The Surface Charge of Isolated Toad Bladder Epithelial Cells

Mobility, effect of pH and divalent ions

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ABSTRACT The surface charge of epithelial cells isolated from the toad bladder has been determined by the microscope method of cell electrophoresis. The cells possess a net negative charge, and a net surface charge density of 3.6×10^4 electronic charges per square micron at pH 7.3. Estimates of net surface charge over the alkaline pH range indicate (*a*) that an average distance of the order of 40 A separates the negatively charged groups, and (*b*) that amino as well as acid groups are present at the electrophoretic surface of shear. A significant increase in mobility following cyanate treatment of the cells suggests that a large proportion of the amino groups are the ϵ-amino groups of lysine. In view of the known effects of calcium and other divalent ions on cell permeability and cell adhesion, the extent of binding of calcium and magnesium to the cell surface was determined by the electrophoretic technique. Mobility was significantly decreased in the presence of calcium or magnesium, indicating that these ions are bound by surface groups. When the pH was lowered from 7.3 to 5.2, calcium binding was markedly decreased, an observation consistent with competition between calcium and hydrogen ions for a common receptor site.

INTRODUCTION

Fixed charges in the vicinity of the cell surface may be of considerable importance in regulating ion movement across the cell, and in promoting cell adhesion. Little is known, however, about the nature or location of these charged groups. It is probable that some are located along pores or channels which extend a considerable distance beneath the cell surface. It is entirely possible, however, that some of the fixed charges of physiologic importance are located at or very near the cell surface itself. Information about the latter groups can be obtained in intact cells by the use of relatively simple techniques, in contrast to the serious experimental problems involved in the identification of

groups far below the surface. Accordingly, we have undertaken a series of studies of the surface charge of a cell whose physiologic properties are well characterized, hoping eventually to correlate certain features of ionic permeability and cell adhesion with surface charge. The epithelial cell of the toad bladder appeared to be suitable for this type of study; large numbers of isolated epithelial cells can be obtained by simply withdrawing calcium from the bathing medium (1), and the surface charge of these cells can be determined under a variety of experimental conditions by the microscope method of cell electrophoresis.

The initial studies to be reported describe the electrophoretic mobility, surface charge density, and acid-base behavior of toad bladder epithelial cells, and the effect of divalent ions on net surface charge.

METHODS

A. Isolation of Epithelial Cells Epithelial cells were isolated from intact toad bladders by rinsing bladder halves briefly in three 30 ml volumes of calcium-free amphibian Ringer's solution (NaCl 112, KCl 3.5, $NaHCO_3$ 2.4 mEq per liter, made up in deionized distilled water). The bladder halves, usually 6 to 8 in number, were then placed in 400 to 600 ml of calcium-free Ringer's solution containing 1.5 mM disodium EDTA per liter. The pH of the EDTA stock solution used was adiusted to 8.0 prior to adding it to the Ringer's solution; the pH of the Ringer's solution was its usual value of approximately 8.2. The bladder halves were gently bubbled in the EDTA-Ringer solution for an hour; each was then picked up with two foreceps, and a hemostat placed across the bladder neck, forming a closed bag containing 1 to 2 ml of Ringer's solution. The bag, (serosal side out), was gently massaged between thumb and forefinger, then cut open, and the contents, containing the detached epithelial cells, collected in a centrifuge tube. The yield of all the bladder halves was collected in a single tube, and the tube was then spun at 2,000 RPM in a table model centrifuge for 15 to 30 sec, yielding a pellet of approximately 0.75 ml volume. The supernatant was carefully pipetted off and approximately 15 ml of fresh calcium-free Ringer's solution without EDTA was added, and the tube vigorously shaken until the cells were completely resuspended. The cells were again spun down and a second washing carried out in the same way as the first. The layer of mucus remaining on the surface of the supernatant was removed following centrifugation. After the second washing, the cells were resuspended in approximately 5 ml of calcium-free Ringer's solution and distributed equally among several centrifuge tubes, the number varying with the experiment to be carried out. Immediately before they were introduced into the cataphoresis apparatus, the cells were washed twice in 15 ml of the appropriate electrophoresis buffer, whose pH was adjusted to the desired value, and resuspended in 1 ml of buffer. This washing procedure proved to be adequate, since cell mobility was not significantly altered if the cells were washed as many as 22 times in electrophoresis buffer. The cataphoresis apparatus was washed through with 300 to 500 ml of buffer and the cell suspension was then introduced into the inflow chamber. The buffer solutions used in the studies contained potassium chloride as the major salt, sucrose, and

