THE SURFACE CHEMISTRY OF THE ERYTHROCYTE AND THROMBOCYTE MEMBRANE

G. V. F. Seaman

Division of Neurology, University of Oregon Medical School, Portland, Oregon 97201

The electrokinetic procedures used for investigation of the nature and distribution of cellular surface charge groups are outlined. The application of ion binding studies, the action of enzymes, and the effects of specific functional group reagents are illustrated by discussion of the results obtained for erythrocytes and thrombocytes. The human erythrocyte has been shown to be a macropolyanion possessing about 10^7 charged groups per cell of which more than 60% are the carboxyl group of N-acetylneuraminic acid; some of the remaining groups at least are the α-carboxyl groups of protein-bound amino acids. The electrokinetic constitution of the human blood platelet is more complex and includes sialic acid carboxyl groups and phosphate groups and at least one other species of unidentified anionogenic groups as well as amino groups. Lastly the effects of neutral and charged polymer adsorption on the electrokinetic properties of blood cells are briefly considered.

Electrophoretic procedures have provided information on the surface structure of red blood cells and thrombocytes without producing significant changes or disruption of the cellular organization. The course of any chemical, enzymatic, immunological, or viral reaction which changes the conformation, topography, or structure of charge groups may be investigated by such procedures. Such information is particularly useful since the peripheral zone or cell envelope is the site of numerous biological processes which relate to the fields of enzymology, immunology, pathology, and virology.

The electrokinetic properties of the blood elements are relevant to the processes of aggregation, adhesion, and rouleaux formation (1, 2). Under physiological conditions platelets and red blood cells carry net negative electrokinetic charges and changes in these charges may play a role in blood element interactions and blood element–vessel wall interactions. Electrophoresis is a method of surface examination of cells and thus the terms cell envelope, periphery, or surface denote the electrokinetic surface of the cell. When a cell moves in an electrical field (electrophoresis) it is probable that there is no sharp transition from the bulk suspending medium to immobile surface. Operationally it is assumed that a hypothetical plane exists at which, if a sharp transition were to occur, the electrophoretic mobility of the cell would be identical to that observed. This plane is located most probably within a few Ångström units of the envelope which describes the position of the terminal ionogenic groups where their numerical density is sufficient to produce a significant change in the optical and rheological properties of the suspending medium. The electrokinetic surface is thus defined in terms of those ionogenic components of the peripheral zone of the cell which contribute to its electrokinetic properties under the particular conditions of measurement. The peripheral zone of the cell is con-

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sidered to be the outer portion of the cell membrane where the volume not occupied by macromolecular structures is accessible to extracellular ions and small molecules.

In general biological surfaces acquire their charge by the ionization of chemical functional groups which are an integral part of the surface structure. Ion adsorption or desorption normally plays at most only a minor role in governing the charge of platelets, red blood cells, and other similar hydrophilic surfaces. The electrokinetic properties of erythrocytes have been reviewed recently by Tenforde (3) and of platelets by Mehrishi (4).

Electrokinetic Theory

It is possible to calculate from an electrophoretic mobility (u) the net electrokinetic charge density (σ) of a cell. This is achieved by calculating a zeta potential (ζ) from the measured electrophoretic mobility and then interpreting this zeta potential in terms of a model of the structure of the electrical double layer to yield an electrokinetic charge density. The zeta potential is the electrical potential at the plane of slippage or plane of hydrodynamic shear between the cell and the suspending medium as the cell undergoes electrophoresis. The zeta potential may be taken as the value of the cellular surface potential (ψ) at the plane of hydrodynamic shear. The zeta potential is of somewhat smaller magnitude than the surface potential.

