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PHYSICOCHEMICAL STUDIES OF MICROBIAL CELL WALLS

I. COMPARATIVE ELECTROPHORETIC BEHAVIOR OF INTACT CELLS AND ISOLATED CELL WALLS

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SUMMARY

The surface properties of intact cells and of the isolated, unfragmented cell walls of four species of bacteria and a yeast have been studied by cell electrophoresis in media with different electrolytes, ionic strengths and pH. The mobilities of isolated walls were as homogeneous and reproducible as those of intact cells and exhibited a similar species-dependent behavior. In general a striking parallelism between the mobilities of isolated walls and the corresponding cells was observed. Over wide ranges of ionic strength and pH the mobilities of walls were lower than those of the cells by 5–15% for bacteria and about 25% for yeast. With some bacterial species less regular differences were observed at low pH. The mobility differences between intact cells and their walls are more likely to be due to a difference in a physical property such as conductivity rather than to real differences in the surfaces.

INTRODUCTION

Recent years have seen a great increase in knowledge of the chemical composition and the biosynthetic and lytic reaction mechanisms of microbial cell walls[1,2]. Less is known about the physical nature of cell walls in their normal aqueous environment, although certain concepts have been established. Cell walls are sufficiently rigid to determine the shape of the cell but they possess considerable flexibility and elasticity[3]. Cell walls from gram-positive organisms have been shown to be open, highly heteroporous, 3-dimensional networks[4,5]. Fixed charged groups on cell wall structures are responsible for physical effects such as volume changes associated with ionic strength and pH changes in the surrounding media[6,7], electrical conductivity[8] and, on the surface, in electrophoretic movement[9] and electrostatic interactions with solid, liquid and gas interfaces[10–12]. Hydrophilic groups are probably responsible for reducing the normal mobility of water in interstitial spaces in cell walls[13]. Further investigation and interpretation of such phenomena in terms of what is known of the macromolecular composition of cell walls can be expected to provide additional understanding of their 3-dimensional structure and functions.

Electrophoresis provides one of the few available ways of probing the nature

of the surfaces of cells in aqueous media. Although electrophoretic studies of microorganisms have been made almost exclusively with intact cells, isolated cell walls might also be profitably investigated by this method. A novel characterization of these well-defined structures would be obtained which might be most useful in a limited exploration of 3-dimensional structure by showing whether or not the properties of the surface are typical of the average bulk properties of the wall. The results might also be applicable to intact cells, provided surface features were not lost during preparation of the walls. Practical advantages might be gained in some cases. Interferences from cytoplasmic or membrane constituents which have been definitely observed in work with intact cells[14] might be more easily eliminated. Such interferences may be a more general source of difficulty than has been recognized, especially where harsh conditions involving pH extremes, non-aqueous solvents and reactive reagents are employed.

For electrophoretic results on isolated walls to be applicable to intact cell surfaces a clear relationship between the two must be established. Eddy and Rudin[15] have shown general agreement between the electrophoretic mobilities of walls and intact cells of several strains of yeast. Boundary electrophoresis was used by Roberson and Schwab[16] as a means of assessing the homogeneity of a bacterial cell wall preparation but no comparisons with intact cells were made. Fisher and Richmond[17] stated that conidia of fungal spores of *Botrytis fabae* gave pH-mobility curves similar to the isolated cell walls but that *Pencillium expansum* walls differed from normal conidia because of the loss during the washing process of a surface constituent carrying phosphate groups. No comparative data were presented, however. A systematic comparison of the electrophoretic behavior of a variety of microorganisms and their respective isolated walls under a wide range of conditions has not yet appeared. The present work attempts to provide this.

