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UCRL-19430

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ELECTROPHORETIC AND CHEMICAL STUDIES ON THE RAT ERYTHROCYTE MEMBRANE INTERFACE

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(Ph. D. Thesis)

December 1969

AEC Contract No. W-7405-eng-48

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ABSTRACT

By combining microelectrophoretic measurements with specific chemical modification of the rat erythrocyte membrane, an attempt was made to identify all of the acidic and basic groups contributing to the electrophoretic mobility at an ionic strength of 0.145. As a means of characterizing ionogenic groups whose dissociation constants lie outside the pH range in which erythrocytes exhibit reversible electrokinetic properties (approximately pH 4 to 10), mobility studies were performed on cells stabilized by fixation with glutaraldehyde, acetaldehyde, and osmium tetroxide. All of these reagents were found to preserve the surface charge properties observed with unfixed cells over the pH range 4 to 10. Only glutaraldehyde fixation, however, produced a reversible electrophoretic behavior from pH 1 to 13. In order to clarify the character of the positive surface charge observed below pH 2, mobility measurements were performed with glutaraldehyde-fixed cells over the pH range 0 to 2 by varying the ionic strength from 0.145 to 1.0.

As a means of studying the effects of glutaraldehyde and osmium tetroxide on various membrane components, an infrared analysis was performed on rat erythrocyte ghosts fixed with these reagents. On this basis, the membrane protein was found to retain primarily an α-helical and/or random coil configuration upon fixation.

In order to detect the presence near the surface of shear of specific ionic moieties, electrophoresis was performed on rat erythrocytes modified chemically by a variety of procedures. These included reaction with p-toluenesulfonyl chloride, 1,5-difluoro-2,4-dinitrobenzene, methanolic hydrochloride, and a water soluble carbodiimide (1-cyclohexyl-3-(2-morpholinylethyl) carbodiimide metho-p-toluenesulfonate); photooxidation in the presence of methylene blue; ethanol extraction of glutaraldehyde-fixed erythrocytes; and reaction with neuraminidase (both from Vibrio cholerae and Clostridium perfringens), asparaginase (Escherichia coli), glutaminase (E. coli), and carboxypeptidase A.

From the amount of sialic acid released by neuraminidase and the composition of saccharides at the cell surface, it was calculated that a maximum of 66% of the rat erythrocyte surface area is composed of carbohydrate. On the basis of electrophoretic data it was possible to account for approximately 80% of the remaining surface area. From surface charge measurements below pH 1, it was estimated that 25% of the cell surface consists of non-ionogenic regions capable of non-specific hydrogen ion adsorption. Electrophoretically detectable proton binding was also shown to be associated with a set of weakly basic groups having an approximate pK_a of 1.6. These bases are probably side chain amides of protein-bound amino acids, and were calculated from their contribution to the surface charge density to occupy 1% of the total surface area. From mobility studies on neuraminidase-treated erythrocytes, it was calculated that two thirds of the negative surface charge density is associated with anionic groups having a surface pK_a in the range 2.5 to 3.0. These appear to be primarily protein-bound α-carboxylic acids,

although some contribution to the mobility might be made by side chain β- and γ-carboxyls. On the basis of electrophoretic data, it was calculated that these carboxylic acids cover 0.8% of the rat erythrocyte surface. No evidence was found for the presence of strong bases, weak acids, or intermediate strength bases near the surface of shear.

CHAPTER 1. INTRODUCTION

A. The Microelectrophoretic Method and Previous Studies on the Surface Charge Properties of Erythrocytes

Free solution electrophoresis provides at present one of the most powerful techniques for studying the interface chemistry of biological membranes. For this purpose the microscope method of electrophoresis devised by Northrup and Kunitz is most commonly employed.[1,2,3] Following its development, microelectrophoresis was used to obtain considerable information on the surface charge properties of living cell membranes.[4,5] Only during the past two decades, however, has the method been used to fullest advantage through the quantitation of changes in surface charge induced by specific chemical alteration of the membrane interface (e.g., through the action of enzymes). In this manner ionic groups responsible for the membrane surface charge may be determined, the most notable success to date being the discovery of sialyl oligosaccharides at the interface of erythrocytes and many tissue cells.

The first evidence that mucoproteins contribute to the surface charge properties of erythrocytes was the observation by Hanig that the negative electrophoretic mobility of human red cells is greatly reduced through the adsorption and subsequent elution of influenza virus.[6] The resulting red 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 mucoprotein 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.[7,8] Despite these early experimental results relating surface charge to mucoproteins, many electrophoreticists contended until as late as 1958 that the mobility of erythrocytes resulted from the ionized phosphate groups of membrane phospholipids. The reasoning of these workers was not altogether unsound, and serves to demonstrate the potential fallibility of the electrophoretic method. For that reason it is worthwhile to briefly review the physical basis and interpretation of electrophoretic measurements before proceeding with a discussion of recent studies on the interface chemistry of erythrocytes.

The electrophoretic mobility of a cell may result from two forms of charging process: (a) the dissociation of ionic groups within approximately one Debye length* of the hydrodynamic surface of shear, and (b) the adsorption of ions onto non-ionogenic regions of the surface. It has also been argued by Glaeser with complete theoretical justification that an internal net charge could contribute to the electrophoretic mobility.[9] In the case of rat erythrocytes, however, he found no experimental evidence to support this contention. Similarly, there does not appear to be any component of the red cell anodic mobility attributable

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* As used here, the term "Debye length" refers to the electrical double layer thickness, which is equal in magnitude to the reciprocal of the Debye-Hückel constant.

to anion adsorption. This follows from the observation by Heard and Seaman that the mobility of human erythrocytes at neutral pH is identical in sodium salt solutions containing anions with differing hydrated radii.[10] An understanding of the anodic mobility of erythrocytes thus reduces to a determination of the ionic groups at the outer membrane surface through a combination of electrophoretic and chemical measurements. It should be remarked, however, that some portion of the cathodic mobility observed at low pH may result from non-specific proton adsorption at the erythrocyte surface. This possibility will be discussed in Chapter 2.

The three basic approaches to identifying ionic groups responsible for the surface charge are the following: (a) approximate dissociation constants may be calculated from the variation of mobility with pH; (b) a charge-reversal spectrum may be determined for the surface of interest and then compared with spectra for charged molecules of known composition, the latter generally being polyacids or polybases adsorbed to quartz or glass beads for convenience in making microelectrophoretic measurements; (c) the surface may be chemically modified through covalent linkage of ionogenic groups, elution of membrane components, or enzymatic cleavage of specific charged groups. Each of these approaches will now be discussed briefly, with particular emphasis on their limitations. An attempt will also be made here to point out the basis for the misleading interpretations of the electrophoretic properties of erythrocytes, which, as previously mentioned, persisted until the late 1950's.

(a) Calculation of Dissociation Constants from Mobility-pH Curves. At the outset it should be stressed that the electrophoretic mobility of a charged colloid at any pH will be a function of the ionic strength of the suspending medium. This follows from the proportionality of the Debye length to the reciprocal square root of the ionic strength. Consequently, as the ionic strength is lowered, the Coulombic screening of charged groups is reduced. In addition, ionogenic 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. In the case of human erythrocytes, 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 Angstroms.[11] At lower ionic strengths, it appears that predominantly basic groups are unmasked, leading to a reduction in the calculated surface charge.[12] It should be pointed out, however, that at low ionic strength the internal conductivity of an erythrocyte can no longer be completely neglected relative to that of the suspending medium, even though the cells are still very nearly perfect insulators.[13] Failure to correct low ionic strength mobility measurements for particle conductivity would in itself lead to a decrease in the calculated surface charge,[14] a fact that was overlooked by Furchgott and Ponder[11] and Bateman and Zellner.[12] The primary consideration of this thesis, however, is the electrokinetic character of erythrocytes in a physiological medium of ionic strength 0.145 (with an associated Debye length of 8 Angstroms), and consequently no further attention will be given to low ionic strength measurements.

The first attempt to characterize the ionization curve of erythrocytes by electrophoretic means was made by Furchgott and Ponder.[11] They found at physiological ionic strength that the mobility-pH curve for human red cells exhibited the remarkable property of being isoelectric at a pH value between 1.5 and 2.0. (The difference between bulk and surface pH[15] does not, of course, influence the isoelectric point.) This result suggested the presence of strongly acidic groups at the membrane interface. Despite the fact that extracted membrane lipids are isoelectric at pH 2.7, Furchgott and Ponder hypothesized that the anodic mobility of human erythrocytes resulted from phosphate radicals associated with membrane phospholipids. Without being aware of the strongly acidic carbohydrate moieties present in the membrane, this was by no means an unreasonable hypothesis. Bangham, Pethica, and Seaman later repeated this work using sheep erythrocytes and concluded that the mobility-pH curve reflected the presence of one ionic group with a pK_a of 2.3, which they also believed to be the phosphate group of phospholipids.[16] Contrary to Furchgott and Ponder's results with human red cells, however, Bangham and co-workers concluded that the sheep erythrocyte does not have a true isoelectric point, indicating that the membrane interface is purely anionic in character. The reason for this discrepancy is not clear, but later workers have generally concluded that most species of erythrocyte possess an extrapolated isoelectric point at approximately pH 2.[17,18]

(b) Charge-Reversal Spectra. The determination of charge-reversal spectra for sheep erythrocytes and other blood cells was undertaken by Bangham, Pethica, and Seaman, who measured the charge-reversal concentrations of several polyvalent cations.[16] By comparing their results

with those of Kruyt on phosphatides and carboxylic acids,[19] they concluded that the spectrum of sheep erythrocytes most closely resembled that of phosphate radicals. This conclusion was later shown to be incorrect, and demonstrates the limitations inherent in the use of charge-reversal spectra.

(c) Chemical Alteration of the Membrane Interface. Chemical modification of membrane surfaces may be accomplished by three basic methods: (i) specific covalent alteration of ionogenic groups, (ii) elution of membrane components by organic solvents, and (iii) enzymatic cleavage of charged groups. The first two methods will be considered in later sections of this thesis; the third has played an important role in elucidating the interface chemistry of erythrocytes and will now be discussed in some detail.

At approximately the same time as Hanig's[6] experiments on viral hemagglutination and surface charge reduction, Burnet and Stone[20,21] 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 that RDE produced a large reduction in the electrophoretic mobility of human erythrocytes.[22] Several years later Gottschalk 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.[23,24] In 1958 Klenk and Uhlenbruck[25] demonstrated conclusively that the product split from red cell membranes by neuraminidase was sialic acid and the suggestion was

put forth by Klenk[26] that the carboxyl group of this molecule was responsible for the surface charge measured electrophoretically. The identification of the enzymatically-split product as sialic acid was of considerable importance since earlier workers had ascribed the reduction in electrophoretic mobility solely to the production of basic groups at the membrane interface.[27] This conclusion had been based on a titration study of neuraminidase-treated urinary sialyl mucoprotein.[28] After treatment the mucoprotein possessed cationic groups with a pK_a of 11.2, but since neuraminidase is specific for the glycosidic bond between sialic acid and its adjacent sugar,[23,24] 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. The exact composition of the oligosaccharide and peptide moieties to which sialic acid is generally bound has not been clarified (compare, for example, Seaman and Cook[29] and Winzler et al.[30]). Nevertheless, a considerable amount of evidence now indicates that sialic acid usually exists as the terminal group of peptide-bound oligosaccharides and is primarily located at the outer membrane surface. Only the work on erythrocytes will be discussed here, the most notable being that of Cook, Heard, and Seaman[31] and Eylar, Madoff, Brody, and Oncley.[32]

Briefly summarizing, the above authors found that neuraminidase reduces the mobility of various species of erythrocyte from 20 to 95 percent. In general the sialic acid released is considerably greater (by roughly a factor of two) than the amount that would be required to account for the surface charge reduction calculated from the equations

of electrophoresis. Several possible explanations have been put forth to explain this discrepancy.[9,32]

First, it is conceivable that the appropriate "electrophoretic radius" might be considerably smaller than the physical radius of the cell. In this regard Eylar and co-workers found that using an electrophoretic radius of approximately 20 Angstroms reconciled the apparent difference between sialic acid removed and the charge reduction calculated from electrophoretic mobility.[32] There does not, however, appear to be any theoretical justification for the use of an electrophoretic radius other than that of the cell.

Secondly, the action of neuraminidase may induce changes in the ionic character of the erythrocyte interface independent of the removal of sialic acid. This might result either from architectural rearrangement of the membrane or from the fact that the surface of shear may reside several Angstroms closer to the membrane matrix after removal of the outermost layer of sialic acid.

A third possible reason for the calculated charge reduction being less than the sialic acid released is the neglect of surface conductance corrections.[33] At physiological ionic strength, this correction should result in a small increase in the calculated surface charge density. In the case of Pyrex glass beads, the increase is negligible at high ionic strength.[34] With bacteria, however, the increase in zeta potential is approximately forty percent at physiological ionic strength.[35] It thus appears that for biological surfaces partial short-circuiting of the electric field in the double layer persists even at high ionic strength. In the case of erythrocytes, the importance of correcting for surface conductance needs to be fully investigated,

and may possibly lead to a somewhat larger estimate of charge reduction upon neuraminidase treatment.

A fourth possible explanation for the difference between sialic acid removed and calculated charge reduction is that a large proportion of the sialic acid released by neuraminidase may be buried in the membrane matrix or located at the inner surface. This does not seem plausible, however, on the basis of two arguments. First, electron microscopic studies on isolated plasma membranes from rat liver cells indicate that sialic acid is located at the outer surface.[36] No evidence was found for the presence of this molecule at the inner surface. In order to demonstrate 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, a fact confirmed by the absence of staining in neuraminidase-treated membranes. Unfortunately, the application of this staining procedure to erythrocyte ghost membranes has not as yet been reported in the literature. Secondly, on the basis of experiments to be described in Chapter 2, the quantitative yield of sialic acid upon treatment of glutaraldehyde-stabilized rat erythrocytes with neuraminidase is closely comparable to that from unfixed cells. Although the molecular weight of purified neuraminidase has not been determined, it is unlikely that any protein molecule could penetrate the rigid structure of a glutaraldehyde-fixed membrane.

Despite these arguments against an internal location for the sialic acid, it is conceivable that the carbohydrate coat of erythrocytes extends considerably deeper than 8 Angstroms from the surface of shear. Glaeser and Mel have calculated that the sialyl oligosaccharide

chains contain roughly five sugar molecules.[37] This, however, is only an average value based on overall composition, and it is quite possible that large variations occur between individual chains. If in fact this is the case, then a considerable number of the terminal sialic acid residues might be located at positions greater than one Debye length from the surface of shear, and thereby contribute only slightly or not at all to the electrophoretic mobility.

A fifth possible reason for the calculated electrophoretic charge reduction being less than the amount of sialic acid released by neuraminidase is the assumption that the membrane surface is impenetrable to counterions. Haydon has shown that charge calculations which assume a rigid surface may be low by as much as a factor of two for a cell interface that is porous and allows free diffusion of counterions.[38] Unfortunately, it is not known to what extent the outer surface of the erythrocyte membrane is penetrable to counterions, so that Haydon's equations cannot at present be applied in surface charge calculations. It seems likely, however, that porosity of the membrane interface may account at least in part for the discrepancy between sialic acid removal by neuraminidase and calculations of charge reduction based on the assumption of a rigid surface.

To summarize, electrophoretic and chemical studies reported to date have supported a model of the erythrocyte membrane interface as composed primarily of carbohydrate with terminal sialic acid residues serving as major contributors to the electrophoretic mobility. Chemical analysis of the sialic acid released upon neuraminidase treatment yields an amount roughly twice that predicted from reduction in the electrophoretic mobility. The basis for this difference has not been clarified

experimentally, but may result from several factors: (a) the appearance of new ionic groups at the surface as a result of physical alteration of non-carbohydrate portions of the membrane; (b) neglect of surface conductance corrections; (c) a non-uniform spatial distribution of terminal sialic acid molecules relative to the surface of shear; and (d) penetrability of the membrane interface to counterions.

B. Purpose and Direction of the Present Research

In addition to the need for further clarification of the role of sialic acid in contributing to the surface charge properties of erythrocytes, two other basic questions remain to be answered. First, the origin of the large residual anodic mobility following neuraminidase treatment has not been thoroughly investigated by previous workers. Secondly, no systematic investigation has been made on the contribution of basic groups to the surface charge. In particular, no attempt has been made to identify the origin of the large cathodic mobility observed below pH 2. It is an approach to these questions that constitutes the primary objective of this thesis.

Broadly speaking, the method of attack consists 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 only stable against hemolysis from pH 4 to 10, 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 chemical 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. The results of this study are presented in Chapter 2, and their implications with regard to the chemistry of the rat erythrocyte surface are fully discussed in the third chapter.

CHAPTER 2. CHEMICAL MODIFICATION OF THE RAT ERYTHROCYTE MEMBRANE: METHODS AND RESULTS

A. Surface Charge Properties of Intact Rat Erythrocytes; Stabilization Against Hemolysis at Extreme pH by Fixation with Glutaraldehyde, Acetaldehyde, and Osmium Tetroxide

The early observations of Furchgott and Ponder on the surface charge properties of human erythrocytes showed that below approximately pH 4 the mobility changes as a function of time.[11] As a means of circumventing this difficulty, Heard and Seaman stabilized erythrocytes by fixation with formaldehyde and acetaldehyde.[39] In this manner they were able to extend mobility studies to pH values slightly below 3, and obtained an extrapolated isoelectric point of 2.4. Glaeser and Mel later showed that fixation with osmium tetroxide could be used to stabilize rat erythrocytes at pH values as low as 1, and directly demonstrated a positive charge character below pH 1.6.[18] Following this work, Haydon and Seaman re-examined the electrophoretic properties of acetaldehyde-fixed human erythrocytes, and concluded that they are polyanionic with no positive mobility branch at low pH.[40] It would appear, therefore, that acetaldehyde reacts with the basic groups responsible for the positive surface charge.

