$, the mobility can be approximated to within 1% by setting $f(\kappa a) = 0.67$ in Eq. (11).

With regard to the electrophoretic motion of particles having geometries other than spherical, an analytical treatment has been given by Henry (68) for infinite cylinders placed either parallel or transverse to an applied electric field. For a cylinder aligned parallel to the field, he found that the Smoluchowski expression (Eq. (5)) is valid for any value of κb, where b is the radius of the cylinder. This result is also independent of the internal conductivity. For an insulating cylindrical particle placed in a transverse field, Henry (68) obtained the result:

$$\mu = \frac{\varepsilon \zeta}{\pi \eta F(\kappa b)} \qquad (12)$$

The function $F(\kappa b)$ has been evaluated graphically by Abramson et al. (3), who calculated ψ in the Debye-Hückel linear approximation. In the limit $\kappa b \gg 1$, $F(\kappa b) = 4$ and Eq. (12) reduces to the Smoluchowski equation.

From the theoretical results presented above for spheres and cylinders, it is clear that the mobility of a non-conducting colloid of any shape can be closely approximated by the Smoluchowski equation if the radius of curvature of the particle is much greater than the double layer thickness. The applicability of the Smoluchowski equation to intact cells at physiological ionic strength is shown by the following calculation for erythrocytes. In electrophoretic measurements conducted at room temperature using aqueous saline solutions of ionic strength 0.145, the Debye length calculated from

Eq. (8) is 8 Å. If the red cell is treated as a rigid sphere with an effective radius of 2 to 3 microns, then a is at least 1000 times larger than $1/\kappa$. An extremely accurate relationship between mobility and zeta potential will therefore be given by the Smoluchowski equation. With smaller particles such as bacteria and viruses, however, it may be necessary to take account of the factor $f(\kappa a)$ in Eq. (11).

(c) Correction Factors. The analysis outlined in the preceding paragraphs constitutes a reasonably complete description of colloid electrophoresis. There are, however, several phenomena that are not taken into account in the theoretical treatment of Henry, and which can contribute substantially to the electrophoretic mobility in certain ranges of particle size, ionic strength, and surface potential. These will now be considered, with a particular view towards evaluating their importance in electrophoretic measurements performed on intact cells at physiological ionic strength.

(i) Relaxation Effect. In outlining the assumptions underlying Henry's derivation of Eq. (3), it was stated that the applied electric field is taken to be superimposed on the field of the double layer. This is not the case physically, however, since a particle and the counterions in its double layer move in opposite directions when an external field is applied. As a result, the particle moves away from its counterion atmosphere, and the symmetry of the double layer is disturbed. Coulomb forces within the double layer tend to restore the original symmetry, but this takes a finite period of time known as the "relaxation time." Consequently, in an applied electric field the counterion atmosphere

constantly lags behind a charged particle and retards its steady state electrophoretic motion. Approximate analytical treatments of the relaxation effect have been given by Overbeek (99) and by Booth (15, 18) for insulating spherical colloids. More recently, Wiersema et al. (143) have performed a computer calculation of the electrophoretic mobility starting from transport equations that include the relaxation phenomenon. The results of this study show that the relaxation effect can exert an appreciable influence on the mobility, especially for values of the zeta potential greater than 50 mV. The effect is negligible, however, for values of $\kappa a > 1000$. Therefore, no correction to the Smoluchowski equation is needed in electrophoretic measurements performed on intact cells at physiological ionic strength.

(ii) Surface Conductance. As the result of ionic conduction through the double layer, the electrical potential gradient across a particle undergoing electrophoretic motion is reduced. This phenomenon, known as "surface conductance," has been examined by Booth (16) and by Henry (69). The results of these authors are similar, and predict that surface conductance can decrease the electrophoretic mobility. This effect is most evident at low ionic strength, as shown by the experiments of Ghosh and Bull (43) on Pyrex glass beads. At ionic strengths above 0.01, however, the influence of surface conductance on the mobility was found by these workers to be negligible. It seems unlikely, therefore, that any correction for this effect needs to be made for intact cells at physiological ionic strength.

(iii) Particle Conductivity. From the analysis of Henry, it is clear that particle conductivity can substantially reduce the electrophoretic mobility (see Eq. (4)). In the case of bacterial particles, the experiments of Einholf and Carstensen (36) show that particle conductivity influences the mobility even at ionic strengths exceeding 0.1. A similar conclusion has been reached by Gittens and James (45), although these workers have interpreted bacterial conductivity as a surface conductance phenomenon. In contrast to the experiments with bacteria, Carstensen et al. (24) have found that erythrocytes behave as nearly perfect insulators. This is in accord with the generally accepted notion that intact cells can be regarded as non-conductors in the interpretation of electrophoretic measurements.

(iv) Dielectric Constant and Viscosity. In deriving the relationship between mobility and zeta potential, changes in dielectric constant and viscosity within the double layer were neglected. However, because of the large local electric fields present in diffuse double layers, ε is expected from theoretical considerations to be smaller than its value in bulk solution, whereas η is predicted to be larger (57, 88). The value of ζ will therefore be underestimated in calculations that assume values of ε and η for bulk aqueous solutions. Unfortunately, the appropriate values of ε and η within the double layer are not known. A thorough discussion of this problem has been given by Haydon (64) and by Overbeek and Wiersema (101). With regard to intact cells at physiological ionic strength, the zeta potential rarely exceeds 25 mV and the double layer electric field is less than 3×10^5 volt/cm. Under these conditions,

both theoretical considerations and the experimental evidence presently available indicate that the calculated value of ζ is not significantly affected by using bulk values for the dielectric constant and viscosity in the equations of electrophoresis.

(v) Other Corrections. In the theoretical treatment outlined above, inertia terms were neglected in the hydrodynamic equations describing the motion of a sphere through a viscous medium. This, however, has been shown by Booth (17) to be completely justified. Similarly, the influence of Brownian motion on the electrophoretic velocity of large colloids can be neglected (143).

(d) Conclusions. The results of this section indicate that the Smoluchowski equation accurately describes the relationship between mobility and zeta potential for non-conducting colloids satisfying the relation $\kappa a > 300$. Although numerous physical assumptions are made in the derivation of Eq. (5), there is no indication that these affect its validity when applied to electrophoretic measurements on intact cells.

2. Calculation of the Surface Charge Density from the Zeta Potential

(a) The Gouy-Chapman Equation. In order to relate the surface charge density of a colloid to its electrophoretic mobility, it is necessary first to express the charge density in terms of ζ. For this purpose, the electrokinetic surface will be treated as a planar interface. This assumption is completely warranted for an intact cell since its radius of curvature is much greater than the double layer thickness. It will be further assumed in the following treatment that

the surface charge is smeared out in a mathematical sense, so that the only variation in potential occurs normal to the surface.

The concentration of cations in the diffuse double layer at the surface is described by the Boltzmann distribution law:

$$n^{+} = n_1 e^{-z_1 e\psi/kT} \tag{13}$$

Here n^+ is the concentration (ions per unit volume) in the neighborhood of the surface, z_1 is the cation valence, and n_1 is the cation concentration in bulk solution. Similarly, the distribution of anions near a charged surface is given by

$$n^{-} = n_2 e^{z_2 e\psi/kT} \tag{14}$$

In Eq. (14) z_2 and n_2 are, respectively, the valence and bulk concentration of anions in the electrolyte medium. It is clear from these equations that in the electrolyte solution adjacent to a charged interface, ions of like sign are repelled, while those of opposite sign are attracted. Using Eqs. (13) and (14), the net charge density per unit volume (ρ) can be expressed as

$$\rho = z_1 e n^{+} - z_2 e n^{-} = e\left[z_1 n_1 e^{-z_1 e\psi/kT} - z_2 n_2 e^{z_2 e\psi/kT}\right] \tag{15}$$

Near a planar surface carrying a uniform charge, the quantities ρ and ψ can also be related by Poisson's equation:

$$\frac{d^2\psi}{dx^2} = -\frac{4\pi\rho}{\varepsilon} \tag{16}$$

Here $\underline{x}$ is the coordinate normal to the surface, with $x = 0$ defining the interface between the surface and the exterior salt solution. The

appropriate boundary conditions on the potential ψ are

$$\psi(x=\infty) = 0 \quad \text{and} \quad \left(\frac{d\psi}{dx}\right)_{x=\infty} = 0 \tag{17}$$

Combining Eqs. (15) and (16) leads to the so-called Poisson-Boltzmann equation:

$$\frac{d^2\psi}{dx^2} = -\frac{4\pi e}{\varepsilon}\left[z_1 n_1 e^{-z_1 e\psi/kT} - z_2 n_2 e^{z_2 e\psi/kT}\right] \tag{18}$$

Using the boundary conditions given by Eq. (17), a first integration may be performed in Eq. (18) to yield a closed expression for $d\psi/dx$:

$$\frac{d\psi}{dx} = \mp\sqrt{\frac{8\pi kT}{\varepsilon}}\sqrt{n_1\left(e^{-z_1 e\psi/kT} - 1\right) + n_2\left(e^{z_2 e\psi/kT} - 1\right)} \tag{19}$$

The upper and lower signs refer, respectively, to positively and negatively charged surfaces.

Equation (19) can now be used to evaluate the net surface charge density (σ) at the surface of shear. Since the system comprised by the charged interface and its exterior electrolyte solution must be electrically neutral as a whole, it follows that the surface of shear (x = a) can be considered to cut the diffuse double layer into two regions containing equal but opposite net charges. The charge density σ at x = a can therefore be calculated from the expression:

$$\sigma = -\int_a^\infty \rho\, dx \tag{20}$$

Since σ represents the charge per unit area evaluated at the surface of shear, it must be regarded as an "electrokinetic" surface charge density. This value of σ will have the same sign, but a smaller magnitude, than

the fixed charge density on the planar interface at x = 0. Inserting Eq. (16) into Eq. (20) and performing a first integration leads to the final result:

$$\sigma = -\frac{\varepsilon}{4\pi}\left(\frac{d\psi}{dx}\right)_{x=a} = \pm\sqrt{\frac{\varepsilon kT}{2\pi}}\sqrt{n_1\left(e^{-z_1 e\zeta/kT}-1\right)+n_2\left(e^{z_2 e\zeta/kT}-1\right)} \quad (21)$$

This relation between σ and ζ was first derived by Gouy (55, 56) and by Chapman (27), and is frequently referred to as the Gouy-Chapman equation. In electrophoresis experiments performed on intact cells, a uni-univalent electrolyte is most commonly employed. For this special case, Eq. (21) reduces to

$$\sigma = \sqrt{\frac{2\varepsilon n_0 kT}{\pi}}\sinh\left(\frac{e\zeta}{2kT}\right) \quad (22a)$$

$$= \frac{\varepsilon kT\kappa}{2\pi e}\sinh\left(\frac{e\zeta}{2kT}\right) \quad (22b)$$

Here n_0 is the bulk salt concentration, and κ is the Debye-Hückel constant defined by Eq. (7b).

If the Smoluchowski relation between ζ and μ (Eq. (5)) is now substituted into Eq. (22b), the resulting equation directly expresses the "electrokinetic" surface charge density σ in terms of the experimentally measured electrophoretic mobility μ:

$$\sigma = \frac{\varepsilon kT\kappa}{2\pi e}\sinh\left(\frac{2\pi e\eta\mu}{\varepsilon kT}\right) \quad (23)$$

Introducing numerical values appropriate to uni-univalent electrolytes in aqueous solutions at 25° C, the value of σ is given by

$$\sigma = \left(0.3514\times10^{5}\right)\sqrt{C}\,\sinh(0.25\mu) \text{ in statcoul/cm}^2 \quad (24a)$$

$$= 0.1171\sqrt{C}\,\sinh(0.25\mu) \quad \text{in coul/m}^2 \quad (24b)$$

In these equations, C is the molar concentration of uni-univalent electrolyte and μ is expressed in the conventional units of micron/sec/volt/cm. It might also be noted that for potentials satisfying the relation $\zeta \ll 2kT/\varepsilon$, Eqs. (22b) and (23) reduce to the simple expressions:

$$\sigma = \frac{\varepsilon \kappa \zeta}{4\pi} \tag{25a}$$

$$= \eta \kappa \mu \tag{25b}$$

In a physiological medium of ionic strength 0.145, the mobility observed for many types of intact cells is approximately -1 micron/sec/volt/cm. The corresponding surface charge density calculated from either Eq. (24b) or (25b) is -1.1×10^{-2} coul/m^2. For a uniform charge distribution, this is equivalent to one elementary charge on every 1460 Å^2 of surface.

When the radius of curvature of a colloid does not greatly exceed the double layer thickness, then a solution of the Poisson-Boltzmann equation must be obtained for a geometry approximating the true particle surface. Unfortunately, an exact solution in analytical form is possible only for a planar interface. For the case of a uniformly charged sphere, Hoskin (72) and Loeb et al. (86) have attempted to overcome this difficulty by performing a numerical integration of the Poisson-Boltzmann equation using electronic computers. The results of Loeb and associates (86) have been conveniently tabulated in book form.

As discussed in Section II, A, 1, b, for small values of ψ (Eq. (6)) the Poisson-Boltzmann equation reduces to the linear form of Eq. (7a). This is known as the Debye-Hückel approximation, and has the exact

solution for a rigid charged sphere given by Eq. (10). Using this expression for ψ, the "electrokinetic" surface charge density can be calculated in the manner outlined above for a planar surface. For a sphere of radius a, the resulting equations relating σ, ζ, and μ are

$$\sigma = \frac{\varepsilon \zeta}{4\pi a}(1 + \kappa a) \tag{26a}$$

$$= \frac{\eta \mu}{a}(1 + \kappa a) \tag{26b}$$

For values of $\kappa a > 100$, these equations reduce to Eqs. (25a) and (25b) with an accuracy of 1%. It might also be noted that Gronwall et al. (58) and MacGillivary (89) have shown that the value of ψ given by Eq. (10) uniformly approximates an exact solution of the Poisson-Boltzmann equation in the limit of zero ionic strength. Therefore, as the electrolyte concentration diminishes, Eq. (26a) becomes a more accurate representation of the surface charge density for arbitrary values of ζ.

(b) Electrophoretic Radius. Under the condition $\kappa a < 100$, it is clear from Eq. (26) that the calculated surface charge density is quite sensitive to the choice of electrophoretic radius. Brinton and Lauffer (21) have illustrated this for the case of filamented and non-filamented bacteria. With intact cells at physiological ionic strength ($\kappa a > 1000$), it is unlikely on theoretical grounds that the surface charge density should depend on particle size or shape. Nevertheless, suggestions have been put forth in the literature (21, 37) that the appropriate electrophoretic radius of an erythrocyte might be less than 100 Å. Brinton and Lauffer (21) have argued that the surface may have a rough texture, and that the appropriate electrophoretic radius might then be the average

radius of curvature of charged structures protruding from the surface. There are, however, indications that surface "roughness" is not an important factor in determining the charge density. For example, the mobility of sphered or sickled red cells is identical to that of normal discoidal erythrocytes (2, 41). Working in collaboration with Mr. John Kroes and Dr. Rosemarie Ostwald, the present author has examined an extreme case of surface alteration in the guinea pig erythrocyte. Following administration of a 1% cholesterol diet to guinea pigs for a period of several weeks, their red cells attain an increased cholesterol and phospholipid content (98), accompanied by the development of large spicules, or burrs, which protrude from the cell surface. The cell morphology in this condition is quite comparable to that of erythrocytes in spur-cell anemia and acanthocytosis (73, 93, 137). Despite this gross alteration in surface structure, the mobility of erythrocytes from cholesterol-fed animals has been found to be identical to that of control cells. In a physiological medium of ionic strength 0.145, the mobility of erythrocytes from eight control animals was 0.79 $\pm$ 0.02 (SD) micron/sec/volt/cm. The average mobility of red cells from six guinea pigs maintained on a 1% cholesterol diet for periods of 4 to 14 weeks was 0.81 $\pm$ 0.03 (SD) micron/sec/volt/cm. It may be concluded from this data that the mobility and net surface charge density at physiological ionic strength are completely insensitive to gross morphological changes in the erythrocyte surface. To this extent, the red cell behaves as a large smooth colloid satisfying the relation $\kappa a > 300$. At an ionic strength of 0.145, the corresponding electrophoretic radius a is greater

than 2400 Å. This is contrary to the conclusion of Brinton and Lauffer (21), and suggests that in the absence of detailed knowledge regarding the architecture of cell surfaces, the most appropriate choice of electrophoretic radius is that of the entire cell.

(c) Counterion Volume. One of the difficulties inherent in the Gouy-Chapman theory is the assumption that counterions in the diffuse double layer are point charges. As a result, the Boltzmann equation overestimates the ionic concentration at the surface of a charged colloid. This in turn leads to a calculated surface charge density that is too high. Several attempts have been made to introduce finite ion volumes into the Boltzmann equation, and are discussed in the reviews of Overbeek and Lijklema (101) and Haydon (64).

In the biological literature, the ionic volume correction that is most commonly employed is based on Gorin's (50) theoretical treatment for a single spherical central ion. Using the Debye-Hückel approximation, Gorin assumed that the central ion is surrounded by a shell containing zero charge and having a thickness equal to the counterion radius. The result of Gorin's investigation has been applied to cell electrophoresis by Furchgott and Ponder (42), who wrote Eq. (25b) in the form:

$$\eta \mu = \sigma \left(\frac{1}{\kappa} + r_i \right) \qquad (27)$$

where r_i is the counterion radius. Using electrophoretic data for human erythrocytes, these workers plotted $\eta\mu$ vs $1/\kappa$ and determined r_i from the intercept. The value obtained in this manner was 1.8 Å. This is approximately the right magnitude for an ionic radius, and was interpreted by Furchgott and Ponder (42) to be a justification for the use of Eq. (27).

Subsequently, several other workers have applied Gorin's correction in calculating the surface charge density of erythrocytes (10, 37, 47).

The use of Gorin's correction has been criticized by Brinton and Lauffer (21), who demonstrated that the value of r_i obtained graphically is extremely sensitive to the choice of electrophoretic radius. This is also clear from the calculations of Overbeek (100). Another difficulty that has been pointed out by Overbeek (100) is the poor agreement in the literature for hydrated ionic radii. The value that should be used for r_i in Eq. (27) is therefore uncertain.

It should also be borne in mind that Gorin's theory was developed for a single central ion, and not for a large colloid containing a distribution of charges on its surface. As Bull (22) has suggested, the correction of Eq. (27) probably overestimates the actual influence of ionic volume on the surface charge density of a large colloid. This follows from the fact that a substantial number of counterions may be hidden in the surface "roughness," and thereby approach fixed surface charges at a closer distance than would be predicted from their radii. In view of the lack of information about the molecular architecture of cell surfaces, as well as the uncertainty in the value of r_i, it seems best at the present time to omit the ionic volume correction in calculations involving the surface charge density of intact cells.

(d) Haydon's Correction. In the Gouy-Chapman theory, the colloid surface is assumed to be rigid and impenetrable to counterions. For intact cells, however, this is not a realistic model since the outermost surface is generally composed of highly porous polysaccharide moieties (106).

Haydon (63) has approached this problem by considering several models of charged surfaces in which the interface is assumed to be partially or wholly penetrable to counterions. He concluded that the surface charge density given by Eq. (21) underestimates the true charge density by a factor equal to $(1 + (1 - \alpha)^{\frac{1}{2}})$, where α is the fraction of the total space within the surface that is not available to counterions. In the case where $\alpha = 0$, the surface is completely penetrable to counterions and the charge density has twice the value calculated from the Gouy-Chapman equation. Unfortunately, an attempt to determine α for human erythrocytes using a titration technique was not successful. Without further knowledge concerning the proper value for α, it is therefore not possible at present to utilize Haydon's correction in surface charge calculations for intact cells.

(e) Other Corrections. Several other factors influence the relation between σ and ζ predicted by the Gouy-Chapman theory. These include the variation of dielectric constant within the double layer, the effect of ion polarization on the distribution predicted from the Boltzmann equation, the influence of counterion charge (the "self-atmosphere" effect), and electrostriction. Each of these effects is discussed in the review by Haydon (64). For intact cells, whose zeta potentials are generally less than 25 mV, all of these corrections can be neglected without introducing significant error into the calculation of surface charge density.

(f) Conclusions. From the discussion given in the preceding paragraphs, it is clear that the Gouy-Chapman equation cannot be

regarded as a completely accurate relationship between surface charge density and zeta potential. When interpreting electrophoretic measurements on intact cells in physiological media, corrections are needed both for counterion volume and for the porosity of the membrane surface. Neglect of the former can lead to roughly a 20% overestimate of the charge density, whereas neglect of the latter can yield a value for σ that is low by as much as a factor of two. Unfortunately, accurate values are not available for the experimental parameters that are needed in order to perform these corrections. Until this difficulty has been overcome, only an approximate value for the charge density can be obtained through use of the Gouy-Chapman equation.