EXPERIMENTAL

Microorganisms

Microbial species and culture conditions were chosen to give homogeneous electrophoretic mobilities, rapid growth rates and high yields. *Micrococcus lysodeikticus* (ATCC 4698) was cultured in Penassay Broth (Difco) with 1% of glucose added; *Escherichia coli* (K-12) was cultured in Trypticase Soy Broth (Difco); *Bacillus megaterium* (ATCC 13632) and *Bacillus subtilis* (var. *niger*) were cultured in a medium of Casamino Acids (Difco), yeast extract and salts given by Edebö *et al.*[18] (Medium No. 3). *Saccharomyces cerevisiae* was Fleischman's commercial bakers' yeast. The bacteria were grown at 37.5 °C in 4.5-l batches in a mass culture apparatus[19] or, occasionally, in Fernbach or 1-l indented flasks on a rotary shaker. No antifoaming agents were employed. Bacterial cells were harvested in the late logarithmic or early stationary phase of growth, and washed three times by centrifugation and resuspension in 0.05 M phosphate or ammonium acetate buffer (pH 7.0). Yeast cells were similarly washed but the upper and lower layers of the pellets were discarded after each sedimentation in order to obtain a more homogeneous preparation.

Cell walls were prepared as described in detail elsewhere[20]. In brief, the cells were ruptured by shaking at 4000 rev./min with 0.2-mm diameter glass beads in an apparatus described by Merkenschlager *et al.*[21] (Braun–Bronwill) at 0–5 °C for

times which resulted in nearly 100% rupture but minimal fragmentation of the walls. The walls were washed at 0–10 °C once in 1 M NaCl and 4–5 times in neutral ammonium acetate buffer and purified by centrifugation at about $20000 \times g$ for 1–2 h on appropriate density gradients: NaBr was used for isolation of *B. megaterium* and *M. lysodeikticus* walls, potassium tartrate for *B. subtilis*, sucrose for *E. coli* and Renografin (Squibb) for yeast. After 5–6 more washes in ammonium acetate buffer, cell wall purity was established by light and electron microscopy and by examination of acid extracts for non-wall constituents absorbing in the ultraviolet[20]. Treatments with trypsin, deoxyribonuclease and ribonuclease were not found to make significant differences by these criteria or in electrophoretic mobilities of isolated walls and were not used routinely. Electron micrographs showed most walls to have a tear at one place in the case of cocci and short rods or a missing end in the case of long rods. In wet mount walls had essentially the same shape as the intact cells.

Electrophoresis

The microelectrophoresis apparatus described in detail elsewhere[22], consisted of a precision-made flat Pyrex observation cell mounted laterally for viewing at $400\times$ with a horizontal microscope equipped with a Zeiss $40\times$, water-immersion, phase-contrast, objective with 1.6-mm working distance. The electrodes were strips of palladium foil electrolytically charged with H_2 before use[23]. The temperature was controlled by immersing the apparatus in water circulated from an auxiliary thermostat kept at 25.00 ± 0.02 °C. Velocity measurements on each particle were made over a distance of 50–100 μm in both directions by reversing the current supplied by a constant-current d.c. generator (Electronics Measurements Co., Model C612A). The conductivity of the suspending medium was determined at 25.00 °C in a dipping cell with a 1000-cycle bridge (Industrial Instruments Co., Model RC-18).

The electrophoretic mobilities were determined on at least 20 individual cells or cell walls (ten at each stationary level) after they were washed at least three times in the chosen medium and diluted to about 10^7 cells or walls/ml. The coefficient of variation was less than $\pm 5\%$ except for yeast where it was as high as $\pm 10\%$. Except at pH extremes media were routinely buffered with sodium barbiturate–sodium acetate[24] and HCl or NaOH ($I=0.001$) with addition of NaCl or other salts to give the desired final ionic strength. Stock solutions of the various salts were filtered through Millipore (HA) filters. Solutions at pH 2.0 and 11.9 were 0.01 M HCl and 0.01 M NaOH, respectively.

RESULTS

In general electrophoretic studies were as readily carried out with isolated cell walls as with intact cells although phase-contrast or dark-field optics are essential for work with bacterial cell walls. The precision of mobility measurements for walls were comparable to those for the cells.