At pH values above 9.5, human erythrocytes are also electrokinetically unstable at physiological ionic strength.[10] Reagents which are reactive with strong bases, e.g., p-toluenesulfonyl chloride[17] and acetaldehyde,[39] do not affect the mobility of human erythrocytes at neutral pH, thereby demonstrating the absence of such groups near the surface of shear. Glaeser and Mel, however, found that rat erythrocytes

fixed with osmium tetroxide exhibit an increase in anodic mobility above pH 11.[18] They concluded that this must result from the dissociation of weakly acidic groups, and proposed that these might be hydroxyl groups associated with sialic acid molecules.

The microelectrophoretic studies described in the following paragraphs were designed to clarify the question of whether a positive charge branch is present in the mobility-pH curve, and also to examine further the existence of weakly acidic dissociating groups near the surface of shear. The fixatives used for stabilization at extreme acidic and alkaline pH were glutaraldehyde, acetaldehyde, and osmium tetroxide. The reactivity of aldehydes with proteins has been well characterized, the primary reaction being a crosslinking of amino groups.[41,42,43] Being a bifunctional reagent, glutaraldehyde has a considerably shorter reaction time than acetaldehyde, and was therefore utilized more extensively in the studies reported below. With osmium tetroxide the reaction time was limited to one minute, so that the primary stabilizing effect was a diester crosslinking of unsaturated fatty acids associated with membrane lipids.[44,45] There is some evidence, however, that oxidation reactions can also occur with proteins over very short periods.[46,47] As a means of assessing the effects of glutaraldehyde and osmium tetroxide on various membrane components, infrared studies were performed using ghosts fixed with these reagents. The results are described in a later part of this section.

Materials and Methods

Blood was drawn from the vena cava of female Sprague-Dawley rats anesthetized with ether, and immediately diluted with twenty volumes of

0.145M NaCl buffered to pH 7.4 with 3×10^{-4}M $NaHCO_3$. This buffer system will be referred to in the following as Standard Buffer. Human blood was obtained by puncture of the forearm and handled in the same manner. Erythrocytes were then spun down by centrifugation at 1100 x g for three minutes, and the supernatent and buffy coat removed by aspiration. The cells were washed three times by suspending them in twenty volumes of Standard Buffer and centrifuging at 1100 x g for three minutes. When erythrocytes were prepared for fixation with glutaraldehyde or osmium tetroxide, the buffer system used in the washing procedure was Sorensen's 0.067M phosphate buffer at pH 7.4. Rat erythrocyte ghosts were prepared by the method of Dodge, Mitchell, and Hanahan.[48] In order to stabilize the ghosts against elution of membrane components, one millimolar $MgCl_2$ was added to the media used for lysis and washing.[49] The resulting ghosts retained a pink color. This was reduced, but not completely removed, by three washes with distilled water.

Fixatives used in these studies were obtained from the following suppliers: osmium tetroxide (0.5 gm vials), Engelhard Industries, Newark, New Jersey; glutaraldehyde (25% wt/vol in water) and acetaldehyde (78.7% wt/vol in water), J. T. Baker Chemical Company, Phillipsburg, New Jersey. Glutaraldehyde was distilled twice by heating at 98° to 100° C and collecting the vapor at room temperature. The distillate was assayed by osmometry and spectrophotometry using the method of Fahimi and Drochmans.[50] The concentration following the second distillation usually ranged from 8 to 10% wt/vol. Acetaldehyde boiling at room temperature (approximately 21-24° C) was collected into a bath at acetone/dry ice temperature and assayed osmometrically. On this basis the distillate had the same purity as the commercial reagent.

Erythrocytes were fixed with glutaraldehyde by mixing equal volumes of a 5% vol/vol cell suspension in pH 7.4 Sorensen phosphate buffer with a 5% wt/vol glutaraldehyde solution in phosphate buffer. Fixation was allowed to proceed for ten minutes at room temperature, after which the cells were pelleted by centrifugation at 1100 × g for three minutes. In order to remove residual fixative, the cells were washed three times by suspension in twenty volumes of Standard Buffer followed by centrifugation at 1100 × g for three minutes. The same procedure was used for fixation with osmium tetroxide except that the cell suspension was 10% vol/vol and the fixative was 1% wt/vol. With osmium tetroxide the reaction time was one minute at room temperature. Fixation with 2% acetaldehyde was for three weeks at 4° C and followed the procedure of Haydon and Seaman.[40] Ghosts were fixed in the same manner as intact erythrocytes.

Microclectrophoresis was performed using the apparatus described by Glaeser with the exception of a few minor changes in the electrode assembly.[9] A rectangular migration chamber (Arthur Thomas Company, Philadelphia, Pa.) was employed in the lateral position. At each of the two stationary levels the velocity of five cells was measured in both directions. The velocity measurements were then averaged and divided by the electric field strength to obtain the electrophoretic mobility. The field strength was calculated from the conductivity, chamber cross-sectional area, and current, and in all experiments was roughly 6 volts/cm. Standard Buffer was used in all measurements, and the pH of a cell suspension adjusted by the addition of 0.145N NaOH or 0.145N HCl, thereby maintaining the ionic strength at 0.145.

Infrared studies were conducted with a Perkin-Elmer Model 421 apparatus. Two or three milligrams of ghosts were washed three times in

distilled water, then layered on a silver chloride plate coated with silver sulfide (Harshaw Chemical Co., Cleveland, Ohio) and allowed to dry on a 40° C plate. Care was taken to protect the silver chloride plate from both light and direct contact with the metal heating pad.

Results

(1) Surface Charge Properties of Unfixed, Glutaraldehyde-fixed, and Osmium Tetroxide-fixed Erythrocytes. The mobility-pH curves for unfixed, glutaraldehyde-fixed, and osmium tetroxide-fixed rat erythrocytes are shown in Figures 1A and 1B.* With both fixatives the mobility over the pH range 4 to 10 was found to be the same as that for unfixed cells. In addition, both curves exhibit an isoelectric point between pH 2.1 and 2.4. At high pH, however, glutaraldehyde-fixed erythrocytes show no evidence for dissociating groups, whereas cells fixed with osmium tetroxide exhibit an increase in negative mobility above pH 11 in agreement with the previous observation of Glaeser and Mel.[18] 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 Figures 1C and 1D. 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. 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. An alternative which cannot be ruled out on

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* Figures are located at the end of the text, beginning on p. 77.

this basis, however, is the possibility that glutaraldehyde may be reactive with these groups, thereby removing their ionogenic character.

A direct method for determining whether a charged group appears at the surface of shear only under extreme alkaline or acidic conditions is to study the reversibility of the surface charge properties as a function of pH. The results of such a study are shown in Figure 2. Cells were incubated successively at the pH values denoted by numerals. The manner in which this was performed was to suspend erythrocytes at a pH value denoted by the numeral 1 and record the electrophoretic velocity. The cells were then centrifuged at 1100 x g for three minutes. The supernatent was removed and its conductivity measured in order to determine the field strength at that particular pH. The packed cells were then resuspended at a pH value denoted by the numeral 2, and the mobility measured in the same manner. This procedure was repeated until the cells were finally returned to a pH value near neutrality.

It is clear from Figures 2A and 2B that the surface charge properties of 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 (Figures 2C and 2D), indicating that the positive branch in the mobility-pH curve is not an artifact resulting from membrane disruption. In

addition, human erythrocytes fixed with either glutaraldehyde or osmium tetroxide were found to exhibit a large cathodic mobility at pH 1.

(2) Infrared Studies on the Effects of Fixation with Glutaraldehyde and Osmium Tetroxide. As a means of assessing the effects of fixation upon various membrane components, an infrared analysis was performed on rat erythrocyte ghosts, ghosts extracted with a 2/1 mixture of chloroform/methanol for twenty minutes at room temperature, and ghosts fixed with glutaraldehyde and osmium tetroxide. The results are shown in Figure 3 and tentative band assignments are made in Table I.* Except for the assignments at 1380-90 and 965 cm^{-1}, the spectral analysis of unfixed ghosts is essentially in agreement with that made by Wallach and Zahler for Ehrlich ascites cell plasma membranes.[51] Two aspects of the spectra of ghosts fixed with glutaraldehyde and osmium tetroxide are particularly worth noting: (a) The Amide I and II bands associated with the conformation of membrane proteins are not shifted in frequency as a result of fixation with either glutaraldehyde or osmium tetroxide. It thus appears that nearly all of the membrane protein retains either α-helical or random coil configuration upon fixation, in contrast to the conclusions of Lenard and Singer based on circular dichroism studies.[52] These workers found a 22% loss of helicity upon glutaraldehyde fixation, and a 63% loss upon fixation with osmium tetroxide. (b) Following fixation with either glutaraldehyde or osmium tetroxide, the absorbance at 720 cm^{-1} is abolished (see Figures 3C and 3D). Chapman, Kamat, and Levene[53] contend that this resonance is associated with the rocking mode of four or more connected methylene groups organized in an all-trans planar configuration. Using human erythrocytes, Chapman and co-workers found that the absorbance associated with this vibrational mode was quite small at room temperature and concluded that methylene groups of lipid hydrocarbons exhibit

* Tables are located at the end of the text, beginning on p. 74.

less all-trans planar character in red cell membranes than is found for phospholipids in a liquid crystalline organization. From Figure 3A, it is clear that rat erythrocyte membranes also exhibit only a small absorption at 720 cm^{-1}. The fact that this resonance is abolished upon reaction of the membranes with glutaraldehyde and osmium tetroxide suggests that fixation may result in the absence of any all-trans planar ordering of fatty acid methylene groups. One difficulty with this interpretation, however, is introduced by the fact that fixation appears to result in some loss of lipid from the membrane. From Figures 3C and 3D, it is clear that the lipid C = O stretching resonance at 1740 cm^{-1} is considerably reduced following reaction of erythrocytes with either glutaraldehyde or osmium tetroxide. This apparent loss of lipid upon fixation might be sufficient to cause a disappearance of the weak band at 720 cm^{-1}. Further experiments are needed to clarify this matter.

(3) Surface Charge Properties of Acetaldehyde-fixed Erythrocytes. Haydon and Seaman have reported that fixation with 2% acetaldehyde for three weeks yielded electrokinetically stable human erythrocytes which gave no indication of a positive surface charge below pH 2.[40] This result is directly contrary to that obtained with rat and human erythrocytes following either glutaraldehyde or osmium tetroxide fixation, and suggests that acetaldehyde might be reactive with basic groups responsible for the positive mobility at low pH. In order to examine this further, several attempts were made to stabilize rat erythrocytes by acetaldehyde fixation. In all cases, the results were similar to those shown in Figures 4A and 4B. 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 direct contradiction to the results of Haydon and Seaman.

As a means of determining whether acetaldehyde is reactive with the groups responsible for the cathodic mobility below pH 2, erythrocytes were treated with acetaldehyde and subsequently fixed with osmium tetroxide. The results are shown in Figure 4C. Erythrocytes handled in this manner are electrokinetically stable at low pH and retain a large positive mobility at pH 1. This result was found to be reproducible with five different preparations of rat erythrocytes. In addition, fixation with 2.5% glutaraldehyde for twenty days at 4° C yielded electrokinetically stable erythrocytes having a large positive mobility at low pH, a result shown in Figure 4D. It thus appears that both mono- and bifunctional aldehydes fail to react over long periods with the basic groups responsible for the positive surface charge.

(4) Electrophoretic Studies on Glutaraldehyde-fixed Erythrocytes at Low pH. On the basis of surface charge properties presented in Figure 1, it is not possible to estimate either the number of basic groups contributing to the positive mobility or their pK_a. This results from the fact that surface charge measurements cannot be extended below pH 0.9 while maintaining the ionic strength at 0.145. In order to overcome this difficulty, mobility studies were made at pH values between 0 and 1 by suspending glutaraldehyde-fixed cells in concentrated 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 (aqueous solutions at 25° C):[33]

$$\sigma = 0.1171\ C^{\frac{1}{2}} \sinh 0.25U$$

Here σ is the surface charge density in coulombs per square meter, C is the molar concentration of uni-univalent electrolyte, and U is the electrophoretic mobility expressed in microns/sec/volt/cm. This equation is

valid for a charged particle whose radius of curvature is at least 300 times greater in magnitude than the double layer thickness, a condition satisfied by rat erythrocytes in the present experiments.

A plot of the surface charge density from pH 0 to 2 is shown in Figure 5. It is clear that an inflection occurs at approximately pH 1, with a large increase in positive charge density appearing at lower values of pH. Between pH 1 and 2 the surface charge density has a logarithmic appearance characteristic of proton binding to one type of weakly basic group, with a maximum contribution to the charge density being +0.009 coulomb per square meter. The pK_a of this group appears to be approximately 1.6. Since the rat erythrocyte zeta potential does not exceed 15 millivolts over the pH range 1 to 2, the difference between bulk and surface pH[15] will not influence this value of the pK_a by more than 0.25 pH units.

Below pH 1 the surface charge density was found to give a reasonably good fit to an adsorption isotherm of the form[33]

$$\sigma = \frac{\sigma_0 K (H^+)_b}{1 + K (H^+)_b}$$

Here σ_0 is the surface charge density when all of the potential binding sites are occupied by protons, K is the binding constant in units of liters per mole, and $(H^+)_b$ is the bulk molar concentration of hydrogen ions. By making a least squares fit of the measured surface charge density over the pH range 0 to 1, the values obtained for K and σ_0 were, respectively, 0.23 ± 0.02(SD) liter per mole and $+0.331 \pm 0.099$ (SD) coulomb per square meter. This value of σ_0 represents the extrapolated surface charge density at infinite hydrogen ion concentration, i.e. when the ionic strength is infinite and the corresponding Debye length is zero. As shown explicitly

in Figure 5, the proton binding exhibited over the pH range 0 to 1 is reversible, thereby indicating that the large increase in positive surface charge does not result from structural rearrangement of the membrane under acidic conditions. From the adsorption isotherm it can also be shown that the surface charge density contributed by these proton-binding groups above pH 1 is small compared to that which is measured electrophoretically. They are therefore distinct from the weakly basic groups of pK_a 1.6.

Using this information, it is worthwhile to speculate on the character of membrane components responsible for the increase in positive surface charge below pH 1. The fact that the charge density is characterized by one binding constant indicates that it may arise through proton binding to a single type of weakly basic group. Alternatively, it may be attributable to non-specific hydrogen ion adsorption onto non-ionic regions of the cell surface. Unfortunately, it is not possible to distinguish between these two alternatives on the basis of surface charge measurements alone. The second proposed charging mechanism, however, is favored by the fact that the contribution of these proton-binding groups to the surface charge density is approximately thirty times as great as that made by any other electrophoretically detectable membrane component. This suggests that below pH 1 the electrical double layer is effectively swamped with hydrogen ions at positions corresponding to non-ionic sites on 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. The maximum possible adsorbed charge is + 0.331 coulomb per square meter, or 2.07×10^{18} hydrogen ions per square meter. Taking the mean corpuscular diameter of

the rat erythrocyte to be 6.3 microns,[54] the surface area calculated by the method of Ponder[55] is 83 square microns. On the basis of these figures, it is possible to adsorb 1.72×10^8 hydrogen ions per cell. Assuming that the hydrogen ion exists in solution as a hydronium ion with a molecular radius of 1.96 Angstroms (the radius of a sphere having equivalent weight and density), the percentage of the surface area composed of potential binding sites is approximately 25%.

Discussion

The information obtained from mobility-pH curves for rat erythrocytes stabilized by fixation with aldehydes and osmium tetroxide may be summarized as follows:

(a) Within three pH units of neutrality, the surface charge properties of aldehyde-fixed erythrocytes are the same as those of unfixed cells. Since aldehydes are reactive with strongly basic groups, in particular the side chain aminos of lysine residues, this result indicates that near neutral pH the rat erythrocyte behaves electrophoretically as a polyanion. This is in accord with previous studies by Heard and Seaman on human erythrocytes.[17]

(b) The existence of weakly acidic dissociating groups reported previously by Glaeser and Mel[18] on the basis of electrophoretic studies on rat erythrocytes fixed with osmium tetroxide appears to be an artifact resulting from structural rearrangement of the membrane under alkaline conditions. This is clear from both the irreversible electrokinetic character of osmium tetroxide-fixed cells at high pH, and from the absence of a dissociating group in this pH range when erythrocytes are fixed with acetaldehyde or glutaraldehyde.

(c) Erythrocytes fixed with both glutaraldehyde and osmium tetroxide exhibit a large cathodic mobility below approximately pH 2. This appears to result from protonation of weakly basic groups with an apparent pK_a of roughly 1.6. The observation of Haydon and Seaman[40] that acetaldehyde-fixed erythrocytes do not have a positive mobility brandh could not be verified. Cells treated with acetaldehyde and subsequently fixed with osmium tetroxide have a reversible electrokinetic character at low pH with a substantial positive surface charge below pH 2. It thus appears that acetaldehyde is not reactive with the weakly basic groups responsible for the cathodic mobility.