B. Experimental Procedures

A schematic diagram of the microelectrophoresis assembly used by the present author is shown in Fig. 2. The electrodes consist of zinc binding posts immersed in a saturated $ZnSO_4$ solution. In order to prevent migration of zinc ions into the sample solution, the electrode chambers are connected through sintered glass disks to side arm solutions containing the same suspending medium as the sample. These intermediate buffer chambers are then connected by means of teflon stopcocks to the sample chamber. Convective exchange at the stopcocks is prevented by adding sucrose to the side arm buffer solutions. The entire electrophoresis assembly shown in Fig. 2 is mounted on a standard microscope containing a monocular eyepiece. For the measurement of particle velocities, the ocular is fitted with a calibrated reticule.

As depicted in Fig. 2, the electrophoresis chamber is a rectangular Northrup-Kunitz cell (Arthur H. Thomas Co., Philadelphia), and is placed in the lateral position. Rectangular chambers with horizontal and vertical orientations have also been employed, as described by Brinton and Lauffer (21). In many laboratories, the cylindrical chamber developed by Bangham and co-workers (9) is presently in use. If an accurate control of temperature is required, the electrophoresis chamber can be mounted within a thermostating jacket.

For a discussion of both the theoretical and practical aspects of electrophoretic measurements using a rectangular cell, the reader is referred to the review by Brinton and Lauffer (21). A thorough description of the cylindrical cell technique has been given by Seaman (117). With adequate care, the experimental error involved in electrophoretic studies on intact cells can be limited to 5% or less. In the case of erythrocytes, the electrophoretic mobility within any given cell population is quite uniform. As a result, an accurate value for the mobility can be obtained by performing individual measurements on ten cells. This procedure was used in all of the mobility measurements reported in Section III for erythrocytes from female Sprague-Dawley rats. In a physiological medium of ionic strength 0.145, the present author has found the mobility of these red cells to be -1.05 micron/sec/volt/cm, with the maximum deviation between measurements being $\pm$ 0.05 micron/sec/volt/cm.

Many of the studies reported in Section III of this chapter involve the measurement of cellular mobility as a function of pH. Several precautions must be observed both in the performance of this type of

experiment, and in the interpretation of the resulting mobility-pH characteristics. First, it is essential that the pH be adjusted after the addition of cells to the suspending medium. Serious error can result from failure to correct for the buffering action of cells, especially when the electrophoresis medium itself is unbuffered. Secondly, in performing mobility measurements at extremely alkaline or acidic values of pH, the surface architecture of intact cells can be irreversibly damaged. In order to assess the effects of pH on the structural integrity of the electrokinetic cell surface, the reversibility of the observed mobility-pH characteristics must be determined. A procedure for the performance of reversibility tests is described in Section III, C, 1, b. A third factor that must be taken into account in the interpretation of mobility-pH curves is the difference between bulk and surface pH (62, 90). At a negatively (positively) charged surface, protons from the suspending medium will be attracted (repelled) in accord with the Boltzmann distribution law (Eq. (13)). By taking the negative common logarithm of both sides of Eq. (13), it is possible to show that

$$(pH)_s = (pH)_b + \frac{e\zeta}{2.303\,kT} \tag{28}$$

Here $(pH)_s$ is the pH evaluated at the surface of shear, and $(pH)_b$ represents the bulk pH. If ζ is expressed in millivolts, then at 25° C the relationship between bulk and surface pH is given by

$$(pH)_s = (pH)_b + \frac{\zeta}{59.1} \tag{29}$$

In studying the variation of surface charge with pH, the electrophoretic mobility is generally plotted as a function of $(pH)_b$. If, however, these mobility-pH characteristics are to be used in the calculation of dissociation constants for ionizable surface groups, then it is necessary to correct for the difference between $(pH)_b$ and $(pH)_s$. In the case of erythrocytes, the magnitude of ζ does not exceed 15 mV over the range $1 < (pH)_b < 13$, so that the difference between $(pH)_s$ and $(pH)_b$ predicted by Eq. (29) is at most 0.25 pH units. For this reason, the surface pH will not be explicitly taken into account in the data presented in Section III of this chapter. It should be understood, however, that the term pH as used here refers to the experimentally measured bulk pH.

III. Studies on the Surface Chemistry of Erythrocytes at Physiological Ionic Strength

A. The Dependence of Surface Charge Properties on Ionic Strength

The electrokinetic behavior of any charged colloid is a distinct function of the ionic strength of its suspending medium. This follows from the proportionality of the Debye length to the reciprocal square root of the ionic strength (see Eq. (8)). Consequently, as the ionic strength is lowered, the Coulombic screening of charged groups is reduced. In addition, ionic groups further from the surface of shear no longer possess counterions that move with the cell as a hydrodynamic unit. These ionic groups are thus "unmasked" and can contribute to the surface charge measured by electrophoretic methods.

The first detailed study on the ionic strength dependence of erythrocyte surface charge was made by Furchgott and Ponder (42) in 1941. Using human erythrocytes, these workers showed that the negative surface charge density at neutral pH remains constant until the ionic strength falls below 0.02, corresponding to a Debye length of approximately 20 Å. At lower ionic strengths, it appears that predominantly basic groups are unmasked, leading to a reduction in the calculated surface charge density. Similar observations have been made by Bateman and Zellner (10) with guinea pig erythrocytes, and by Glaeser and Mel (47) with rat erythrocytes.

The most extensive study on the electrokinetic behavior of erythrocytes as a function of ionic strength has been made by Heard and Seaman (66). These workers examined the effects of varying both pH and ionic strength on the electrophoretic mobility of human erythrocytes. The results are

shown diagrammatically in Fig. 3. Three electrokinetic "states" were defined: (1) a "stable" state in which no adsorption of hemolysate occurs; (2) a "metastable" state characterized by a reversible adsorption of hemolysate; and (3) an "unstable" state in which the erythrocyte membrane exhibits an irreversible adsorption of hemolysate or more general hemolytic degradation. In the states characterized as "stable" or "metastable," the cells were considered to be uninjured. In the regions of pH and ionic strength where erythrocytes were characterized as "stable," the electrophoretic mobility was found to remain constant for a period of at least two hours after initial preparation of the cell suspension. In the "metastable" region, erythrocytes were observed to undergo a slow decrease in mobility. Heard and Seaman (66) attributed this to the adsorption of hemoglobin present in solution as a result of gradual hemolysis of part of the red cell population. They found that the initial mobility observed in a "metastable" region could be restored upon washing the cells with saline at neutral pH, and subsequently resuspending them at values of pH and ionic strength lying in the "metastable" region. In ranges of pH and ionic strength characterized as "unstable," changes in mobility with time could not be reversed by a saline wash, and were attributed to actual alteration of the surface structure rather than adsorption of hemolysate.

In the remainder of this chapter, the discussion of erythrocyte surface chemistry will be restricted to electrophoretic measurements performed at an approximately physiological ionic strength of 0.145. At this ionic strength the region of electrokinetic stability extends from pH 4.5 to 9.0. As the ionic strength is lowered, the "stable"

region shrinks until, at an ionic strength of 0.0029, the erythrocyte exhibits a constant mobility only at pH 7.4.

In a medium having an ionic strength of 0.145, the thickness of the ionic double layer associated with charged groups at the cell surface is eight angstroms. The electrophoretic properties measured at this ionic strength will thus reflect ionic groups residing within approximately eight angstroms of the surface of shear. In the case of erythrocytes, it has been well established for a decade that a large number of the charged groups meeting this condition are sialic acid molecules associated with carbohydrate at the cell surface. A brief review of the experimental evidence in support of this view is given in the following subsection.

B. The Role of Sialic Acid in Determining Electrophoretic Mobility; Surface Carbohydrates

The first evidence that acidic polysaccharides contribute to the surface charge properties of erythrocytes was the observation by Hanig (61) that the negative electrophoretic mobility of human red cells is greatly reduced through the adsorption and subsequent elution of influenza virus. The modified cells no longer possess the ability to adsorb virus. The inference drawn from these experiments was that the virus attaches itself to a receptor substance at the erythrocyte surface. By virtue of an enzyme embedded in the virus coat, the receptor substance is then altered in such a way that it will no longer bind the attacking virus. Tentative identification of the receptor substance as a polysaccharide was based on

the observation that both urinary and submaxillary gland mucoproteins can inhibit the process of viral hemagglutination. Chemical analysis of the products split from the erythrocyte surface by the viral enzyme system later confirmed this in a conclusive manner (51, 54).

At approximately the same time as Hanig's experiments on viral hemagglutination and surface charge reduction, Burnet and Stone (23, 126) discovered an enzyme system from Vibrio cholerae which they appropriately named "receptor-destroying enzyme" (RDE). Following treatment with RDE, erythrocytes were no longer susceptible to agglutination by viruses, suggesting that the viral receptor in the erythrocyte membrane had been released enzymatically. It was also noted by Ada and Stone (4) that RDE produced a large reduction in the electrophoretic mobility of human erythrocytes. Several years later Gottschalk (52, 53) recognized that RDE was a glycosidase, and renamed the enzyme "neuraminidase" because of its ability to liberate N-acetyl and N-glycolyl neuraminic acid (sialic acid) through hydrolysis of an α-glycosidic bond. The structure of this molecule in its N-acetylated form is shown in Fig. 4. In 1958 Klenk and Uhlenbruck (77) demonstrated conclusively that the product split from red cell membranes by neuraminidase was sialic acid, and the suggestion was put forward by Klenk (75) that the carboxyl group of this molecule was responsible for the surface charge measured electrophoretically. This identification of the enzymatically split product as sialic acid was of considerable importance since earlier workers had ascribed the reduction in mobility solely to the production of strongly basic groups at the membrane interface (11). This conclusion had been

based on a titration study of neuraminidase-treated urinary sialyl mucoprotein (33). After treatment the mucoprotein possessed dissociating groups with a pK_a of 11.2, but since neuraminidase is specific for the glycosidic bond between sialic acid and its adjacent sugar (52, 53), it has generally been held that action of the enzyme *per se* is unlikely to yield basic groups.

Following the work of Klenk, several exhaustive studies were made on the presence of sialic acid in the membranes of erythrocytes and several types of tissue cells. Only the work on erythrocytes will be discussed here. A recent review discussing the presence of sialic acid at the interface of other cell types has been prepared by Weiss (138).

In 1961 Cook *et al.* (31) measured both the reduction in negative mobility of human erythrocytes and the release of sialic acid resulting from reaction of the red cells with neuraminidase. The surface charge density at neutral pH was reduced by approximately 75% from the value observed with untreated erythrocytes. From a chemical analysis, the enzymatic yield of free sialic acid was found to be approximately twice the "theoretical" yield predicted from reduction in mobility. Similar results were later obtained by Eylar and co-workers (37), who studied the effects of neuraminidase on six species of erythrocyte. With the exception of horse red cells, they found a reduction in negative surface charge density ranging from 67% to 94% following treatment with neuraminidase. These workers also found roughly a twofold difference between the chemically measured and theoretically predicted yields of sialic acid. Since calculations based on the Gouy-Chapman equation can lead to a substantial underestimate of the surface charge density (see Section II, A, 2, d),

this discrepancy may not be significant. The possibility that sialic acid susceptible to release by neuraminidase might exist at positions within the plasma membrane other than the outer surface seems unlikely. Steck et al. (124) have recently demonstrated that the amount of sialic acid released enzymatically from the inner aspect of human erythrocyte membranes is extremely small. This is supported by the electron microscopic observations of Benedetti and Emmelot (12) on isolated plasma membranes from rat liver cells. These workers demonstrated that sialic acid is located at the outer surface, but found no evidence for the presence of this molecule on the inner membrane surface. In order to observe this asymmetric distribution of sialic acid, plasma membranes were incubated in solutions of colloidal iron hydroxide below pH 2. In this pH range only the dissociated carboxyls of sialic acid ($pK_a = 2.6$) should be stained electrostatically, an hypothesis confirmed by the absence of staining in neuraminidase-treated membranes.

With regard to the types of neuraminic acids at the erythrocyte surface, evidence has been obtained only for the presence of N-acetyl and N-glycolyl forms (37, 120). Other derivatives such as N,O-diacetylneuraminic acid have not been detected. Both the N-acetyl and N-glycolyl derivatives are susceptible to release by neuraminidase.

The membrane components with which sialic acid is associated are classified as gangliosides or glycoproteins, depending upon the mode of saccharide linkage to the erythrocyte surface. The gangliosides (sialylglycolipids) are ceramide hexosides that contain sialic acid either at terminal or internal chain positions, or both. It has been

well established that the ceramide linkage is to glucose through a β -glycosidic bond (25). Nelson (94) has found, however, that the gangliosides from many species of red cell are a complex mixture, and at present the relative amounts of mono-, di-, and tri-sialyl forms have not been quantitated. It should also be noted that only a limited amount of the sialic acid contained in erythrocyte gangliosides is susceptible to release by neuraminidase (145), in contrast to glycoprotein-linked sialic acid. Some electrophoretic evidence in support of this is presented in Table I. In species of erythrocyte with a high ganglioside content, only a small reduction in surface charge is observed following reaction with neuraminidase.

In the case of glycoproteins, substantial evidence exists for the linkage of sialyl oligosaccharides through O-glycosidic bonds to the hydroxyls of serine and threonine residues, and through glycosidic ester bonds to the γ-carboxyls of glutamic acid residues (29). This has been inferred primarily from the predominance of these residues in the glycopeptides extracted by proteolytic enzymes from erythrocyte membranes (28, 30, 147). By effecting the cleavage of glycosidic bonds under alkaline conditions and in the presence of tritium-labelled sodium borohydride, Winzler and co-workers (147) have obtained evidence that the sugar involved in O-glycosidic linkage to serine and threonine is primarily N-acetylgalactosamine.

Seaman and associates (65, 118) have also employed microelectrophoretic techniques to study the mode of saccharide-protein linkage at the surface of human erythrocytes. Red cells were first stabilized by fixation with

acetaldehyde, and then subjected to mild alkaline hydrolysis in order to break glycosidic bonds between sialyl oligosaccharides and the terminal peptide residue. Electrophoretic measurements on erythrocytes treated in this manner showed that the carboxyls of sialic acid had been replaced nearly one-for-one by acidic groups with a pK_a of approximately 3.95. This result was interpreted as being an indication that sialyl oligosaccharides had been released from glycosidic ester linkage to glutamic acid, and that the γ-carboxyl involved in this bonding was now free to contribute to the electrophoretic mobility. The fact that there was a one-for-one replacement of the sialic acid carboxyl by the glutamic acid side chain carboxyl suggests that all of the electrophoretically detectable sialyl oligosaccharides at the human erythrocyte surface are linked by glycosidic ester bonds to glutamic acid. This would not be expected from biochemical evidence, which favors the presence of large numbers of O-glycosidic bonds to serine and threonine.

Using glutaraldehyde-fixed rat erythrocytes, the present author has also examined the effects of mild alkaline hydrolysis on the negative surface charge at neutral pH. Following incubation in 0.145N NaOH for two hours at 50° C, the anodic mobility of three cell samples was 0.81 ± 0.05 (SD) micron/sec/volt/cm. This represents a 26% reduction from the control value of -1.10 micron/sec/volt/cm. Lengthening the time of hydrolysis did not lead to a further reduction in the surface charge. As will be discussed in Section III, C, 6, a, reaction of glutaraldehyde-fixed rat erythrocytes with neuraminidase results in a

36% reduction in the anodic mobility at neutral pH. A comparison of this figure with the reduction resulting from alkaline hydrolysis suggests that at most 30% of the electrophoretically detectable sialyl oligosaccharides at the surface of rat erythrocytes are linked by glycosidic ester bonds to glutamic acid residues.

In concluding this section, it is worthwhile to present some calculations on the total carbohydrate at the erythrocyte surface. In addition to sialic acid, the sugars found in erythrocyte stroma are primarily glucose, galactose, glucosamine, and galactosamine (87, 107, 115, 147). All of the amino sugars are thought to exist in N-acetylated form (74). Other hexoses such as mannose and fucose have been found only in small amounts (87, 108), but appear to be important components of the A and B surface antigens (135).

Three sets of chemical data on the saccharide moieties of human erythrocyte stroma are presented in Table II. Using this data, it is possible to calculate the average number of neutral sugars associated with each molecule of sialic acid at the cell surface. Letting m and n be the number of molecules of hexose and N-acetylhexosamine, respectively, associated with each sialic acid molecule, then from the data of Table II,

$$\frac{m}{n} = \frac{2.44/180}{1.43/221} \qquad (30)$$

and

$$\frac{1.2}{309} = \frac{5.07}{309 + 180m + 221n} \qquad (31)$$

Here the molecular weights of sialic acid, hexose, and N-acetylhexosamine have been taken as 309, 180, and 221, respectively. Solving these equations gives m = 3.45 and n = 1.65. Consequently, in addition to a terminal sialic acid, the oligosaccharide chains at the human erythrocyte surface contain roughly five neutral sugars.

Using this information, it is possible to calculate the proportion of the human erythrocyte surface occupied by carbohydrate. From molecular models, Glaeser (46) has estimated the "face-on" area associated with molecules of hexose and sialic acid to be 40 $Å^2$ and 60 $Å^2$, respectively. Taking the total number of molecules of sialic acid at the surface of one cell to be 2.4×10^7 (37), then from calculations presented in the preceding paragraph, the number of neutral sugars is 1.2×10^8. The total "face-on" area occupied by carbohydrate is thus 62.4 μ^2. Since the area of the human red cell is 163 μ^2 (103), this represents about 38% of the total cell surface. In making this calculation, it has been assumed that saccharides lie flat against the membrane, so that 38% must be regarded as the maximum percentage of the human erythrocyte surface that could be occupied by carbohydrate moieties.

C. Chemical Modification of the Erythrocyte Surface in Combination with Microelectrophoresis

The fact that enzymatic cleavage of sialic acid fails to remove the entire negative surface charge strongly suggests that other anionic groups exist near the erythrocyte surface of shear. In addition, the

erythrocyte membrane contains large numbers of basic groups associated with protein and phospholipid moieties. Quantitative calculations of the acidic and basic protein, lipid, and carbohydrate groups present in the human erythrocyte membrane are summarized in Table III. Polar amino acid residues clearly predominate, with their relative numbers ranging from approximately 10^8 to 5×10^8 per cell. Ionogenic groups associated with sialic acid and phospholipids are present in numbers of 10^7 to 10^8 per cell. Because of their hydrophilic character, there is no a priori basis for excluding the possibility that acidic and basic groups of non-carbohydrate membrane components might exist at the outer surface and contribute to the electrophoretic mobility.

As a means of identifying the ionic groups responsible for the electrophoretic properties of red cells, the most fruitful method of attack has been the use of chemical modification of the membrane interface in combination with electrophoretic measurements. In this manner, a charged group may be detected through a change in mobility induced either by enzymatic cleavage of the group or chemical alteration of its ionogenic character. This approach is complicated, however, by the fact that at physiological ionic strength the erythrocyte is electrokinetically stable only from pH 4.5 to 9.0, whereas charged groups that contribute to the electrophoretic mobility have dissociation constants lying outside this range. As a consequence, in order to study the result of certain types of chemical modification, it has been necessary to stabilize the erythrocyte over a broader pH range by fixation using reagents that

do not alter the electrokinetic character of the membrane interface. Only through this combined use of fixation and chemical modification has it been possible to undertake a systematic investigation of all acidic and basic groups near the surface of shear.

(1) Stabilization of Erythrocyte Membranes Using Aldehydes and Osmium Tetroxide; Mobility-pH Relations for Human and Rat Erythrocytes

(a) The Chemistry of Fixation. As a means of stabilizing erythrocytes against time-dependent changes in mobility and against hemolysis at extreme values of pH, the fixatives most commonly employed have been formaldehyde, acetaldehyde, glutaraldehyde, and osmium tetroxide. All of these reagents preserve the surface charge properties observed with unfixed cells in the "stable" pH range of 4.5 to 9.0 at an ionic strength of 0.145. Before discussing their utility in extending mobility studies from pH 1 to 13, it is appropriate to review the mechanisms by which these reagents serve to stabilize membranes against structural alteration under extreme acidic or alkaline conditions.