The electrophoretic mobility (u) is defined as the electrophoretic velocity (v) of a cell per unit electrical field strength (x). It is usually calculated as either μm sec^{-1} V^{-1} or cm^2 statvolt^{-1} sec^{-1}. The blood elements when suspended in physiological media have dimensions which are large in comparison to the extension or effective thickness of the electrical double layer which surrounds them. The electrophoretic mobilities will depend mainly therefore upon the properties of the peripheral zone of the cells and not upon their shape or orientation in the applied electrical field (1). Since platelets and red blood cells are nonconductors and the dielectric constant and viscosity are the same within the electrical double layer, as in the bulk medium, then the relationship between the electrophoretic mobility and zeta potential is described reasonably accurately by the Helmholtz-Smoluchowski equation

$$\frac{v}{x} = u = \frac{\zeta \epsilon}{4\pi\eta} \tag{1}$$

where v is the electrophoretic velocity in μm sec^{-1}, x is the electrical field strength in ordinary volts cm^{-1}, and η and ϵ are the viscosity in poise and dielectric constant, respectively, within the electrical double layer.

The electrokinetic charge density depends only on the nature of the cell surface and on the ionic composition of the suspending medium. The calculated zeta potentials may be converted into surface charge densities by application of the Gouy–Chapman equation which for predominantly uni-univalent electrolytes takes the following form:

$$\sigma(\text{statcoul. cm}^{-2}) = 2\left[\frac{Nkt}{2000\pi}\right]^{1/2} (\epsilon I)^{1/2} \sinh\left[\frac{e\zeta}{2kT}\right] \tag{2}$$

in which N is Avogadro's number, k the Boltzmann constant, T the absolute temperature, e the charge on the electron, and I the ionic strength which is defined as

$$I = \frac{1}{2} \sum c_i z_i^2$$

where c_i is the molar concentration of ion of type i and z_i is the valency of charge of ion i.

The applicability and limitations of the electrokinetic theory has been reviewed by Haydon (5). The Gouy–Chapman equation assumes a surface which is impenetrable to ions, but this is known not to be the case for the blood elements. As a consequence Haydon (6) introduced into the equation a term, α, which was defined as the fraction of total space within the peripheral zone unavailable to counterions; the modified Gouy–Chapman equation takes the form

$$\sigma = [1 + (1-\alpha)^{1/2}]\ 2\left[\frac{NkT}{2000\pi}\right]^{1/2} (\epsilon I)^{1}\lambda \sinh\left[\frac{e\zeta}{2kT}\right] \qquad (3)$$

When α is unity for a system impenetrable to counterions the equation reduces to eq. (2).

Under normal circumstances electrophoretic analysis of the peripheral zones of cells yields only the net electrokinetic charge and further it assumes that this charge is distributed uniformly throughout the region. At physiological ionic strength (0.15 M uni-univalent electrolyte) the effective thickness of the electrical double layer is ~ 8 Å and for a surface which is penetrable to counterions, ionogenic groups up to about 10 Å beneath the slip plane may contribute to a significant degree to the electrophoretic properties of the cell.

Procedures for the Surface Analysis of Cells

The nature and distribution of cellular surface charge groups may be investigated by studying the dependence of the electrophoretic mobility on (1) pH of the suspending medium; (b) ionic strength of the suspending medium; (c) the influence of a series of ions; (d) treatment with a variety of chemical reagents; and (e) treatment with enzymes.

In designing appropriate procedures for obtaining data about surface ionogenic groups it is useful to have an idea of the possible types of groups which may be present. Extensive chemical analyses of both platelet and red blood cell membranes have identified many potentially relevant components. An approach which at first sight appears to be attractive is to study the electrokinetic properties of well-characterized model compounds known to be present in membranes or which possess functional groups thought to be in such membranes. By analogy between the electrokinetic behavior of the blood cells and the model compound it may be possible to deduce if the compound plays any significant role in the electrokinetic behavior of the cell. Unfortunately the approach is severely limited by the large number of potentially relevant components which have been identified in blood cell membranes and the possibility that the complexity of the peripheral zone of blood cells may lead to interactions between components which would result in their displaying electrokinetic behavior significantly different from that observed in model systems. In addition relevant model compounds have not been available in high enough purity to enable unambiguous assignment of their observed electrokinetic properties to them rather than to impurities. For example, a concentration of 0.1 % w/w of a foreign unknown ionogenic material, if surface active, could be primarily responsible for the electrokinetic behavior of the model compound (7).