(d) Below pH 1 glutaraldehyde-fixed rat erythrocytes exhibit a large increase in positive surface charge density. This appears to result from non-specific proton adsorption onto non-ionic regions occupying approximately 25% of the total surface area.

(e) An infrared analysis of membranes stabilized by reaction with glutaraldehyde and osmium tetroxide indicates that protein conformation is not affected, in contrast to the conclusions of Lenard and Singer[52] based on circular dichroism studies.

B. Chemical Modification via Covalent Linkage of Ionogenic Groups

The results of the previous section indicate that strong bases do not contribute to the surface charge properties of erythrocytes at high pH. Also Heard and Seaman have shown that treatment of human erythrocytes with p-toluenesulfonyl chloride, a reagent reactive with amino groups, produces no change in the anodic mobility at neutral pH.[17] In

extending this approach to rat erythrocytes, experiments have been performed with both p-toluenesulfonyl chloride and 1,5-difluoro-2,4-dinitrobenzene. The latter compound is reactive with amino and histidyl bases, and also with weakly acidic sulfhydryl and tyrosyl residues. Like glutaraldehyde, the difluoro reagent is bifunctional and serves to stabilize erythrocytes against hemolysis in distilled water.[56]

Another question which has been approached through chemical modification of the erythrocyte surface is the character of the anionic groups responsible for the negative mobility near neutral pH. As discussed in Chapter 1, only part of the negative surface charge appears to be associated with sialic acid carboxyls. Haydon and Seaman have proposed that the charge remaining on human erythrocytes after neuraminidase treatment is attributable to α-carboxyls of protein-bound amino acids.[40] Their argument was based in part on the fact that the anodic mobility at neutral pH can be reduced to zero by methylation. In this section, the results of a similar experiment with rat erythrocytes is reported, as well as an attempt to react carboxylic acids at the cell surface with a water-soluble carbodiimide.

Materials and Methods

p-toluenesulfonyl chloride (tosyl chloride) was obtained from the J. T. Baker Chemical Company, Phillipsburg, New Jersey. Rat erythrocytes were treated with tosyl chloride using the method of Seaman and Heard.[17] Tosyl chloride was dissolved in Standard Buffer at a concentration of one mg/ml by vigorous shaking at room temperature for six hours. The solution was then buffered to pH 7.4 by the dropwise addition of 0.1M $NaHCO_3$. Reaction with erythrocytes was carried out by mixing

four volumes of the tosyl chloride solution with one volume of packed cells, followed by incubation at 37° C for thirty minutes. The cells were then washed three times with Standard Buffer. A stock of control erythrocytes was also incubated at 37° C for thirty minutes, followed by three washes in Standard Buffer.

1,5-difluoro-2,4-dinitrobenzene (DFNB) was obtained from the J. T. Baker Chemical Company, Phillipsburg, New Jersey. Reaction of rat erythrocytes with DFNB followed the procedure of Berg, Diamond, and Marfey.[56] A 10% wt/vol solution of DFNB in methanol was prepared by shaking at room temperature for one hour. A total of 0.57 ml of the 10% DFNB stock was added to 99.43 ml of Standard Buffer, making the final concentration of DFNB 2.8 mM. One volume of packed erythrocytes was mixed with twenty volumes of the DFNB solution, and the reaction allowed to proceed for one hour at room temperature (21-24° C) with occasional stirring. The erythrocytes were then spun down and washed three times with Standard Buffer.

Methylation of surface carboxyls was performed with glutaraldehyde-fixed rat erythrocytes using the method of Haydon and Seaman.[40] Cells were washed once by suspending them in thirty volumes of 0.05N HCl and centrifuging at 1100 × g for three minutes, and then twice in absolute methanol using the same procedure. One volume of packed cells was incubated for two hours at 37° C in twenty volumes of a methylating solution composed of an equivolume mixture of absolute methanol and 4N HCl. Finally, the cells were washed twice in absolute methanol and three times in Standard Buffer.

The water soluble salt 1-cyclohexyl-3-(2-morpholinylethyl) carbodiimide metho-p-toluenesolfonate (CMC) was obtained from the Aldrich

Chemical Company, Milwaukee, Wisconsin. The structure of this carbodiimide will be represented here as $R' - N = C = N - \overset{+}{R}''$, where R′ is the cyclohexyl ring and R′′ the N-methylmorpholinylethyl group. The (+) denotes the quaternary ammonium of the N-methylmorpholinylethyl group. Reaction with surface carboxyls was performed using glutaraldehyde-fixed rat erythrocytes. Twenty volumes of 0.1M CMC dissolved in Standard Buffer was mixed with one volume of packed cells. The suspension was then warmed to 37° C, and the pH adjusted to 4.75 by the dropwise addition of 1N HCl. The reaction was allowed to proceed at 37° C for varying lengths of time with continuous stirring. (A more detailed description of the kinetics will be given later.) The reaction was then stopped by spinning down the cells and washing them three times with Standard Buffer.

Results and Discussion

(1) p-toluenesulfonyl chloride and 1,5-difluoro-2,4-dinitrobenzene. Following tosylation, the electrophoretic mobility of unfixed rat erythrocytes was -1.03 micron/sec/volt/cm, compared with a control value of -1.06. Both measurements were made at pH 7.45 ± 0.10. This difference in mobility is insignificant in relation to the estimated experimental error of 5%.

After reaction with DFNB, the mobility of unfixed rat erythrocytes was -1.04 micron/sec/volt/cm, compared with a control value of -1.07. Both measurements were made at pH 7.35 ± 0.10. Again, the change in mobility is not significant.

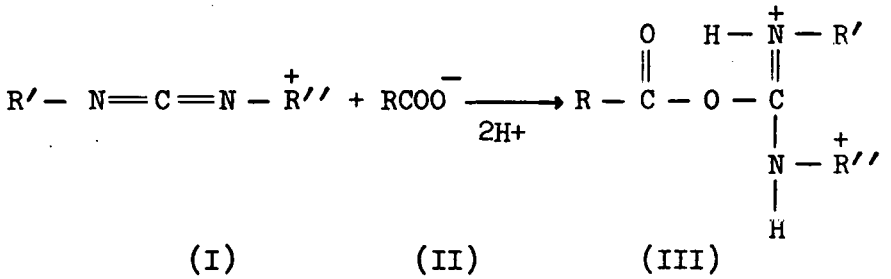
On the basis of these results, it may be concluded that reagents reactive with strongly basic groups produce no change in the surface charge properties of rat erythrocytes near neutral pH. Therefore, no

strong bases reside within approximately one Debye length of the surface of shear.

(2) Methylation and Reaction with Carbodiimide. The mobility of glutaraldehyde-fixed rat erythrocytes was examined at two different stages in the methylation procedure of Haydon and Seaman.[40] Cells washed with 0.05N HCl, then twice with absolute methanol, and finally three times with Standard Buffer had a mobility of -0.80 micron/sec/volt/cm at pH 6.90. This represents roughly a 25% reduction from the usual mobility of glutaraldehyde-fixed erythrocytes at neutral pH. A brown material was leached from the cells during the methanol washes, possibly resulting in some structural rearrangement of the membrane.

Following the complete methylation procedure (see Materials and Methods), the mobility at pH 6.86 was -0.55 micron/sec/volt/cm. This is a 50% decrease from the normal value for glutaraldehyde-fixed erythrocytes. Because the preliminary acid and methanol washes in themselves produced a 25% reduction in the net negative surface charge, the interpretation of this result is not clear. It does suggest, however, that at least 25% and at most 50% of the anionic groups contributing to the electrophoretic mobility are carboxylic acids susceptible to methylation. It is also possible that the esterification was not complete, but no attempt was made to study the reduction in mobility after longer periods of treatment with methanol/hydrochloric acid.

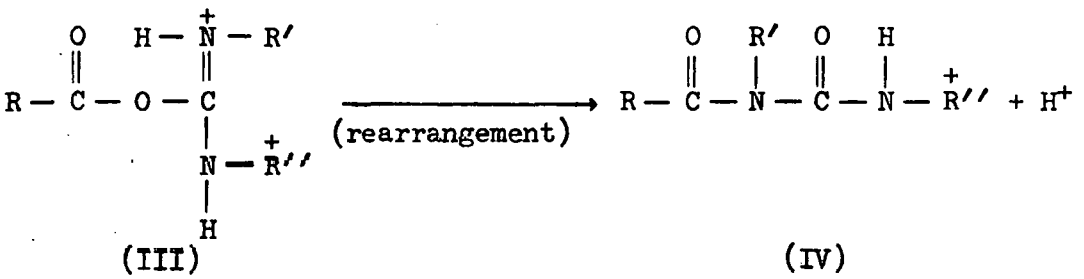
The initial reaction of surface carboxyls (denoted here as $RCOO^-$) with CMC is of the form:[57]



(I) (II) (III)

As a result, the anionic carboxylic acid (II) is converted to an acylisourea (III) having a cationic character by virtue of both the N-methylmorpholinylethyl group and the basic amidine group. Figure 6A shows the kinetics of surface charge reduction upon reaction with CMC. After approximately three hours, the surface charge is reduced to zero and remains at that level following longer periods of reaction.

Other workers who have studied the reaction of carboxylic acids with water soluble carbodiimides suggest that the reaction product (III) shown in the previous paragraph is unstable, and rearranges to give an acylurea (IV):[57,58]



(III) (IV)

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 Figure 6B. 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 tosyl chloride was reacted with glutaraldehyde-fixed erythrocytes previously treated with CMC for three hours. As shown in Figure 6A, 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.[57] 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-pH characteristics shown in Figure 6B might be mentioned at this point, namely, the proton-binding exhibited below pH 6. This results from the association of hydrogen ions with anionic groups which have not reacted with CMC, and from protonation of the weakly basic groups of pK_a 1.6.

In summary, methylation results in the esterification of 25 to 50% of the surface anions, while CMC reacts with a total of 35% of the anionic groups contributing to the mobility at neutral pH. This suggests that 35% of the negative surface charge results from carboxylic acids residing within approximately one Debye length of the surface of shear. It is also possible that phosphate groups associated with membrane lipids are chemically modified by these procedures,[57] but arguments will be given in Section D of this chapter against the involvement of phospholipid headgroups in determining surface charge properties.

C. Photo-oxidation in the Presence of Methylene Blue

The electrophoretic curves presented in Section A of this chapter gave no indication of a dissociating group with a pK_a close to neutral pH. On the other hand, Sanui, Carvalho, and Pace have performed a titration study on human erythrocyte ghost fragments, and concluded that

there were titrable groups with pK_a values of 6.1 and 7.1.[59] They proposed that membrane components having pK_a values in this range might be the phenolic hydroxyl of diiodotyrosine, the imidazole ring of histidine, and secondary phosphoryl groups of certain phospholipids. Of these possibilities, histidine is of particular interest since it may contribute to the positive surface charge at low pH.

A direct method of testing whether histidine groups contribute to the electrophoretic mobility is to alter their base properties by photo-oxidation in the presence of methylene blue.[60] A decrease in the pK_a of the histidine imidazole ring following photo-oxidative splitting has been demonstrated by the experiments of Lukton, Weisbrod, and Schlesinger.[61] These workers found that rate constants for the photo-oxidation process could be calculated from the pH change of an unbuffered histidine solution, and that these agreed quite well with rate constants determined by a colorimetric assay for ring cleavage. Consequently, photo-oxidation of histidine residues in the erythrocyte membrane should produce a measurable change in surface charge properties if these groups lie within a Debye length of the surface of shear.

Materials and Methods

Rat erythrocytes were mixed with twenty volumes of pH 7.4 Sorensen phosphate buffer containing methylene blue (Matheson, Coleman, and Bell, Norwood, Ohio) at a concentration of 0.01 mg/ml. The suspension was placed in a 37° C bath and subjected to illumination from a 150 watt lamp at a distance of six inches. Irradiation was allowed to proceed for ninety minutes with continuous stirring. The cells were then washed twice in phosphate buffer and fixed with glutaraldehyde. Extensive

hemolysis accompanied the photo-oxidative process. This phenomenon is known as photodynamic hemolysis, and has been shown to result from the photo-sensitized destruction of membrane components.[62] In addition, there was considerable blackening of the cells during the course of irradiation. Photo-oxidation of glutaraldehyde-fixed cells followed the same procedure as unfixed cells. In this case, however, no hemolysis accompanied the irradiation.

Results and Discussion

As shown in Figure 7, the erythrocyte surface charge properties below neutral pH are unaltered as a result of the photo-oxidative process. Both the shape of the mobility-pH curve and the isoelectric point are comparable to those obtained for glutaraldehyde-fixed cells not subjected to irradiation. The fact that cell blackening and photodynamic hemolysis accompanied the process indicates that oxidation actually occurred. From these results it appears that no contribution is made to the mobility at low pH by histidine bases.

D. Ethanol Extraction of Glutaraldehyde-fixed Erythrocytes

An attempt was made to examine the involvement of phospholipid head-groups in determining the surface charge properties of glutaraldehyde-fixed rat erythrocytes by making mobility measurements on cells after their lipid had been extracted with ethanol. This procedure was suggested by the studies of Korn and Weisman on the elution of lipids from amoebae during the dehydration procedures involved in preparing specimens for electron microscopy.[63] These workers found that ethanol extraction of glutaraldehyde-fixed amoebae causes a 95% loss of lipid.

Materials and Methods

Glutaraldehyde-fixed rat erythrocytes were washed twice with distilled water and suspended in ten volumes of absolute ethanol for one hour at room temperature (21-24° C). The cells were then pelleted by centrifugation at 1100 × g for three minutes, and the faintly yellow supernatent removed for lipid analysis. The packed cell volume was unchanged after ethanol extraction, indicating that the extent of hemolysis was negligible. Under the phase microscope the morphology of the extracted cells appeared to be normal except for the presence of numerous blebs ranging in diameter from roughly 0.5 to 1.0 micron. Prior to electrophoretic measurements, the extracted cells were washed three times in Standard Buffer.

For a gravimetric analysis of the lipid extract, the supernatent was transferred to a glass vial and blown down under a stream of nitrogen gas at 40° C. On the basis of two experiments the average amount of lipid extracted was 4.22 ± 0.97 mg per ml of packed cells. The total lipid content of the rat erythrocyte membrane is 4.86 mg per ml of packed cells,[64] so that an average of 87% by weight of the membrane lipid was eluted by ethanol. 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 and then layered 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.

Results and Discussion

The mobility-pH characteristics of glutaraldehyde-fixed rat erythrocytes extracted with absolute ethanol are shown in Figure 8. The surface

charge properties 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.

The fact that nearly complete lipid extraction does not alter the anodic mobility at neutral pH indicates that phosphate and carboxyl ions associated with phospholipid moieties are not located at positions close to the surface of shear. In addition, the appearance of a large positive charge at pH 1 is an indication that the quaternary ammonium associated with choline does not contribute to the electrophoretic mobility. Recent nuclear magnetic resonance studies on erythrocyte membranes suggest that choline has a large rotational mobility and may be located in an aqueous environment at the cell surface.[65] From the electrophoretic results presented here, however, it appears that choline does not reside within a Debye length of the surface of shear.

E. Enzymatic Cleavage of Ionic Groups from Carbohydrate and Protein Moieties:

1. Neuraminidase

From a titration study on neuraminidase-treated sialyl mucoprotein, Curtain concluded that action of the enzyme results in the appearance of cationic groups with a pK_a of 11.2.[28] Studies by Cook, Heard, and Seaman on the electrophoretic properties of unfixed erythrocytes treated with neuraminidase showed both the presence of ionogenic groups at high pH and a change in the extrapolated isoelectric point from pH 2 to pH 3.[31] These workers also found that upon post-fixation with acetaldehyde, the mobility of neuraminidase-treated human erythrocytes increased

by roughly 25%. Since acetaldehyde is reactive with basic groups, they contended that a small portion of the surface charge reduction observed after enzyme treatment resulted from the appearance of cationic groups near the surface of shear. A different conclusion was later reached by Haydon and Seaman, who argued against the appearance of basic groups if neuraminidase treatment was performed on acetaldehyde-fixed cells and the enzyme preparation was free of proteolytic contaminants.[40]

Previous studies have also raised a question as to the character of the anionic groups responsible for the negative surface charge observed after neuraminidase treatment. Haydon and Seaman contend in the case of human erythrocytes that these groups have a pK_a of 3.35 and are probably α-carboxyls associated with protein-bound amino acids.[40] It has not been clarified, however, whether these groups are present at positions near the surface of shear prior to neuraminidase treatment, or appear only as a side effect associated either with enzyme attachment at the cell surface or the presence of proteolytic contaminants in the enzyme preparation. In addition, no attempt has been made using a species of erythrocyte other than human to estimate the pK_a of these anionic groups.

In the following paragraphs microelectrophoretic studies are presented on the surface charge properties of neuraminidase-treated rat erythrocytes previously stabilized by fixation with glutaraldehyde. An attempt has been made to clarify the question of whether enzyme action results in the appearance of dissociating groups at high pH. Observations have also been made on the anionic groups giving rise to the anodic mobility remaining after neuraminidase treatment. In addition, several studies have been directed towards determining whether the

weak bases responsible for the cathodic mobility at low pH are acetamido groups associated with sialic acid molecules.