The first use of aldehydes to produce electrokinetically stable erythrocytes was made by Heard and Seaman (67), who reacted human red cells with formaldehyde and acetaldehyde. The effectiveness of these reagents as fixatives is thought to result primarily from a crosslinking of membrane protein through reaction with nitrogenous amino acid side chains. Evidence also exists that the amino group of phosphatidyl ethanolamine can participate in crosslinking reactions (114). From the work of Fraenkel-Conrat and Olcott (38, 39), it appears that

formaldehyde first reacts with an amino group, and then condenses further with either a neighboring amide or guanidinium group. In the case of acetaldehyde, only amino and guanidinium groups appear to be involved in the crosslinking reaction (92). It is probable that both types of crosslinks are labile under acidic conditions (20, 110).

The aldehyde that acts most effectively as a fixative is the bifunctional reagent glutaraldehyde (116). Only amino bases appear to be involved in crosslinking, with the glutaraldehyde serving to form a five-carbon bridge between neighboring groups. Evidence has been presented that phosphatidyl serine and phosphatidyl ethanolamine, as well as lysine residues, can participate in the crosslinking reactions (44, 114). The amino groups involved are thought to be converted to secondary amines rather than Schiff bases. Two observations that support this are the following. First, the pK_a's of modified lysine residues in β-lactoglobulin reacted with glutaraldehyde lie in the range 8.0 to 8.5 (14). If Schiff bases were formed, the expected pK_a values would be no higher than 5. Secondly, the reaction product is stable under extremely acidic conditions (19, 20, 105). Again, this would not be expected if Schiff base formation were involved. From nuclear magnetic resonance studies, Richards and Knowles (109) have concluded that glutaraldehyde exists in solution in the form of cyclic polymers containing unsaturated bonds. The product proposed by these workers for the reaction of glutaraldehyde with amino bases is shown in Fig. 5.

The stabilizing effect of osmium tetroxide is thought primarily to involve a cyclic diester crosslinking of neighboring unsaturated fatty acid chains in membrane phospholipids. Criegee et al. (32) considered the

reaction product to contain osmium in the hexavalent state, whereas Korn (78) has presented evidence for a tetravalent state. These two types of crosslinked structures are depicted in Fig. 5. Riemersma (112) has considered a third alternative, namely, cyclic osmic monoesters. Evidence also exists for the interaction of osmium tetroxide with the polar choline moiety of lecithin (111, 113), and with reducing groups of proteins (6, 60). Reactions of this nature are of significance in terms of the staining properties of osmium tetroxide, but it is unlikely that they contribute substantially to the effectiveness of this compound as a crosslinking reagent.

One other important aspect of stabilization using aldehydes and osmium tetroxide is the possibility that membrane structures may become disordered during the fixation process. From circular dichroism measurements, Lenard and Singer (84) concluded that fixation with 4% osmium tetroxide for 30 min at 4° C resulted in a 63% loss of helicity in human erythrocyte membrane protein. Following fixation with 6% glutaraldehyde for two hours at room temperature, they found a 22% loss of helix. Working in collaboration with Dr. Norman K. Freeman and Dr. George Oster, the present author (129) has studied the effects of fixation on rat erythrocyte membranes using infrared techniques and somewhat milder fixation procedures, namely 1% osmium tetroxide for one minute at room temperature and 2.5% glutaraldehyde for ten minutes at room temperature. Under these conditions, the membrane protein was found to retain primarily an α-helical and/or random coil configuration upon fixation, with no appearance of β-structure. It is likely, therefore, that the loss of helicity results from a conversion to a

more random conformation, without the occurrence of extensive unfolding characteristic of protein in the β-configuration.

(b) Mobility-pH Relations for Human and Rat Erythrocyte Membranes at Physiological Ionic Strength. The first detailed analysis of erythrocyte mobility-pH characteristics was made by Furchgott and Ponder (42). Their data for human erythrocytes at an ionic strength of 0.172 has been plotted in Fig. 6. In order to obtain mobilities at pH values less than 4, i.e. outside of the range of electrokinetic stability, it was found necessary to make measurements immediately after preparation of a cell suspension. Heard and Seaman (67) later attempted to overcome this difficulty by making electrophoretic measurements on human erythrocytes that had previously been fixed with 1.5% formaldehyde or 2% acetaldehyde for 20 days. In this manner they succeeded in extending the lower limit of electrokinetic stability to pH 2.8 $\pm$ 0.1 at an ionic strength of 0.145, while at the same time reproducing the surface charge properties observed for unfixed cells over the stable pH range 4.5 to 9.0. The extrapolated isoelectric point obtained by these workers was 2.5, in reasonable agreement with Furchgott and Ponder's (42) value of 1.7.

More recently, Haydon and Seaman (65) have re-examined the electrokinetic properties of acetaldehyde-fixed human erythrocytes. In this study, the acetaldehyde medium was buffered to pH 7.4 in order to protect against a drop in pH during fixation. The resulting red cells were found to exhibit a stable behavior at pH values as low as 1. Contrary to previous observations, human erythrocytes fixed with 2% acetaldehyde under these conditions did not possess a true isoelectric point, as shown in Fig. 7. Haydon and Seaman (65) have interpreted these mobility-pH properties as an indication that only anionic groups exist near the erythrocyte surface of shear.

Two other fixatives that preserve the surface charge properties observed for unfixed red cells at physiological ionic strength are glutaraldehyde and osmium tetroxide (129). These reagents provide the advantage that stabilization of erythrocytes can be achieved with a reaction time far less than the 20 day period required for acetaldehyde fixation. The mobility-pH relations at ionic strength 0.145 are shown in Figs. 8A and 8B for rat erythrocytes reacted with 2.5% glutaraldehyde at room temperature for 10 minutes, and with 1% osmium tetroxide at room temperature for one minute. The electrophoretic properties of unfixed rat red cells over the pH range 4 to 10 are included for comparison. Following fixation with these reagents, rat erythrocytes exhibit an isoelectric point between pH 2.1 and 2.4, below which they acquire a large cathodic mobility. In the alkaline pH range, glutaraldehyde-fixed erythrocytes show no evidence for dissociating groups, whereas cells fixed with osmium tetroxide exhibit an increase in negative mobility above pH 11. As a means of clarifying the character of the mobility curve at high pH, electrophoretic data was obtained using cells fixed with both glutaraldehyde and osmium tetroxide. The results are shown in Figs. 8C and 8D. Following dual fixation with these reagents, there is no evidence for dissociating groups at high pH, suggesting that the result with osmium tetroxide is an artifact resulting from structural rearrangement of the membrane under alkaline conditions. This is further supported by the observation that erythrocytes fixed with osmium tetroxide hemolyze within approximately two minutes after exposure to pH values above 12.

A direct method for assessing whether a charged group appears at the surface of shear only under extreme alkaline or acidic conditions is to determine the reversibility of the surface charge properties as a function of pH. Experiments of this nature are shown in Fig. 9. Each mobility curve was obtained using a single suspension of erythrocytes incubated successively at the pH values denoted by numerals. It is clear from Figs. 9A and 9B that the surface charge properties of rat erythrocytes fixed with osmium tetroxide exhibit an irreversible character at high pH, while cells fixed with glutaraldehyde show complete reversibility. This indicates that the dissociating groups observed with the former result from disruption of the membrane at high pH, possibly as a result of alkaline hydrolysis of osmium diester linkages between unsaturated fatty acids.

At low pH values rat erythrocytes fixed with either glutaraldehyde or osmium tetroxide exhibit reversible surface charge properties (Figs. 9C and 9D), indicating that the positive branch in the mobility-pH curve is not an artifact resulting from membrane disruption. The presence of a large cathodic mobility at pH 1 has been confirmed for erythrocytes from several other species of mammals. This data is tabulated in Table IV. In all cases the negative mobility at neutral pH was unaffected by fixation with either glutaraldehyde or osmium tetroxide, and the cells reversibly acquired a positive surface charge at low pH.

The data presented in Figs. 9C and 9D and in Table IV are in conflict with the mobility characteristics at low pH measured by Haydon and Seaman (65) for acetaldehyde-fixed human erythrocytes (Fig. 7). The fact that these

workers did not observe a positive mobility branch suggests that acetaldehyde might be reactive with the groups responsible for this surface charge property. In order to examine this further, several attempts were made by the present author (129) to stabilize rat erythrocytes by fixation with 2% acetaldehyde. In all cases, the results were similar to those shown in Figs. 10A and 10B. At pH values below 3.2, the mobility is time dependent and exhibits an irreversible character. As a result, a large positive branch appears in the mobility-pH curve. A similar behavior was observed with acetaldehyde-fixed human erythrocytes, in contrast to the results of Haydon and Seaman (65).

As a means of determining whether acetaldehyde is reactive with the groups responsible for the cathodic mobility below pH 2, erythrocytes were treated with 2% acetaldehyde and subsequently fixed with 1% osmium tetroxide. Erythrocytes handled in this manner are electrokinetically stable at low pH and retain a large positive mobility at pH 1, as shown in Fig. 10C. A similar result was obtained with several preparations of human erythrocytes. In addition, fixation with 2.5% glutaraldehyde for 20 days at 4° C yielded electrokinetically stable rat erythrocytes having a large positive mobility at low pH, a result shown in Fig. 10D. It thus appears that both mono- and bifunctional aldehydes fail to react over long periods with the groups responsible for the positive surface charge at pH 1. In a later section of this chapter (III, C, 6, c), evidence will be presented that the cathodic mobility observed from pH 1 to 2 is primarily associated with protonation of the weakly basic acetamido group of N-acetylneuraminic acid. Using N-acetylglucosamine, Dr. Neal Castagnoli and I have studied the reactivity of acetaldehyde

with N-acetylated sugars by means of nuclear magnetic resonance spectroscopy. After treatment of N-acetylglucosamine with 2% acetaldehyde for 20 days at 4° C, the acetamido resonance at δ = 2 ppm (relative to a tetramethyl silane internal standard) was unaffected, thereby indicating the absence of any reaction. In view of the fact that neuraminic acid at the surface of human erythrocytes exists almost entirely in N-acetylated form (37), the above results are clearly in conflict with the observation by Haydon and Seaman (65) that no proton binding occurs below pH 2 following acetaldehyde fixation.

One difficulty that arises in describing the positive mobility branch below pH 2 is the fact that surface charge measurements cannot be extended below pH 0.9 while maintaining the ionic strength at 0.145. In order to characterize further the electrophoretic properties at low pH, mobility studies were made at pH values between 0 and 1 by suspending glutaraldehyde-fixed cells in hydrochloric acid solutions having ionic strengths ranging from 0.145 to 1.0. The surface charge was then calculated from the mobility using the Gouy-Chapman equation (Eqn. (24b)). A plot of the surface charge density from pH 0 to 2 is shown in Fig. 11. It is clear that there is an inflection in the curve at about pH 1, below which the cells acquire an extremely large positive surface charge. Several points can be made with regard to this lower branch of the cathodic mobility. (1) As will be discussed in Sections III, C, 4 and III, C, 5, there are no cationic protein, carbohydrate, or phospholipid moieties present near the surface of shear, so that the positive charge expressed at low pH is not attributable to strongly basic groups. (2) As shown in Fig. 11, the surface charge

properties of glutaraldehyde-fixed cells below pH 1 are reversible, indicating that no structural alteration of the surface occurs even at pH values as low as zero. (3) The positive surface charge density from pH 0 to 1 does not fit a Langmuir adsorption isotherm for statistical hydrogen ion adsorption onto one set of proton-binding sites. (4) The magnitude of the surface charge density at pH 0 is at least 15 times as great as that associated with any other electrophoretically detectable membrane component. Taken together, these facts suggest that the large increase in positive surface charge below pH 1 represents a non-specific proton adsorption onto non-ionogenic regions of the membrane surface. Assuming this to be the case, a calculation can be made of the proportion of the rat erythrocyte surface capable of non-specific proton adsorption. From Fig. 11 the maximum adsorbed charge appears to be approximately +0.14 coulomb/m^2, or 8.74×10^{17} hydrogen ions/m^2. Taking the mean corpuscular diameter of the rat erythrocyte to be 6.3μ (104), the surface area calculated by the method of Ponder (103) is 83μ^2. On the basis of these figures, it is possible to adsorb 7.25×10^7 hydrogen ions per cell. Assuming that the hydrogen ion exists in solution as a hydronium ion with a molecular radius of 1.96 Å (the radius of a sphere having equivalent weight and density), the percentage of the surface area composed of potential binding sites is approximately 11%.

2. Studies on Anion Adsorption as a Possible Charging Mechanism

Before proceeding with a discussion of ionogenic groups near the erythrocyte surface of shear, it is appropriate to present studies directed towards the possibility that the anodic mobility of red cells may arise in part through anion adsorption. Heard and Seaman (66) have reported that the surface charge density of human erythrocytes at neutral pH is independent of the anion contained in the suspending medium. This was tested for values of the ionic strength ranging from approximately 0.002 to 0.145. In addition, the present author has found that the negative surface charge of rat erythrocytes is identical in 0.145M electrolyte media composed of NaSCN, NaI, NaBr, NaCl, or NaF. Since these anions have differing hydrated radii, the extent to which they are adsorbed at an interface will not be identical. The fact that the surface charge of human and rat erythrocytes is unaffected by the presence of different anions thus indicates that adsorptive charging does not occur. The surface charge of rat erythrocytes at neutral pH and ionic strength 0.145 has also been found to be independent of the cation present in the suspending medium, indicating the absence of any desorptive process at the cell interface.

3. Susceptibility of Surface Groups to Methylation and Reaction with a Water Soluble Carbodiimide

Another indication that the negative surface charge of erythrocytes is attributable solely to acidic groups near the surface of shear is the observation by Haydon and Seaman (65) that methylation of acetaldehyde-fixed

human erythrocytes reduces their mobility to zero. However, an attempt by the present author (129) to reproduce this using glutaraldehyde-fixed rat erythrocytes was unsuccessful. Following incubation of rat red cells in methanolic hydrochloride for two hours at 37° C, the anodic mobility at neutral pH was reduced by only 50%. It was also observed that a 25% reduction in the mobility at pH 7 results from successive washes of glutaraldehyde-fixed cells with 0.05N HCl and absolute methanol, suggesting that a combination of alcohol and acidic conditions leads to some structural alteration of the membrane independent of the methylation of surface anions.

Because of the drastic conditions necessary for methylation, an attempt has been made to "titrate" surface anions under mild conditions using a water soluble carbodiimide salt: 1-cyclohexyl-3(2-morpholinylethyl)carbodiimide metho-p-toluenesulfonate (CMC). The structure of CMC is shown in Fig. 12. This reagent is reactive with a large number of groups, including carboxyls, phosphates, sulfhydryls, and hydroxyls (95). In the following discussion, it will be assumed that all of the electrophoretically detectable surface anions that might be reactive with CMC are carboxylic acids. Evidence in support of this will be presented in the following three subsections (III, C, 4 - 6).

The reaction of CMC with a carboxylic acid is shown in Fig. 12. In the first step of the reaction, an anionic carboxylic acid (I) is converted to an acylisourea (III) having a cationic character by virtue of both the N-methylmorpholinylethyl group and the basic amidine group. Fig. 13A shows the kinetics of surface charge reduction upon incubation of

glutaraldehyde-fixed rat erythrocytes in a saline solution containing 0.1M CMC. The temperature was maintained at 37° C and the pH at 4.75 during the reaction. After approximately three hours, the surface charge was reduced to zero and remained at that level following longer periods of incubation.

Other workers who have studied the reaction of carboxylic acids with water soluble carbodiimides suggest that the reaction product III is unstable, and rearranges to give an acylurea (40, 95). This compound is denoted as IV in Fig. 12. In order to test whether III or IV represents the final product, the alkaline stability of the reaction product was examined electrophoretically. The acylisourea should hydrolyze in the alkaline pH range, whereas the acylurea should be stable under the conditions that electrophoretic measurements are made (i.e. at room temperature and pH values less than 13). The mobility-pH characteristics of glutaraldehyde-fixed cells reacted with CMC for three hours are shown in Fig. 13B. When returned to neutral pH after a fifteen minute exposure to pH 12.75, the anodic mobility is approximately 80% of the usual value observed for glutaraldehyde-fixed cells. This indicates that roughly 80% of the surface charge reduction results from the formation of alkali-labile acylisoureas (III), and 20% from the conversion of carboxyls to stable acylureas (IV).

In order to quantitate the percentage of electrophoretically detectable surface carboxyls converted to products III and IV, an experiment was performed in which p-toluenesulfonyl chloride (tosyl chloride) was reacted with glutaraldehyde-fixed erythrocytes previously treated with CMC

for three hours. As shown in Fig. 13A, tosylation under these conditions produces a mobility of -0.33 micron/sec/volt/cm. This results from covalent linkage to acylisourea amidine groups, thereby removing their cationic character. From this information, it may be deduced that 30% of the surface carboxyls (corresponding to a mobility of -0.33 micron/sec/volt/cm) react with CMC to form product III. This accounts for 90% of the total surface charge reduction since the formation of III results in the replacement of an anionic group with two cationic groups. The remaining 10% of the charge reduction is accounted for by the conversion of 5% of the surface carboxyls (corresponding to -0.05 micron/sec/volt/cm) to the acylurea (IV). The formation of this product results in the replacement of one anionic group by one cationic group. A total of 35% of the surface carboxyls therefore appear to be reactive with CMC, with 86% of the products being acylisoureas and 14% being acylureas.

Another possible mechanism by which CMC could reduce the negative mobility at neutral pH is through the formation of carboxylic anhydrides between neighboring carboxyls at the cell surface (95). This product would be labile in the alkaline pH range, and could account for the irreversible electrokinetic properties at high pH. An argument against the extensive production of anhydrides, however, is the observation that tosylation partially reverses the reduction in negative surface charge resulting from treatment with CMC. This would be expected only if amines were present near the surface of shear by virtue of the covalent linkage of CMC to carboxylic acids.

One other feature of the mobility characteristics shown in Fig. 13B might be mentioned at this point, namely, the proton-binding exhibited below pH 6. This results from the association of hydrogen ions with carboxylic acids that have not reacted with CMC.

In summary, the fact that the anodic mobility of acetaldehyde-fixed human erythrocytes can be reduced to zero by methylation indicates that the negative surface charge arises entirely through the dissociation of acidic groups at the membrane interface, with no contribution from adsorptive or desorptive charging processes. In the case of glutaraldehyde-fixed rat erythrocytes, the number of electrophoretically detectable carboxylic acids esterified by methanolic hydrochloride is at most 50% of the total. However, in the mobility-pH curve shown in Fig. 13B for CMC-treated cells, the binding of protons to unreacted carboxylic acids is clearly indicated by the reversible increase in positive charge density below neutral pH. Therefore, the fact that two thirds of the negative surface charge density is unaffected by treatment with CMC is attributable to the limited reactivity of this reagent with surface carboxyls, and cannot be regarded as an indication that this portion of the charge density arises from anion adsorption.

The physical basis for the incomplete methylation of anionic groups at the rat erythrocyte surface, in contrast to the results reported for human red cells, is not clear. It is possible that a large portion of the carboxylic acids contributing to the mobility of rat erythrocytes are localized in tightly packed "islands." As a consequence, steric hindrance could prevent the majority of these groups from being methylated

or chemically modified by reagents such as CMC. Another possible explanation would be that large numbers of anions may exist in cul-de-sacs at the cell surface and contribute to the mobility by virtue of their inaccessibility to counterions. It is quite reasonable to assume that groups of this nature would not be accessible to CMC.

4. Evidence Against Dissociating Groups Above Neutral pH

From the mobility-pH curves presented in Section III, C, 1, b for erythrocytes stabilized by aldehyde fixation, there is no indication of dissociating groups over the pH range 5 to 13. The increase in negative mobility above pH 11 exhibited by cells fixed with osmium tetroxide was shown to be an artifact resulting from structural alteration of the surface under alkaline conditions. The absence of electrophoretically detectable dissociating groups above neutral pH indicates that no contribution is made to the surface charge properties by strongly basic or weakly acidic membrane components. Some further experiments that support this conclusion are reported in the following paragraphs.

The basic membrane components having pK_a's near neutral pH or above are the amino groups of lysine residues, phosphatidyl serine, and phosphatidyl ethanolamine; the guanidinium group of arginine; and the imidazole ring of histidine (see Table III). A reagent that reacts with these groups and removes their basic character through the formation of sulfonamides is p-toluenesulfonyl chloride (tosyl chloride). If strongly basic groups contribute to the surface charge properties of erythrocytes, the removal of their proton-binding capacity by tosylation should produce an increase in the negative mobility at neutral pH. This

has not been observed with either human or rat erythrocytes (119, 129). Results obtained with the latter are summarized in Table V. Another reagent that blocks amino and histidyl groups is 1,5-difluoro-2,4-dinitrobenzene. The mobility of rat erythrocytes is not affected by reaction with this compound, as demonstrated in Table V. Finally, as shown by data presented in Section III, C, 1, b, acetaldehyde fixation preserves the surface charge properties of both human and rat erythrocytes. As discussed previously (Section III, C, 1, a), this compound is reactive with amino and guanidyl groups. Weiss et al. (139) have also demonstrated that no change in the anodic mobility results from reaction of human and mouse erythrocytes with the amino blocking reagents formaldehyde, 2,4,6-trinitrobenzenesulfonic acid, 2-chloro-3,5-dinitropyridine, and 2-chloro-3,5-dinitrobenzoic acid.