Native blood cells display reversible or partially reversible electrokinetic behavior over only a limited range of conditions. The ranges of ionic strength and pH of the suspending medium over which the human red blood cell yields reversible changes in electrokinetic behavior have been documented by Heard and Seaman (8) (Fig. 1). Many potentially useful reagents for the modification of blood cell surface groups may not be employed

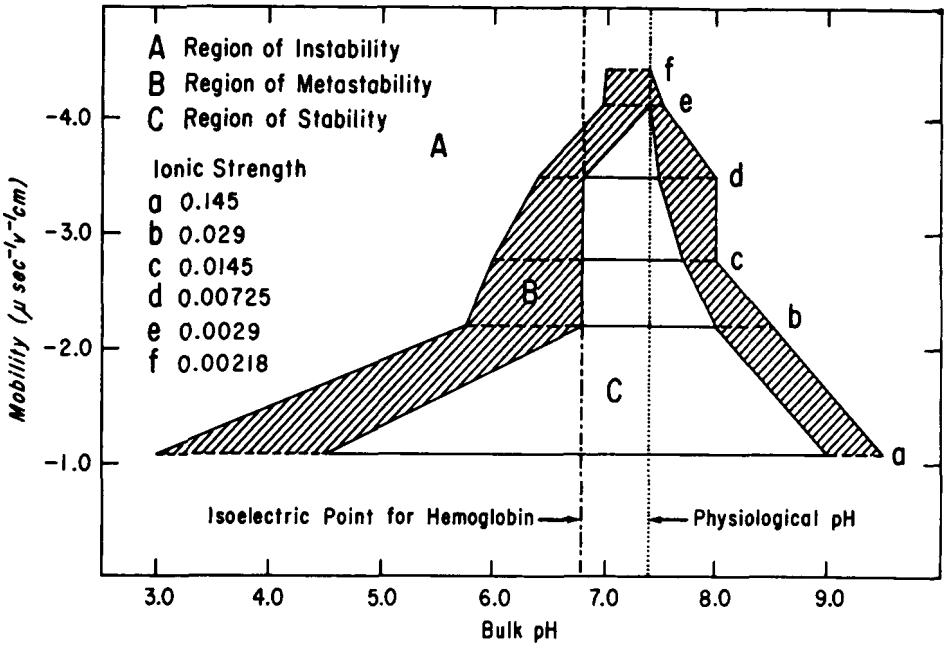

Fig. 1. Electrokinetic stability diagram for human red blood cells. (A) region in which irreversible changes in the electrophoretic mobility occur; (B) region in which adsorption of hemoglobin takes place, but where this may be reversed by washing in standard saline; (C) region where no significant time-dependent changes in electrophoretic mobility arise.

because the reactions require nonphysiological conditions which produce irreversible changes in the electrophoretic properties of erythrocytes or thrombocytes. However, the electrokinetic stability of blood cells may be greatly increased by treatment with aldehydes (9, 10). Such aldehyde treatment produces no change in the electrophoretic mobility of the human erythrocyte over the ranges of pH and ionic strength for which the native erythrocyte is stable (Fig. 1, Region C) but does produce a significant increase in the anodic mobility of thrombocytes (11).

MATERIALS AND METHODS

The standard saline used as the suspending medium for many of the blood cells suspensions consisted of 0.15 M aqueous NaCl solution made 3×10^{-4} M with respect to $NaHCO_3$, pH 7.2 ± 0.2. Standard washed suspensions of red blood cells and platelets were prepared as previously described (9, 11). Sources of reagents and enzymes are given in earlier publications: salts (8, 12), amino group reagents (8, 11), carboxyl group reagents (13), and proteolytic enzymes and neuraminidase (11, 14).

Microelectrophoresis was carried out with a cylindrical tube apparatus equipped with Ag/AgCl electrodes as described by Seaman and Heard (15). Treatment of blood cells with aldehydes was conducted as reported by Vassar et al. (10). Chemical modification of human erythrocyte surface groups was performed as previously outlined (13, 16). Details of the treatment of red blood cells and platelets with neuraminidase and proteolytic

enzymes are given by Seaman and Uhlenbruck (14) and Seaman and Vassar (11). The effects of pharmacological agents on the electrokinetic properties of platelets are given by Seaman (2) and Seaman and Brooks (17). The methodology and analysis of the effects of neutral polymers on the electrokinetic properties of blood cells is given by Seaman and Brooks (17) and Brooks and Seaman (18).