Materials and Methods

Two types of neuraminidase were used in these studies: (1) RDE, a crude extract from Vibrio cholerae, was obtained from Microbiological Associates (Bethesda, Maryland); (2) chromatographically purified neuraminidase from Clostridium perfringens was obtained from Worthington Biochemical Corporation (Freehold, New Jersey).

The commercial RDE solution was mixed with an equal volume of Standard Buffer. One volume of glutaraldehyde-fixed rat erythrocytes was suspended in two volumes of the enzyme solution and incubated at 37° C for one hour. The suspension was stirred by pipetting at ten minute intervals. The erythrocytes were then pelleted by centrifugation at 1100 × g for three minutes, and the supernatent removed for determination of sialic acid. Prior to electrophoretic examination, the cells were washed three times with Standard Buffer.

Glutaraldehyde-fixed rat erythrocytes were reacted with Worthington neuraminidase under the following conditions. One volume of packed cells was mixed with two volumes of a 0.250 mg/ml enzyme solution in 0.067M Sorensen phosphate buffer at pH 5.35. The incubation was extended for one hour at 37° C with stirring at ten minute intervals. Higher concentrations of enzyme did not result in a greater reduction of the negative surface charge at neutral pH. Following enzyme treatment, the remaining preparative procedures for cells and supernatent were the same as those described in the preceding paragraph.

Sialic acid was determined by the method of Warren.[66] Crystalline N-acetylneuraminic acid (Sigma Chemical Company, St. Louis, Missouri)

was used as a standard. Appropriate enzyme blanks were carried through the preparative and assay procedures. Cell counts were made with a hemocytometer.

Experiments were also performed with glutaraldehyde-fixed rat erythrocytes in which sialic acid was cleaved from the cell surface by hydrolysis with acid using the procedure of Haydon and Seaman.[40] Cells were suspended in ten volumes of 0.1N sulfuric acid for one hour at 80° C with stirring at ten minute intervals. The erythrocytes were then washed four times with Standard Buffer.

Results

The mobility-pH curves for glutaraldehyde-fixed rat erythrocytes reacted with RDE and Worthington neuraminidase are shown in Figures 9A and 10A, respectively. In addition to the reduction in negative surface charge at neutral pH, there are distinct changes in the electrokinetic properties at both high and low pH relative to untreated glutaraldehyde-fixed erythrocytes (see Figure 1A). Several aspects of the surface charge properties of the enzyme-treated cells will now be discussed in detail.

(a) Surface Charge Reduction at Neutral pH. Following treatment with either RDE or Worthington neuraminidase, the mobility of glutaraldehyde-fixed erythrocytes at pH 7 is reduced to -0.70 micron/sec/volt/cm, a 36% decrease from the control value of -1.10. This is comparable to the 42% reduction in mobility at neutral pH found by Glaeser and Mel for unfixed rat erythrocytes reacted with RDE.[18] The experimental conditions used by these workers were the same as those employed in the studies reported here.

In two experiments, the amount of sialic acid released from glutaraldehyde-fixed cells by RDE was 0.29 ± 0.03 micromole per 10^{10} cells. A value of 0.22 ± 0.05 was obtained in two experiments with Worthington neuraminidase. On the basis of four experiments with unfixed rat erythrocytes, Glaeser found a yield of 0.22 ± 0.03 micromole of sialic acid per 10^{10} cells upon treatment with RDE.[9] Since it is unlikely that neuraminidase could penetrate deeply the rigid structure of a glutaraldehyde-fixed membrane, the comparable activity of this enzyme with fixed and unfixed cells suggests that its substrate is located primarily at the outer surface. As discussed in Chapter 1, the concept that sialic acid is localized at the outer periphery of plasma membranes is supported by the electron microscope studies of Benedetti and Emmelot.[36]

(b) Appearance of Dissociating Groups at High pH. From the mobility-pH curves shown in Figures 9A and 10A, it is clear that glutaraldehyde-fixed cells reacted with neuraminidase exhibit a reversible increase in negative surface charge above approximately pH 10. This property remains after post-fixation of the enzyme-treated cells with either osmium tetroxide or glutaraldehyde (see Figures 9B, 10B, and 10C). Since glutaraldehyde is reactive with the side chain moieties of cysteine, tyrosine, and lysine residues,[43] it appears that these groups are not responsible for the increase in anodic mobility. Under the fixation conditions employed in these experiments, glutaraldehyde and osmium tetroxide should not react with either the strongly basic guanidinium group of arginine or the weakly acidic hydroxyls of sugar moieties.[42,47,67] These groups are therefore possible sources of the surface charge increase at high pH.

(c) Electrophoretic Properties at Low pH. Glaeser and Mel have suggested that acetamido groups associated with N-acetylneuraminic acid molecules may be the weak bases responsible for the positive surface charge observed at low pH.[18] In addition, all of the glucosamine and galactosamine molecules contained in sialyl oligosaccharides at the erythrocyte surface exist in N-acetylated form.[68] The acetamido groups of these molecules may lie sufficiently close to the surface of shear to contribute to the electrophoretic mobility. An argument which might be advanced against this hypothesis is the fact that the pK_a of a substituted amide is generally less than zero,[69] whereas results presented in Section A of this chapter indicate that the weakly basic groups near the surface of shear have an approximate pK_a of 1.6.

As a means of determining whether sialic acid contributes to the cathodic mobility, electrophoretic studies were extended to pH 1 using glutaraldehyde-fixed rat erythrocytes reacted with RDE and Worthington neuraminidase. Similar studies have previously been reported by Haydon and Seaman using erythrocytes stabilized by fixation with acetaldehyde.[40] As discussed earlier, these workers contend that acetaldehyde-fixed human red cells do not exhibit a true isoelectric point, and have presented data indicating that this property persists after neuraminidase treatment. Electrophoretic results presented in Section A of this chapter, however, are in contradiction to the observations of Haydon and Seaman. As a consequence, no attempt has been made to investigate the effects of neuraminidase on rat erythrocytes fixed with acetaldehyde.

From the mobility-pH curve presented in Figure 9A, it is clear that glutaraldehyde-fixed erythrocytes treated with RDE are electrokinetically unstable at low pH. The anodic mobility at neutral pH is greatly reduced

after exposure of the cells to pH 1 for a period of approximately 15 minutes, indicating the appearance of new basic groups near the surface of shear. After post-fixation with osmium tetroxide, the RDE-treated cells exhibit reversible surface charge properties at low pH. As shown in Figure 9B, cells handled in this manner have a cathodic mobility at pH 1 comparable to that observed for untreated cells (see Figure 1A). This suggests that removal from the cell surface of acetamido groups associated with sialic acid molecules does not alter the positive surface charge characteristics at low pH.

The results of similar studies using Worthington neuraminidase are shown in Figures 10A and 10B. Glutaraldehyde-fixed cells reacted with this preparation of neuraminidase also exhibit irreversible surface charge properties at low pH. In this case, however, cells exposed to pH 1 show an increase in anodic mobility when returned to neutral pH. This indicates that electrokinetic instability at low pH is associated primarily with the appearance of new anionic groups near the surface of shear. As with RDE, stability of the enzyme-treated cells under acidic conditions may be achieved by post-fixation with osmium tetroxide. The resulting surface charge properties at low pH are considerably different, however, from those observed after reaction with RDE. As shown in Figure 10B, the mobility at pH 1 is only +0.23 micron/sec/volt/cm, representing approximately a 75% reduction from the value obtained with untreated erythrocytes (see Figure 1A).

Further electrophoretic studies were performed in which glutaraldehyde-fixed rat erythrocytes were treated with Worthington neuraminidase, and then post-fixed with glutaraldehyde. From Figure 10C 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 which were not fixed subsequent to enzyme action (see Figure 10A). 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 Figure 10D. 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 (see Figure 10A). The initial reduction in mobility at pH 1 is in reasonable agreement with measurements made on enzyme-treated cells post-fixed with osmium tetroxide.

Briefly summarizing, at low values of pH the electrophoretic properties of glutaraldehyde-fixed rat erythrocytes reacted with Worthington neuraminidase are distinctly different from those observed with RDE-treated cells. When stabilized against acidic conditions by post-fixation with osmium tetroxide, cells reacted with Worthington neuraminidase exhibit a cathodic mobility at pH 1 which is substantially reduced from the value obtained with untreated cells. Reaction with RDE, however, does not produce a reduction in the positive mobility at low pH. The former result indicates that the weak bases responsible for the positive surface charge at pH 1 may be acetamido groups of sialic acid molecules, whereas the studies with RDE argue to the contrary. A direct method of resolving this question would be to examine the electrophoretic properties of glutaraldehyde-fixed erythrocytes reacted with other commercial preparations of neuraminidase. Such studies, however, have not been undertaken.

(d) Studies on the Character of the Negative Surface Charge Remaining After Neuraminidase Treatment. After reaction of glutaraldehyde-fixed rat erythrocytes with either RDE or Worthington neuraminidase, the mobility at pH 7 is -0.70 micron/sec/volt/cm (see Figures 9A and 10A). The fact that two thirds of the negative surface charge remains after enzyme treatment suggests that sialic acid carboxyls play only a secondary role in determining the anodic mobility at neutral pH. Some portion of this residual charge, however, may arise as a result of enzyme action at the cell surface. The appearance of dissociating groups at high pH upon treatment with neuraminidase adds weight to this possibility. The contribution of new acidic groups to the electrophoretic mobility after treatment with neuraminidase could occur in either of two ways:

(i) 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 the human erythrocyte, Haydon and Seaman contend that 38.5% of the surface charge density at neutral pH is attributable to such groups, and further propose that their contribution to the mobility is equal for untreated and neuraminidase-treated cells.[40] 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 which are commercially available at the present time are active only on free amino acids,[70] so that this type of experiment cannot be performed.

(ii) A second means by which acidic groups might appear near the surface of shear upon treatment with neuraminidase is through structural alteration of the existing cell surface. This could occur, for example, through the cleavage of peptide bonds by proteolytic contaminants in the enzyme preparation. As an indirect test of whether the enzyme induces extensive rearrangement of the membrane surface, electrophoretic measurements were performed on glutaraldehyde-fixed erythrocytes from which sialic acid was removed by a non-enzymatic method, namely, through mild acid hydrolysis. Using human erythrocytes, Haydon and Seaman have shown that the release of sialic acid from the surface of acetaldehyde-fixed cells through this form of treatment is approximately equal to that resulting from reaction with neuraminidase.[40] After incubation in 0.1N sulfuric acid for one hour at 80° C, the mobility of glutaraldehyde-fixed rat erythrocytes was found to be -0.62 micron/sec/volt/cm at pH 6.86. This represents a 44% reduction from the control value of -1.10, and is closely comparable to the 36% decrease in surface charge observed after reaction with neuraminidase. With acetaldehyde-fixed human erythrocytes, Seaman and Cook found a 63% reduction in mobility following either form of treatment.[29] Taken together, these results suggest that reaction with neuraminidase does not result in the appearance of a substantial

number of new anionic groups either through proteolysis or other forms of structural alteration of the membrane surface.

From electrophoretic studies on cells reacted with neuraminidase, it is possible to determine the pK_a of the anions responsible for the negative surface charge remaining after enzyme treatment. In the case of human erythrocytes, Haydon and Seaman have calculated the pK_a of these anions to be 3.35.[40] From data reported here for rat erythrocytes, it is difficult to make an accurate estimate of the pK_a because of the proton binding attributable to weakly basic groups. As judged from the surface charge characteristics presented in Figure 10B for cells reacted with Worthington neuraminidase, the groups responsible for the anodic mobility remaining after enzyme treatment exhibit a pK_a in the range 2.5 to 3.0. Recalling from Section D of this chapter that carboxyl and phosphate radicals of phospholipid moieties do not appear to contribute to the electrophoretic mobility, this value for the pK_a indicates that the anionic groups are probably ionized carboxyls associated with membrane protein.

It should be mentioned at this point that Weiss and Mayhew have reported that phosphate groups of ribonucleic acid contribute to the electrophoretic mobility of Ehrlich ascites tumor cells and a line of cultured mammalian cells, RPMI no. 41.[71] These workers did not, however, find electrophoretic evidence for the presence of RNA at the membrane interface of human, mouse, or embryonic chick erythrocytes. This was confirmed for rat erythrocytes by examining the electrophoretic properties of cells reacted with Ribonuclease A (Worthington Biochemical Corporation, Freehold, New Jersey). The enzyme concentration was 0.1 mg/ml and the reaction carried out at pH 5.0 for thirty minutes at 37° C. The mobility

of ribonuclease-treated cells at pH 7.51 was -1.00 micron/sec/volt/cm. A value of -1.01 micron/sec/volt/cm was obtained at pH 7.47 for untreated erythrocytes subjected to the same experimental conditions.

Discussion

The results of studies presented in this section on the surface charge properties of glutaraldehyde-fixed rat erythrocytes reacted with neuraminidase may be summarized as follows:

(a) Upon treatment of glutaraldehyde-fixed cells with either RDE or Worthington neuraminidase, the chemically determined yield of sialic acid and the reduction in electrophoretic mobility at neutral pH are closely comparable in magnitude to values obtained with unfixed erythrocytes.

(b) Reaction of glutaraldehyde-fixed cells with either preparation of neuraminidase results in the appearance of electrophoretically detectable dissociating groups at high pH. This property is unaffected by post-fixation with glutaraldehyde, thereby indicating that the groups responsible are not sulfhydryls, phenolic hydroxyls, or aminos. Other possible sources for the observed increase in anodic mobility above pH 10 are guanidinium groups and sugar hydroxyls.

(c) Erythrocytes reacted with Worthington neuraminidase exhibit a 75% reduction in positive mobility at pH 1, suggesting that acetamido groups associated with sialic acid molecules may be the weak bases responsible for the positive surface charge. On the contrary, cells treated with RDE show no change in cathodic mobility, thereby indicating that acetamido groups are not the origin of the positive branch in the mobility-pH curve. Some support is given to the result obtained with

RDE-treated cells by the fact that acetylated amides generally have pK_a's less than zero,[69] whereas the weakly basic groups contributing to the electrophoretic mobility have a pK_a of approximately 1.6.

(d) Hydrolytic removal of sialic acid by heating aldehyde-stabilized erythrocytes in dilute sulfuric acid results in a surface charge reduction comparable to that obtained upon neuraminidase treatment. This indirectly suggests that the large anodic mobility observed after reaction with neuraminidase does not arise from structural alteration of the membrane surface. The anionic groups responsible for the negative mobility remaining after enzyme treatment exhibit a pK_a in the range 2.5 to 3.0, and are probably carboxylic acids associated with membrane protein. Because the appropriate amino acid decarboxylases are not available, it is impossible at the present time to determine whether these groups contribute to the electrophoretic mobility prior to neuraminidase treatment. It is conceivable that they are situated at depths approximating one Debye length from the surface of shear only after the outermost layer of sialic acid has been enzymatically removed.

2. Asparaginase and Glutaminase

In the preceding subsection it was shown that the mobility-pH characteristics of erythrocytes reacted with different commercial preparations of neuraminidase give conflicting evidence with regard to the contribution of sialic acid acetamido groups to the surface charge at low pH. It is well documented, however, that the pK_a of an N-substituted amide is typically lower by two pH units than the value of 1.6 observed for weakly basic groups near the erythrocyte surface of shear.[69] For this reason it would seem that a more probable origin of the cathodic mobility at pH 1 is proton binding by amidated amino acids associated

with membrane protein. One basis for this hypothesis is the fact that primary amides generally have higher pK_a's than amides which are N-substituted. For example, values of +0.37 and +0.63 have been reported for acetamide,[69,72] compared with -0.46 for N-methylacetamide.[69] A second factor which makes this hypothesis attractive is the large number of amidated amino acid residues found in plasma membranes. In the case of the Ehrlich ascites cell plasma membrane, Wallach and Zahler have found that asparagine and glutamine comprise 14.7% of the total amino acid residues.[51] Taking the mean residue weight to be 115 and assuming that the plasma membrane contains approximately 10^{-12} gram of protein,[48] this would correspond to 7.7×10^8 amidated amino acids per membrane. In comparison, it can be calculated from the amount of sialic acid released by neuraminidase that only 1.4×10^7 sialic acid acetamido groups are present at the surface of a rat erythrocyte membrane.

The objection might be raised at this point that the fixatives used to stabilize cells at low pH prior to electrophoretic measurements may be reactive with amides, thereby altering their base properties. Under the fixation conditions employed here, however, acetaldehyde,[41] glutaraldehyde,[42] and osmium tetroxide[67] should not react with either glutamine or asparagine side chains. In addition, all electrophoretic measurements were performed at room temperature (21-24° C), so that no acid hydrolysis should have occurred during experiments conducted at low pH.

On the basis of these arguments, a series of experiments were undertaken to study the possible involvement of amidated amino acids in determining the surface charge below pH 2. In this subsection, the results of a chemical analysis for the activity of amidohydrolases on

rat erythrocyte membranes are reported, along with electrophoretic measurements on the enzyme-treated cells.

Materials and Methods

Asparaginase and glutaminase from Escherichia coli were obtained from Worthington Biochemical Corporation (Freehold, New Jersey). The reagent used for preparation of Nessler's solution was obtained from Sigma Chemical Company (St. Louis, Missouri) under the trade name "Sigma Ammonia Color Concentrate."

