Salt-induced Contraction of Bacterial Cell Walls

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Intact *Bacillus megaterium* cells were found to contract as much as 26% in terms of dextran-impermeable volume when transferred from water to unbuffered, non-plasmolyzing NaCl solutions. This shrinkage appeared to be primarily due to electrostatic wall contraction rather than to any osmotic response of the cells. A variety of salts (but not sucrose) added to water suspensions of isolated cell walls caused protons to be released from the walls with resultant lowering of suspension pH and contraction of the structures. In effect, *B. megaterium* walls behaved as flexible, amphoteric polyelectrolytes, and their compactness in aqueous suspensions was affected by changes in environmental ionic strength and pH. Isolated walls were most compact in low ionic strength media with a pH of about 4, a value close to the apparent isoelectric pH of wall peptidoglycan. Electrostatic attractions appeared to play a major role in determining the compactness of highly contracted walls, and the walls responded to increased environmental ionic strength by expanding. In contrast, electrostatic repulsions were dominant in highly expanded walls, and increased environmental ionic strength induced wall contraction. Walls of whole bacteria also shrank when the cells were plasmolyzed. This second type of contraction seemed to result from relief of wall tension during plasmolysis, and it could be induced with nonionic solutes. Thus, cell wall tone in *B. megaterium* appeared to be set both by mechanical tension and by electrostatic interactions among wall ions.

Many investigations have shown shrinkage of whole bacterial cells after transfer from dilute to concentrated salt solutions. For example, Johnson and Harvey (13) concluded from measurements of packed cell volume and light scattering that gram-negative *Achromobacter* (*Photobacterium*) *fischeri* cells shrank as much as 27% in volume when transferred from seawater to 2.5 times concentrated seawater. Salt-induced contraction of many other gram-negative bacteria has been reported (3, 4, 12, 15), and there appeared to be a close association between whole cell shrinkage and observable plasmolysis.

Gram-positive bacteria also can be made to shrink in salt solutions, even though they are not readily plasmolyzed. Dutky (*unpublished data*, cited in reference 14) found that *Bacillus megaterium* cells contracted from 15 to 25% when transferred from normal broth to broth containing 2 M NaCl. Mitchell and Moyle (18) indicated that *Staphylococcus aureus* cells contracted from 2 to 3% in linear dimensions, without becoming visibly plasmolyzed, when transferred from 0.1 to 1.0 M NaCl solution.

Salt-induced shrinkage of bacteria has been interpreted as an osmotic response of the cells

mainly because the effect can be produced in easily plasmolyzed, gram-negative organisms with either electrolytes or nonelectrolytes (3, 4, 13, 15). Our results, however, indicate that salt-induced contraction of gram-positive *B. megaterium* cells does not primarily involve osmotic plasmolysis, but that it is caused mainly by electrostatic contraction of amphoteric, polyionic, cell wall polymers.

MATERIALS AND METHODS

Bacteria. The asporogenous KM strain of *B. megaterium* was grown at 30°C in aerated 2% Oxoid peptone broth. Cells were harvested near the end of the exponential-growth phase by centrifugation in the cold.

Cell wall isolation. The harvested cells (in chilled suspensions containing 10% by weight cells and 10% homogenizing glass beads) were broken with a Branson model S125 sonic oscillator operated at full power for 15 min. The walls were separated from other cell debris by repeated differential centrifugation and were treated with trypsin to remove contaminating proteins (21).

The homogeneity of the preparations was assessed by phase microscopy, electron microscopy, and testing the sensitivity of the walls to lysozyme. When lysozyme was added to water suspensions of trypsin-treated

walls, the optical density (700 m μ) decreased more than 95%. Also, absorbance-scattering spectra (220 to 700 m μ) of wall suspensions showed no absorption peaks.

Walls were used shortly after preparation or were frozen until needed.