either imidazole, imidazole acetic acid hydrochloride, potassium bicarbonate, or potassium phosphate as buffer salts. The solution most frequently used contained KCl 29 mEq, imidazole 0.8 to 2.0 mEq per liter, and sucrose 56 g per liter. Small amounts of HCl or KOH were used to adjust the pH to the desired value. The ionic strength of this solution varied in different experiments from 0.030 to 0.032, but was kept identical in any given experiment. Cell mobility showed the expected linear relationship to the reciprocal of the square root of the ionic strength (6), over a wide range of ionic strengths (0.134 to 0.009). The relative viscosity, as determined with an Ostwald viscosimeter, was 1.15 relative to water at 25°C. The conductivity of the buffer solutions at 25° was determined with a Radiometer model CDM2 conductivity meter (Radiometer Company, Copenhagen, Denmark).

B. *Determination of Electrophoretic Mobility* The cataphoresis apparatus consisted of a rectangular glass cell of the Northrop-Kunitz type (Arthur H. Thomas Co., Philadelphia, Pennsylvania) fixed to the microscope stage in the "lateral" position (2). The depth of the cells used was between 500 and 600 microns. The sidearms of the cell were attached by Tygon tubing to silver-silver chloride electrodes bathed in 42.5 mEq per liter KCl, which was used rather than saturated KCl to more closely approximate the ionic strength of the electrophoresis buffer. This, plus the distance between electrodes and chamber, minimized the effects of any contamination of the buffer by the electrode solution. There was no difference in the observed mobilities when saturated KCl was used in the electrodes. The cell was kept at constant temperature by placing it in a lucite water bath whose temperature was maintained at 25° ± 0.1 by a Haake circulating pump; the microscope objective and sidearms from the electrodes entered the bath through gasketed holes. Several experiments were carried out at room temperature; when this was the case, it will be noted in the text. The values obtained at room temperature did not differ appreciably from those obtained at 25°, although a small effect on mobility did appear when temperature was varied over a sufficiently large range (see Table II). The apparatus was cleaned daily with 10% KOH in 70% alcohol followed by 1% HCl. The movement of the cells in the chamber was observed under phase contrast with a Leitz binocular microscope (PV 20 objective with a 2.0 mm working distance, and ×10 periplan eyepieces, giving a 250-fold magnification including a factor of 1.25 from the binocular prisms). A Heine phase contrast condenser was used; light passed through a cooling cell and a green filter. The microscope was focused on cells at the first stationary layer, 0.21 times the distance between the two inner walls of the glass chamber. Only those cells in sharp focus were run. At the magnification used, it was not usually possible to distinguish between the various types of epithelial cells of the toad bladder, with the exception of the small basal cells. These were excluded from our experimental series. A current, generally 1 ma, was passed through the chamber, and the rate of movement of the cells between two lines of an eyepiece micrometer disc was determined to the nearest 0.2 sec. The current was then reversed by means of a switch, and the rate of movement in the opposite direction determined. The mean value was then calculated. If the difference between the two determinations was greater than 20%, the observation was discarded. Drift was corrected by clamping the outlet hose of the apparatus. Ten to twenty cells were observed to determine a single experimental point. Our mobility value for human erythrocytes

at 25° (NaCl–Na phosphate–sucrose buffer, pH 6.4, ionic strength 0.072) was 15.9 ± 0.2 × 10^{-5} cm^2 sec^{-1} v^{-1}, 3 % less than the value of 16.4 × 10^{-5} cm^2 sec^{-1} v^{-1} reported by Eylar and associates (3), who used an apparatus similar to ours.

Electrophoretic mobility was calculated from the equation:

$$U = \frac{ADK}{It} \tag{1}$$

where U is the electrophoretic mobility, A the cross-sectional area of the chamber, D the distance travelled by the cell in time t, K the specific conductance of the electrophoresis buffer, and I the current passed across the chamber. Mobility measurements were corrected to water at 25° by multiplying by 1.15, the relative viscosity of the KCl-sucrose buffer.

Electrophoretic mobility is proportional to net surface charge density (σ), and is regarded as being independent of particle size. The simplest form of the expression for mobility in terms of charge density, valid for large smooth particles is:

$$U\eta = \sigma\left(\frac{1}{k}\right) \tag{2}$$

Correction for the size of the ions in the double layer leads to the expression:

$$U\eta = \sigma\left(\frac{1}{k} + a_i\right) \tag{3}$$

where η is the viscosity in poises, σ the net surface charge density in electrostatic units per cm^2, k the Debye-Hückel function in cm^{-1}, and a_i the hydrated radius of the counter ion, potassium in our case. The value for k at 25° is 0.327 × 10^8 $\sqrt{\Gamma/2}$ (3), where $\Gamma/2$ is the ionic strength. Considerable disagreement exists regarding the hydrated radius of potassium; we have expressed our calculated σ as a range, employing the value of Padova (4) (2.17 A) and Nightingale (5) (3.31 A) for the hydrated radius. It is important to note that equation (3) assumes the cell to have a large radius of curvature. However, the radius of curvature of the mobility-determining structures on the surface of biological particles may be considerably less than that estimated from the size of the particle itself (6); for this reason estimates of surface charge density may be lower than the true charge density of biologically "rough" surfaces.