Reaction of rat erythrocytes with asparaginase was carried out in the following manner. One milliliter of packed cells was mixed with 2.5 ml of Standard Buffer containing 25 units of asparaginase per ml. The cell suspension was incubated at 37° C for one hour with stirring at ten minute intervals. The erythrocytes were then pelleted by centrifugation at 1700 × g for five minutes. Two milliliters of supernatent were removed and 0.1 ml of 1.5M trichloroacetic acid added to precipitate the enzyme and any hemolytic products. This solution was then centrifuged at 1700 × g for fifteen minutes, and a 1.6 ml aliquot of the supernatent removed for determination of ammonia. The enzyme-treated erythrocytes were washed three times with Standard Buffer prior to electrophoretic examination.

Analysis of the supernatent for ammonia was performed by adding 0.2 ml of Nessler's reagent. The solution was mixed by pipetting and allowed to stand at room temperature for ten minutes, at which time the optical density was recorded at 415 nm. A blank containing only Standard Buffer, an enzyme blank, and a suspension of control erythrocytes were also carried through the preparative and assay procedures. The net

absorbance at 415 nm attributable to ammonia released from erythrocyte membranes was then calculated from the optical densities of these solutions and the value obtained for the supernatent from asparaginase-treated cells. The relationship between optical density at 415 nm and ammonia concentration was determined by preparing a standard curve using solutions of ammonium sulfate in Standard Buffer. Trichloroacetic acid and Nessler's reagent were added in the manner described above. Nessler's method was found to be sufficiently sensitive to detect ammonia at concentrations of 10^{-5}M. Activity of the commercial enzyme preparation was checked using L-asparagine.H_2O (Sigma Chemical Company, St. Louis, Missouri).

An identical procedure was used in treating erythrocytes with glutaminase, except that the enzyme solution was prepared at 10 units/ml. This reduction in activity per milliliter relative to that employed in experiments with asparaginase was necessitated by the extremely low activity per milligram dry weight of the commercial glutaminase preparation, i.e., approximately 0.5 unit/mg. The concentration of glutaminase, however, was still sufficiently high to interfere with a determination of ammonia using Nessler's reagent. Even after repeating the trichloroacetic acid precipitation step several times, the addition of Nessler's reagent resulted in visible turbidity and a large, time-varying optical density at 415 nm. As a consequence, attempts to assay for glutaminase activity through detection of ammonia were unsuccessful at the high levels of enzyme concentration employed in these experiments.

Results and Discussion

In two experiments using Nessler's reagent, no ammonia was found to be released from the membranes of erythrocytes reacted with 25 units/ml

asparaginase. This indicates either that asparagine is inaccessible to the enzyme, or that asparaginase is inactive when its substrate is bound to membrane protein. For reasons discussed in the Materials and Methods section, attempts to assay for glutaminase activity were unsuccessful.

Despite this chemical evidence for the inactivity of amidohydrolases at the erythrocyte surface, electrophoretic studies were performed at neutral pH and at pH 1 on cells reacted with 25 units/ml asparaginase and 10 units/ml glutaminase. The results are tabulated in Tables II and III. It is clear that treatment of erythrocytes with either asparaginase or glutaminase resulted in no significant change in the anodic mobility at neutral pH or the cathodic mobility at pH 1.

In view of the fact that asparaginase, and presumably also glutaminase, fail to act on the erythrocyte membrane, the question of whether amidated amino acids contribute to the positive surface charge at low pH remains unresolved. Before electrophoretic studies can be effectively directed towards this problem, amidohydrolases must be developed that are capable of reacting with protein-bound substrates in the cell membrane.

3. Carboxypeptidase A

In the discussion of electrophoretic properties exhibited by neuraminidase-treated rat erythrocytes, evidence was presented that -0.70 micron/sec/volt/cm, or 65%, of the anodic mobility at neutral pH may be contributed by carboxylic acids associated with membrane protein. The fact that these groups have an approximate pK_a of 2.5 to 3.0 suggests that they are primarily α-carboxyls, since the pK_a's of protein β- and γ-carboxyls generally lie in the range 3.0 to 4.7.[73] Two types of α-carboxylic acids might be expected to contribute to the electrophoretic

mobility: (i) As discussed previously, the carboxyl radicals may exist on C-terminal protein-bound amino acids that are linked covalently through their side chains to sialyl oligosaccharides at the cell surface. (ii) The α-carboxyl groups may exist on "free" C-terminal residues of polypeptide chains. In the case of sheep erythrocytes, no C-terminals were found in membrane protein solubilized by treatment with sodium borohydride and iodoacetamide in the presence of sodium dodecyl sulfate.[74] Despite this evidence, it was considered worthwhile to carry out experiments to determine whether "free" C-terminal residues are present at the rat erythrocyte membrane interface. For this purpose, both chemical and electrophoretic tests were performed following reaction of rat erythrocytes with carboxypeptidase A.

Materials and Methods

In preparation for electrophoretic measurements, one volume of rat erythrocytes was mixed with an equal volume of Standard Buffer containing 10 units/ml of carboxypeptidase A (Worthington Biochemical Corporation, Freehold, New Jersey). The cell suspension was allowed to stand at room temperature (21-24° C) for thirty minutes with stirring at ten minute intervals. The erythrocytes were then pelleted by centrifugation at 1100 × g for three minutes, and washed three times with Standard Buffer prior to electrophoretic examination.

Activity of the enzyme was determined by using ninhydrin to assay for the release of amino acids. Carboxypeptidase A was reacted with two milliliters of packed erythrocytes in the manner described above. After the erythrocytes were spun down by centrifugation at 1100 × g for three minutes, the supernatent was removed and dialyzed against two milliliters of fresh saline for 24 hours at 4° C. Three drops of the

dialyzate were placed at the center of a five inch sheet of Whatman No. 42 filter paper and dried by heating at 85° C for five minutes. A 1-butanol solution containing 0.25% ninhydrin (Sigma Chemical Company, St. Louis, Missouri) was sprayed lightly onto the paper and dried for one hour at 85° C. As a control, a two milliliter volume of Standard Buffer containing 10 units/ml of carboxypeptidase A was allowed to stand at room temperature for thirty minutes with stirring at ten minute intervals. This solution was then processed in the same manner as the supernatent from enzyme-treated cells. The qualitative relationship between concentration and ninhydrin coloration was determined using 10^{-4}M to 10^{-3}M solutions of several amino acids.

Results and Discussion

The electrophoretic mobility of unfixed rat erythrocytes treated with 10 units/ml carboxypeptidase A was -1.00 micron/sec/volt/cm, compared with a control value of -1.01. Both measurements were made at pH 7.48 ± 0.02. The absence of a change in the mobility following treatment with carboxypeptidase A does not in itself constitute evidence that the enzyme is inactive at the cell surface. This follows from the fact that action of the enzyme results in a one for one replacement of α-carboxyls on terminal residues by those of adjacent residues. It would be expected, however, that digestion of some polypeptide chains might proceed to a point where their terminal residues no longer reside within a Debye length of the surface of shear. This would influence the electrophoretic mobility if a contribution is made by "free" C-terminal amino acids.

In three experiments, the dialyzates from enzyme-treated erythrocytes and carboxypeptidase A control solutions exhibited levels of

ninhydrin coloration corresponding to amino acid concentrations in the range 10^{-4}M to 10^{-3}M. In all cases, however, equal coloration was observed for the dialyzates from sample and control solutions. This suggests that the presence of any amino acids in the sample dialyzates resulted from autolysis of the enzyme. When the carboxypeptidase A concentration was lowered to 0.1 unit/ml, no ninhydrin coloration was observed with either the sample or control dialyzate.

Taken together, the electrophoretic and chemical results indicate that "free" C-terminal amino acids, if present in the rat erythrocyte membrane, are inaccessible to carboxypeptidase A. It is probable, therefore, that any protein α-carboxylic acids contributing to the electrophoretic mobility are associated with C-terminal residues linked covalently through their side chains to carbohydrate moieties at the cell surface. The peptide linkage of this form of C-terminal residue would not be cleaved by carboxypeptidase A.

CHAPTER 3. THE IONIC CHARACTER OF THE RAT ERYTHROCYTE MEMBRANE INTERFACE AT PHYSIOLOGICAL IONIC STRENGTH

In this chapter the effects of specific chemical modification of the rat erythrocyte membrane will be briefly summarized, with an effort being made to characterize the ionic groups at the surface. This will be approached by first grouping the predominant ionic moieties within the membrane according to their acid or base strength, and then restating experimental results which indicate the presence or absence of a specific group at the cell interface.

(A) Strong Bases and Weak Acids*

The strong bases that might be expected to contribute to the erythrocyte surface charge are amino groups associated with phosphatidyl serine, phosphatidyl ethanolamine, and membrane proteins (especially lysine side chains), guanidinium groups of arginine residues, and the choline moieties of lecithin and sphingomyelin. Treatment of rat erythrocytes with acetaldehyde (2A3), glutaraldehyde (2A1), p-toluenesulfonyl chloride (2B1), and 1,5-difluoro-2,4-dinitrobenzene (2B1) was carried out under experimental conditions where all four reagents should be highly reactive with amino bases, and acetaldehyde and p-toluenesulfonyl chloride with guanidinium bases.[17,41,43,56] The anodic mobility at neutral pH was unchanged following reaction with each of these reagents, thereby

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* In referring to methods and results described in the previous chapter, the relevant section and (when appropriate) subsection will be quoted. As an example, the notation 2A1 would be used in reference to a result discussed in Chapter 2, Section A, Subsection 1.

indicating that strongly basic amino and guanidinium groups are not present near the surface of shear. From the fact that no change was observed in the mobility-pH characteristics following ethanol extraction of glutaraldehyde-fixed erythrocytes (2D), it appears that no contribution is made to the surface charge by quaternary ammonium groups of choline-containing phospholipids.

The weak acids that might be expected to contribute to the electrophoretic mobility at high pH are the side chain moieties of tyrosine and cysteine residues. Mobility measurements on glutaraldehyde-fixed rat erythrocytes gave no evidence for the existence of dissociating groups having pK_a's in the alkaline pH range (2A1). Since studies on model compounds indicate that glutaraldehyde is only partially reactive with phenolic hydroxyls,[43] this result suggests that tyrosine residues are not present near the erythrocyte surface of shear. 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.[43] However, the absence of cysteine from positions near the surface of shear may be inferred from the fact that fixation with osmium tetroxide does not alter the negative surface charge at neutral pH (2A1). If these 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 cysteine sulfhydryls to sulfonic acids.[47]

(B) Intermediate Strength Bases

Contrary to the results of a titration study on human erythrocyte membranes,[59] the mobility-pH curves of both human and rat red cells show

no evidence for a contribution from dissociating groups with pK_a's near neutrality. The intermediate strength bases present within the membrane in sufficient quantity to make a substantial contribution to the surface charge are the imidazolium groups of histidine residues. However, following photo-oxidation of erythrocytes under conditions where the basicity of these groups should be substantially altered (2C), no change was observed in the surface charge properties over the pH range 1 to 8. In addition, with the fixation procedures employed in these studies, glutaraldehyde should be partially reactive with imidazolium groups whereas osmium tetroxide should not.[43,47] Erythrocytes fixed with these two reagents, however, exhibit identical surface charge properties below neutral pH (2A1). These results thus indicate that histidine bases are not located within a Debye length of the surface of shear.

(C) Weak Bases

Rat erythrocytes fixed with either glutaraldehyde or osmium tetroxide exhibit an isoelectric point at approximately pH 2, below which they reversibly acquire a large cathodic mobility (2A1). Over the pH range 1 to 2, this positive surface charge is primarily the result of proton binding by a set of weakly basic groups with an approximate pK_a of 1.6 (2D). Below pH 1 glutaraldehyde-fixed cells exhibit an additional large increase in positive charge density that appears to arise from non-specific proton adsorption (2D). This will be discussed further in Section E of this chapter.

Two types of weakly basic moieties present within the membrane in sufficient quantity to account for the cathodic mobility are the acetamido groups of sialic acid and N-acetylated hexosamine sugars, and the side

chain amides of asparagine and glutamine residues. Mobility studies were performed on glutaraldehyde-fixed rat erythrocytes from which sialic acid had been removed through reaction with neuraminidase (2E1). These experiments did not give consistent results with regard to the role of sialic acid acetamido groups in determining the positive charge character. The cathodic mobility at pH 1 was unchanged following reaction of erythrocytes with RDE, whereas treatment with Worthington neuraminidase produced a 75% reduction in the positive surface charge (2E1). Amidohydrolases were found not to be reactive with amidated residues bound to membrane protein (2E2). No attempt was made to modify amides by non-enzymatic methods, for example, through hydrolysis, conversion to primary amines (Hofmann reaction), or dehydration to nitriles.[75] It is probable that the experimental conditions required for these procedures, such as heating or pH extremes, would result in some disruption of the membrane structure.

On the basis of results presented here, it is therefore not possible to identify the membrane components responsible for the weakly basic character of the cell interface at low pH. Two factors, however, suggest that protein side chain amides are a more probable source of the cathodic mobility than the N-acetylated amides of sialyl oligosaccharides. First, the pK_a of an N-substituted amide is generally less than zero, whereas that of an unsubstituted amide lies in the range zero to one (see p. 49). The latter is more closely comparable to the approximate value of 1.6 observed for weakly basic groups at the cell surface. Secondly, the number of amidated amino acid residues is greater by an order of magnitude than the total number of acetamido groups associated with sialic acid and N-acetylated amino sugars.

From Figure 5, the maximum surface charge density associated with weakly basic groups at the cell surface is +0.009 coulomb per square meter. If it is assumed that this is attributable to hydrogen ion binding by protein side chain amides, and the area per residue is taken to be 17 square angstroms,[76] then it may be calculated that 1% of the cell surface is occupied by amidated amino acids. Taking the number of amidated residues within the membrane to be 7.7×10^8 (see p. 49), then the number required at the surface in order to account for the positive charge density is approximately 0.6% of the total.

(D) Strong Acids

The role of sialic acid as the major contributor to the surface charge of erythrocytes has been well established for a decade.[31,32] From studies on rat erythrocytes reacted with neuraminidase, however, it appears that these anions account for only -0.40 micron/sec/volt/cm, or 36%, of the negative mobility at neutral pH (2E1). This suggests that some portion of the anionic groups responsible for the large mobility of neuraminidase-treated cells (i.e., -0.70 micron/sec/volt/cm) may reside near the surface of shear only after reaction with the enzyme, thereby obscuring the actual contribution of sialic acid carboxyls to the surface charge. In this regard it was observed that cleavage of sialic acid molecules by mild acid hydrolysis produces a surface charge reduction comparable to that resulting from reaction with neuraminidase (2E1). This indirectly suggests that new anionic groups do not appear near the surface of shear as a side effect associated with enzyme treatment (e.g., through the activity of proteolytic contaminants in the enzyme solution). It is possible, however, that the surface of shear resides several

angstroms closer to the physical membrane surface after removal of sialic acid molecules from the terminal positions of oligosaccharide chains. As a consequence, a new set of anionic groups might effectively be "unmasked" and thereby contribute to the electrophoretic mobility. Arguments both for and against this possibility will now be considered.

The one fact suggesting that neuraminidase treatment results in a contribution to the anodic mobility from new anionic groups is the large discrepancy between the release of sialic acid measured chemically and the yield calculated from surface charge reduction. The average release of sialic acid was found by Warren's method[66] to be 0.24 micromole per 10^{10} cells (2E1), or 1.44×10^{7} molecules per cell. From the Gouy-Chapman equation (p. 21), the surface charge density associated with the contribution of sialic acid to the mobility (-0.40 micron/sec/volt/cm) is -0.0045 coulomb per square meter. Taking the area of the rat erythrocyte to be 83 square microns (p. 24), this charge density corresponds to 2.33×10^{6} sialic acid molecules per cell. The chemically determined release of sialic acid is therefore six times as great as that calculated from the reduction in surface charge. This difference is difficult to reconcile even allowing for the uncertainties involved in calculations based on the equations of electrophoresis (see Chapter 1), and suggests that some portion of the mobility measured after reaction with neuraminidase may arise from new anionic groups near the surface of shear.

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: (i) The surface charge densities of both rat[18] and human[11] erythrocytes remain constant as the Debye length is increased from eight to twenty angstroms. A further increase in the

Debye length results in a reduction of the negative charge density, suggesting that predominantly basic groups are unmasked. (ii) Haydon and Seaman 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 angstroms.[40] 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 anions 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. Therefore, despite the fact that new anionic groups would partially explain the large discrepancy between the chemically measured and the electrophoretically calculated yields of sialic acid, the available evidence argues strongly against their appearance. As a consequence, it is likely that the negative mobility remaining after reaction with neuraminidase is attributable to anions present near the surface of shear prior to enzyme treatment.