Electrophoretic mobilities of cells and isolated walls are shown plotted against ionic strength in Fig. 1 and against media pH in Fig. 2. In studies of the pH–mobility behavior it was found, after exposure to pH extremes, that both cells and walls of *E. coli* and *B. megaterium* and also yeast cells did not give the normal control mo-

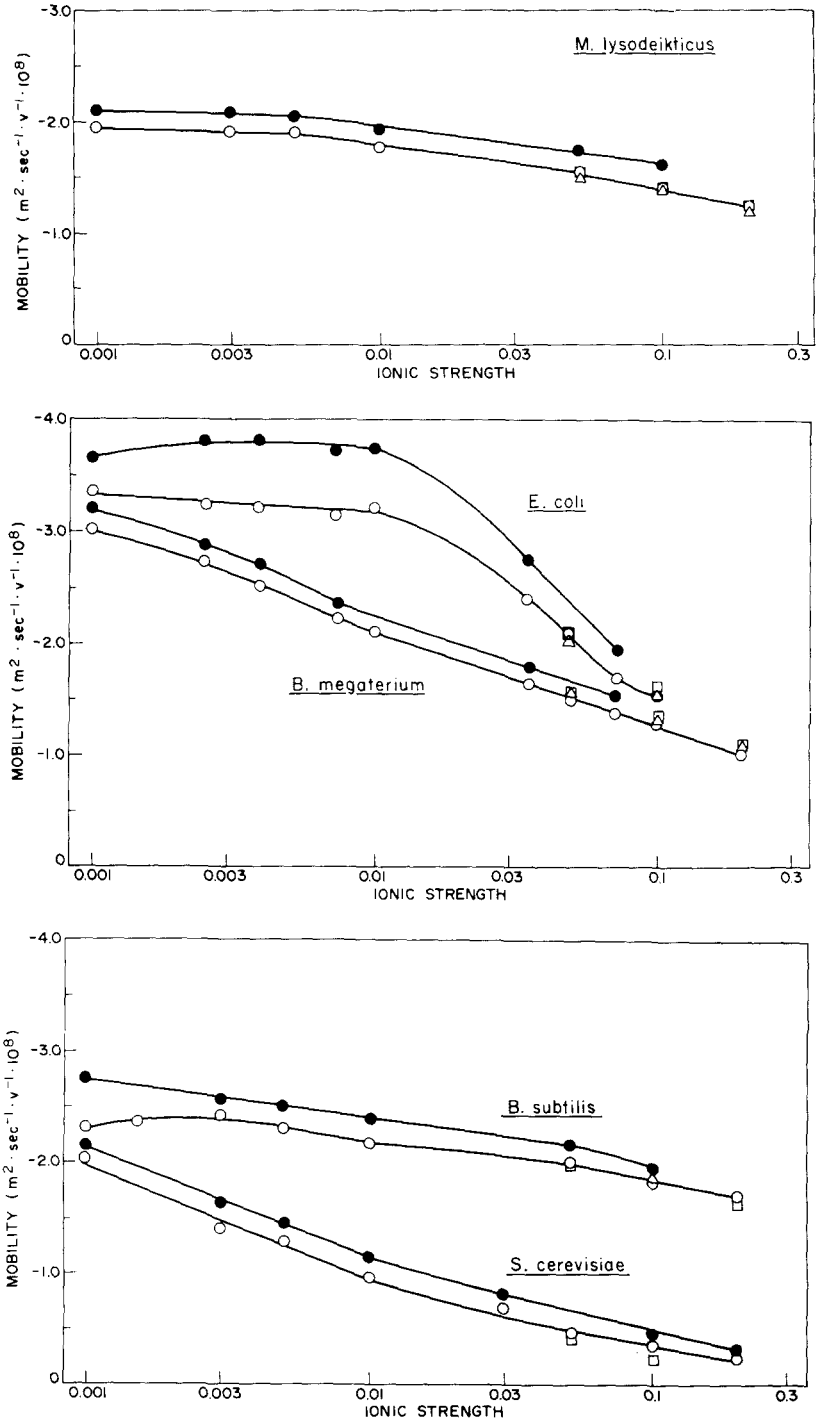

Fig. 1. The electrophoretic mobility of intact microbial cells (●——●) and isolated cell walls (○——○) as a function of ionic strength of NaCl solutions containing a constant amount of barbiturate buffer ($I \approx 0.001$, pH 7.0). Mobility values of walls in solutions containing NaSCN (□——□) and NaI (△——△) in place of NaCl are also shown.