RESULTS

A. *Mobility* The epithelial cells possess a net negative charge at pH 7.3. Table I shows the mean of 17 determinations of cell mobility carried out at 25°C on 13 different days, in KCl–imidazole–sucrose buffer, ionic strength 0.030. The range of daily values was from −9.2 to −13.9 × 10^{-5} cm^2 sec^{-1} v^{-1}, with a mean of −11.3 ± 0.3 × 10^{-5} (SE) cm^2 sec^{-1} v^{-1}. Included in the table for comparative purposes are mobilities determined in our apparatus for human and toad erythrocytes, and a value from the studies of Cook and

associates (7) for Ehrlich ascites tumor cells, the latter interpolated to an ionic strength of 0.030. The exposure to EDTA used in the preparation of the cells for electrophoresis did not appear to influence their mobility; cells scraped from the bladder and washed twice in electrophoresis buffer had mobilities identical to those prepared with EDTA. The distribution of mobility values for toad bladder epithelial cells at 25° and ionic strength 0.030 is shown in Fig. 1. The scattergrams represent single runs of from 30 to 50 cells, two of which were carried out at pH 7.3 and one at pH 3.0, where the net surface charge is positive. At pH 7.3, the mobilities appeared to vary about a single mean; at 3.0, the same appeared to be true, although the existence of two or more populations of cells with almost identical mobilities could not be ruled out.

TABLE I

ELECTROPHORETIC MOBILITY AND NET SURFACE CHARGE DENSITY, DETERMINED BY THE MICROSCOPE METHOD AT 25°

Cell	Mobility	Net surface change density
	$cm^2\ sec^{-1}\ v^{-1} \times 10^5$	(*electronic charges*/μ^2) $\times 10^{-4}$
Toad bladder epithelial cell	−11.3±0.3 (SEM)	3.4–3.6
Human erythrocyte	−18.9±0.3	5.6–6.0
Toad erythrocyte	−15.8±0.2	4.7–5.0
Ehrlich ascites tumor cell	−14.8	4.6

Determinations 1 to 3 carried out in KCl—imidazole—sucrose buffer, ionic strength 0.030, pH 7.3.
Ehrlich ascites tumor cell data from Cook *et al.* (7).
A range of values is given for net surface charge density, corresponding to differing values for the hydrated radius of potassium (see Methods).

B. *Surface Charge Density* The net surface charge density calculated for toad bladder epithelial cells is shown in Table I. Since net surface charge density is a direct function of mobility, the toad bladder epithelial cells again show a lower value than the erythrocytes and Ehrlich ascites tumor cells.

C. *Temperature Dependence of Mobility* Brinton and Lauffer (6) have pointed out that as temperature (T) increases, there is a compensating decrease in the dielectric constant (D) of water, so that the term $\sqrt{DT}$ changes very little in comparison to the viscosity. If σ is assumed to be constant, the following relationship should serve to correct the mobility to any temperature:

$$\eta_1 U_1 = \eta_2 U_2$$

Studies of the mobility of mineral oil droplets and paraffin wax particles in water (8) have confirmed this prediction. However, the mobilities of both horse serum albumin (9) and human erythrocytes (10) appear to show an

additional temperature dependence, which has been attributed to changes in the structure of the protein and the cell surface, respectively. Toad bladder epithelial cells exhibited an increase in mobility in two experiments when the temperature of the bath surrounding the chamber was raised from 15 to 30° (Table II). The effect was a small one, however (0.6% per °C).

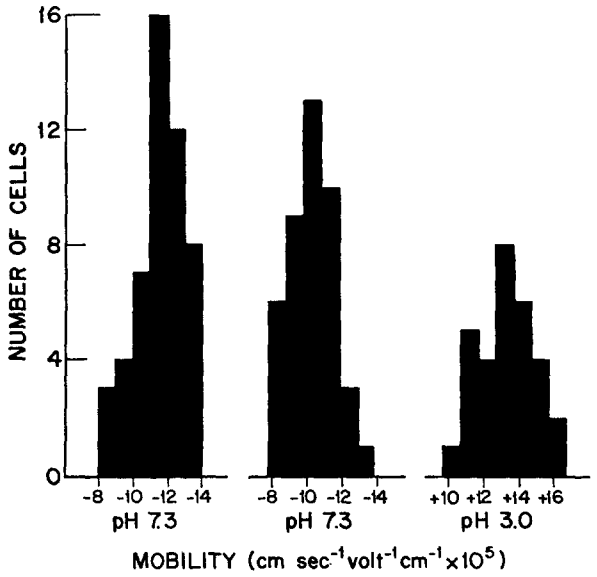

FIGURE 1. Distribution of mobilities of isolated toad bladder epithelial cells in KCl–imidazole–sucrose buffer, ionic strength 0.030. Two determinations at pH 7.3, and one at pH 3.0 are shown.