With regard to the identity of these groups, experimental results presented in Chapter 2 indicate that they are probably carboxylic acids associated with membrane protein. Other types of acidic groups present within the membrane in sufficient quantity to make a substantial contribution to the mobility are those associated with membrane phospholipids. However, the fact that the negative charge density of glutaraldehyde-fixed cells is unaffected by ethanol extraction (2D) argues against a contribution from lipid carboxyl or phosphate radicals. In addition, since only neutral sugars have been detected,[30] the acidic character of

carbohydrates at the cell surface appears to be attributable solely to sialic acid. Finally, the possibility that anion adsorption might serve as a charging mechanism is unlikely in view of the observation that the anodic mobility is independent of the monovalent anion present in the suspending medium.[10]

On the basis of electrophoretic measurements on glutaraldehyde-fixed erythrocytes following neuraminidase treatment, the pK_a of the remaining anionic groups appears to lie in the range 2.5 to 3.0 (2E1). This low value of the pK_a suggests that they are protein-bound α-carboxylic acids, rather than side chain β- or γ-carboxyls. The inactivity of carboxypeptidase A at the cell surface (2E3) indicates that none of the α-carboxylic acids contributing to the mobility exist as "free" C-terminals. It is probable, therefore, that any electrophoretically detectable α-carboxyls are associated with C-terminal polypeptide residues linked covalently through their side chains to sialyl oligosaccharides. It is also conceivable that C-terminal residues might be covalently bonded to lipid moieties. This seems unlikely, however, since membrane lipids are readily extracted at 0° C with a 2/1 mixture of chloroform/methanol.[64]

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 a terminal group in mucopolysaccharide carbohydrate.[77] If this is correct, then each of the oligosaccharide moieties at the cell surface should contain sialic acid as a terminal group. 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

(i.e., -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 associated with other types of carboxylic acids, in particular, the side chain carboxyls of glutamic and/or aspartic acid residues. One argument which might be advanced against this idea is the fact that the anodic mobility of neuraminidase-treated cells does not show an inflection at low pH (see Figures 9B and 10B). This would seem to indicate that only one type of anionic group is contributing to the surface charge. It must be borne in mind, however, that the titration curve of a dibasic acid will show an inflection only if the ratio of the two dissociation constants exceeds 60.[33] Consequently, the absence of an inflection in the mobility-pH curve for neuraminidase-treated cells does not exclude the possibility that the negative surface charge may be determined by a mixture of α-, β-, and γ-carboxyls with pK_a's separated by less than two pH units. However, the fact that the average surface pK_a of these groups lies in the range 2.5 to 3.0 clearly indicates a preponderance of α-carboxylic acids.

At this point it is worthwhile to calculate the proportion of the rat erythrocyte surface occupied by acidic protein and carbohydrate moieties. Assuming that the contribution made to the anodic mobility by protein-bound amino acid carboxyls is -0.70 micron/sec/volt/cm, then from the Gouy-Chapman equation (p. 21) the surface charge density associated with these groups is -0.0078 coulomb per square meter. If the area per amino acid is taken to be 17 square angstroms,[76] it may be calculated from the charge density that 0.8% of the cell surface area is occupied by electrophoretically detectable acidic residues.

From studies on human erythrocyte ghosts, Rosenberg and Guidotti have found that hexose, N-acetylhexosamine,* and sialic acid constitute, respectively, 4.0, 2.0, and 1.2% of the membrane weight.[78] Using this information it can be calculated that, on the average, each molecule of sialic acid is linked to eight molecules of sugar. If an approximate molecular area of 60 $Å^2$ is assigned to sialic acid and 40 $Å^2$ to each sugar molecule,[9] the total surface area covered by one sialyl oligosaccharide unit is then 380 $Å^2$. This calculation, however, assumes that polysaccharides are flattened out at the cell surface, so that 380 $Å^2$ must be regarded as the maximum area covered by one unit. From a chemical analysis of the sialic acid released by neuraminidase, the number of these molecules at the rat erythrocyte surface is 1.44×10^7 (see p. 61). On the basis of this information, the maximum area per cell occupied by carbohydrate is 54.7 square microns, or 66% of the total surface area.

(E) Ion Adsorption

As discussed in Chapter 1, Heard and Seaman have shown that the anodic mobility of human erythrocytes is independent of the monovalent anion present in the suspending medium.[10] Since the anions tested have differing hydrated radii, this observation indicates that no component of the negative surface charge density is attributable to anion adsorption. Haydon and Seaman have also found that the anodic mobility of acetaldehyde-fixed human erythrocytes can be reduced to zero by methylation,[40] a result which would not be expected if anion adsorption served as a charging mechanism. Similar experiments with glutaraldehyde-fixed rat erythrocytes

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* Rosenberg and Guidotti referred to this class of sugars as hexosamines. Kathan and Adamany have shown, however, that amino sugars at the erythrocyte surface are acetylated.[68]

were inconclusive (2B2). The number of electrophoretically detectable anionic groups esterified by methanolic hydrochloride was found to be 25 to 50% of the total, and the number reactive with a water soluble carbodiimide (CMC) was 35% of the total (2B2). However, in the mobility-pH curve shown in Figure 6B for CMC-treated cells, the binding of protons to unreacted anionic groups 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 anions, and cannot be regarded as an indication that this portion of the charge density arises from anion adsorption. The physical basis for the incomplete reactivity of methanolic hydrochloride and CMC with ionic groups at the rat erythrocyte surface is not clear. It is possible that a large portion of the carboxylic acids contributing to the mobility are localized in tightly packed "islands." As a consequence, steric hindrance could prevent the majority of these groups from being chemically modified by reagents such as CMC. Another possible explanation would be that large numbers of anionic groups may exist in cul-de-sacs at the cell surface and contribute to the mobility by virtue of their inaccessibility to counterions (see Chapter 1, p. 10). It is quite reasonable to assume that groups of this nature would be inaccessible to CMC.

Below pH 1, glutaraldehyde-fixed rat erythrocytes exhibit a large increase in positive surface charge density (2A4). This proton binding can be fit reasonably well to a Langmuir adsorption isotherm with a single binding constant. The maximum charge density associated with this set of hydrogen ion binding sites is +0.331 coulomb per square meter, a

value which is approximately thirty times as large as the surface charge density attributable to any other membrane component. This fact suggests that the charging mechanism involved is probably non-specific hydrogen ion adsorption onto non-ionogenic regions of the membrane, as opposed to proton binding by a single class of weakly basic groups. From the charge density it can be calculated that approximately 25% of the total surface area of the rat erythrocyte is composed of non-specific hydrogen ion binding sites (2A4).

(F) Summary

At this point it is appropriate to summarize briefly the available chemical and electrophoretic data in the form of a model of the rat erythrocyte membrane interface. Based on a chemical analysis of sialic acid, hexose, and N-acetylhexosamine, it appears that at most 66% of the rat erythrocyte surface is covered by sialyl oligosaccharides. At present, the composition of the remainder of the surface cannot be accounted for in terms of specific chemical information. Nevertheless, some speculations can be put forth on the basis of electrophoretic studies.

After removal of sialic acid molecules by neuraminidase treatment or mild acid hydrolysis, approximately two thirds of the negative surface charge remains. The ionic groups responsible for this portion of the total negative charge have been tentatively identified as protein-bound carboxylic acids. From the fact that their average surface pK_a lies in the range 2.5 to 3.0, it would appear that they are predominantly α-carboxyls, although as many as 40% of these anions may be side chain β- and/or γ-carboxyls. The α-carboxyls are probably associated with C-terminal

residues involved in protein-carbohydrate linkage at the cell surface. From their contribution to the mobility, it can be calculated that the proportion of the rat erythrocyte surface area occupied by protein-bound carboxylic acids is 0.8% of the total.

Electrophoretic studies at low pH on glutaraldehyde-fixed rat erythrocytes indicate the presence near the surface of shear of weakly basic groups with an approximate surface pK_a of 1.6. These are probably protein-bound side chain amides, although available information does not rule out the possibility that they are acetylated amides associated with sialic acid and N-acetylhexosamine molecules. If these weak bases are indeed amide groups of asparagine and/or glutamine residues, then it may be calculated from their contribution to the positive charge density that they occupy 1% of the total cell surface area.

Below pH 1, glutaraldehyde-fixed rat erythrocytes exhibit a large increase in positive surface charge density, the origin of which is probably non-specific hydrogen ion binding onto non-ionogenic regions of the cell surface. From the charge density it can be calculated that these proton binding sites constitute approximately 25% of the total surface area.

The electrophoretic information summarized in the preceding paragraphs accounts for roughly 80% of the non-carbohydrate regions of the rat erythrocyte surface. In this regard it must be borne in mind that the surface charge measured at physiological ionic strength is determined by ionogenic properties localized within approximately eight angstroms of the surface of shear. Consequently, some of the chemical groups located at the physical membrane surface may not be detectable by electrophoretic means. Also, in making calculations based on the equations of

electrophoresis, it was assumed that the cell surface is impenetrable to counterions and that surface conductance corrections could be neglected. As discussed in Chapter 1, both of these factors can lead to underestimates of the actual surface charge density.

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TABLE I: INFRARED BAND ASSIGNMENTS

Vibration Frequency in cm^{-1}	Probable Origin of Band
1740	C = O stretch; lipids
1650	Amide I (C = O stretch); peptide amides in α-helical and/or random coil configuration; sphingomyelin
1540	Amide II (N - H bend); peptide amides in α-helical and/or random coil configuration; sphingomyelin
1450 - 1465	Methylene scissors; asymmetric methyl bending
1380 - 1390	Symmetric methyl bending; symmetric carboxyl stretch
1235	P = O stretch; asymmetric C - O - C stretch; lipids
1165	Symmetric C - O - C stretch; lipids
1060 - 1080	P - O - C stretch; lipids
965	N - C stretch; choline
720	Methylene rock; lipids

TABLE II: ELECTROPHORETIC STUDIES ON RAT ERYTHROCYTES TREATED WITH ASPARAGINASE

Sample	Treatment	pH	Mobility $\left(\frac{\text{microns/sec}}{\text{volt/cm}}\right)$
A	Control erythrocytes incubated at 37° C for one hour	7.20	-1.11
B	Same as Sample A with subsequent glutaraldehyde fixation	7.56	-1.07
C	Erythrocytes reacted with 25 units/ml asparaginase at 37° C for one hour	7.53	-1.09
D	Same as Sample C with subsequent glutaraldehyde fixation	7.50	-1.09
E	Same as Sample B	1.05	+1.15
F	Same as Sample D	1.10	+1.01

TABLE III: ELECTROPHORETIC STUDIES ON RAT ERYTHROCYTES TREATED WITH GLUTAMINASE

Sample	Treatment	pH	Mobility $\left(\frac{\text{microns/sec}}{\text{volt/cm}}\right)$
A	Control erythrocytes incubated at 37° C for one hour	7.30	-1.01
B	Same as Sample A with subsequent glutaraldehyde fixation	7.35	-1.05
C	Erythrocytes reacted with 10 units/ml glutaminase at 37° C for one hour	7.18	-1.05
D	Same as Sample C with subsequent glutaraldehyde fixation	7.64	-1.03
E	Same as Sample B	1.15	+1.08
F	Same as Sample D	1.14	+1.07

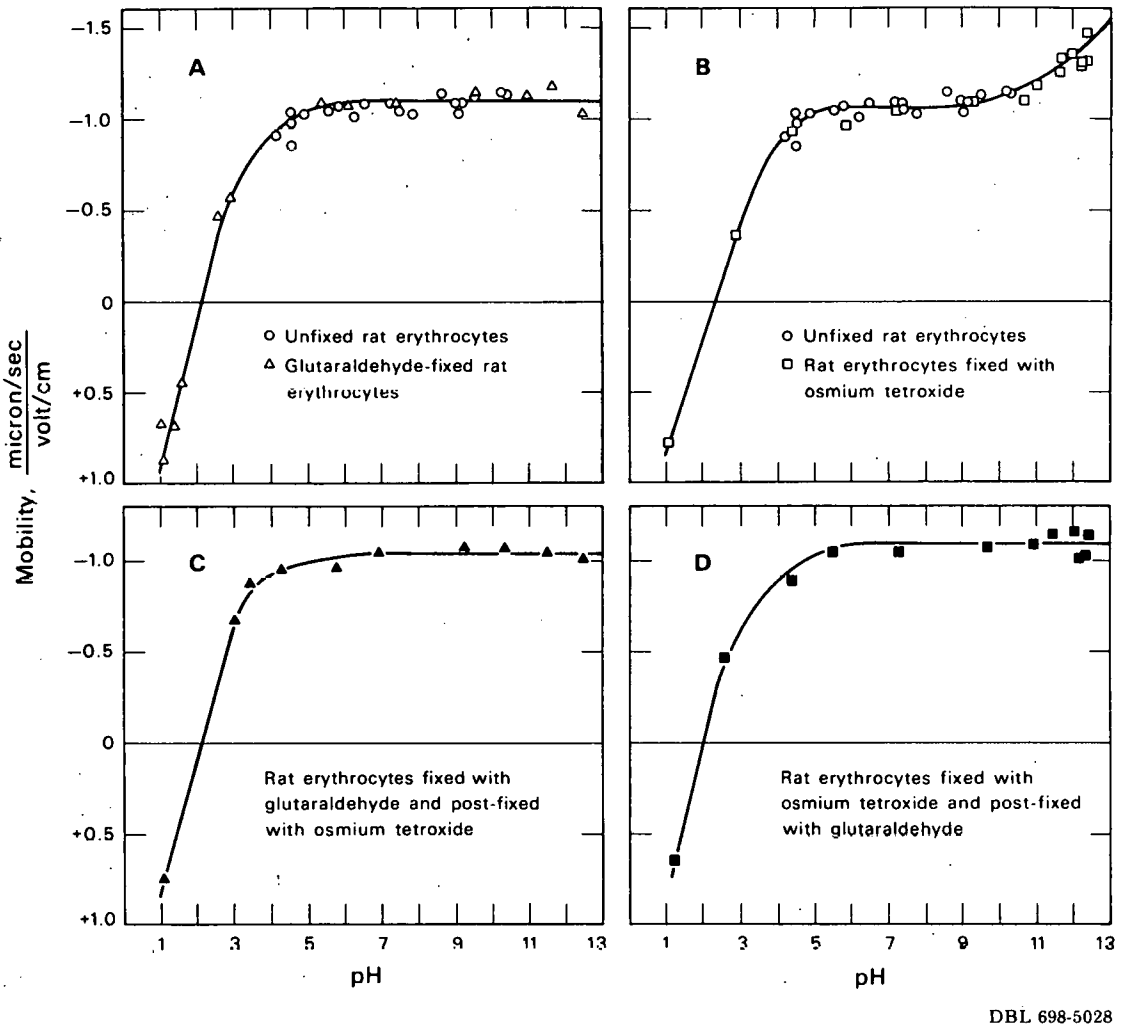
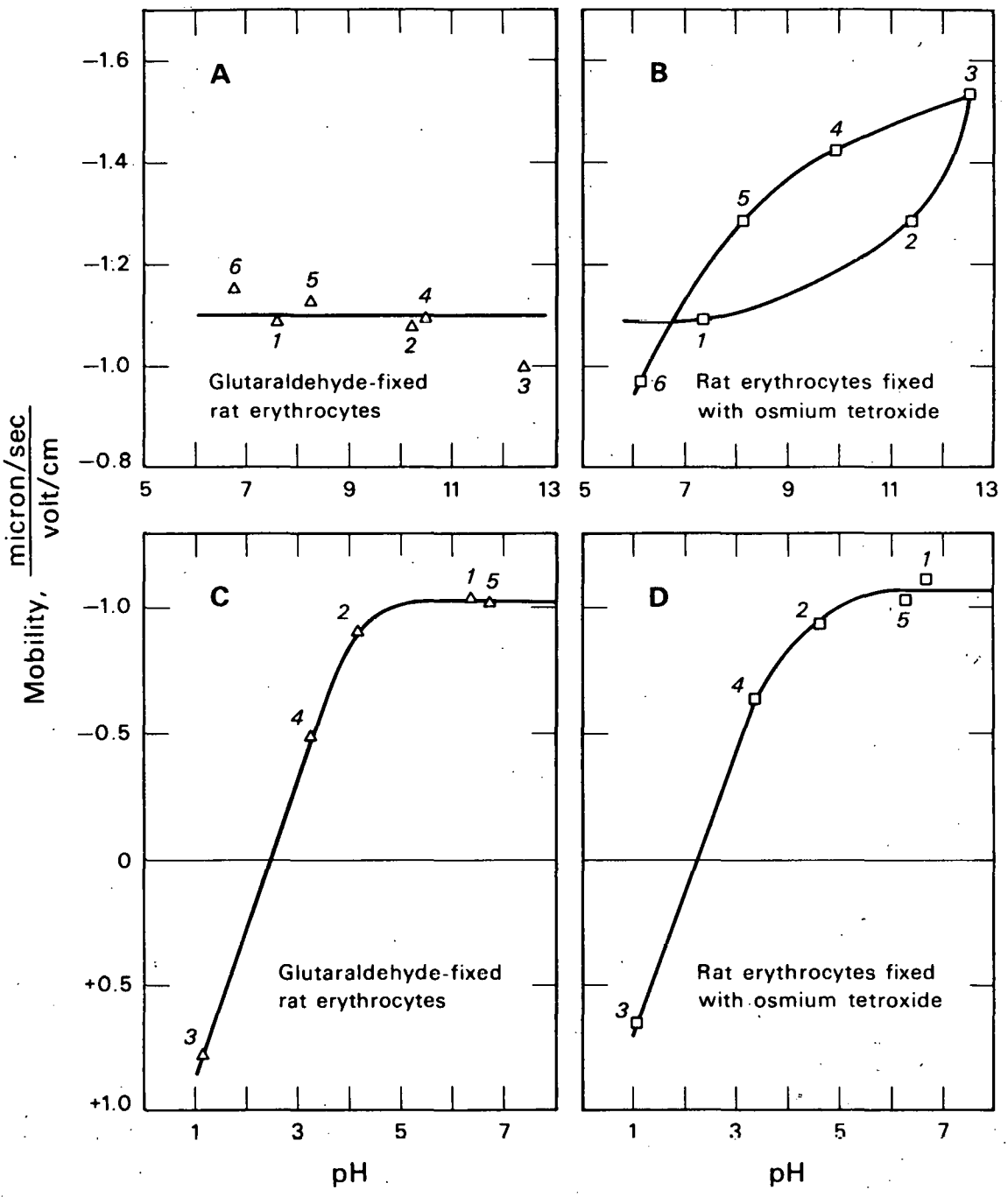


FIGURE 1. Mobility-pH curves at ionic strength 0.145 are shown in A and B for unfixed rat erythrocytes and erythrocytes fixed with 2.5% glutaraldehyde and 1% osmium tetroxide. Fixation with both reagents was carried out at pH 7.4 and room temperature (21-24° C). With glutaraldehyde the reaction was allowed to proceed for ten minutes, and with osmium tetroxide for one minute. In C and D, the surface charge properties are shown following dual fixation with 2.5% glutaraldehyde and 1% osmium tetroxide.