bility on return to pH 7.0 buffer. Mobility values obtained as quickly as possible (about 0.5 h) after exposure to media causing such irreversible change are nevertheless included in the data to show trends and are denoted by the broken lines in Fig. 2. Possible causes of these changes will be discussed in a later paper. The somewhat greater sensitivity to pH extremes of cells of *E. coli* and yeast compared to walls might possibly be due to surface adsorption of released cytoplasmic or membrane constituents[14] but this was not established.

An electronegative charge dominates the surface of all organisms investigated except in some cases at low pH. This is typical of nearly all microorganisms on which data are available[9]. It is generally recognized that such a charge can originate from

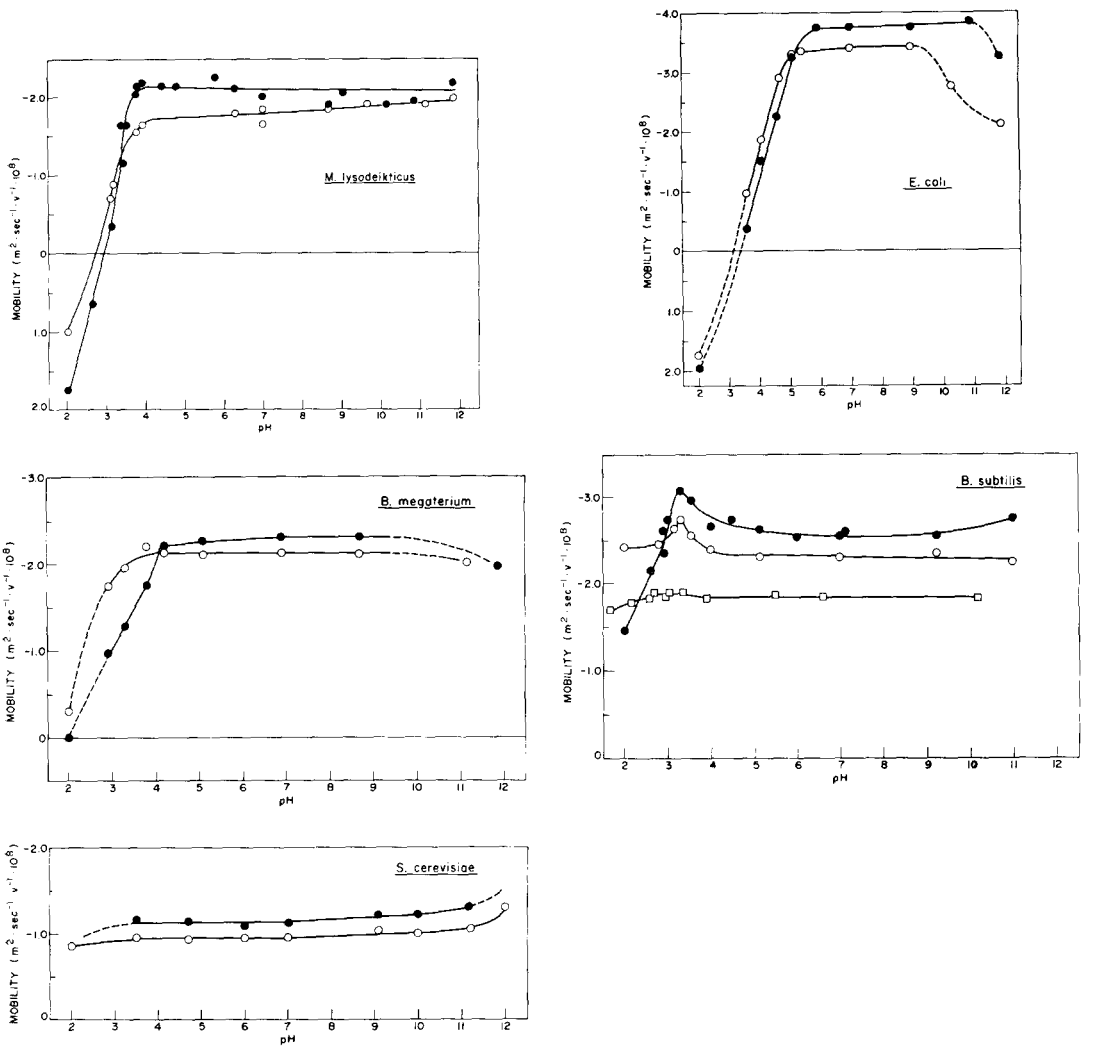

Fig. 2. The electrophoretic mobility of intact microbial cells (●——●) and isolated cell walls (○——○) as a function of pH (I=0.01). Mobilities for *B. subtilis* walls at I=0.1 are also shown (□——□).

the dissociation of acidic groups which are part of the surface structure or from surface adsorption of anions of the electrolyte. In order to determine whether the barbiturate buffer which was routinely included in the media might be contributing to surface charge, a number of experiments were carried out using NaCl solutions (I=0.01) with addition only of HCl or NaOH for pH adjustment. In no case were the mean mobilities significantly different from the values obtained in buffered media. The substitution of Cl^- in the media by the highly adsorbable anions, SCN^- and I^-, also caused no significant differences in mobilities. Fig. 1 includes some of these results at higher ionic strength for cell walls; similar agreement was found for intact cells. Thus it appears possible to rule out the possibility of surface charge contributions by anion adsorption in the experiments reported.

A comparison of the mobilities of microbial cells with their respective isolated cell walls shows a remarkable consistency. The mobility–ionic strength curves for all isolated walls are nearly parallel to those for intact cells, falling 5–15% below the cells in the case of bacteria and about 25% below in the case of yeast (Fig. 1). The pH–mobility curve for yeast walls is also parallel to that for the intact cells over the whole pH range and with bacteria the same consistency holds from the strongly basic to weakly acidic regions (Fig. 2). However, at low pH the curves for all bacterial cell walls cross those of the intact cells and in the case of *E. coli* and *M. lysodeikticus* continue parallel at lower pH. Only with *B. megaterium* and *B. subtilis* at low pH is there much difference in the behavior of walls and intact cells.