TABLE II

THE EFFECT OF TEMPERATURE ON MOBILITY

Experiment	Mobility 15°	Mobility 30°	Δ	p
	$cm^2\ sec^{-1}\ v^{-1} \times 10^5$			
1	−10.4±0.3 (SEM)	−11.2±0.2	0.8	<0.02
2	−11.1−0.2	−12.2±0.2	1.1	<0.01

Determinations carried out in KCl—imidazole—sucrose buffer, pH 7.3, ionic strength 0.030.

D. *Effect of pH* The effect of pH on epithelial cell mobility was studied over a range of 10.6 to 2.5. Imidazole, imidazole acetic acid hydrochloride, bicarbonate, or phosphate were used as buffers in a large number of experiments, which were carried out both at 25° and at room temperature. The curves obtained were all comparable; values obtained in four experiments are shown in Fig. 2. Between pH 7.3 and 4.0 there was a decrease in mobility

of approximately 20% from -10.8 to -8.6×10^{-5} cm^2 sec^{-1} v^{-1}. Below pH 4.0, mobility decreased sharply; the cells became isoelectric at a pH of approximately 3.5, and then assumed a net positive charge, moving relatively rapidly toward the cathode at pH 3.0. Mobility showed little increase between pH 7.3 and 9.6; above 9.6, however, there was a sharp increase in net negative charge.

To determine whether irreversible changes took place at the cell surface at pH 3.0 and 10.6, the cells were placed in electrophoresis buffer adjusted to these values for 20 min; they were then washed twice in buffer of pH 7.3 and

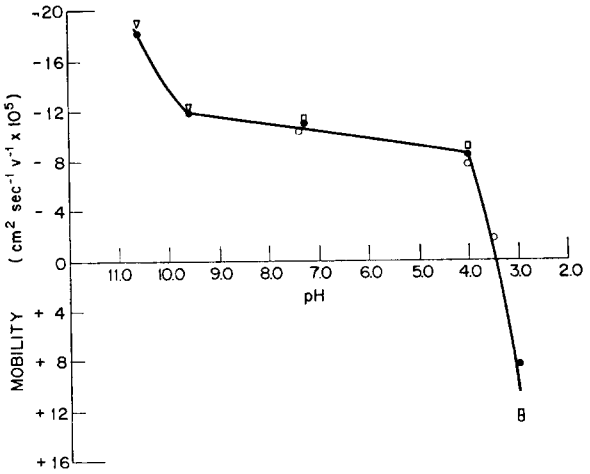

FIGURE 2. Effect of pH on mobility. The points show were obtained at 25° in KCl–sucrose, with the following buffers: 1 mM imidazole, total ionic strength 0.030 (●);2 .5 mM imidazole acetic acid hydrochloride, total ionic strength 0.030 (□); 2.0 mM potassium bicarbonate, total ionic strength 0.032 (▽); and, at room temperature, 1.2 mM phosphate buffer, total ionic strength 0.027 (○).

their mobilities compared with those of control cells which had remained at pH 7.3. The results are shown in Table III, part A; the mobilities of cells exposed to high or low pH returned to control values when the cells were returned to pH 7.3 buffer.

Experiments at ionic strength 0.067 and 0.078 showed the same isoelectric point, within experimental error, as those at 0.030; below pH 3.5, cells at the higher ionic strengths again exhibited a net positive charge.

E. *Reversibility of Effect of Ionic Strength on Mobility* To determine whether large alterations in ionic strength produced irreversible changes at the cell surface, cells were placed in buffer of ionic strength 0.006 for 10 min, than washed twice in buffer of ionic strength 0.120, and their mobilities compared to control cells which had remained at 0.120. The results are shown in Table III,

part B; there was no significant difference between control cells and cells exposed to ionic strength 0.006 buffer for 10 min.