FIGURE 2. Studies are presented on the reversibility of the surface charge properties observed for rat erythrocytes fixed with 2.5% glutaraldehyde and 1% osmium tetroxide. The ionic strength in these experiments was maintained at 0.145.

Erythrocytes were incubated successively at the pH values denoted by numerals. The manner in which this was performed was to suspend erythrocytes at a pH value denoted by the numeral 1 and record the electrophoretic velocity. The cells were then centrifuged at 1100 x g for three minutes. The supernatent was removed and its conductivity measured in order to determine the field strength at that particular pH. The packed cells were then resuspended at a pH value denoted by the numeral 2, and the mobility measured in the same manner. This procedure was repeated until the cells were finally returned to a pH value near neutrality.

From the data presented in A and B, it is clear that glutaraldehyde-fixed cells exhibit a reversible electrokinetic character in the alkaline pH range, whereas erythrocytes fixed with osmium tetroxide do not. As shown in C and D, erythrocytes fixed with either reagent have a reversible behavior in the acid pH range. Although the data is not presented here, it was found that dual fixation with these reagents yielded cells having reversible surface charge properties at both high and low pH.



DBL 698-5029

FIGURE 2. The descriptive caption for this figure is given on the preceding page.

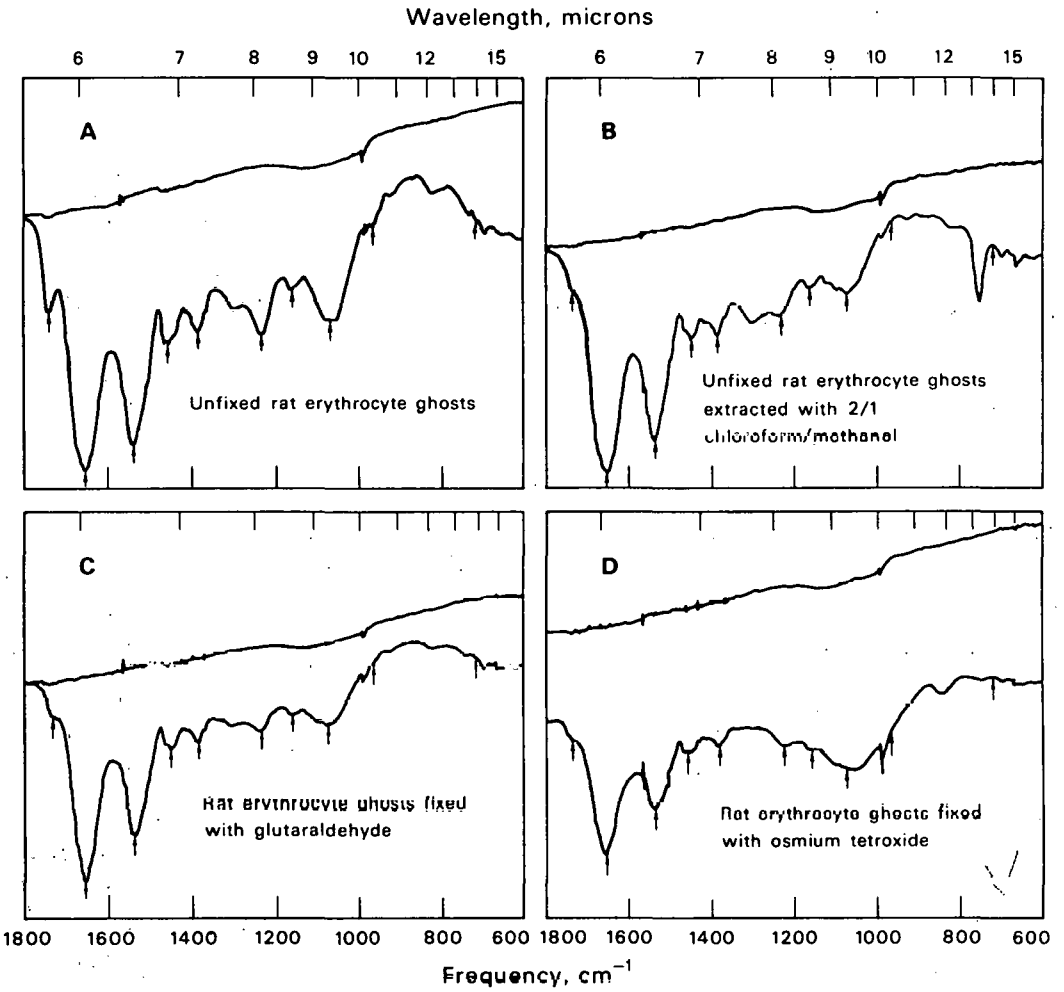


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FIGURE 3. Infrared spectra are shown for unfixed rat erythrocyte ghosts (A), ghosts extracted with a 2/1 mixture of chloroform/methanol for twenty minutes at room temperature (B), ghosts fixed with 2.5% glutaraldehyde (C), and ghosts fixed with 1% osmium tetroxide (D). A silver chloride plate coated with silver sulfide was used as a substrate. Approximately two to three milligrams of ghosts suspended in distilled water were layered on a plate and dried at 40° C.

The transmittance scale is linear, with the baseline of each figure corresponding to 0% transmittance and the top to 100% transmittance. In each figure the transmission characteristics are shown for the silver chloride plate used as a substrate. In B the absorbance at 755 cm^{-1} is associated with the C - Cl stretching mode of residual chloroform contained in the extracted ghosts. The arrows refer to absorbances for which tentative origins are assigned in Table I, p. 74.

FIGURE 4. In A the surface charge properties at ionic strength 0.145 are shown for rat erythrocytes reacted with 2% acetaldehyde in phosphate-buffered saline, pH 7.4. The fixation medium was changed at the end of the first day, after which the cells were allowed to fix for a further twenty days. The temperature was maintained at 4° C throughout the period of fixation. The numerals 1, 2, and 3 refer to a reversibility study at low pH utilizing the technique described in the caption for Figure 2 (p. 78). The primed numerals refer to 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.

The surface charge properties of acetaldehyde-fixed erythrocytes following post-fixation with 1% osmium tetroxide are shown in C.

A mobility-pH curve is shown in D for rat erythrocytes reacted with 2.5% glutaraldehyde in pH 7.4 Sorensen phosphate buffer for twenty days. The temperature was maintained at 4° C throughout the fixation.

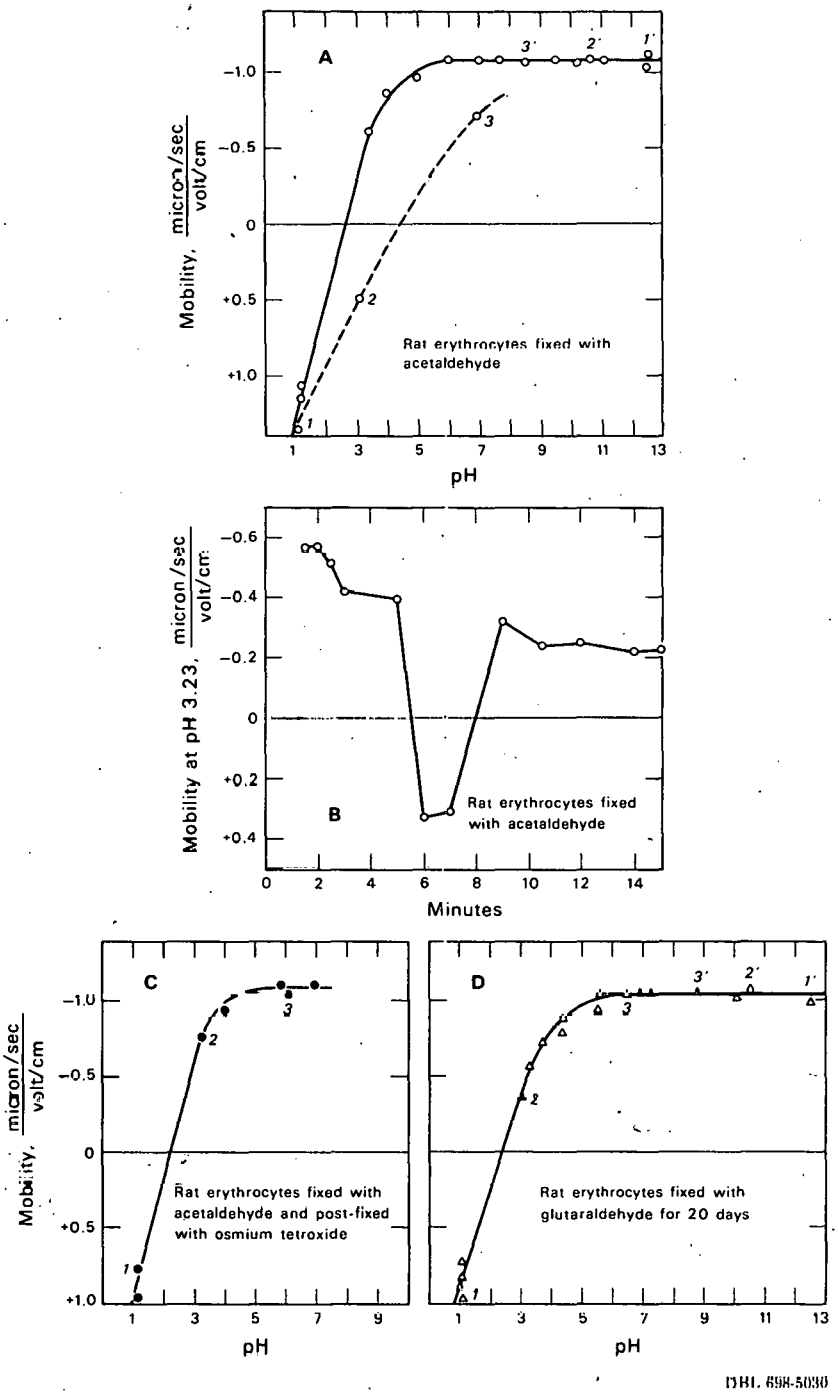

FIGURE 4. The descriptive caption for this figure is given on the preceding page.

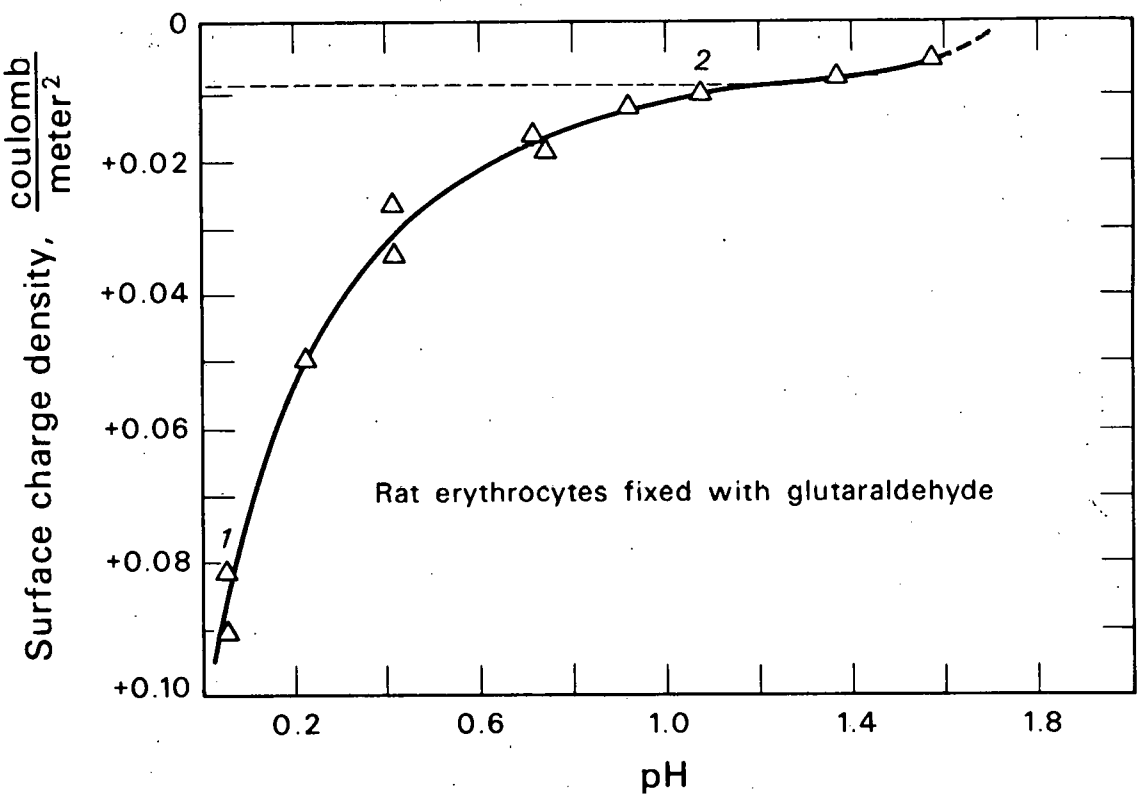

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FIGURE 5. The surface charge density is plotted as a function of pH for rat erythrocytes reacted with 2.5% glutaraldehyde. The numerals 1 and 2 refer to a reversibility study. The charge density was calculated from the electrophoretic mobility using the Gouy-Chapman equation (see p. 21). 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 6. The reduction in electrophoretic mobility at ionic strength 0.145 and pH 7.4 is shown in A for rat erythrocytes fixed with 2.5% glutaraldehyde and then reacted with 0.1M CMC for varying lengths of time. CMC is the water soluble compound 1-cyclohexyl-3-(2-morpholinylethyl) carbodiimide metho-p-toluene-sulfonate. 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 0.1M 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.