RESULTS AND DISCUSSION

Although there is no a priori reason to expect the mobility of blood cells to vary from one animal species to another, it has been found that they vary markedly from one species to another but are very reproducible for any particular species of animal. Data for mammalian red blood cells are presented in Table I. Although the information on platelets is more limited the electrokinetic behavior of platelets roughly parallels that of the red blood cells (2). The highly reproducible nature of the electrophoretic mobility of human red blood cells in standard saline or phosphate buffer (8) has led to their use as calibration particles for checking the correct alignment of microelectrophoresis apparatus. The mobility of blood cells have been found to be independent of the method of blood collection, the age, sex, and race of the donor and also of blood group. Danon and

Table I. Electrokinetic Properities of Mammalian Erythrocytes

Animal	Electrophoretic Mobility ($\mu m\ sec^{-1}\ V^{-1}\ cm$) 25°C	
	M/15 Phosphate buffer, pH 7.4	Standard saline pH 7.2 ± 0.2
Man	1.31 (40)*	1.10 (41) 1.08 (43) 0.94 (3)
O, MN	–	1.08 (43)
A_1 MN	–	1.08 (43)
A_2 MN	–	1.08 (43)
B,MN	–	1.08 (43)
AB, MN	–	1.08 (43)
Cat	1.39 (40)	1.27 (4)
Chicken	–	0.82 (44)
Chimpanzee	–	1.18 (44)
Dog	1.65 (40)	1.28 (44)
Golden hamster	–	1.10 (41)
Guinea pig	1.11 (40)	–
Harbor seal	–	1.04 (3)
Horse	–	1.16 (44)
Killer whale	–	0.94 (3)
Mouse	1.40 (4)	1.10 (41)
Opossum	1.07 (40)	–
Ox	–	0.96 (44)
Pig	0.98 (40)	0.88 (44)
Rabbit	0.55 (40)	0.48 (41)
Rhesus monkey	1.33 (40)	1.12 (41)
Sheep	–	1.14 (44)
Sloth	0.97 (40)	–
Rat	1.45 (40)	1.18 (41) 1.28 (42) 1.25 (45) 1.00–1.10 (3)

*Numbers in parentheses refer to references.

Marikowsky (19) reported that young blood cells have a higher electrophoretic mobility than older blood cells. Yaari (20) subsequently found that the electrophoretic mobility of human red blood cells decreased with increasing age of the cell, a phenomenon which may well be of importance in the removal of effete red blood cells from the circulation.

Ion Binding Studies

Information on the nature of ionogenic groups in the cellular periphery may be obtained by a detailed examination of the effects of one or two specific cations or anions on the electrokinetic properties of the cell. A study of calcium ion binding by human red blood cells has shown the presence of at least three types of anionic charge configurations in the peripheral zone of these cells. The calculated electrochemical free energies of binding for calcium ions to the surface anions indicate in all cases weak binding. Thus calcium ions would be poor ligands for direct red cell–red cell bridging (21). An investigation of the influence of calcium and magnesium ions on the electrokinetic behavior of blood platelets has shown relatively weak binding of calcium ions to the peripheral zone of the human platelet and even weaker binding of magnesium ions. Some of the anionic sites exhibit little affinity for divalent cations, perhaps because of screening by positive sites, and a significant proportion of the anionic sites are univalent, thus facilitating reversal of charge by divalent cations (22). The interaction of larger cations such as methylene blue have been used to locate anionogenic groups in the peripheral zone of the red blood cell (23). By this means the carboxyl groups in the ultrastructure of the human red blood cell were shown to be all situated in approximately the same superficial plane in the peripheral zone (13, 23).