F. *Effect of Acetaldehyde and Cyanate on Mobility* The migration of cells toward the cathode below pH 3.5 provides evidence for the existence of positively charged groups at the cell surface. The sharp increase in cell mobility above pH 9.6 indicates that the pK's of these positive groups are in the range of the basic amino acids. Additional evidence for the presence of amino groups was obtained by treating the epithelial cells with acetaldehyde and potassium cyanate. Acetaldehyde, like formaldehyde, combines with amino groups of proteins (11), and may be presumed to form methylolamine groups initially, in a manner similar to that suggested by Levy (12) for formaldehyde. Cyanate

TABLE III

REVERSIBILITY OF EFFECT OF pH AND IONIC STRENGTH ON MOBILITY AT 25°

Experiment	Mobility
	$cm^2\ sec^{-1}\ v^{-1} \times 10^5$
A. Effect of pH (ionic strength 0.030)	
Mobility at pH 7.3	−13.9±0.5 (SEM)
Mobility at pH 3.0	+12.4±0.5
Mobility at pH 10.6	−21.3±0.6
Mobility at pH 7.3 after 20 min at pH 3.0	−13.9±0.5
Mobility at pH 7.3 after 20 min at pH 10.6	−14.4±0.7
B. Effect of ionic strength (pH 7.3)	
Mobility at ionic strength 0.120	−7.1±0.4
Mobility at ionic strength 0.006	−16.2±0.5
Mobility at ionic strength 0.120 after 10 min at 0.006	−7.0±0.3

has been shown to combine with the positively charged ε-amino group of lysine to form an uncharged carbamyl group (13). Treatment of cells with either acetaldehyde or cyanate would then be expected to increase electrophoretic mobility. Fig. 3 shows the mobility of cells exposed to 2.0% acetaldehyde for 12 days at 4°C. Acetaldehyde-treated cells exhibited a higher mobility than paired controls run at pH 7.5 and 3.5, and the isoelectric point decreased from 3.5 to 2.2.

The effect of 0.2 M cyanate on mobility is shown in Table IV; cells exposed to cyanate for 3 hr showed a 36% increase in mobility over paired controls. The effect of pH on the mobility of cyanate-treated cells is shown in Fig. 4. At pH 7.3 and 9.6 a large increase in mobility over that of control cells was apparent. Between pH 9.6 and 10.6 the usual upward inflection of the curve was lost. Of interest is the fact that cyanate-treated and control cells had identical mobilities at pH 4.0 and 3.0 (see Discussion).

G. *Effect of Calcium and Magnesium on Mobility* Divalent ions, notably calcium, have a variety of effects on the movement of ions and water across cells (14-16); calcium also promotes cell adhesion in the toad bladder, as in other tissues. For these reasons, it was of interest to determine the effect of calcium and other divalent ions on the mobility of toad bladder epithelial cells. A decrease in mobility would be expected if there were a significant degree of binding of divalent ions to negatively charged surface sites. Accordingly, we determined the mobility of epithelial cells, first in the absence of the divalent ion, then in its presence. The buffer was adjusted so that the pH and ionic

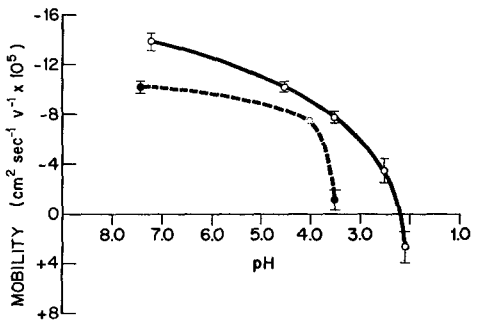

FIGURE 3. The effect of acetaldehyde on mobility. (○—○), cells treated with 2% acetaldehyde at 4°C for 12 days; (●—●), control cells. The point at pH 4 (○) was interpolated and is representative of control cell mobility at this pH. The standard error of the mean is shown for each point.

TABLE IV

THE EFFECT OF CYANATE ON MOBILITY AT 25°

Experiment	Mobility		Δ	*p*
	Control	Cyanate		
	cm² sec⁻¹ v⁻¹ × 10^5			
1	−10.6±0.4 (SEM)	−13.9±0.3	3.3	<0.001
2	−10.9±0.3	−15.0±0.3	4.1	<0.001
3	−10.6±0.2	−14.7±0.3	4.1	<0.001

Cells incubated in 0.2 M KCNO or (as control), 0.2 M KCl, with 0.1 M *n*-methylmorpholine buffer, for 3 hr.

Electrophoresis carried out in KCl—imidazole—sucrose buffer, pH 7.3, ionic strength 0.032.

strength in both control and test solutions were identical. Following the second determination of mobility, the apparatus was washed out with cleaning solution to ensure the absence of small amounts of the divalent ion. An additional control run was then carried out; in this way, any changes in mobility due to changes in the apparatus with time would become apparent as a discrepancy between the two control runs.