It should be pointed out that the generally similar behavior of walls and cells observed in this investigation may be found to hold only for relatively undamaged walls which retain their normal bag shape and not necessarily for fragmented walls produced, for example, by ultrasonic methods[16]. The latter have interior surfaces exposed which may have electrokinetic characteristics different from the wall exterior. It is for this reason, however, that fragmented walls might be worth future investigation.

The failure of isolated cell walls to show electrophoretic behavior identical with that of the intact cells may obviously be due to a real difference in surface composition. Such a difference could arise from the removal of surface features of intact cells such as flagella, fimbriae or capsules by the extreme shearing stresses involved in cell wall preparation. Underlying wall material with different electrokinetic properties might then be exposed[25,26]. This possibility is made unlikely, however, by the fact that electron micrographs of the intact cells selected for this investigation showed no surface appendages except for an occasional flagellum in the case of *E. coli*. It seems likely that the high-speed centrifugation and vigorous shaking used to resuspend pellets during routine washing procedures removed non-wall surface appendages from intact cells before electrophoretic examination. It should be mentioned, however, that a capsule was visible on freshly harvested *B. subtilis* but was apparently removed during the washing procedures. It is possible that vestiges of this capsular material, undetected in electron micrographs, still adhered to the washed cells but were removed during cell wall preparation to expose dissociable groups of lower pK than those carried by the capsule. Such a mechanism might explain the observation that these walls exhibited a considerably higher mobility than the cells at pH 2.0. In general, however, it appears improbable that such mechanisms could satisfactorily explain the regularity of the differences in

behavior between walls and cells observed in all the species of microorganisms examined.