Action of Neuraminidases on Blood Cells

Treatment of native or aldehyde-modified human erythrocytes or thrombocytes with neuraminidases results in the release of virtually all of the membrane-bound sialic acid and to a marked decrease in their electrophoretic mobilities (11, 24). The general structure of the sialic acids is depicted in Fig. 2. They are linked glycosidically via carbon 2 usually to another sugar or amino sugar, indicated as R_1. R_2 may be a hydrogen or an acetyl group. R_3 is either acetyl or glycolyl. Neuraminidases from various sources exhibit differences in substrate specificity. Three types of α-ketosidic linkage have been shown to exist for sialic acids, namely, 2→3, 2→6, and 2→8. Vibrio chlorae neuraminidase will split all three

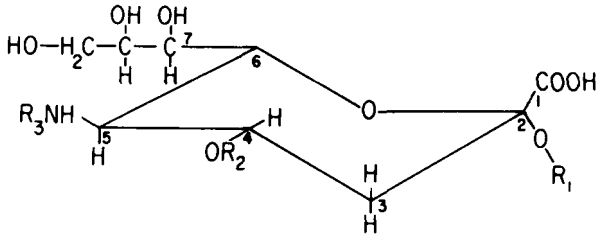

Fig. 2. Conformation of glycosidically linked sialic acid. R_1 = adjacent sugar or amino sugar moiety; R_2 = CH_3CHO- or $-H$; R_3 = CH_3CO- or CH_2OHCO-. Carbons numbered from carboxyl group (pK 2.6).

Table II. Ionogenic Groups of Human Blood Platelets and Erythrocytes

Groups	Platelet		Eyrthrocyte	
	Groups/cell $\times 10^{-5}$	Mean group Separation Å*	Groups/cell $\times 10^{-5}$	Mean Group Separation Å*
Sialic acid carboxyl	9.4–18.8	55–39	77–154	46–32
Other carboxyl	?	?	52–104	56–40
Phosphate	6.5–13.0	66–47	None (≤4–8)	–
Unidentified negative	7.0–14.0	64–45	None	–
Amino	5.3–10.6	74–52	None (≤4–8)	–
Sulfhydryl	2.9–5.9	99–69	None	–
Total negative	22.9–45.8	35–25	129–258	35–25
Total positive	5.3–10.6	73–52	None	–
Total positive and negative**	28.2–56.4	32–22	129–258	35–25

*Surface area; erythrocyte 163 μm^2, platelet 28.3 μm^2.

**Excludes sulfhydryl.

types of linkage between N-acetylneuraminic acid and other carbohydrates whereas the neuraminidases from some myxoviruses split 2→6 linked sialic acid much more slowly than 2→3 linked molecules, and fowl plague virus neuraminidase is very ineffective on 2→8 linkages in disialyllactose (25).

It has been estimated that over 60% of the electrokinetic charge of aldehyde-fixed human erythrocytes originates from the ionized carboxyl group of N-acetylneuraminic acid (see Table II). The quantity of sialic acid which is released by the action of neuraminidase is about double that calculated from the change in electrophoretic mobility, assuming the simple Gouy–Chapman equation is applicable and that the carboxyl group of every sialic acid molecule is fully effective at the electrokinetic surface of the cell. Since a calculation employing the simple Gouy–Chapman equation can underestimate the electrokinetic charge density by up to a factor of two (see Table II) the difference between the amount of sialic acid estimated by chemical assay and that computed from the change in electrophoretic mobility may not be significant (16).

It is suggested that the presence of the charged carboxyl groups of N-acylated neuraminic acids will both confer "structural rigidity" to the protein backbones of surface glycoproteins and in addition restrict the translational movement of such molecules by electrostatic repulsion between neighboring molecules. There is some evidence that reduction in the surface charge of erythrocytes by neuraminidase may be accompanied by an increase in their deformability (2). Furthermore evidence in support of a fluid dynamic membrane model (26) is accumulating. Such a model permits the random arrangement of components by free translational planar diffusion or movements mediated or induced by interaction with components added to the ambient medium (27, 28).

Action of Other Enzymes

In theory the use of enzymes of known specificity under essentially physiological conditions coupled with an examination of their reaction products represents one of the most valuable tools available for the elucidation of the molecular structure of the peripheral zone of blood cells.