The predominant weakly acidic moieties contained in erythrocyte membranes are the phenolic hydroxyls of tyrosine residues and the sulfhydryl groups of cysteine residues (see Table III). The absence of tyrosine residues from positions near the surface of shear is indicated by the fact that glutaraldehyde-fixed rat erythrocytes give no evidence for dissociating groups in the alkaline pH range. Since studies on model compounds have demonstrated that glutaraldehyde exhibits only a very limited reactivity with tyrosine (59), the weakly acidic character of the majority of these residues should be preserved following glutaraldehyde fixation. A similar conclusion does not follow for cysteine residues in view of the fact that glutaraldehyde is highly reactive with these groups, thereby removing their weakly acidic character (59). As a means

of testing whether cysteine sulfhydryls reside near the surface of shear, rat erythrocytes were reacted with p-chloromercuribenzoate (PCMB). This compound converts sulfhydryls to mercaptides which, by virtue of the benzoate carboxyl, behave as strong acids. As a result, the presence of sulfhydryls should be detectable through an increased negative surface charge at neutral pH. As shown by the data of Table VI, the mobility of rat erythrocytes at ionic strength 0.145 is unchanged following treatment with PCMB. On this basis, it appears that cysteine sulfhydryls are not present at positions lying within approximately 8 Å from the surface of shear. This conclusion also follows from the fact that fixation with osmium tetroxide does not alter the negative surface charge at neutral pH (see Section III, C, 1, b). If cysteine residues were present at the membrane interface, then an increase in the anodic mobility should occur upon reaction with osmium tetroxide through the rapid conversion of sulfhydryls to sulfonic acids (6).

It might be remarked that the absence of sulfhydryl groups from positions near the erythrocyte surface of shear is in contrast to the finding of Mehrishi and Grassetti (91) that electrophoretically detectable sulfhydryls exist at the surface of ascites tumor cells, human blood platelets, and human lymphocytes. These workers found an increased negative surface charge at neutral pH following reaction with the sulfhydryl blocking reagent 6,6'-dithiodinicotinic acid. It should also be noted that studies presented here fail to support the conclusion of Vansteveninck and co-workers (133) that sulfhydryl groups involved in glucose transport exist at the outer surface of red cells (see Section I).

The electrophoretic results obtained with rat erythrocytes suggest that groups of this nature, if present at the cell interface, are located somewhat deeper than 8 Å from the surface of shear.

5. Studies on Possible Phospholipid Involvement in Erythrocyte Surface Charge Properties

Until as late as 1958, many workers contended that the anodic mobility of erythrocytes was attributable to the strongly acidic phosphate groups of membrane phospholipids. Furchgott and Ponder (42) concluded this from a comparison of the mobility-pH characteristics for human erythrocytes (Fig. 7), their lipid-extracted stromal residues, and an emulsion of the extracted lipids. The electrophoretic characteristics of membrane lipids were found to resemble those of intact red cells, in distinct contrast to the surface charge properties observed for lipid-extracted stroma. At about the same time as the work of Furchgott and Ponder (1940-1), the reversal of surface charge by various cations was studied by Winkler and Bungenberg de Jong (144), who also concluded that phospholipid headgroups were present at the outer surface of red cells. In 1958, Bangham and associates (8) measured the charge-reversal concentrations of several polyvalent cations for sheep erythrocytes and other blood cells. By comparing their results with those of Kruyt (82) on phosphatides and carboxylic acids, they concluded that the spectrum of sheep red cells most closely resembled that of phosphate radicals. Shortly after this investigation, the contribution of sialic acid to the negative surface charge was firmly established (see Section III, B), and no detailed studies have subsequently been reported on the possible involvement of phospholipids in erythrocyte surface charge properties.

As a means of approaching this question, the present author has performed electrophoretic measurements on glutaraldehyde-fixed rat erythrocytes following extraction of lipids by suspension of the cells in absolute ethanol for one hour at room temperature. This procedure was suggested by the studies of Korn and Weisman (79) on the elution of lipids from amoeba during the dehydration procedures involved in preparing specimens for electron microscopy. These workers found that ethanol extraction of glutaraldehyde-fixed amoeba causes a 95% loss of lipid.

The amount of material extracted from glutaraldehyde-fixed rat erythrocytes has been determined from a gravimetric analysis of three samples to be 3.83 ± 0.07 (SD) mg per 10^{10} cells. This corresponds exactly to the total lipid content of rat red cells as measured by Parpart and Dziemian (102). The possibility that all of the lipid was removed by ethanol seems unlikely in view of the evidence for several types of tissue that phosphatidyl serine and phosphatidyl ethanolamine cannot be extracted with organic solvents following glutaraldehyde fixation, presumably as the result of crosslinking to protein amino groups (44, 114). In the case of rat erythrocytes, it can be calculated from the data of Nelson (94) that these phosphatides constitute roughly 22% by weight of the total membrane lipid. In order to determine whether any protein was present in the extracted product, the ethanol supernatent from approximately 1.5 ml of packed erythrocytes was concentrated under a stream of nitrogen gas at 40° C, and subsequently dried on a AgCl plate for infrared analysis. The spectrum gave clear evidence for the presence of phospholipids, but the Amide I and II bands characteristic of protein were absent. It is also possible that

intracellular leakage products such as salt were present in the extract, although an attempt was made to minimize this form of contamination by washing the cells twice in large volumes of distilled water prior to incubation in ethanol. Nevertheless, the leakage of small amounts of non-lipid material would severely limit the accuracy of the gravimetric analysis reported here.

The mobility-pH characteristics of glutaraldehyde-fixed rat erythrocytes extracted with absolute ethanol are shown in Fig. 14A. The surface charge properties at ionic strength 0.145 are identical in all respects to those of untreated glutaraldehyde-fixed cells except for the appearance of irreversible changes in mobility after exposure to extreme alkaline and acidic pH. The presence of lipid thus appears to be necessary for electrokinetic stability of the membrane under these conditions. Despite the extraction of substantial amounts of lipid, it was found that a reversible electrophoretic behavior could be obtained over the pH range 1 to 7 by post-fixation with osmium tetroxide. This is shown in Fig. 14B. From this data it is clear that both the isoelectric point and the magnitude of the positive charge branch are unaffected by lipid extraction.

These results indicate that acidic and basic moieties associated with membrane phospholipids do not contribute to the surface charge properties of erythrocytes at physiological ionic strength. Despite this, the suggestion put forth by earlier workers that lipid polar groups exist at the red cell surface cannot be completely disregarded. On the basis of nuclear magnetic resonance studies, it appears that choline groups associated

with membrane phospholipids have a large rotational mobility, and may exist in an aqueous environment at the erythrocyte surface (26). From electrophoretic data reported here, however, it can be concluded that phospholipid headgroups, if present at the cell interface, are not located at positions lying within 8 Å of the surface of shear.

6. Mobility-pH Characteristics of Neuraminidase-Treated Erythrocytes

As discussed in Section III, B, all species of erythrocyte examined thus far have exhibited a residual negative surface charge following treatment with neuraminidase. In some species, such as equine, feline, and rat red cells, more than one half of the anodic mobility is retained (37, 48, 131). As a means of examining the electrokinetic character of the surface following enzymatic cleavage of sialic acid, several workers have performed mobility-pH measurements on red cells following reaction with neuraminidase (31, 37, 47, 121). Seaman and associates (65, 118) have also found that neuraminidase is active at the surface of human erythrocytes following acetaldehyde fixation, and the present author (129) has obtained a similar result with glutaraldehyde-fixed rat erythrocytes. This is advantageous since it reduces the extent of structural damage to the membrane surface induced by action of the enzyme. In addition, mobility measurements can be performed on the neuraminidase-treated cells over a broad pH range. For these reasons, the discussion here will be primarily restricted to the electrophoretic properties of erythrocytes stabilized by aldehyde fixation prior to reaction with neuraminidase.

Also, because of the somewhat complex electrokinetic behavior of the enzyme-treated cells, various aspects of their surface charge characteristics will be approached on an individual basis.

(a) Surface Charge Reduction at Neutral pH. The two types of neuraminidase that have received widest use in the investigation of cell surfaces are the purified extracts from cultures of the bacterial strains *Vibrio cholerae* and *Clostridium perfringens*. Of these two, the former is preferable in cellular studies since it does not appear to possess hemolytic or cytotoxic contaminants. The neuraminidase from *Cl. perfringens* has been shown to possess phospholipase activity, and to cause considerable structural degradation of membranes (81). When acting on erythrocytes previously stabilized by glutaraldehyde fixation, however, there is no hemolysis and the cell morphology as judged by phase microscopy is unaffected. Both types of neuraminidase appear to be nearly free of proteolytic contaminants. This can be demonstrated by their inability to degrade a collagen-dye complex, as shown by data reported in Table VII.

The activity of these two types of neuraminidase on unfixed and glutaraldehyde-fixed rat erythrocytes is summarized in Table VII. The surface charge reduction observed at neutral pH was 36% of the control value following reaction of glutaraldehyde-fixed cells with either enzyme. In the case of *V. cholerae* neuraminidase, an identical result was obtained for the unfixed cells. Because of extensive hemolysis that accompanied the reaction, no attempt was made to study the surface charge properties of unfixed rat red cells following treatment with *Cl. perfringens* neuraminidase. The chemically determined yield of sialic acid from glutaraldehyde-fixed erythrocytes was found to be 0.14 micromole per 10^{10} cells with both types

of neuraminidase. This represents only 64% of the sialic acid released from unfixed rat red cells by V. cholerae neuraminidase (0.22 micromole per 10^{10} cells). The reduced yield of sialic acid from glutaraldehyde-fixed cells suggests that a substantial fraction of these molecules may reside relatively deep within the erythrocyte peripheral zone, and thereby become inaccessible to neuraminidase following aldehyde fixation. The comparable reduction in surface charge, however, argues that the outermost layer of surface carbohydrate is unaffected by the fixation process.

(b) Appearance of Dissociating Groups at High pH. The surface charge properties of glutaraldehyde-fixed rat erythrocytes following reaction with Cl. perfringens and V. cholerae neuraminidase are shown in Figs. 15A and 16A, respectively. In both cases, the enzyme-treated cells exhibit a reversible increase in negative surface charge above approximately pH 10. Reaction of the neuraminidase-treated cells with p-toluenesulfonyl chloride was found to produce no change in the surface charge at neutral pH, indicating that the dissociating groups in the alkaline pH range are not nitrogenous bases. In addition, when cells treated with Cl. perfringens neuraminidase were post-reacted with either glutaraldehyde and p-hydroxymercuribenzoate, the dissociation at high pH was unaffected. The result with glutaraldehyde is shown in Fig. 15C. Since both of these compounds are effective sulfhydryl blocking agents, it is unlikely that cysteine side chains are responsible for the increase in anodic mobility at high pH. Also, since glutaraldehyde exhibits a small but measurable reactivity with phenolic hydroxyls (59), the absence of any effect following post-fixation with this reagent suggests that no contribution is made by tyrosine residues.

One set of weakly acidic groups at the cell surface that would not be affected by glutaraldehyde post-fixation are the hydroxyls of terminal sugars remaining after the enzymatic cleavage of sialic acid. Glucose, for example, has a pK_a of 12.0 (140), in reasonable agreement with the weakly acidic dissociating groups at the surface of neuraminidase-treated cells. It seems probable, therefore, that groups of this nature are responsible for the mobility characteristics at high pH following reaction with neuraminidase.

(c) Electrophoretic Properties at Low pH. As demonstrated explicitly in Figs. 15A and 16A, the electrophoretic properties of glutaraldehyde-fixed rat erythrocytes treated with neuraminidase are irreversible at low pH. This difficulty can be overcome by post-fixation with osmium tetroxide, as shown in Figs. 15B and 16B. It is clear from this data that cleavage of sialic acid molecules from the erythrocyte surface leads to a substantial reduction in the cathodic mobility at pH 1. Following treatment with Cl. perfringens neuraminidase, the positive surface charge at pH 1 is reduced by approximately 75% from the control value of +0.80 micron/sec/volt/cm. With V. cholerae neuraminidase, the reduction is about 50%.

Further electrophoretic studies were performed in which glutaraldehyde-fixed erythrocytes were treated with Cl. perfringens neuraminidase, and then post-fixed with glutaraldehyde. From Fig. 15C it is clear that cells handled in this manner are electrokinetically unstable at low pH, with the character of the instability being similar to that observed for cells that were not fixed subsequent to enzyme action (cf. Fig. 15A). After glutaraldehyde post-fixation, however,

it was noted that the cathodic mobility at pH 1 changed as a function of time in the manner shown in Fig. 15D. The extrapolated mobility at time t = 0, i.e. the instant at which the cells were suspended at pH 1, was +0.41 micron/sec/volt/cm. After approximately five minutes the mobility increased to +0.90 micron/sec/volt/cm, corresponding closely to the value observed instantaneously at this pH when post-fixation was not employed (cf. Fig. 15A). The initial reduction in mobility at pH 1 is in reasonable agreement with measurements made on enzyme-treated cells post-fixed with osmium tetroxide. A comparable experiment with V. cholerae neuraminidase has not been attempted.

On the basis of these results, it appears that the weakly basic acetamido groups of N-acetylneuraminic acid are primarily responsible for the positive mobility branch between pH 1 and 2. In addition, it might also be proposed that the remaining positive charge is attributable to N-acetylneuraminic acid molecules not susceptible to release by neuraminidase, or to N-acetylated amino sugars present as terminal groups of oligosaccharide chains following the enzymatic cleavage of sialic acid. An argument that might be advanced against this is the fact that acetylated amides generally have pK_a's of zero or less. N-methylacetamide, for example, has a pK_a of -0.46 (49). It is conceivable, however, that the basicity of a sialic acid acetamido group may be somewhat greater because of its proximity to a strongly acidic carboxyl, or by virtue of its environment at the cell surface. In this sense, the base strength of N-methylacetamide cannot be regarded as a proper analog.

(d) The Negative Surface Charge Remaining After Neuraminidase Treatment. From the mobility-pH characteristics shown in Figs. 15B and 16B, the acidic groups contributing to the mobility of rat erythrocytes after neuraminidase treatment have an approximate pK_a in the range 2.5 to 3.0. A similar result has been reported by Haydon and Seaman (65) for human red cells fixed with acetaldehyde and subsequently reacted with neuraminidase. As shown in Fig. 17, the mobility-pH characteristics observed by these workers following enzyme treatment fit a theoretical titration curve for anionic groups with a pK_a of 3.35. They contend that these groups contribute 38.5% of the surface charge density of human erythrocytes at neutral pH, and propose that they are α-carboxyls associated with C-terminal peptide-bound amino acids linked covalently through their side chains to saccharides at the cell surface. This is shown diagrammatically in Fig. 18.

With regard to rat erythrocytes, one conceptual difficulty arises in this analysis of the mobility properties of neuraminidase-treated cells. Although not conclusive, the bulk of experimental evidence indicates that only sialic acid serves as the terminal group in mucopolysaccharide carbohydrate (146). If this is correct, then each of the peptide-linked oligosaccharide moieties at the cell surface should contain a terminal sialic acid residue. As a consequence, the total contribution made to the mobility by α-carboxylic amino acids involved in carbohydrate linkage should not exceed that of sialic acid, namely, -0.40 micron/sec/volt/cm. Since the mobility remaining after neuraminidase treatment is -0.70 micron/sec/volt/cm, it would appear on this basis that at least -0.30 micron/sec/volt/cm must be attributable to other types of strongly acidic

groups. From results presented in Section III, C, 5, it is unlikely that these groups are associated with phospholipid moieties. Also, both chemical and electrophoretic studies have shown that carboxypeptidase A is inactive at the surface of unfixed rat erythrocytes, thereby indicating the absence of "free" C-terminal residues from positions near the surface of shear (129). In view of the fact that the average pK_a of the charged groups remaining after neuraminidase treatment lies in the range 2.5 to 3.0, it is also improbable that a contribution is made by protein β- and γ-carboxyls. These groups generally exhibit pK_a's in the range 3.0 to 4.7 (141). Another possibility is that the remaining charged groups are sialic acid residues not susceptible to attack by neuraminidase. Since the pK_a of sialic acid is 2.6 (128), this would be consistent with the low pK_a observed for the surface anions of the enzyme-treated cells. As discussed in Section III, B, neuraminidase does not effect the complete release of sialic acid from gangliosides at the erythrocyte surface. Since rat red cells have a large ganglioside content (see Table I), it is reasonable to propose that a contribution is made to the surface charge by sialic acid associated with mucolipids. This would not be expected for human erythrocytes, however, since their content of lipid-bound sialic acid is extremely small (80, 148).

It might be noted at this point that ethanol extraction of lipids from glutaraldehyde-fixed rat erythrocytes does not affect the anodic mobility, as discussed in Section III, C, 5. This indicates that mucolipids, if present in the eluted material, are not contributors to the surface charge. No tests have been performed, however, for the presence of sialic

acid in the extract. As described in Section III, C, 3, sequential washing of glutaraldehyde-fixed rat red cells with acid and methanol leads to a 25% reduction in the negative surface charge. Since methanol solubilizes several forms of ganglioside (80), it is possible that the observed decrease in mobility resulted from the partial extraction of mucolipid. A series of experiments are planned for the near future in which glutaraldehyde-fixed cells will be treated with organic solvents that effect a nearly complete removal of gangliosides (e.g. chloroform/methanol (94, 127)). By combining electrophoretic measurements with a chemical characterization of the extracted products, it is hoped that direct evidence will be obtained on the role of lipid-bound sialic acid in determining erythrocyte surface charge properties.

In interpreting the electrokinetic properties of neuraminidase-treated erythrocytes, the question arises as to whether any portion of the residual negative charge is associated with new anionic groups appearing at the cell surface as the result of reaction with the enzyme. This could arise, for example, through the activity of proteolytic, lipolytic, or other forms of enzyme in the commercial neuraminidase preparations. Since the purified extract from V. cholerae appears to be free of such contaminants, it is unlikely that this enzyme induces extensive alteration of the membrane surface apart from the cleavage of sialic acid. It should also be noted that the electrophoretic properties of glutaraldehyde-fixed erythrocytes reacted with Cl. perfringens neuraminidase are closely comparable to those observed following treatment with the purified enzyme from V. cholerae. This indicates that the electrokinetic surface of glutaraldehyde-fixed

red cells is not seriously disrupted through the activity of contaminants in the Cl. perfringens extract.

Another mechanism by which new acidic groups could contribute to the electrophoretic mobility after treatment with neuraminidase is through a change in the location of the surface of shear relative to the physical membrane surface. After removal of sialic acid molecules from the terminal positions of oligosaccharide chains, the effective surface of shear may reside several angstroms closer to the membrane surface. A contribution to the electrophoretic mobility might then be made by anions which, prior to neuraminidase treatment, were located at positions greater than one Debye length from the surface of shear. In particular, this may apply to α-carboxyls associated with C-terminal peptide-bound amino acids that are linked covalently through their side chains to saccharides at the cell surface. In the case of acetaldehyde-fixed human erythrocytes, Haydon and Seaman (65) have proposed that such groups contribute equally to the mobility of untreated and neuraminidase-treated cells. A direct method of testing this hypothesis would be to observe the electrophoretic mobility following enzymatic decarboxylation of amino acids at the membrane surface. If Haydon and Seaman are correct, amino acid decarboxylation should result in roughly a 40% reduction in the anodic mobility of the human erythrocyte irrespective of neuraminidase treatment. Unfortunately, bacterial decarboxylases that are commercially available at the present time are active only on free amino acids (132), so that this type of experiment cannot be performed.

Two observations which indicate that a new set of anionic groups do not contribute to the electrophoretic mobility subsequent to neuraminidase treatment are the following. First, the surface charge densities of

several species of erythrocyte have been found to remain constant as the Debye length is increased from 8 to 20 Å (10, 42, 47). A further increase in the Debye length results in a reduction of the negative charge density, suggesting that predominantly basic groups are unmasked. Secondly, Haydon and Seaman (65) have found that the electrophoretically detectable binding of methylene blue to the human erythrocyte remains essentially constant as the Debye length is varied from 12 to 88 Å. This would not be expected if an increase in the Debye length were accompanied by a contribution to the surface charge density from new groups of titrable anions. Both of these observations are clearly inconsistent with the possibility that a new set of acidic groups might contribute to the erythrocyte mobility if the surface of shear were moved closer to the physical membrane surface through removal of sialic acid from the cell periphery.