Information about the kind of groups which might dissociate to contribute a negative surface charge can be obtained from a comparison of the pH–mobility behavior with that of model particles of known constitution[27–29]. Carboxyl groups appear to be the main source of anions on the surfaces of *E. coli* and *M. lysodeikticus* (Fig. 2). This is in agreement with the results of Davies *et al.*[30] on a different strain of *E. coli* and of Gittens and James[31] on *E. coli* (Strain K-12). We also observe positive mobilities at very low pH, as did these investigators, indicating that cationic groups are also present on the surface. Few *et al.*[32] concluded that carboxyl groups were solely responsible for the negative charge on *M. lysodeikticus* cells; however, in contrast to our results, they found a substantial rise in negative mobility beginning at about pH 8.5 and proceeding to a higher plateau. This indicated a loss of cationic charge of basic, probably amino, groups on the surface whereas with the cells used here cationic groups are apparent only at low pH. It must be remembered in making such comparisons that mobility behavior can vary with the strain of organism used and the culture conditions.

The *B. megaterium* surface appears largely dominated by carboxyl groups but the low apparent p*K* for walls does suggest an admixture of a stronger acid group, possibly phosphate. The results of Douglas[33] on another strain of *B. megaterium* were similar to our results with intact cells.

The results for yeast cells agree well with certain strains studied by Eddy and Rudin[15] and their conclusion that phospho-diester groups are present on the surface accounts satisfactorily for the retention of negative charge at very low pH.

The mobility maximum which we observe in the acid region with *B. subtilis* is unusual. However, James and Brewer[34] also observed this kind of behavior with *Staphylococcus aureus* and attributed it to a pH-dependent reversible rearrangement at the surface of an acidic phosphate polymer, teichoic acid. The same explanation may apply for *B. subtilis* (var. *niger*) since a teichoic acid is a known constituent of these walls[35]. The presence of a strong acid group is certainly indicated by the anionic charge at pH 2.0. The greatly lowered mobility maximum obtained for walls at high ionic strength may be due to conformational changes in superficial, pendant polyelectrolyte chains occurring as a result of reduced electrostatic repulsion between anionic charges[36]. This is a subject of continuing investigation.

DISCUSSION

The electrophoretic mobilities of isolated cell walls are as homogeneous and reproducible as those of intact cells. In general walls and cells show the same species-dependent behavior and only for *B. subtilis* and *B. megaterium* in media at low pH is there a significant departure from close parallelism. Thus isolated cell walls can be precisely characterized by microelectrophoresis and studies of their surface constitution by this method are clearly feasible. Walls may be particularly useful where intact cells leak cytoplasmic constituents which interfere by adsorbing on the cell surface or where it is desired to examine the basic wall structure without influence of the flagella, fimbriae or capsular materials which are removed during cell wall preparation.

The differences between the mobilities of cells and walls, though small, were invariably observed and call for some explanation. It is improbable that the consistent difference between the mobilities of cells and walls can be explained by an equally consistent difference in net surface charge density in all five species of microorganisms examined. It is much more likely that the surfaces of the two are essentially the same and that the differences are due to some common physical factor affecting the electrophoretic measurement which is not the same for cells and walls. Such a difference may obviously derive from the absence of cytoplasmic membranes in the walls. It is conceivable that the presence of a cytoplasmic membrane in intact cells may increase cell mobilities relative to walls by enhancing the zeta potential. For example, with intact cells the ionic charge density on the membrane might be sufficient to act through the wall and increase the external concentration of diffusible counter ions above that necessary to compensate the fixed charges on the wall material alone. This would result in higher zeta potentials and mobilities for cells than for walls. However, studies on protoplasts indicate that the charge on cytoplasmic membranes is approximately the same[37,38], or much lower[32] than cell-wall surfaces and, therefore, unlikely to exert a significant effect through the thickness of a cell wall. Such mechanisms also appear improbable when the relative thickness of cell walls and electrokinetic double layers is considered. The thickness of the thinnest wall used here, *E. coli*, is about 100 Å in electron micrographs[39] but at least 600 Å in the water-swollen state[40]. This is considerably greater than the 100-Å double-layer thickness calculated from the Debye–Hückel quantity, $1/\kappa$, at $I=0.001$ (assuming the dielectric constant to be unchanged within the wall, *cf.* ref. 8). Thicker walls and higher ionic strengths make such an explanation even less attractive. A more likely possibility derives from theoretical derivations and experimental observations showing that conducting particles have lower mobilities than insulating particles with the same surface charge[41]. Intact cells would be expected to have lower electrical conductivity than cell walls because their interiors are insulated by the poorly conducting cytoplasmic membrane.