Treatment of human blood platelets with alkaline phosphatase has shown the presence

of susceptible phosphate groups (Table II) (29). Sialic acid is released in bound form from human red blood cells by treatment with proteases (trypsin, ficin, papain, etc.), with often a concomitant decrease in the electrophoretic mobility (14). A correlation exists between the amount of sialic acid released and the corresponding decrease in the electrophoretic mobility. However the changes in mobility could have arisen not only from the direct and specific action of the enzyme but also from other factors including adsorption of the enzyme or products of its autolysis, enzymatic action of impurities and adsorption of cellular degradation, or leakage components. Rearrangements of ionogenic groups may also occur as well as changes in cellular parameters which influence electrophoretic mobility, for example, position of the plane of shear or conductivity of the blood cell.

Structural analysis of components liberated from the peripheral zone of blood cells by enzyme action is useful in obtaining knowledge concerning the molecular anatomy of the peripheral zone. The structure of some of the glycopeptides which possess weak M and N hemagglutinating inhibitory activity and which are removed by trypsin from human erythrocytes have been partially elucidated (30). Little work has so far been carried out along these lines for the human blood platelet.

Action of Functional Group Reagents on Blood Cells

Many of the conditions used for the modification of functional groups are markedly nonphysiological and thus increase the possibility of undesirable conformational or structural changes. The use of mild reversible reagents can provide good evidence that changes in mobility result from the primary effects of chemical modification of specific functional groups. The evidence may be further strengthened by showing that removal of the modifying moiety, including its chemical identification, is associated with restoration of the original electrokinetic properties.

The continued existence of an appreciable negative electrokinetic charge density after removal of all the sialic acid from erythrocytes and thrombocytes implies that other anionic groups are effective at the electrophoretic surface. Possible groups include the carboxyl groups of acidic amino acid residues, phosphate groups from phospholipids, or nucleic acids and perhaps sulfate groups. Positively-charged groups may originate from side chain amino groups of lysine and hydroxylysine residues, the terminal α amino groups of peptide chains, imidazolic groups of histidine residues, guanidine groups of arginine, phospholipid and glycolipid amines, and the quaternary ammonium groups of choline.

The normal human erythrocyte under physiological conditions behaves as a macropolyanion, that is, any cationogenic groups if present at the electrophoretic surface number less than 2 or 3% of the number of anionogenic groups. (see Table II). The evidence for polyanionic character is as follows: (a) the absence of variation in red cell mobility in the range pH 7 to 10 (pK range for amino groups) (8); (b) the lack of specificity toward the anions bromide, chloride, iodide, and thiocyanate (8); and (c) the absence of any change in electrophoretic mobility following treatment with acetaldehyde and formaldehyde (9), p-toluenesulfonyl chloride (8), 2:4-dinitrofluorobenzene (16), 2, 4, 6-trinitrobenzene sulfonic acid, 2-chloro-3, 5-dinitropyridine, and 2-chloro-3, 5-dinitrobenzoic acid (31).

The ionogenic constitution of the human blood platelet (Table II) was obtained from the following experimental evidence: (a) the marked decrease in the electrophoretic mobility produced by neuraminidase with the concomitant release of N-acetylneuraminic acid (11) indicating the presence of surface sialic acid moieties; (b) the increase in anodic

mobility produced by acetaldehyde (11), citraconic anhydride, and 2, 3-dimethyl maleic anhydride (32) implicating the presence of surface amino groups; (c) the finding that alkaline phosphatase produces a significant decrease in the anodic mobility, thus implying the presence of phosphate groups susceptible to alkaline phosphatase (29); and (d) the increase in the electrophoretic mobility of blood platelets following treatment with 6, 6′-dithiodinicotinic acid thus demonstrating the presence of surface sulfhydryl groups (33).

The ionogenic features derived from the above data for both human erythrocytes and thrombocytes are presented in Table II. The lower value for the groups per cell was calculated from eq. (2) and the upper value from eq. (3) with α assumed to be zero; the values thus represent the extreme limits for a nonpenetrable and totally penetrable cellular peripheral zone. The surface areas, although probably in error, were taken from Mehrishi (4). It should be noted that at best these figures are approximate and, furthermore, very artificial in that each species of ionogenic group is assumed to be distributed uniformly at the cell surface.