(d) Summary. The results of studies presented in this section on the surface charge properties of aldehyde-fixed erythrocytes may be summarized as follows:

(i) Upon treatment of glutaraldehyde-fixed rat erythrocytes with neuraminidase from *V. cholerae* and *Cl. perfringens*, the reduction in electrophoretic mobility at neutral pH is identical to the 36% reduction observed with unfixed red cells. The chemically determined yield of sialic acid, however, is only 64% of that obtained with unfixed cells. The comparable decrease in surface charge indicates that the outermost layer of polysaccharide is not affected by the fixation process. On the other hand, the reduced yield of sialic acid suggests that a substantial

amount of surface carbohydrate lies deep relative to the surface of shear, and becomes inaccessible to neuraminidase upon fixation with glutaraldehyde.

(ii) Reaction of glutaraldehyde-fixed cells with neuraminidase results in the appearance of electrophoretically detectable dissociating groups at high pH. These are unaffected by reaction of the enzyme-treated cells with blocking reagents that mask amino and guanidyl bases, phenolic hydroxyls, and sulfhydryls. At the present time, it seems probable that the increase in negative surface charge above pH 10 is attributable to weakly acidic hydroxyls of terminal sugars remaining after the enzymatic cleavage of sialic acid.

(iii) Following post-fixation with osmium tetroxide, the enzyme-treated cells exhibit reversible mobility characteristics at low pH. Relative to control erythrocytes, the positive charge at pH 1 is reduced by 50 to 75%, indicating that the proton-binding observed from pH 1 to 2 is primarily attributable to the weakly basic acetamido groups of N-acetylneuraminic acid residues.

(iv) From electrophoretic studies on acetaldehyde-fixed human erythrocytes treated with neuraminidase, Haydon and Seaman (65) have concluded that 38.5% of the negative surface charge is associated with acidic groups having a pK_a of 3.35. They propose that these are α-carboxyls of C-terminal protein residues linked covalently through their side chains to oligosaccharide units at the cell surface. In the case of glutaraldehyde-fixed rat erythrocytes, nearly two thirds of the negative charge remains following reaction with neuraminidase. This is attributable

to acidic groups having an average pK_a in the range of 2.5 to 3.0. The large ganglioside content of rat erythrocyte membranes suggests that a substantial number of these anionic groups may be sialic acid residues not susceptible to release by neuraminidase. A contribution to the mobility may also be made by protein-bound α-carboxylic acids involved in peptide-saccharide linkage at the rat erythrocyte surface.

D. Summary: The Ionic Character of the Erythrocyte Membrane Surface at Physiological Ionic Strength

On the basis of microelectrophoretic studies, the erythrocyte in physiological media appears to behave as a polyanion, with the negative surface charge determined primarily by glycoprotein and ganglioside moieties at the cell interface. For many species of erythrocyte, a major part of the surface charge is associated with neuraminidase-susceptible sialic acid. Those species that show only a small reduction in mobility following reaction with neuraminidase generally contain a large number of sialic acid residues bound to gangliosides. The limited activity of neuraminidase against mucolipids suggests that the residual surface charge may be attributable to sialic acid. This is supported by the low pK_a (ca. 3) of the surface anions remaining after neuraminidase treatment. Electrophoretic evidence also supports the possibility that a contribution to the negative surface charge is made by α-carboxyls associated with C-terminal residues involved in protein-carbohydrate linkage at the cell surface.

Below approximately pH 2, erythrocytes exhibit a large positive surface charge. From studies on neuraminidase-treated rat red cells,

the cathodic mobility from pH 1 to 2 appears to be primarily attributable to the weakly basic acetamido groups of N-acetylneuraminic acid residues. Below pH 1, rat erythrocytes acquire an extremely large positive charge density, the magnitude of which is at least 15 times as great as that of any other electrophoretically detectable membrane component. The characteristics of this mobility branch suggest that it may arise from a non-specific proton adsorption onto non-ionogenic regions of the cell surface. The identity of these hydrogen ion binding sites has not been determined, but they appear to occupy at least 10% of the rat erythrocyte surface.

With regard to membrane components that are absent from the interfacial zone of erythrocytes, no evidence has been found for a contribution to the mobility at physiological ionic strength by dissociating groups with pK_a's in the range of neutral pH or above. In addition, a contribution from protein β- and/or γ-carboxyls seems unlikely in view of the fact that anionic groups at the red cell surface exhibit pK_a's of approximately 3 or less. With the possible exception of α-carboxylic acids involved in saccharide linkage, it thus appears that none of the surface charge properties of erythrocytes are attributable to protein-bound amino acid residues. From electrophoretic studies following lipid extraction, a similar conclusion has been drawn for acidic and basic groups associated with membrane phospholipids.

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TABLE I: Erythrocyte Membrane Ganglioside Content and the Mobility Reduction Following Neuraminidase Treatment

Erythrocyte Species	Ganglioside Content (% of Total Lipid)	% Reduction in Mobility Following Neuraminidase Treatment
Human	Not Detectable[a]	94[c]
Pig	3.3[b]	67[c]
Cat	8.8[b]	45[d]
Rat	6.3[b]	36[e]
Horse[f]	15.5[b]	20[c]

(a) From Yamakawa and Suzuki (148).

(b) From Nelson (94).

(c) From Eylar et al. (37).

(d) From Uhlenbruck et al. (131).

(e) From Table VII, Section III, C, 6, a.

(f) A rough calculation of the amount of lipid-bound sialic acid present in the horse erythrocyte membrane can be made in the following manner. All of the ganglioside will be assumed to be of the "hematoside" type containing sphingosine: fatty acid: hexose: N-glycolylneuraminic acid in the ratio 1: 1: 2: 1 (Ref. (76)). Small amounts of other gangliosides exist as well (149), but will be neglected here. Using Nelson's (94) data for the total lipid content (2.58×10^{-13} gm/cell) and percentage of gangliosides (15.5%), it can be calculated that the amount of lipid-bound sialic acid is 1.95×10^{7} molecules per cell. In comparison, the number of molecules released by neuraminidase is 0.59×10^{7} per cell (37).

TABLE II: Carbohydrates in the Human Erythrocyte Membrane

	Sialic Acid[a]	Hexose[a]	N-acetylhexosamine[a]
Ludewig[b]	1.2%	2%	1.2%
Rosenberg and Guidotti[c]	1.2%	4%	2%
Relander[d]	Not Measured	1.33%	1.1%
Average Values:	1.2%	2.44%	1.43%

(a) % of dry stromal weight.

(b) Ref. (87).

(c) Ref. (115).

(d) Ref. (107).

TABLE III: Major Ionic Components of the Human Erythrocyte Membrane[a]

	Amino Acids[b]		Carbohydrate[c]	
Strongly Acidic	Glutamic	41.9×10^7	Sialic Acid	2.4×10^7
	Aspartic	29.3×10^7		
Weakly Acidic	Tyrosine	8.3×10^7	**Phospholipids[d]**	
	Cysteine	3.7×10^7	Lecithin	10.7×10^7
Basic	Lysine	18.0×10^7	Phosphatidyl Ethanolamine	9.3×10^7
	Arginine	15.6×10^7	Phosphatidyl Serine	5.4×10^7
	Histidine	8.4×10^7	Sphingomyelin	8.6×10^7

(a) Expressed as the number of molecules per cell membrane.

(b) Calculated from the amino acid composition given by Rosenberg and Guidotti (107). The weight of membrane protein was taken as 6.6×10^{-13} gm/membrane (35).

(c) From Ref. (37).

(d) Calculated from the data of Ways and Hanahan (136).

TABLE IV: Mobility-pH Data for Human, Harbor Seal, and Killer Whale Erythrocytes[a]

Species	Mobility of Unfixed Cells	Mobility of Glutaraldehyde-fixed Cells[b]		Mobility of Osmium Tetroxide-fixed Cells[c]	
	pH 7.3	pH 7.3	pH 1.2	pH 7.3	pH 1.2
Human	-0.91	-0.87	+1.28[d]	-0.86	+1.04[g]
Harbor Seal	-1.01	-0.99	+1.34[e]	-1.00	+1.00[h]
Killer Whale	-0.91	-0.91	+0.75[f]	-0.97	+0.49[i]

(a) Mobilities expressed in units of micron/sec/volt/cm.

(b) Fixation was performed with 2.5% glutaraldehyde for 10 minutes at room temperature. The fixation medium was buffered to pH 7.4.

(c) Fixation was performed with 1% osmium tetroxide for one minute at room temperature. The fixation medium was buffered to pH 7.4.

(d) When brought to neutral pH after a 15 minute exposure to pH 1.2, the mobility of this cell suspension was -0.91 micron/sec/volt/cm. This value is closely comparable to the control mobility (-0.87 micron/sec/volt/cm), and indicates that no structural damage was sustained by the cell surface during exposure to low pH.

(e) When reversed to neutral pH, the mobility was -1.03 micron/sec/volt/cm.

(f) When reversed to neutral pH, the mobility was -0.96 micron/sec/volt/cm.

(g) When reversed to neutral pH, the mobility was -0.89 micron/sec/volt/cm.

(h) When reversed to neutral pH, the mobility was -0.99 micron/sec/volt/cm.

(i) When reversed to neutral pH, the mobility was -0.89 micron/sec/volt/cm.

TABLE V: Mobility Properties of Rat Erythrocytes Treated with p-toluenesulfonyl chloride and 1,5-difluoro-2,4-dinitrobenzene

	Unfixed Rat Erythrocytes (Control)	Treated with p-toluenesulfonyl chloride[a]	Treated with 1,5-difluoro-2,4-dinitrobenzene[b]
Mobility in micron/sec/volt/cm at pH 7.4	-1.06	-1.03	-1.04

(a) Tosylation was performed by the method of Seaman and Heard (119). One packed volume of cells was mixed with four volumes of a pH 7.4 solution containing 1 mg/ml p-toluenesulfonyl chloride. The reaction was carried out for 30 min at 37° C.

(b) Reaction of rat erythrocytes with 1,5-difluoro-2,4-dinitrobenzene (DFNB) followed the procedure of Berg et al. (13). One packed volume of cells was mixed with 20 volumes of saline containing 2.8 mM DFNB. The reaction was carried out for one hour at room temperature.

TABLE VI: Mobility Properties of Rat Erythrocytes Treated with p-chloromercuribenzoate

	Unfixed Rat Erythrocytes (Control)	Treated with p-chloromercuribenzoate[a]
Mobility in micron/sec/volt/cm at pH 7.5	-1.01	-1.02

(a) One packed volume of cells was mixed with 20 volumes of a saline solution containing 10^{-4}M p-chloromercuribenzoate. The reaction was carried out at pH 7.5 for one hour at room temperature.

TABLE VII: Effects of Neuraminidase on Glutaraldehyde-fixed Rat Erythrocytes

	Surface Charge Reduction at pH 7 for Unfixed Cells— % of Control Mobility[a]	Surface Charge Reduction at pH 7 for Glutaraldehyde-fixed Cells— % of Control Mobility[a]	Sialic Acid Released from Unfixed Cells— micromoles per 10^{10} cells[b]	Sialic Acid Released from Glutaraldehyde-fixed Cells— micromoles per 10^{10} cells[b]	Proteolytic Activity— Equivalent Trypsin Units[c]
Clostridium perfringens Neuraminidase[d] (Worthington)	Not Measured[f]	36%	Not Measured[f]	0.14 ± 0.02 (SD)	0.03
Vibrio cholerae Neuraminidase[e] (Behringwerke)	36%, 42%[g]	36%	0.22 ± 0.01 (SD), 0.29 ± 0.03[g]	0.14 ± 0.02 (SD)	0

(a) Control mobility = -1.10 micron/sec/volt/cm.

(b) Sialic acid was measured by the method of Warren (134). Determinations were made on three cell samples.

(c) Proteolysis was determined by the "Azocoll" method (Calbiochem). This procedure involves a colorimetric measurement of the amount of dye released by a proteolytic enzyme from an insoluble collagen-azo dye complex. After incubation at 37° C for 15 min, the insoluble substrate is removed by filtration, and the supernatent optical density read at 580 nm. As a standard, a determination is made of the amount

TABLE VII: Continued

of dye solubilized by a 0.1 mg/ml trypsin solution at pH 7.4. An "equivalent trypsin unit" is the ratio of the optical density obtained with an unknown enzyme preparation to that of the 0.1 mg/ml trypsin standard.

(d) One packed volume of cells was incubated with two volumes of an enzyme preparation containing 0.25 mg/ml neuraminidase. The reaction was carried out at pH 5.35 for one hour at 37° C.

(e) One packed volume of cells was incubated with two volumes of a 100 units/ml enzyme solution (prepared by dilution of the 500 units/ml commercial stock with a 0.9% NaCl solution containing 1.0 mg/ml $CaCl_2$). The reaction was carried out at pH 5.5 for 30 min at 37° C.

(f) Under the reaction conditions employed here, hemolysis of unfixed cells occurred within a few minutes.

(g) Values obtained by Seaman et al. (121).

FIGURE CAPTIONS

FIGURE 1. A schematic representation of the diffuse double layer at a negatively charged surface. The surface potential is denoted by ψ; its value at the surface of shear is termed the zeta potential.

FIGURE 2. A microelectrophoresis assembly employing a rectangular chamber in the lateral position. Details of this design are described in the text.

FIGURE 3. The electrokinetic stability diagram of Heard and Seaman (J. Gen. Physiol. 43, 635 (1960)). The terms "stability," "metastability," and "instability" are defined in the text.

FIGURE 4. The ring structure of N-acetylneuraminic acid. Another form of sialic acid known to be present at the surface of several species of erythrocyte is N-glycolylneuraminic acid.

FIGURE 5. Proposed reactions of glutaraldehyde and osmium tetroxide. This form of crosslinking reaction between polymeric glutaraldehyde and two primary amino groups has been proposed by Richards and Knowles (J. Mol. Biol. 37, 231 (1968)). The amino groups are converted to secondary amines connected by a five-carbon bridge. The action of osmium tetroxide as a fixative is thought to result from the crosslinking of unsaturated fatty acid chains of membrane phospholipids. Following reaction, the osmium is believed to exist in either a hexavalent or tetravalent state.

FIGURE 6. The mobility-pH characteristics of human erythrocytes as reproduced from the data of Furchgott and Ponder (J. Gen. Physiol. 24, 447 (1941)). An ionic strength of 0.172 was maintained at all values of pH. The erythrocytes were observed to become isoelectric at pH 1.7.

FIGURE 7. The mobility-pH characteristics at ionic strength 0.145 obtained by Haydon and Seaman for human erythrocytes fixed with 2% acetaldehyde (Arch. Biochem. Biophys. 122, 126 (1967)). Fixation was carried out for 20 days with the pH maintained at 7.4. The dashed line represents a theoretical titration curve for a surface containing two types of anionic groups: 61.5% with a pK_a of 2.6, and 38.5% with a pK_a of 3.35.

FIGURE 8. Mobility-pH curves at ionic strength 0.145 are shown in A and B for unfixed rat erythrocytes and erythrocytes fixed with 2.5% glutaraldehyde and 1% osmium tetroxide. Fixation with both reagents was carried out at pH 7.4 and room temperature. With glutaraldehyde the reaction was allowed to proceed for ten minutes, and with osmium tetroxide for one minute. In C and D, the mobility properties are shown following dual fixation with 2.5% glutaraldehyde and 1% osmium tetroxide. The electrophoresis medium was 0.145M NaCl buffered with 3×10^{-4}M $NaHCO_3$. Adjustment of pH was performed by adding 0.145N NaCl or 0.145N NaOH.

FIGURE 9. Studies on the reversibility of surface charge properties as a function of pH are presented for rat erythrocytes fixed with 2.5% glutaraldehyde and 1% osmium tetroxide. The ionic strength was maintained at 0.145. In each of the four experiments shown here, a single cell suspension was incubated successively at the pH values denoted by numerals.

FIGURE 10. In A the mobility-pH characteristics are shown at ionic strength 0.145 for rat erythrocytes fixed with 2% acetaldehyde. Cells were incubated in the fixation medium for 20 days at pH 7.4 and 4° C. The numerals 1, 2, and 3 refer to a reversibility test at low pH; the primed numerals denote a reversibility test at high pH. In B the mobility of acetaldehyde-fixed erythrocytes is shown as a function of time at pH 3.23. A mobility-pH curve for acetaldehyde-fixed cells following post-fixation with 1% osmium tetroxide is shown in C. In D the mobility characteristics are presented for rat erythrocytes fixed with 2.5% glutaraldehyde for 20 days. The temperature was maintained at 4° C throughout the fixation.

FIGURE 11. The surface charge density is plotted as a function of bulk pH for rat erythrocytes fixed with glutaraldehyde. The numerals 1 and 2 refer to a reversibility study. In order to extend mobility measurements below pH 1, erythrocytes were suspended in hydrochloric acid solutions having ionic strengths ranging from 0.145 to 1.0.

FIGURE 12. Reactions are shown for a carboxylic acid (I) and a water soluble carbodiimide (II). The carbodiimide (CMC) is 1-cyclohexyl-3-(2-morpholinylethyl)carbodiimide metho-p-toluenesulfonate. The cationic portion of this salt is represented here as $R' - N = C = N - \overset{+}{R}''$. The first reaction product is an acylisourea (III). This compound can then rearrange to form an acylurea (IV).

FIGURE 13. The reduction in electrophoretic mobility at ionic strength 0.145 and pH 7.4 is shown in A for rat erythrocytes fixed with glutaraldehyde and then reacted with 0.1M CMC for varying lengths of time. CMC

is the water soluble carbodiimide shown in Fig. 12. Reaction of erythrocytes with CMC was carried out at pH 4.75 and 37° C. The effect of reacting CMC-treated cells with p-toluenesulfonyl chloride (tosyl chloride) is also shown. Tosylation was performed by mixing the cells with a one mg/ml solution of tosyl chloride at pH 7.4. The reaction was allowed to proceed for thirty minutes at 37° C. In B the mobility-pH characteristics are shown for glutaraldehyde-fixed rat erythrocytes reacted with CMC for three hours. The primed and unprimed numerals refer to reversibility tests at high and low pH, respectively. Above neutral pH the curve is drawn as a dashed line to indicate the alkaline lability of a large portion of the reaction products formed by CMC with surface anions.

FIGURE 14. The mobility-pH characteristics at ionic strength 0.145 are shown in A for glutaraldehyde-fixed rat erythrocytes after incubation in absolute ethanol for one hour at room temperature. The primed and unprimed numerals refer to reversibility studies at high and low pH, respectively. In B the mobility properties at low pH are plotted following post-fixation of the ethanol-extracted cells with osmium tetroxide.

FIGURE 15. In A the mobility-pH characteristics at ionic strength 0.145 are shown for rat erythrocytes fixed with glutaraldehyde and then reacted with *Clostridium perfringens* (Worthington). The enzyme concentration was 0.25 mg/ml and the reaction was performed at pH 5.35 for one hour at 37° C. The primed and unprimed numerals refer to reversibility tests

at high and low pH, respectively. In B and C the mobility properties are shown following post-fixation of the neuraminidase-treated cells with osmium tetroxide and glutaraldehyde. The mobility of the glutaraldehyde post-fixed cells at pH 1.03 is shown as a function of time in D.

FIGURE 16. Mobility-pH characteristics at ionic strength 0.145 are shown in A for rat erythrocytes fixed with glutaraldehyde and subsequently reacted with *Vibrio cholerae* neuraminidase (Behringwerke). The enzyme concentration was 100 units/ml and the reaction was carried out at pH 5.5 for 30 min at 37° C. The enzyme solution contained 1 mg/ml $CaCl_2$. Primed and unprimed numerals refer to reversibility tests at high and low pH, respectively. In B the mobility properties are shown following post-fixation of the enzyme-treated cells with osmium tetroxide.

FIGURE 17. The mobility-pH characteristics at ionic strength 0.145 observed by Haydon and Seaman for human erythrocytes fixed with acetaldehyde and then reacted with *Vibrio cholerae* neuraminidase (*Arch. Biochem. Biophys.* 122, 126 (1967)). The dashed line corresponds to a theoretical titration curve for anionic groups of $pK_a = 3.35$.