The effect of 1 and 5 mM per liter of magnesium and calcium on mobility is shown in Table V. The first experiment (A) was carried out at 25°, the second and third (B) at room temperature. A control period preceded and followed

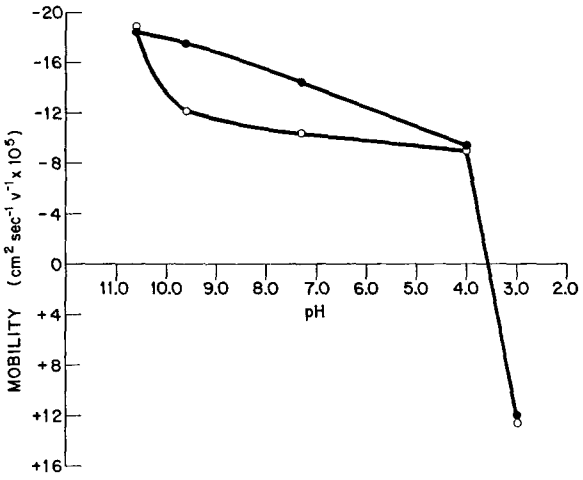

FIGURE 4. The effect of 0.2 M cyanate on mobility. (●—●), cyanate-treated cells; (○—○), control cells.

the run with each divalent ion. Both magnesium and calcium decreased net surface charge in these and twelve additional experiments.

The effect of calcium on mobility was easily reversed by washing the cells exposed to calcium in calcium-free buffer.

The effect of pH on the extent to which calcium decreased mobility was determined in a series of paired experiments in which the mobility of a given batch of isolated cells was determined at pH 7.3 (imidazole buffer) and pH 5.2

TABLE V

THE EFFECT OF MAGNESIUM AND CALCIUM ON MOBILITY

Experiment	Mobility	Δ	p
	$cm^2\ sec^{-1}\ v^{-1} \times 10^5$	(*mean of 2 controls-test ion*)	
A. 1 mM magnesium and calcium; ionic strength 0.011			
Control	−11.7±0.3 (SEM)		
1 mM magnesium	−8.1±0.2	3.7	<0.001
Control	−11.8±0.2		
1 mM calcium	−9.0±0.2	2.5	<0.001
Control	−11.2±0.2		
B. 5 mM magnesium and calcium; ionic strength 0.032			
Control	−12.0±0.4		
5 mM magnesium	−10.7±0.3	2.1	<0.001
Control	−13.5±0.4		
Control	−10.5±0.5		
5 mM calcium	−8.9±0.4	2.3	<0.001
Control	−11.8±0.5		

Mobilities determined in KCl—imidazole—sucrose buffer, pH 7.3.

(acetate or phthalate buffer), in the presence and absence of 5 mM per liter calcium. The ionic strength of all buffers used was identical, and the experiments were carried out at room temperature. The results are shown in Table VI. At pH 5.2, the effect of calcium on mobility was considerably diminished, and indeed was not significant statistically.

TABLE VI

THE EFFECT OF pH ON CALCIUM BINDING AT ROOM TEMPERATURE. 5 PAIRED EXPERIMENTS

Buffer	Mobility	
	pH 7.3	pH 5.2
	$cm^2\ sec^{-1}\ v^{-1} \times 10^5$	
Control	10.7	9.4
5 mM calcium	8.4	8.6
Δ	2.3±0.5 (SEM)	0.8±0.4
p	=0.01	<0.2

Experiments carried out in KCl—imidazole acetic acid hydrochloride buffer, pH 7.3, and KCl-phthalate or acetate-sucrose buffer, pH 5.2. The ionic strength of all buffers was 0.032.

DISCUSSION

Since the early observations of Jurgenson (17) on the mobility of frog erythrocytes in an electric field, a considerable body of experimental data has accumulated on the electrophoretic mobility of a variety of cells. Although most studies have been carried out on cells which are isolated under ordinary circumstances, there are some reports on the mobilities of cells detached from organs such as liver (18), kidney (19), and colon (20). The epithelial cells of the toad bladder fall into this latter category. In the intact toad bladder, they are tightly apposed near their luminal surfaces by a junctional complex (21, 22), which appears to conform to the tripartite structure recently described by Farquhar and Palade (23). Below the junctional complex, the lateral and basal surfaces of adjoining cells are thrown into convoluted folds; desmosomes also contribute to cell attachment in this region. In the absence of calcium (with or without EDTA present) the attachments between cells break down, and, with the help of gentle massaging, the cells detach completely from the bladder. Electron microscopic study of the isolated cells at this point (1) showed the surface structures to be intact; these structures include the microvilli of the luminal surface and their fine filamentous covering, and the finger-like convoluted folds of the lateral and basal surfaces.