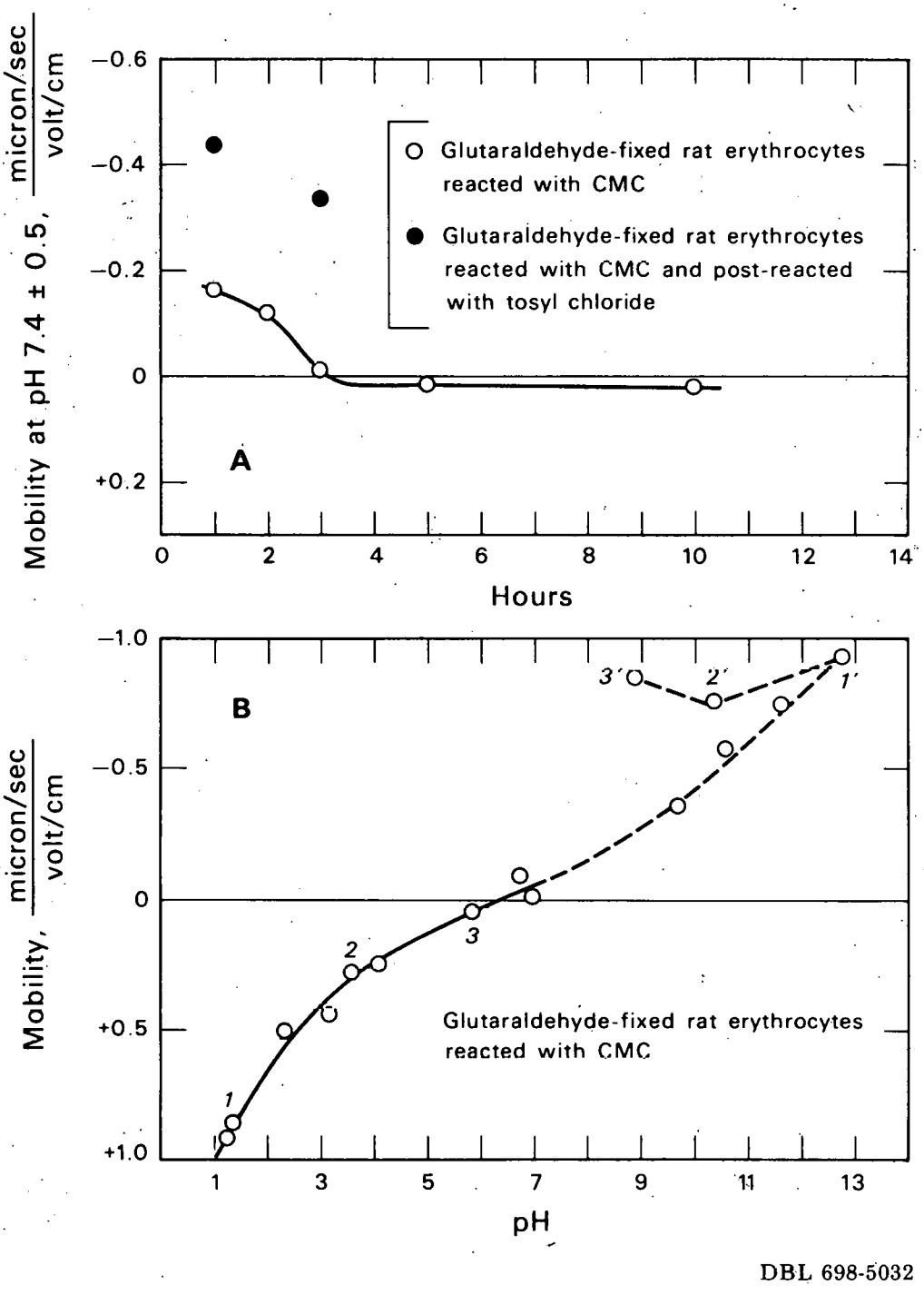
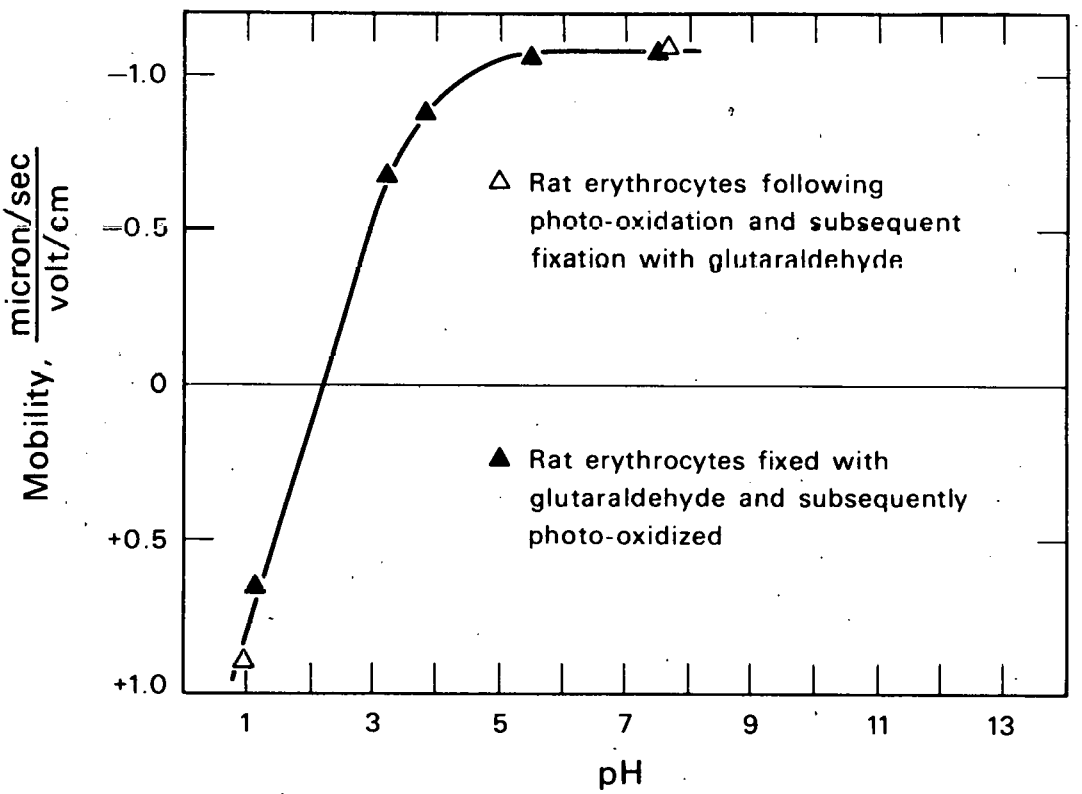
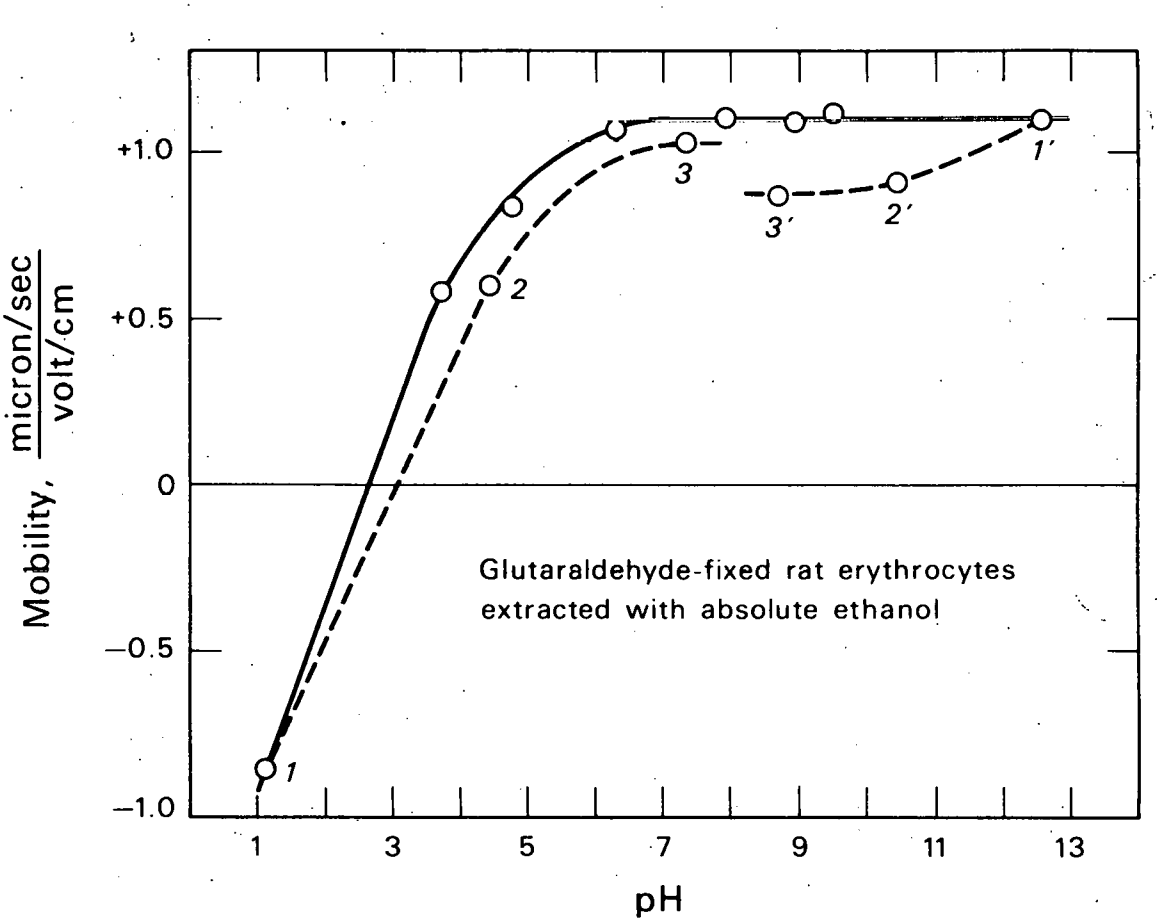


FIGURE 6. The descriptive caption for this figure is given on the preceding page.



DBL 698-5033

FIGURE 7. The electrophoretic mobility at ionic strength 0.145 is shown as a function of pH following photo-oxidation of rat erythrocytes in the presence of 0.01 mg/ml methylene blue. The cell suspension containing methylene blue was placed in a 37° C bath and subjected to illumination from a 150 watt lamp at a distance of six inches. The irradiation was allowed to proceed for ninety minutes with continuous stirring. Erythrocytes were fixed with 2.5% glutaraldehyde either prior or subsequent to the photo-oxidative process.



DBL 698-5034

FIGURE 8. The mobility-pH characteristics at ionic strength 0.145 are shown for glutaraldehyde-fixed rat erythrocytes after incubation in absolute ethanol for one hour at room temperature (21-24° C). The primed and unprimed numerals refer to reversibility studies at high and low pH, respectively.

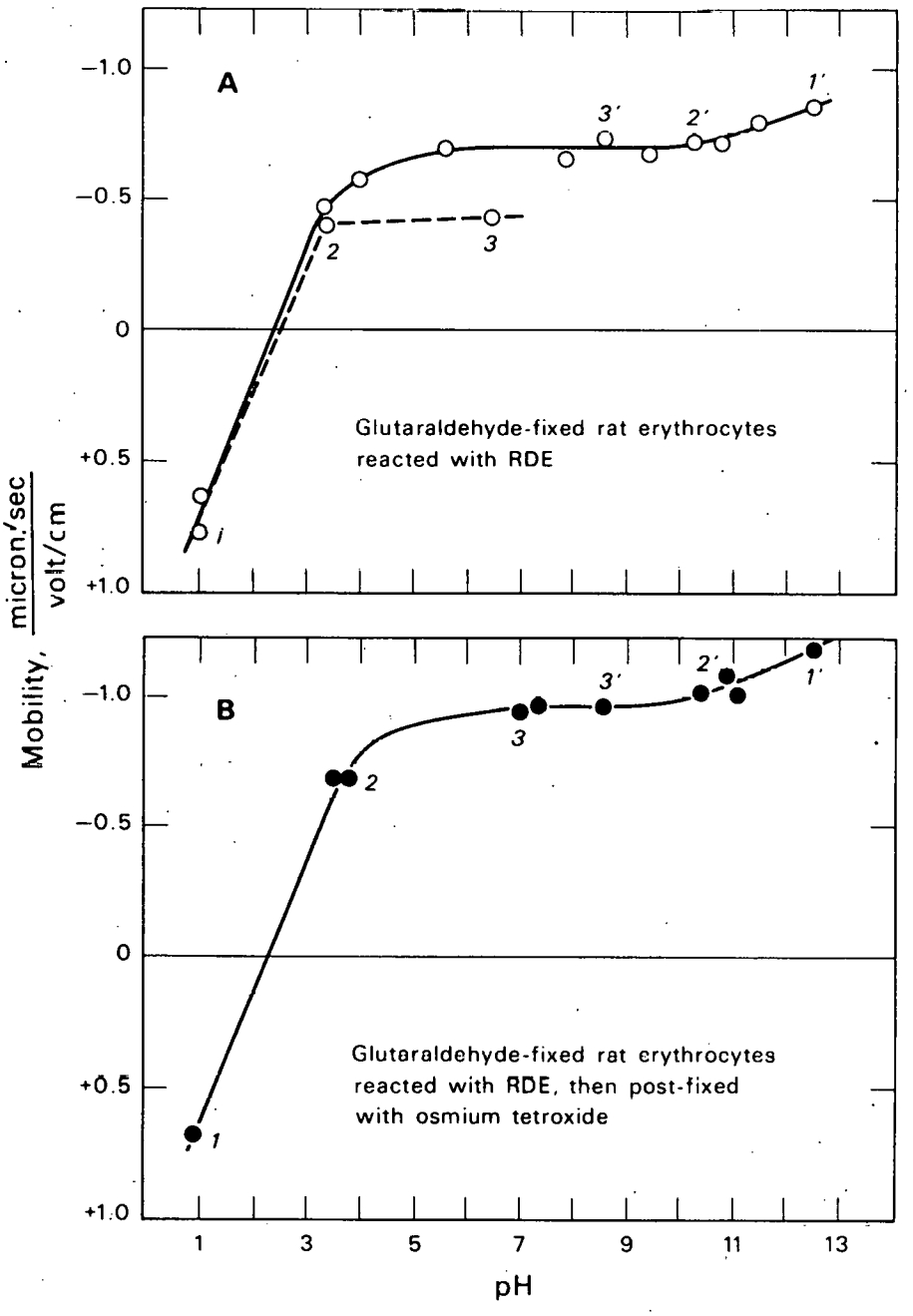


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FIGURE 9. The surface charge properties at ionic strength 0.145 are shown in A for rat erythrocytes fixed with 2.5% glutaraldehyde and then reacted with RDE (receptor-destroying enzyme). This preparation of neuraminidase is a crude extract from Vibrio cholerae. Reaction conditions were adjusted to achieve a maximal release of sialic acid.

In B the mobility-pH characteristics are shown for RDE-treated cells following post-fixation with 1% osmium tetroxide.

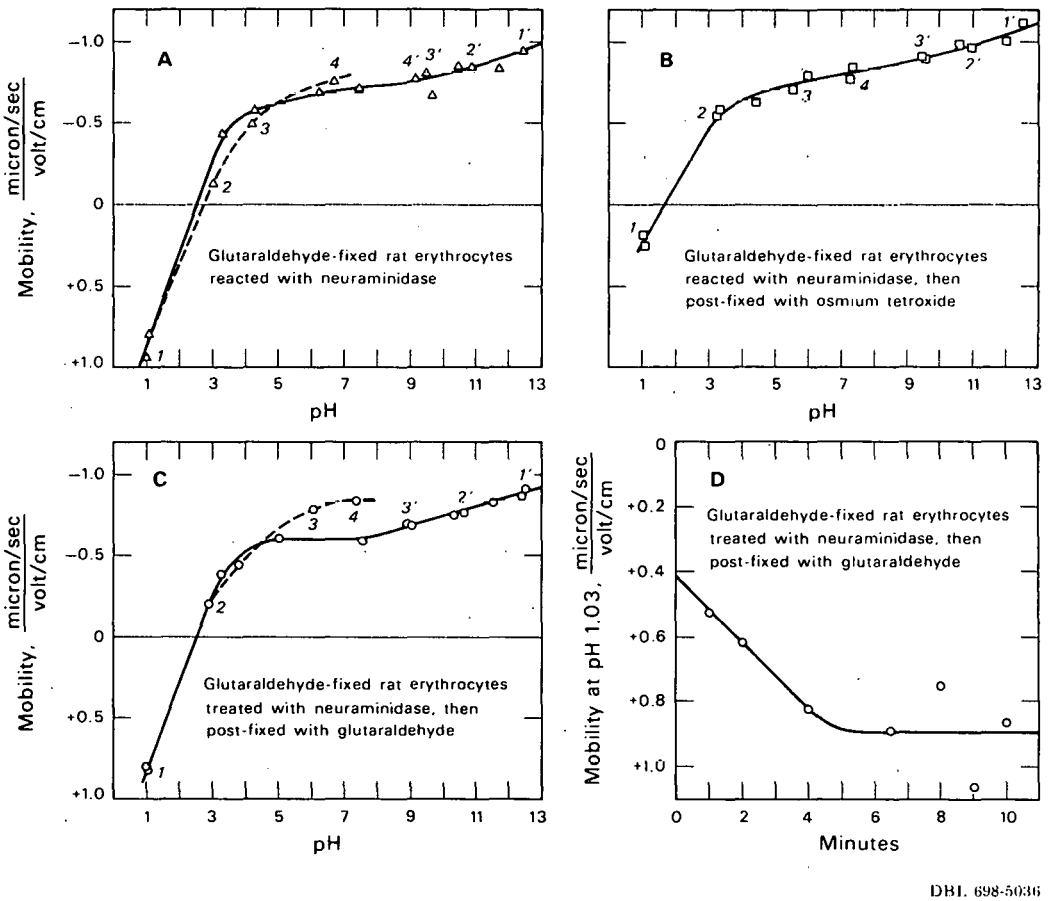

FIGURE 10. In A the mobility-pH characteristics at ionic strength 0.145 are shown for rat erythrocytes fixed with 2.5% glutaraldehyde and then reacted with chromatographically purified neuraminidase from Clostridium perfringens. The enzyme concentration was 0.25 mg/ml and the reaction was performed at pH 5.35 for one hour at 37° C. These conditions were determined to effect a maximal release of sialic acid. The primed and unprimed numerals refer to reversibility tests at high and low pH, respectively.

In B and C the surface charge properties are shown following post-fixation of the neuraminidase-treated cells with 1% osmium tetroxide and 2.5% glutaraldehyde. The mobility of the glutaraldehyde post-fixed cells at pH 1.03 is shown as a function of time in D.

ACKNOWLEDGMENTS

The author wishes to express appreciation to the members of his research committee, Dr. Robert M. Glaeser, Dr. Alexander V. Nichols, and Dr. A. D. McLaren, for advice and encouragement during the period of this research. The suggestions and collaboration of many others have contributed substantially to my efforts. In particular, I would like to thank Dr. Robert I. Macey, Dr. Norman K. Freeman, Dr. Neal Castagnoli, Mr. John Kroes, Dr. Donald L. Puppione, and Dr. George Oster. The facilities provided by the Lawrence Radiation Laboratory greatly aided my research.

Support was received during the period Fall 1963 through Summer 1965 from a training fellowship administered under the Biophysics Training Section of the National Institutes of Health, Grant No. 5-T1-GM-829. From the Fall of 1965 through the Summer of 1968 the author was the holder of a National Institutes of Health pre-doctoral fellowship, No. 5-F1-GM-31. The position of Research Assistant at the Lawrence Radiation Laboratory was held during the period Fall 1968 through Summer 1969.

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