FIGURE 18. A schematic representation of the linkage of sialyl oligosaccharides in erythrocyte membrane mucoproteins. The peptide-saccharide linkage is believed to involve glycosidic linkages of N-acetylgalactosamine to serine, threonine, and glutamic acid side chains (see Section III, B). Haydon and Seaman propose that these residues are C-terminals, and that their α-carboxyls contribute 38.5% of the human erythrocyte surface charge (*Arch. Biochem. Biophys.* 122, 126 (1967)).

FIGURE 1

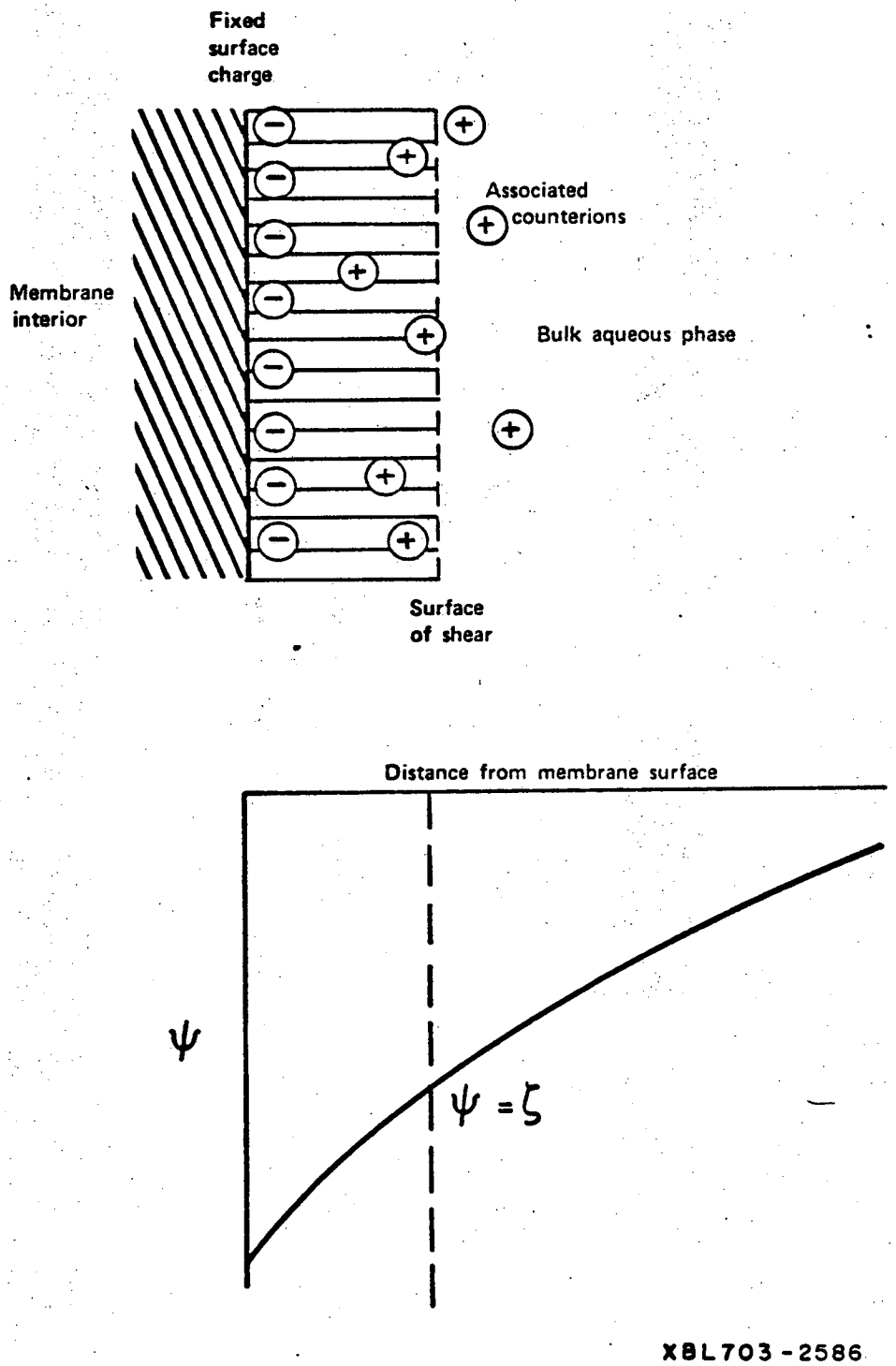

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FIGURE 2

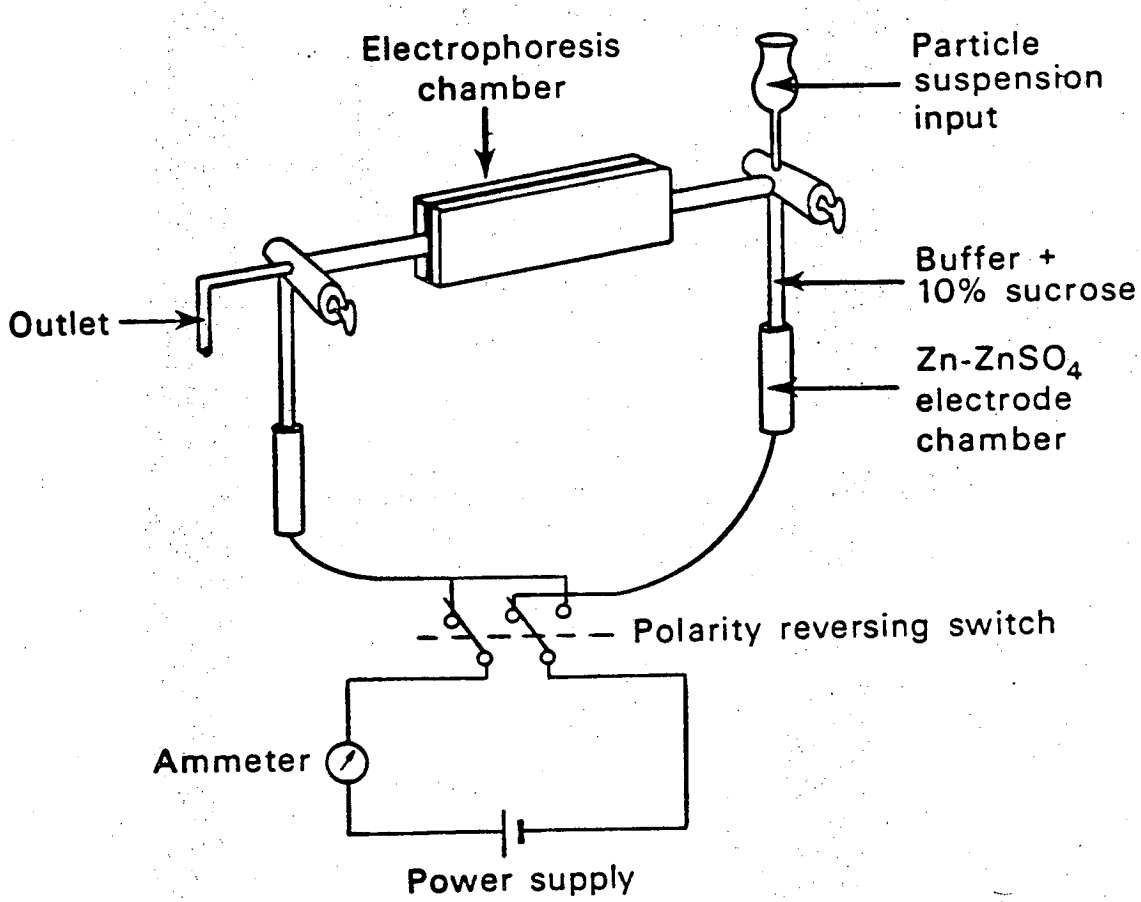

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FIGURE 3

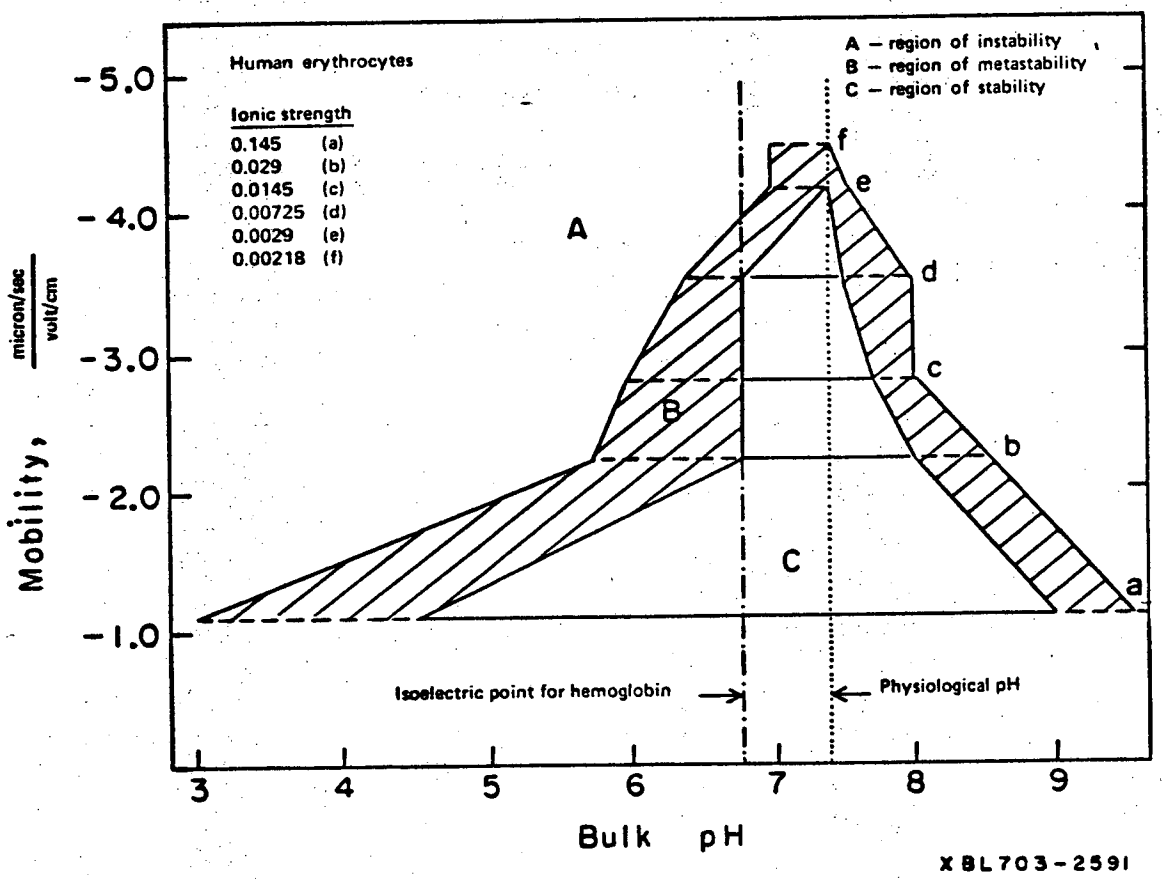
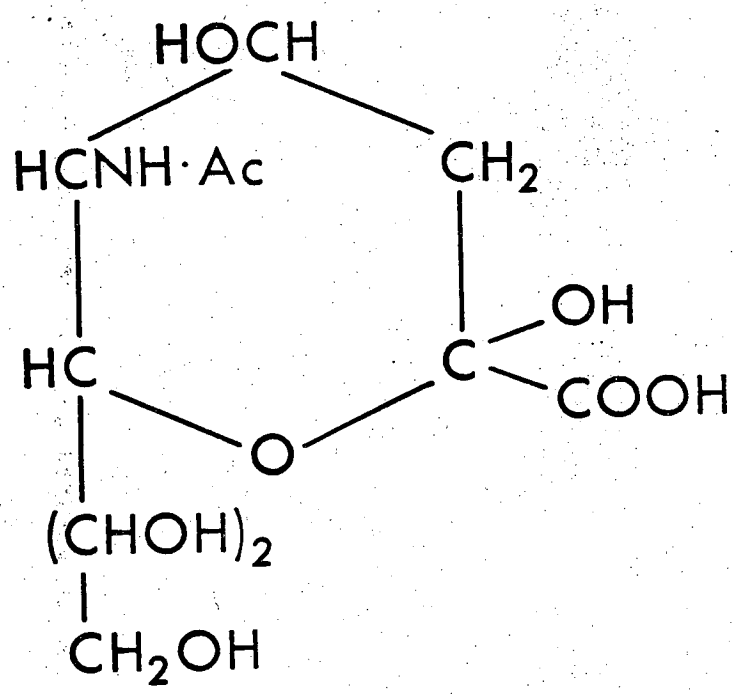

Human erythrocytes
Ionic strength
0.145 (a)
0.029 (b)
0.0145 (c)
0.00725 (d)
0.0029 (e)
0.00218 (f)
A – region of instability
B – region of metastability
C – region of stability
A
B
C
a
b
c
d
e
f
Isoelectric point for hemoglobin
Physiological pH
Mobility, micron/sec / volt/cm
-5.0
-4.0
-3.0
-2.0
-1.0
3
4
5
6
7
8
9
Bulk pH
XBL703-2591

FIGURE 4



MU-31331

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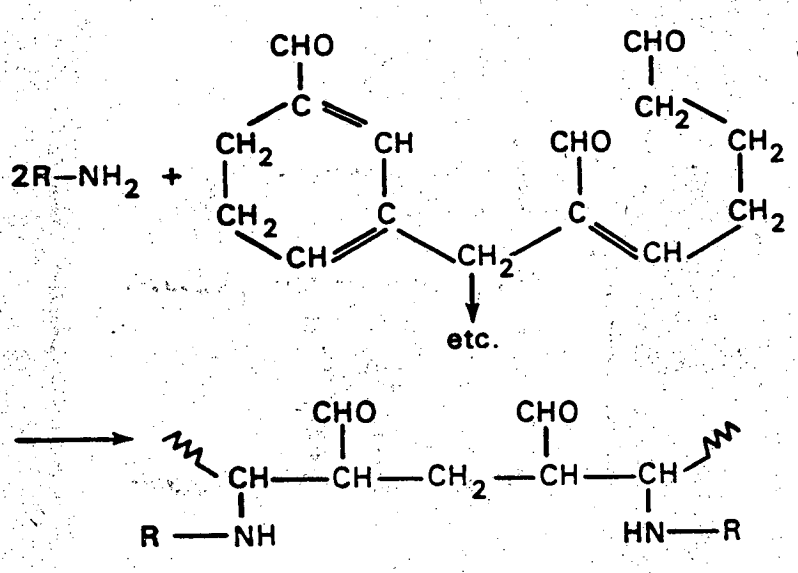