It is clear that both histologically and functionally, there are important differences between the luminal and serosal surfaces of the cell. Studies by Leaf and associates (24, 25) have shown that the permeability of the luminal

surface is lower than that of the serosal, and that a barrier at or near the luminal surface appears to control the rate of entry of a variety of substances including sodium, water, and urea. Vasopressin alters the permeability of this barrier, permitting this restricted group of substances to enter the cell at a greater rate. The lateral and serosal surfaces, on the other hand, are involved in cell attachment. Active extrusion of sodium entering across the luminal surface is believed to take place across the serosal surface.

In addition to the differences between the surfaces of any given cell, there are well recognized differences in the cell types which are present in the epithelial layer of the toad bladder (21, 22). The dominant cell type is the granular cell, which has been estimated to comprise about 75% of the major cell types of the epithelial layer (22). Mitochondria-rich cells and mucous cells are present as well, each to the extent of about 12%. Approximately 15 to 20% of the total surface of the granular cell faces the lumen; an even smaller proportion of the mitochondria-rich cell does so, since the bulk of this cell lies deep, communicating with the surface by a slender neck. Basal cells are present near the basement membrane; these small cells, believed to be immature epithelial cells, could be recognized with the phase microscope and their mobility was not included in any of our experimental series.

Clearly then, our mobility values represent an over-all value for several cell types, and the fact that the values appear to vary about a single mean (at least at pH 7.3) may reflect the similarity in net surface charge of the lateral and basal surfaces of all cells, regardless of type. On the other hand, small differences in mobility between, for example, granular and mitochondria-rich cells could be obscured by the fact that the granular cells are far more numerous, and a second small population of mobilities could not be detected in the 30 to 50 determinations in the scattergrams. More precise information about the mobilities of the several cell types should be obtainable when we can work with a suitable phase system at higher magnification.

The calculation of net surface charge density must be accepted with caution, since uncertainty still exists regarding the relation of mobility to charge density. In studies of the red cell, for example, where neuraminic acid is responsible for most of the surface charge, Eylar and associates (4) found that as much as four times more neuraminic acid was released by neuraminidase from horse erythrocytes than would have been predicted from the observed decrease in erythrocyte mobility. This discrepancy, and smaller ones in erythrocytes of other species, required explanation. The most likely interpretation was that the radius of curvature of the mobility-determining structures at the cell surface was considerably smaller than infinity. Under these conditions, the correction factor of Henry (26) had to be applied. This yielded values for surface charge density closer to that predicted from the loss of sialic acid. Additional considerations, based on possible distributions of sialic acid molecules a

few angstroms from the surface of shear, led to a further increase in estimated surface charge density. For reasons similar to the above, our estimate of net surface charge density is probably low.

The effect of temperature on mobility was a small one, of the order of 0.6% per °C over the restricted temperature range employed (15 to 30°). Sachtleben (10), working with human erythrocytes over a wider range of temperature (1 to 37°), found a higher degree of temperature dependence, averaging 1.7% per °C. The slope of the line relating temperature to mobility was not constant; at least three different slopes were apparent in his study. The cause of this temperature dependence is not known, but may be related to structural changes in the cell surface itself (10). The observed effect of temperature on the mobility of toad bladder epithelial cells was felt to be small enough to permit the inclusion of observations carried out at room temperature.

The studies on the effect of pH on mobility provide evidence for the presence of positive as well as negative groups at the electrophoretic surface of shear. The movement of the cells toward the cathode below pH 3.5 indicates the presence of positive groups, and the sharp upward inflection of the curve above 9.6 suggests that a considerable proportion are amino groups. This amphoteric behavior is characteristic of a number of cells whose electrophoretic mobility has been studied, including mouse lymphocytes and liver cells (18), and Ehrlich ascites tumor cells (7). Human erythrocytes, on the other hand, are believed to have a completely anionic surface of shear, showing no charge reversal at low pH (27). Additional evidence for this property of human erythrocytes is the observation that formaldehyde has little or no effect on mobility (4, 28). Rat erythrocytes, on the other hand, have been reported to show significant charge reversal below pH 3.5 (29), although a detailed account of this particular electrophoretic determination was not given by the authors.

Thus, the relatively low electrophoretic mobility of the toad bladder epithelial cell at pH 7.3 is due to the presence of a significant number of cationic groups. At pH 10.6, where the bulk of the amino groups lose their protons, the net negative charge density becomes 5.9×10^4 electronic charges/μ^2, equaling that of the human erythrocyte. Assuming an even distribution of negative charges over the entire cell surface, one may estimate the mean distance between negative groups as 40 A. Further corrections for radius of curvature (as previously discussed) would place these groups somewhat closer together. Using Abramson and Moyer's assumption that an ion occupies an area of about 1×10^{-15} cm^2 (30), approximately 0.6% of the total cell surface would be negatively charged under these conditions. This calculation, of course, does not take into account the fact that there may be charged groups below the electrophoretic surface of shear; these groups may be numerous, and may be of importance in determining the permeability properties of the

cell, yet they would not contribute to electrophoretic mobility. Clearly, the number and importance of these other groups cannot yet be assessed.