An estimation of the magnitude of the effect of conductivity on mobilities of *M. lysodeikticus* can be made from the conductivity measurements of Carstensen and co-workers[8,42]. For a spherical particle with radius much greater than the thickness of the surrounding diffuse double layer of counter ions.

$$\zeta = \frac{u4\pi\eta}{X\varepsilon}\left(\quad + \frac{\sigma_2}{2\sigma_1}\right)$$

where u is the electrophoretic mobility, ζ is the potential at the surface of shear with respect to the bulk medium (zeta potential), X is the applied field strength, ε is the dielectric constant, η is the viscosity and δ_2 and δ_1 are the conductivities of the particles and suspension medium respectively. If the surfaces of cells and walls are the same then $\zeta_{walls}=\zeta_{cells}$ and if a conductivity correction is appropriate then

$$\frac{\zeta_{walls}}{\zeta_{cells}} = \frac{u_{walls}\left(1+\frac{\sigma_{walls}}{2\sigma_1}\right)}{u_{cells}\left(1+\frac{\sigma_{cells}}{2\sigma_1}\right)} = 1$$

Using mobilities for *M. lysodeikticus* from Fig. 1 and the conductivity values of Carstensen *et al.*[8,42] for the same organism, the ratio is 1.02 at 0.1 M NaCl and 1.64 at 0.01 M NaCl. Thus the conductivity correction brings the zeta potential ratio into agreement at high ionic strength but over corrects at low ionic strength. The result, nevertheless, encouraged a careful investigation using walls and cells from identical cultures which we will report shortly.

A feature of the pH–mobility curves common to all bacterial organisms examined is that wall mobilities cross those of the cells as the pH is lowered through the region where mobilities start to drop sharply from the plateau. At still lower pH, wall mobilities are more negative than cells and with *M. lysodeikticus* and probably *E. coli* the curves for walls and cells continue parallel through a zero point of charge and into a region where positive mobilities for cells exceed those of the walls. This is consistent with the lower negative mobility of walls at high pH and might also be explained by a higher conductivity for cell walls. The crossover of the pH–mobility curves for cells and walls may thus be a necessary consequence of a decreasing pH which changes the net surface charge from negative to positive. The reason for a crossover above the zero point of charge is not known but may be more easily interpreted when data on conductivity variations of cells and walls with pH are available. The failure to attain positive mobilities for *B. subtilis* and yeast within the pH region examined may be due to the low p*K* of their surface anionic groups; in the case of *B. megaterium* there may be no cationic groups near the surface.

It is obvious from this and other electrophoretic work with microorganisms that mobility behavior is often difficult to correlate with the presence or absence at the surface of specific polymer moieties known to be present in the wall from biochemical and serological analyses. The use of isolated walls rather than intact cells can offer the advantages of a more stable organelle whose surface properties are less likely to be altered by other cell constituents. The usefulness of electrophoresis could also be greatly enhanced if it were carried out concurrently with other experimental techniques. Douglas[43] and Gittens and James[31], for example, have given an indication of how specific chemical and enzymatic reactions can be advantageously used in electrophoretic studies on bacteria. We have continued this approach using specific reactions and extractive procedures with isolated walls and plan to present the results in a forthcoming report.

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