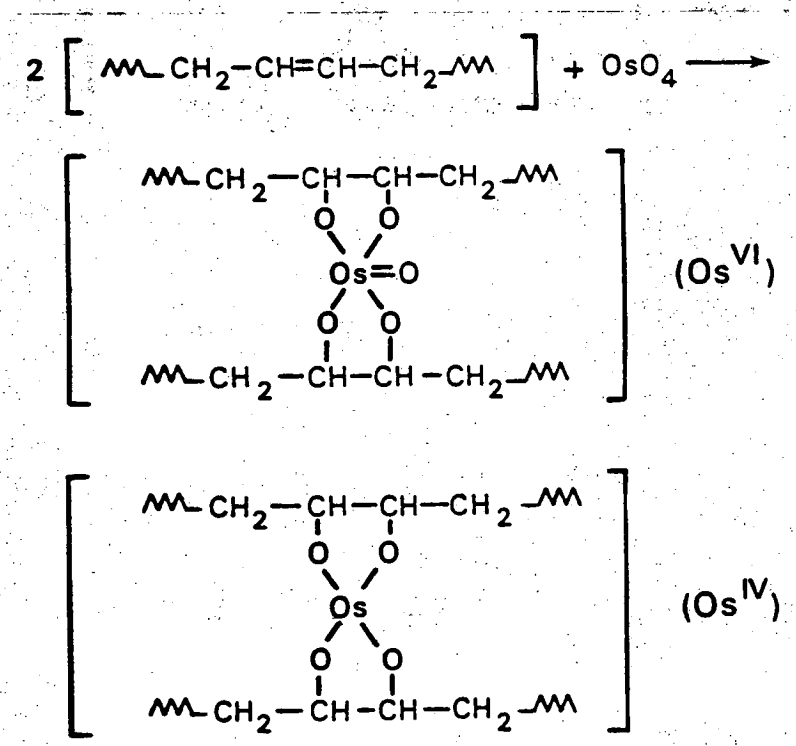

DBL 704-5667

FIGURE 6

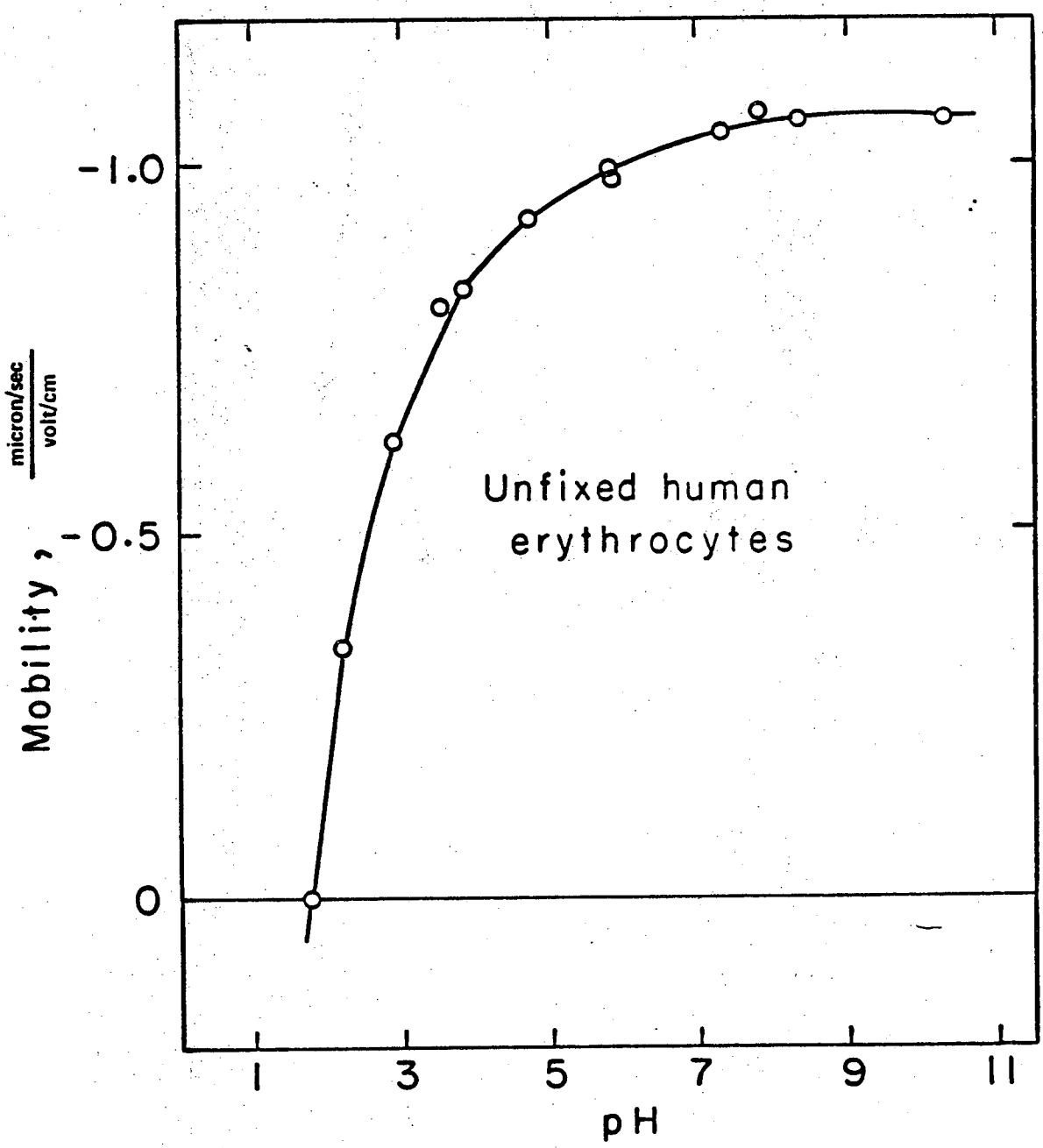

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FIGURE 7

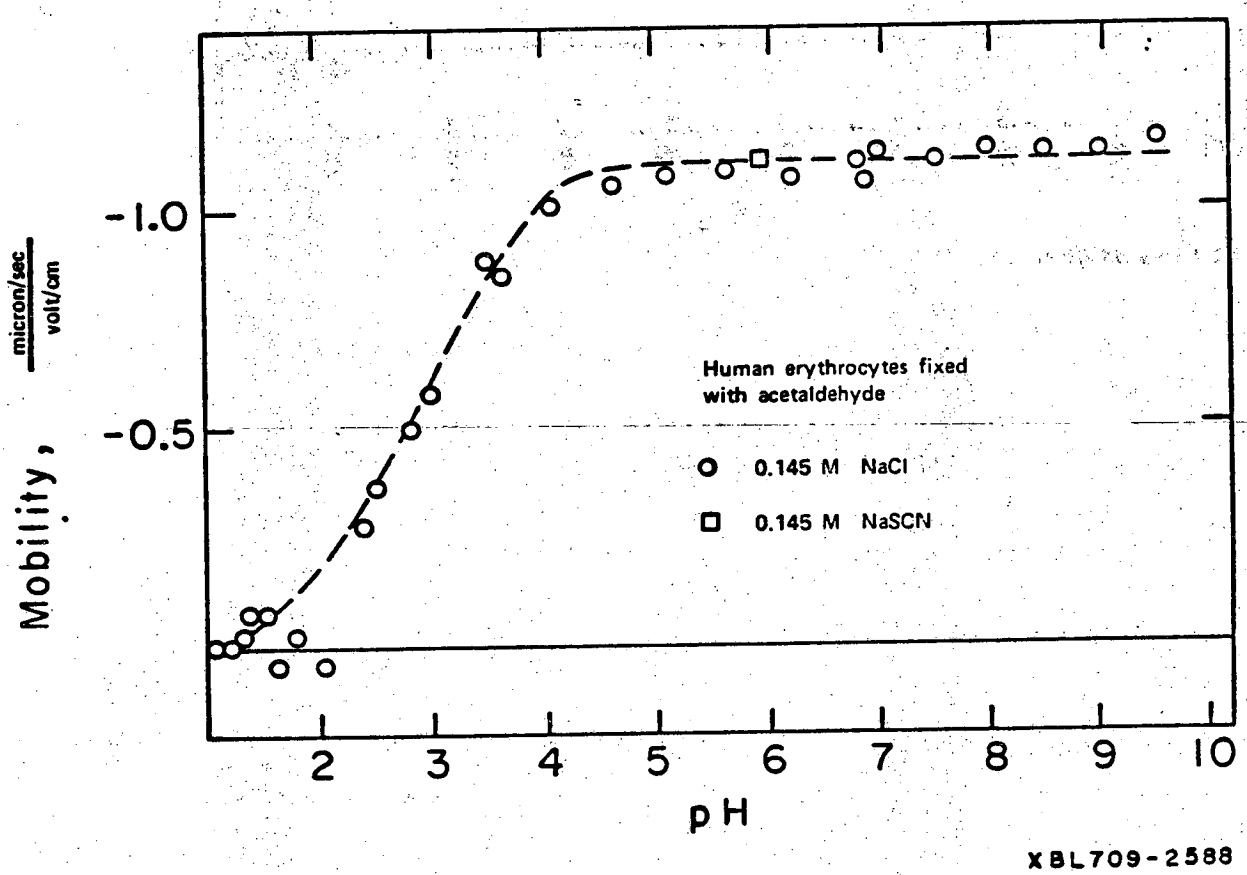
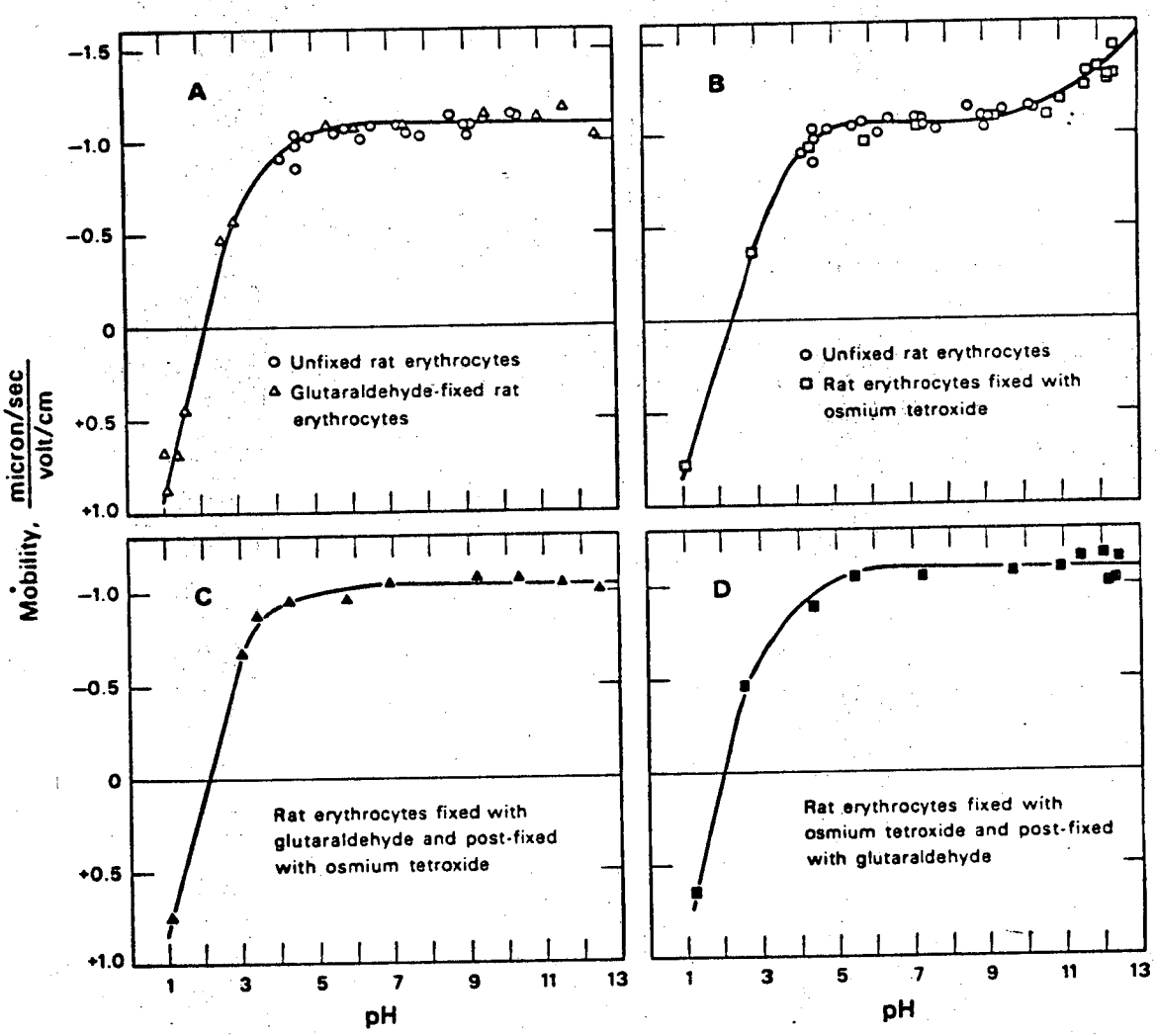

Mobility, micron/sec / volt/cm
-1.0
-0.5
Human erythrocytes fixed with acetaldehyde
0.145 M NaCl
0.145 M NaSCN
2
3
4
5
6
7
8
9
10
pH
XBL709-2588