The titration curve also gives some information about the pK's of the acid surface groups. It is likely that one or more groups are present with pK's in the 4.0 to 7.0 range, and that at least one group is titrated at a significantly lower pH. The curve obtained after acetaldehyde treatment suggests that the latter group may have a pK in the 2.5 to 3.0 range. More precise assignment of pK values is difficult because of the possibility of interactions between neighboring surface groups.

Both acetaldehyde and cyanate increase mobility at pH 7.3, providing additional evidence for the presence of amino groups. Cook and associates (7) have observed similar increases in electrophoretic mobility following acetaldehyde treatment of Ehrlich ascites tumor cells. The cyanate experiments suggest that the ϵ-amino groups of lysine are present at the surface of shear. Since cyanate can add to —SH groups, and —NH_2 groups which are present as terminal residues of proteins (13), it is possible that these reactions took place as well. The finding that the cyanate-treated and control cells had identical mobilities at pH 4.0 and 3.0 was unexpected, since the loss of positive charge alone should have resulted in a decrease in the positive mobility of the cells at low pH. It is unlikely that at low pH there was a reversal of carbamylation and regeneration of the amino group; cyanate-treated cells suspended in a buffer at a pH as low as 1.8 for 50 min still exhibited a more rapid mobility than paired controls when returned to pH 7.3. We cannot at present account for this finding.

The effect of calcium and magnesium on electrophoretic mobility is of interest in view of the relation of these and other multivalent ions to the adhesion of toad bladder epithelial cells (31). Several points deserve emphasis; first, the decrease in mobility was small, especially when it is realized that the concentration of calcium used in this experiment (10^{-3} M) was approximately fifty times that necessary to maintain cell adhesion in the intact bladder (32). The decrease in cell mobility in the presence of 10^{-4} M calcium was so small as to be within the experimental error of the electrophoresis technique. One might therefore conclude from these results that a small percentage of the cell surface is involved in calcium-mediated cell adhesion. It is possible, on the other hand, that both the nature and extent of calcium binding between *adjoining* cells may differ greatly from that seen in single cells. Any conclusions drawn from the electrophoresis experiments must, therefore, be tested in the intact preparation.

Our findings also indicate that neutralization of surface charge alone is not sufficient to promote cell adhesion. Magnesium, for example, which is incapable of promoting adhesion, effectively decreases surface charge. The same is true of barium (31). It therefore appears possible that a specific complex is

formed between the calcium ion and negatively charged groups of adjoining cells, and that certain ions, such as magnesium and barium, are incapable of forming such a complex. Other investigators have suggested such a role for calcium (18, 33).

Although a direct linkage between adjoining cells *via* the calcium ion may be required for close cell adhesion, it may be necessary as well for negative surface groups in the vicinity of the intercellular bridge to be neutralized, since the repulsive forces between cells might not ordinarily permit the tight intercellular junction described in this and similar tissues. Studies by Katchalsky (34) for example, have shown that polylysine of a low degree of polymerization was capable of agglutinating red blood cells by forming bridges between them. However, a significant suppression of net surface charge by the polylysine had to take place before agglutination could occur. Such charge neutralization in our system could be brought about by calcium ions not participating in the intercellular bridge.

Significantly more calcium appears to be bound to the cell surface at pH 7.3 than at pH 5.2. In this respect, our results are similar to those obtained in studies of the binding of calcium (35, 36), manganese (37), and zinc (38) to serum albumin. It is possible that the increase in calcium binding at the higher pH is due to the higher over-all surface negativity; this has been offered as the explanation of the enhanced binding of calcium by human serum albumin when the pH is raised above 6.0 (36). Alternatively, our results are consistent with the binding of calcium to a specific site or group of sites with pK's in the 5.0 to 7.0 range. Imidazole or carboxylate groups, for example, could serve as ligands for calcium at the cell surface. Evidence for imidazole as a ligand for manganese and zinc has been obtained in studies of serum albumin by Mildvan and Cohen (37) and Gurd and Goodman (38). Similar results have been reported by Tanford (39) for the binding of a number of metals by bovine serum albumin. Under these conditions, the effect of pH on metal binding may be viewed as a true competition between the hydrogen and metal ion for specific binding sites.

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