Effect of Polymers on Blood Cells

Ponder (34) reported that addition of dextran to saline suspensions of human erythrocytes resulted in an increase in the zeta potential as calculated from the electrophoretic mobility. Other neutral polymers such as ficoll and polyvinylpyrrolidone have also been reported to produce a zeta potential increase in cell suspensions (35). A consideration of possible artefacts which could be introduced by the presence of the polymer suggests that the experimental results are real and that polymer adsorption is implicated in the phenomenon. Previous explanations which involve either a rearrangement of the peripheral zone of the cell to increase the number of charge groups at the electrophoretic

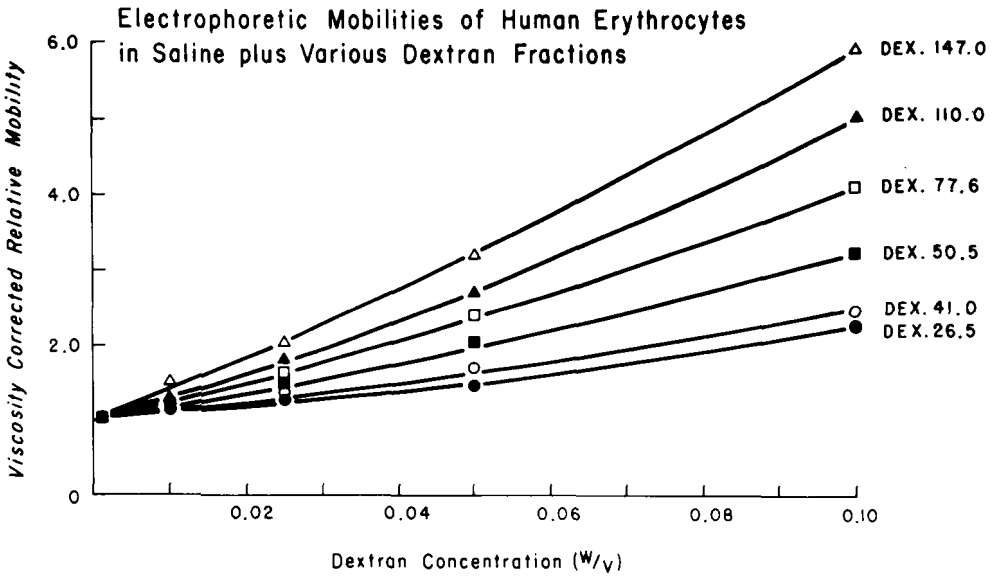

Fig. 3. Dependence of the viscosity corrected relative electrophoretic mobility of human erythrocytes in standard saline with increasing dextran concentration and molecular weight. • $\overline{M}_w$26,500; ○, $\overline{M}_w$41,000; ■, M_w 50,500; □, M_w 77,600; ▲, M_w 110,000; △, M_w 147,000.

surface or a dielectric constant change within the electrical double layer can be shown to be insufficient to account for the magnitude of the effects observed. A model has been proposed to account for the increase in zeta potential of blood cells in neutral polymer solutions (36). In the model the presence of adsorbed neutral polymer is predicted to produce an expansion of the electrical double layer as a result of generalized excluded volume effects, thus at constant charge density the surface potential will increase. As long as the electrophoretic plane of shear is not shifted too far from the cell surface the zeta potential is predicted to increase in the presence of adsorbed neutral polymer.

Addition of dextrans to erythrocytes or thrombocytes suspended in saline can produce aggregation, probably by the mechanism of polymer bridging. Increasing the polymer concentration past that required for initial aggregation produces first an increase, then eventually a decrease in the degree of aggregation until at a critical dextran concentration and a critical zeta potential the cells disaggregate. The disaggregation concentration, but not that required to induce aggregation, is a function of the ionic strength of the suspending medium. The data are consistent with a model in which intercellular electrostatic repulsion can be sufficiently enhanced by the presence of adsorbed neutral polymer to overcome the interactions required for the maintenance of polymer bridging (37). The critical concentration of dextran necessary to effect disaggregation is an increasing function of molecular weight. The increases in the electrophoretic mobility of human erythrocytes as a function of dextran concentration and molecular weight are illustrated in Fig. 3.

The electrokinetic properties of erythrocytes and thrombocytes can also be changed by adsorption of charged macromolecules such as polylysine (38), DEAE–dextran, and polybrene (39). It is possible also with these polymers to study the influence of electrokinetic charge on the aggregation process.

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