FIGURE 8



DBL 698-5028

FIGURE 9

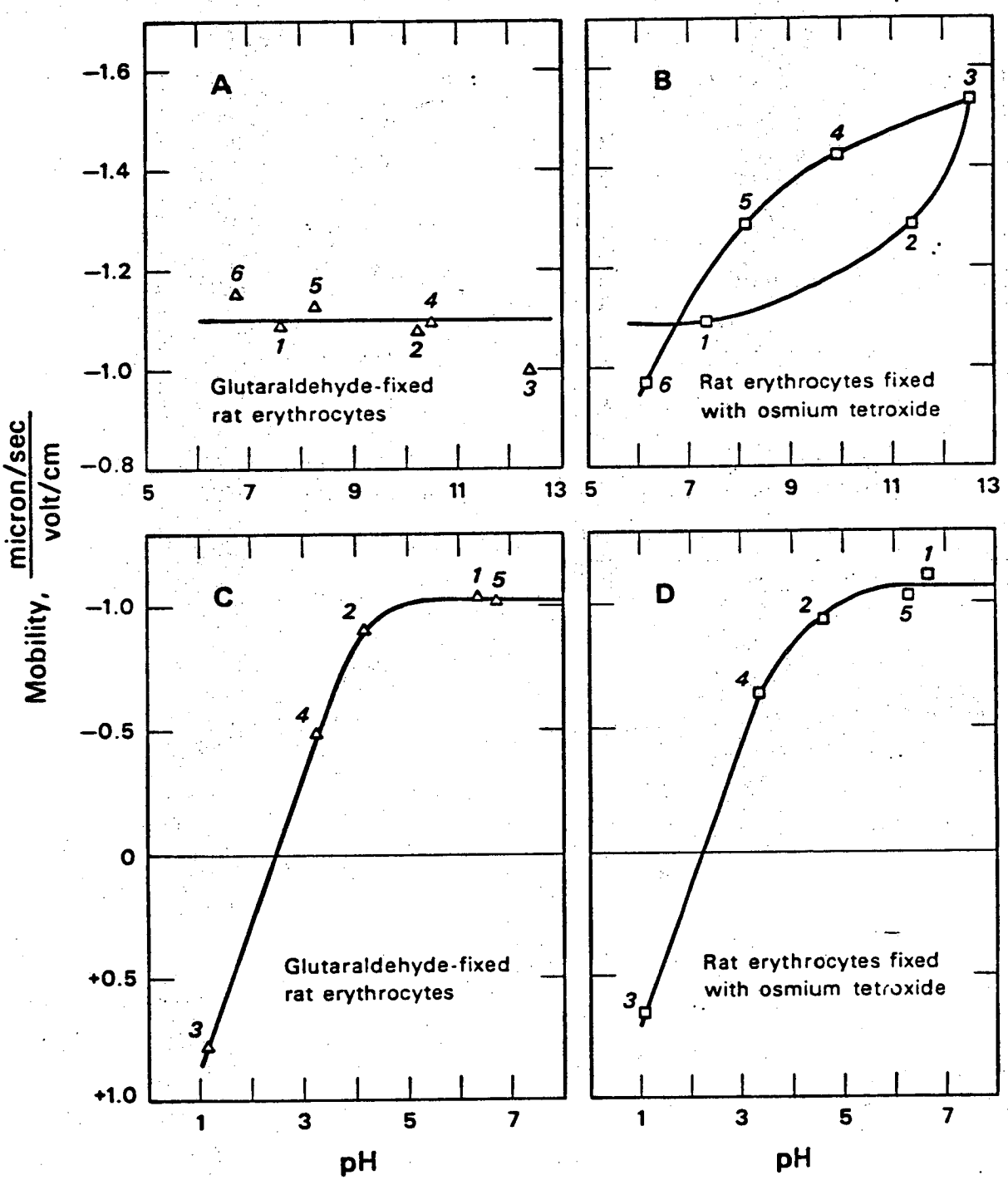


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FIGURE 10

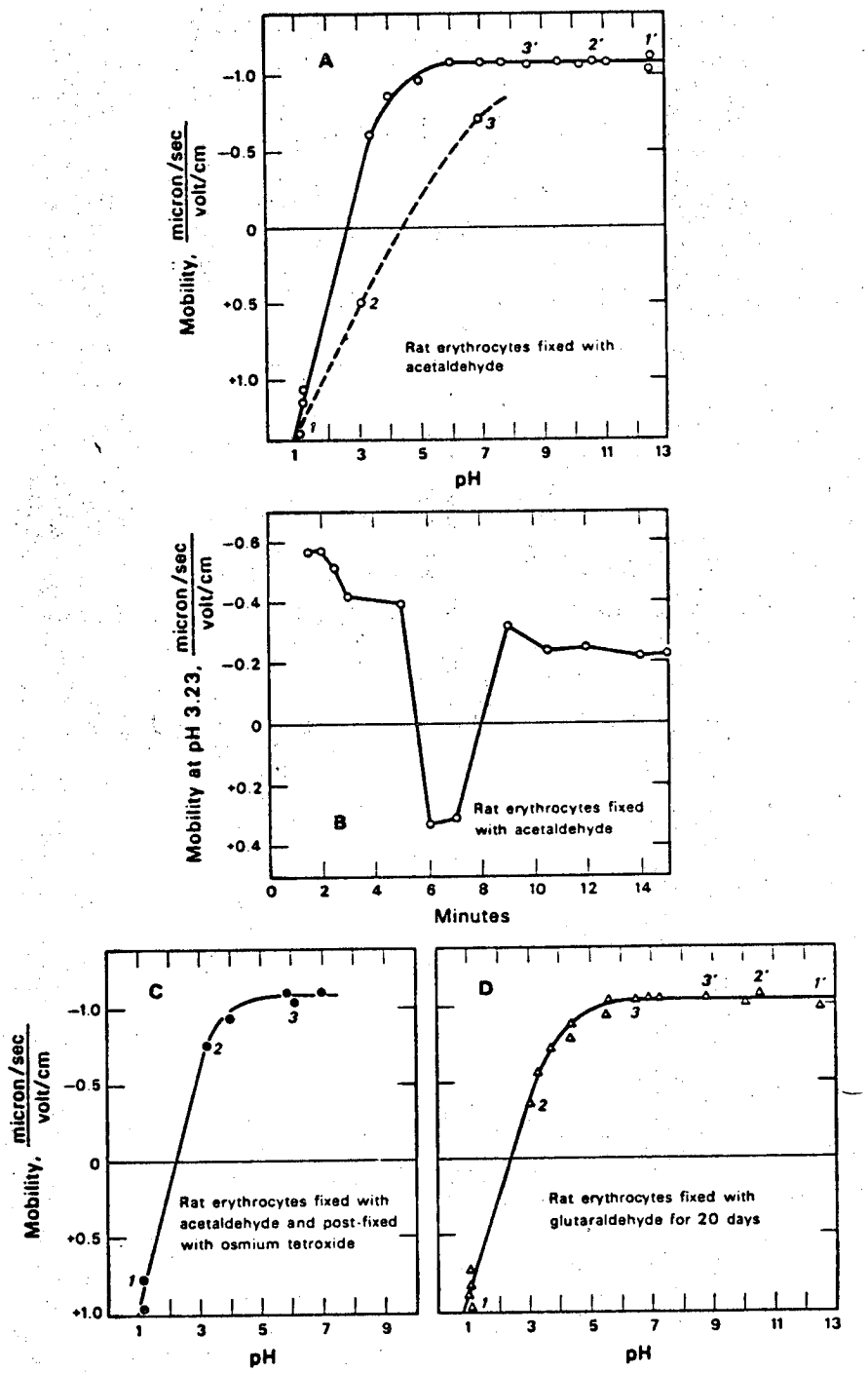

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FIGURE 11

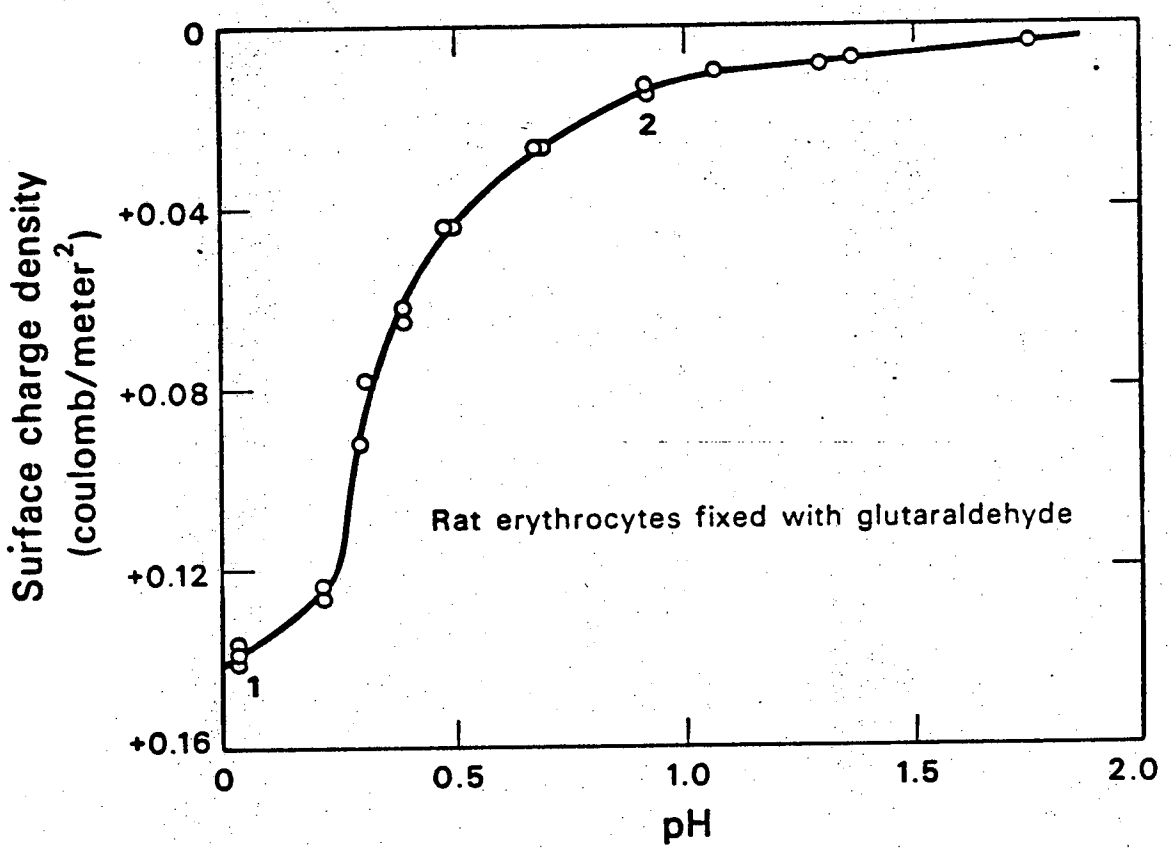

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FIGURE 12

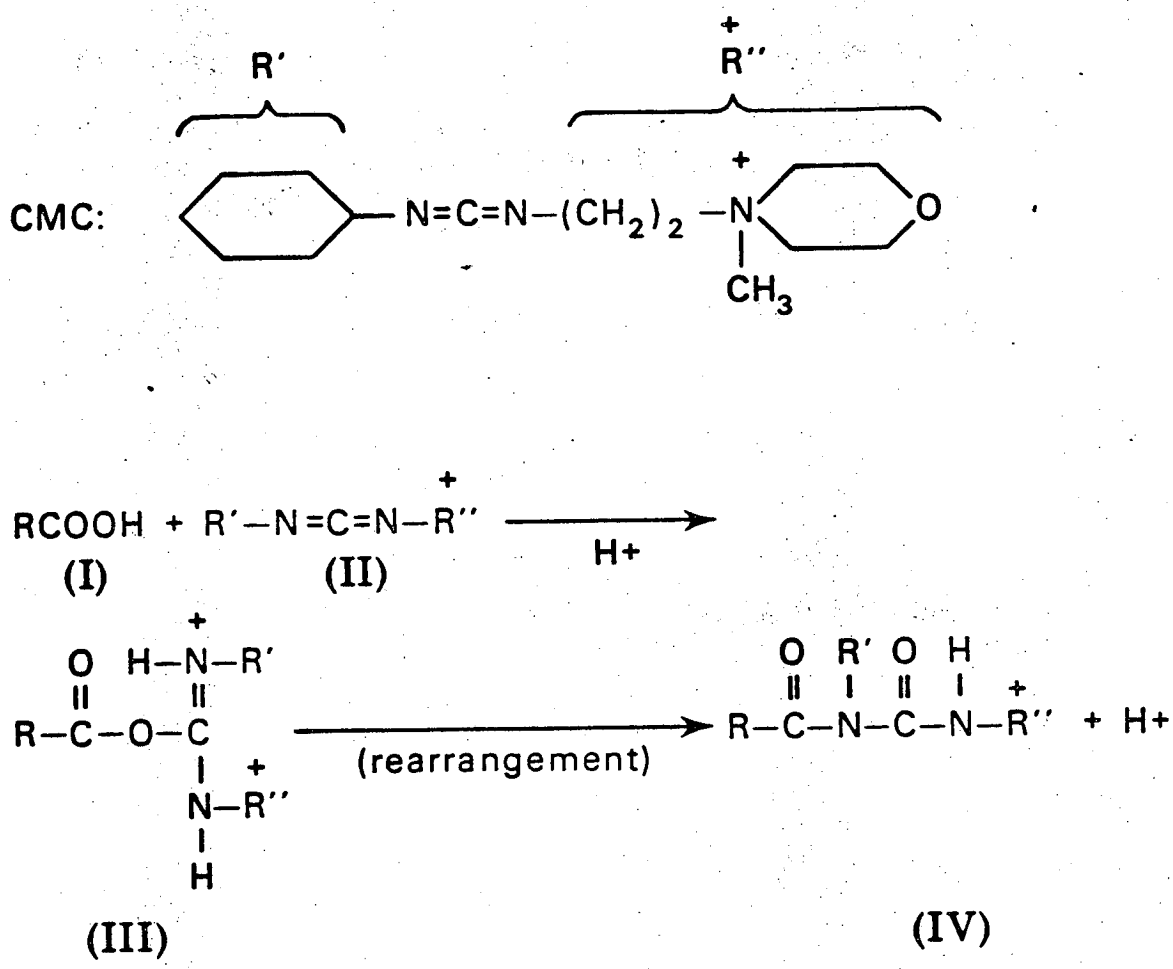

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FIGURE 13

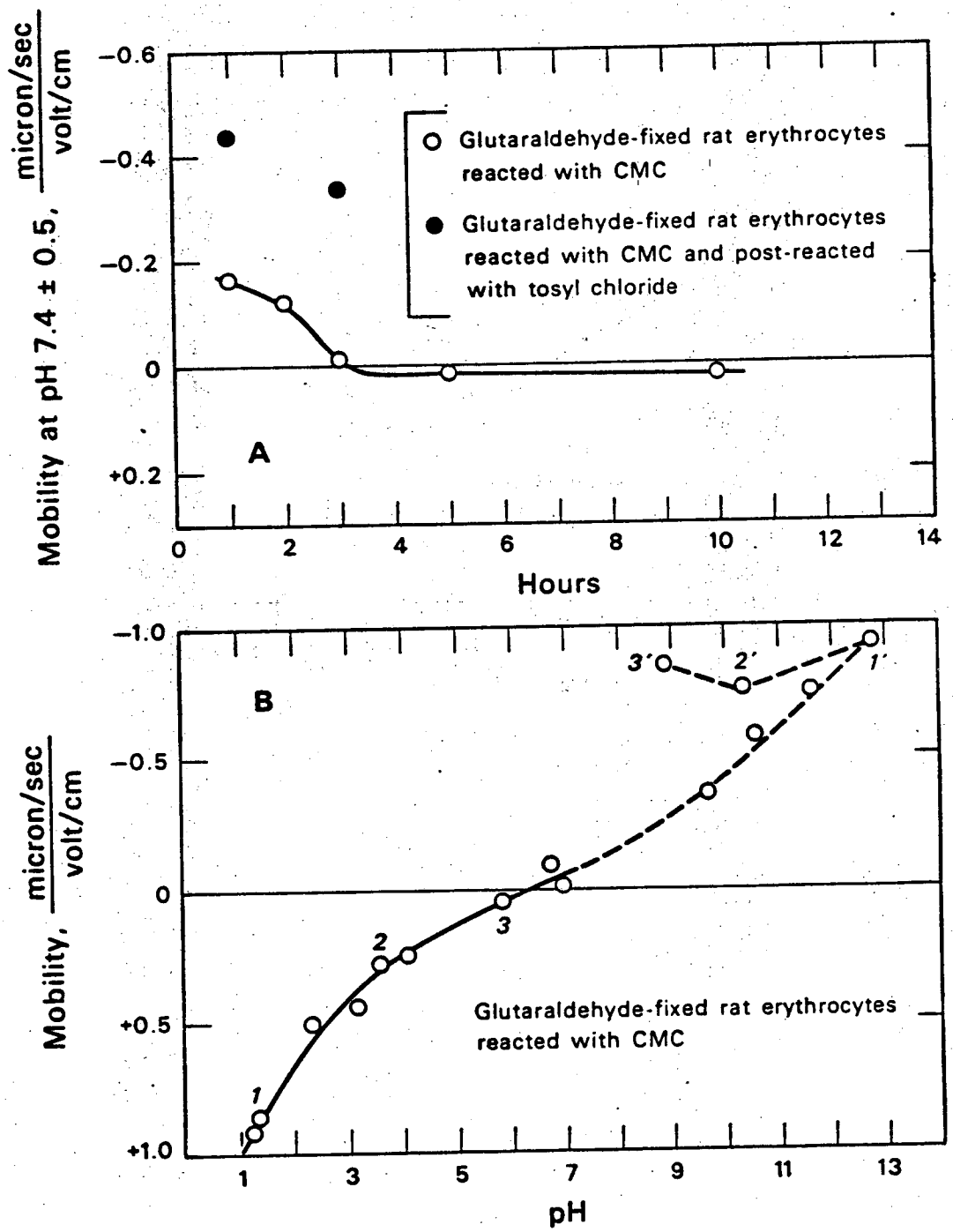

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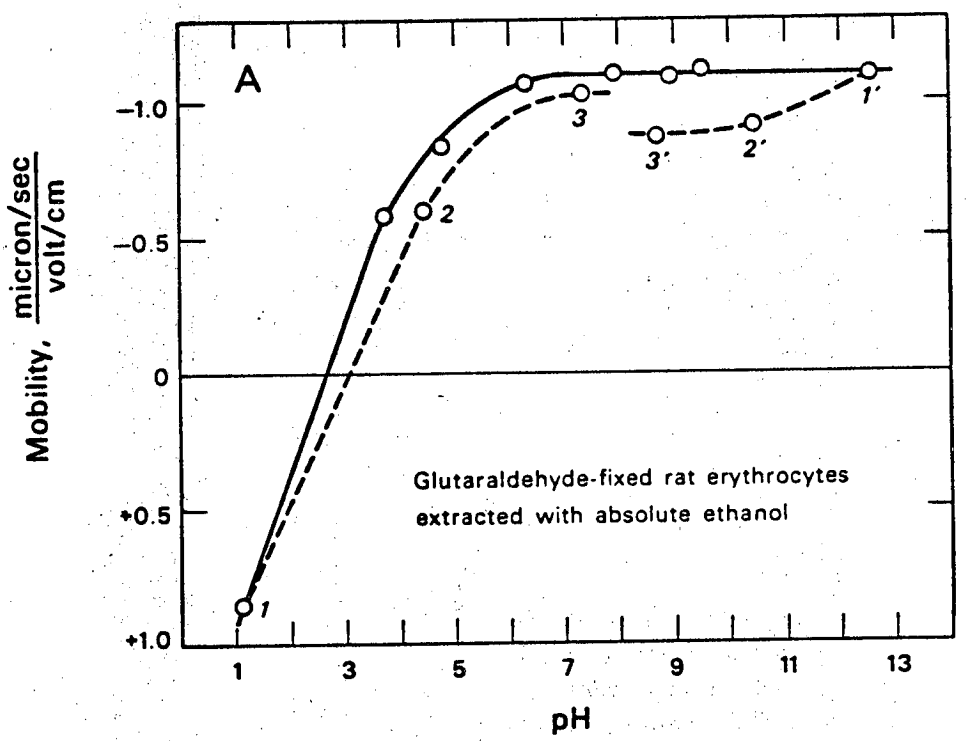
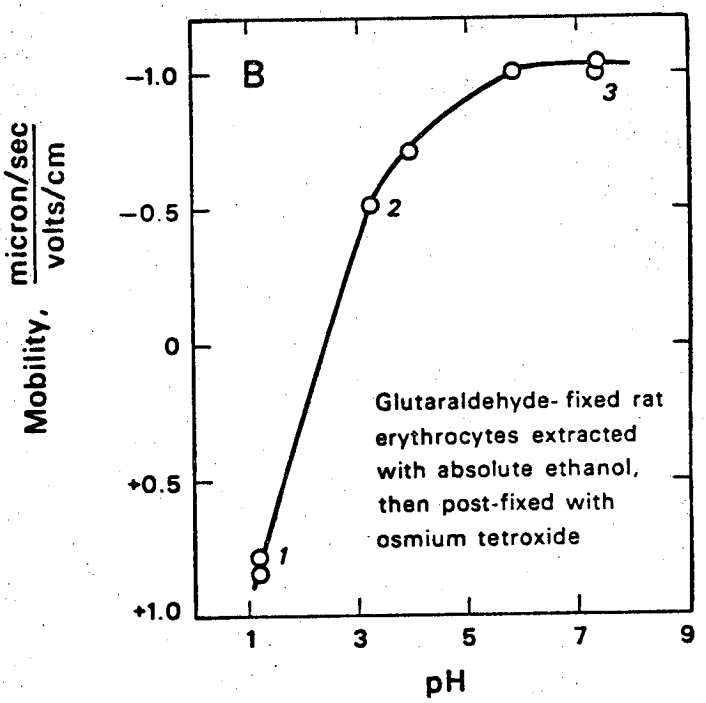
FIGURE 14





DBL 704-5664

FIGURE 15

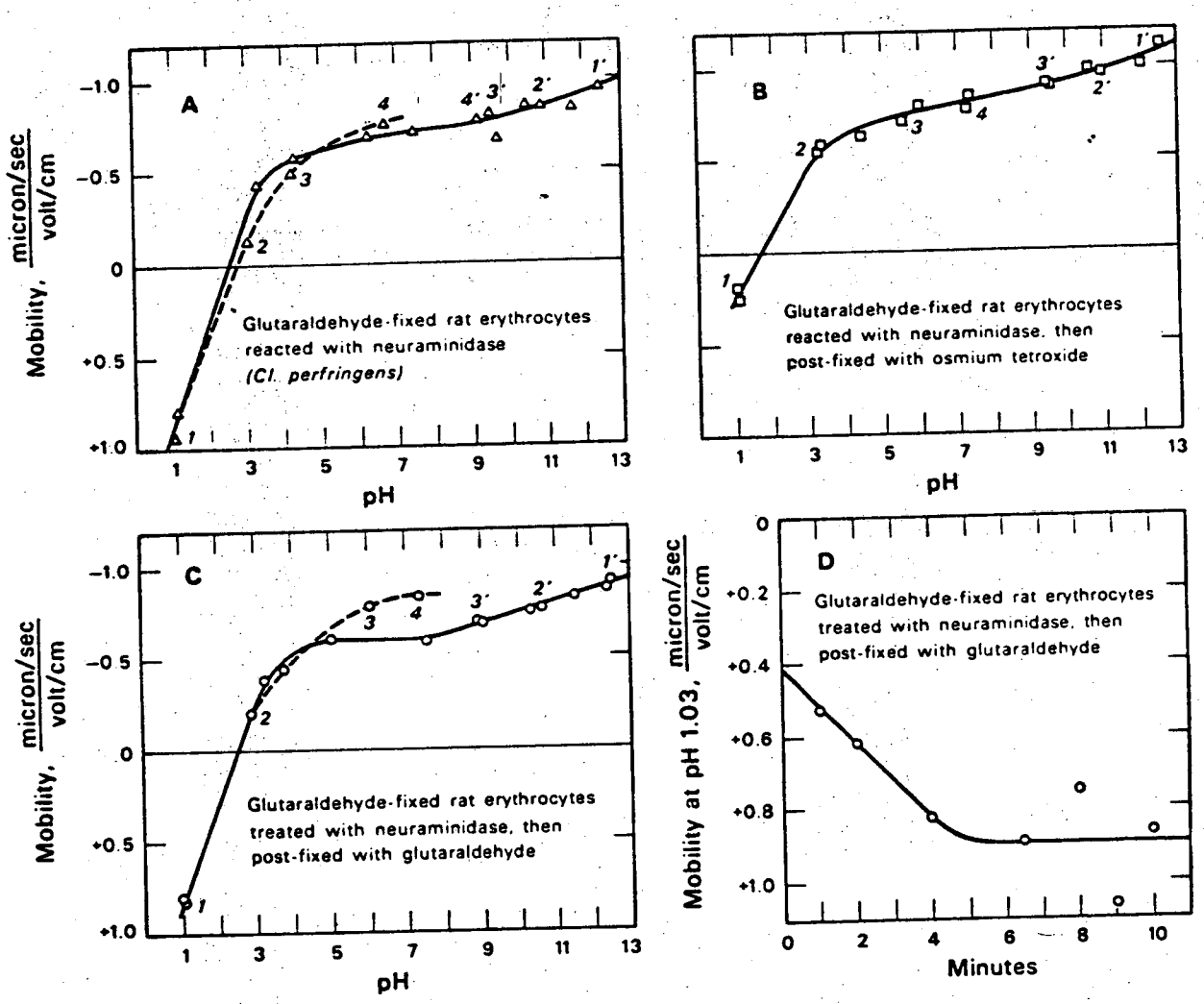


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FIGURE 16

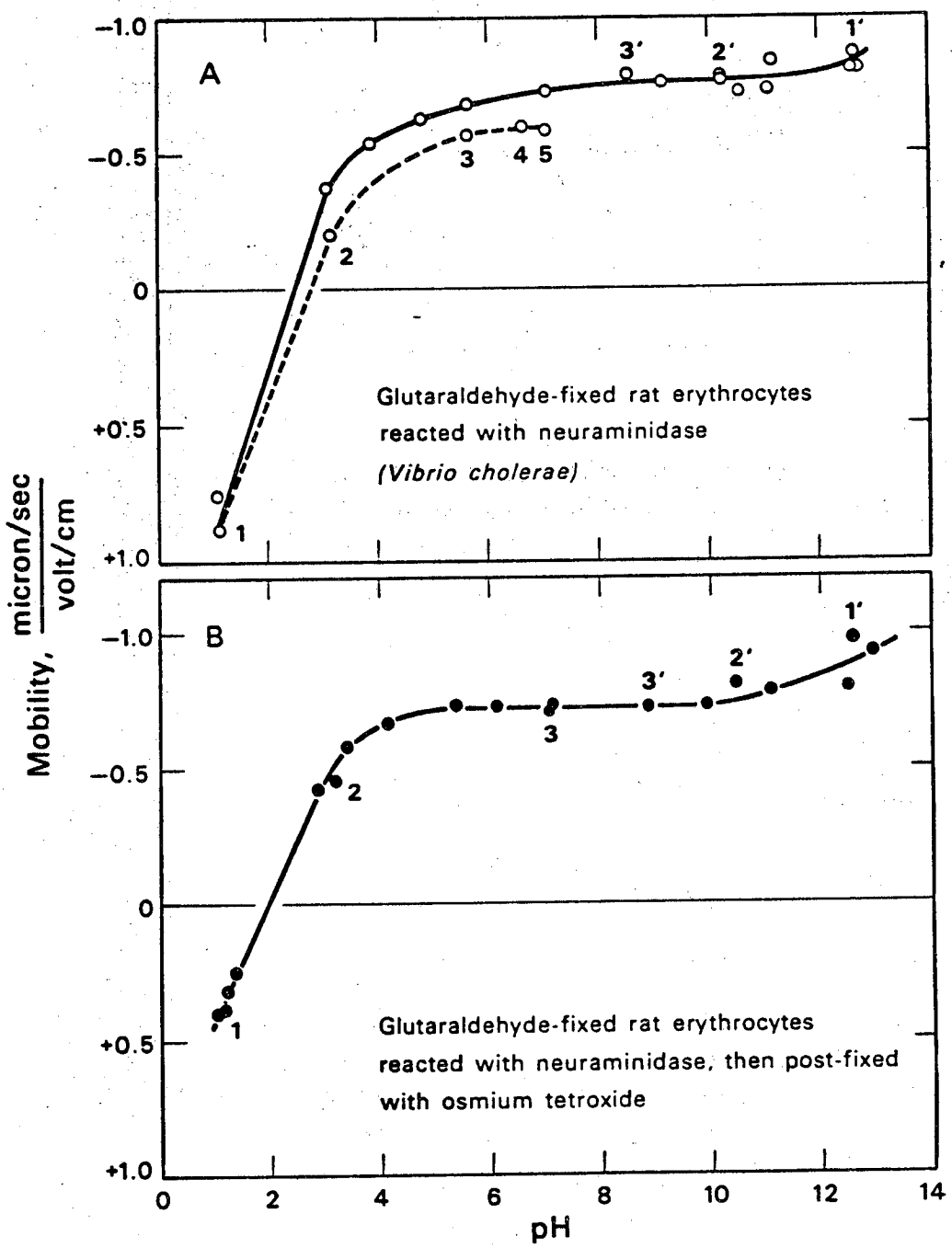

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FIGURE 17

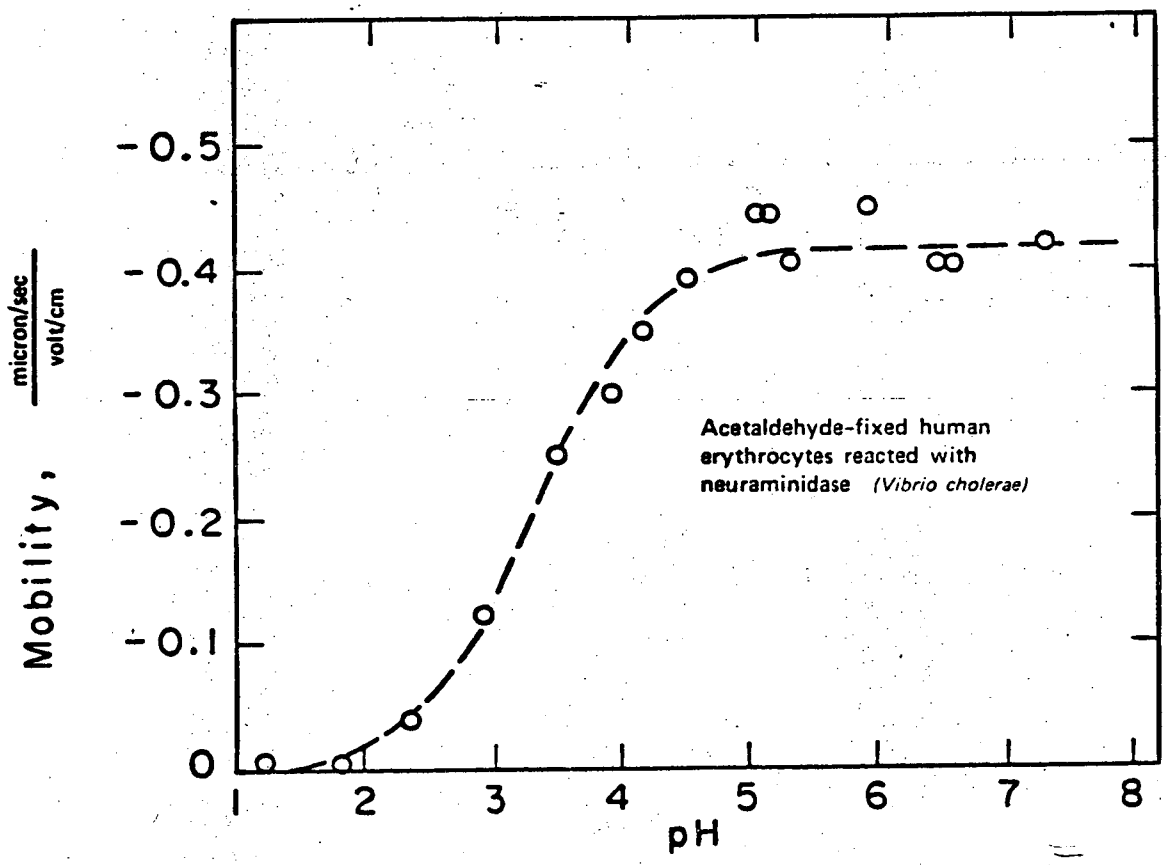

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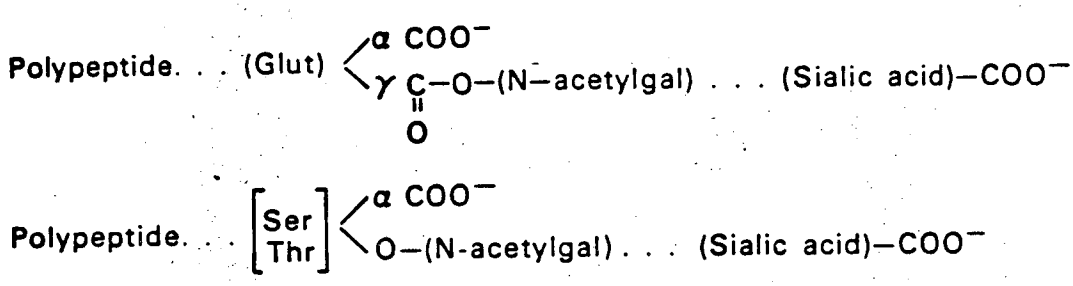
FIGURE 18



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