Dextran-impermeable volume measurements. The compactness of water-soluble polyelectrolytes in solution was assessed previously from properties such as intrinsic viscosity or angular dependence of light scattering (19). For suspensions of insoluble cell walls, it was possible to estimate wall volume by measuring the volume of the suspension inaccessible to dextran molecules. *B. megaterium* cell walls were found (10) to exclude dextrans with average molecular weights greater than about 57,000. For the experiments reported here, very large dextran molecules with average molecular weights of about 2 million (Dextran 2000, from Pharmacia Inc., New Market, N.J.) were used.

The experimental procedure involved centrifuging chilled, water suspensions of water-washed cells or isolated walls at $33,000 \times g$ for 35 or 90 min, respectively, to obtain pellets weighing 3 to 6 g. A measured volume (approximately equal to the pellet volume) of chilled test solution containing 1% (w/v) dextran was added to each pellet; the pellets were thoroughly suspended; the suspensions were placed in an ice bath for about 30 min and then recentrifuged. Supernatant fluids were carefully decanted and saved for dextran analyses. At least one pellet in each group of pellets was suspended in a solution containing no solute other than dextran; the dextran-impermeable volume of this pellet was considered to be equal to the dextran-impermeable volume of the material in water suspension.

A commonly used equation (5, 17) for calculating dextran-permeable space or volume is

$$R = \frac{V_s}{V_p} \left(\frac{C_0}{C_f} - 1 \right)$$

where R is the dextran-permeable volume (i.e., the fractional volume of the pellet of test material into which dextran molecules can diffuse), V_s is the volume of dextran solution added, V_p is the pellet volume, C_0 is the initial dextran concentration, and C_f is the final concentration. The dextran-impermeable volume is then $1 - R$.

V_p is difficult to measure accurately, and thus W_p , the pellet wet weight, is often used instead of V_p . Black and Gerhardt (5) described in detail a procedure for determining the densities of cells or isolated walls and for using these density values to correct W_p so that it more closely approximates V_p . This correction procedure was the one used to calculate the dextran-impermeable volumes presented in this paper. The densities of cells and walls were found to be 1.06 and 1.04 g/ml, respectively, when the materials were suspended in distilled water. The degree of correction involved was relatively small, and the true dextran-permeable volume was only slightly less than the value calculated with W_p multiplied by the density of the material (5).

Many of the test solutes used, e.g., NaCl, caused whole cells and isolated walls to contract so that their densities increased. However, the densities required for calculations were those of the materials before exposure to test solutes. Thus, when whole cells or walls contracted in NaCl solutions, water was squeezed out into the suspending medium, and this water became accessible to dextran molecules. The extent of contraction could then be related to changes in dextran-permeable (or dextran-impermeable) volume.

Dextran assays. Dextran concentrations were assayed gravimetrically after drying samples overnight at 105 C or by the anthrone reaction (20). For dry weight determinations, large dextran molecules were separated from small molecules by dialysis. A measured volume of supernatant fluid or standard solution containing dextran was placed in a sac of cellophane dialysis tubing; the sac was sealed and placed in a large flask of distilled water for at least 48 hr. The water was changed frequently during this period. With samples of standard dextran-sucrose or dextran-NaCl solutions, it was possible to free completely the solutions of sucrose or NaCl and still recover all of the dextran.

Osmolalities. Solution osmolalities were calculated from molalities by use of reported osmotic coefficients, primarily those given for sucrose and NaCl by Scatchard, Hamer, and Wood (22).

Contribution of the cell wall to cell weight and volume. Chemical, morphological, and serological studies (25, 28) indicated that lysozyme treatment completely removes walls from *B. megaterium* KM cells. Therefore, the fraction of cell weight represented by wall materials can be estimated by measuring the weight of wall fragments released during lysozyme treatment of whole cells suspended in protoplast-stabilizing media. Weibull (27) found that these wall fragments are very large, water-soluble polymers.

Lysozyme (2 mg/ml) was added to cells suspended in 2 molal sucrose solutions, and these suspensions were kept at room temperature until essentially all of the cells were converted to protoplasts. The suspensions were centrifuged, and supernatant fluids were carefully removed. The inorganic phosphate contents of these fluids and their capacities to absorb ultraviolet light (240 to 340 m μ wavelength) indicated that no leakage of intracellular solutes occurred during lysozyme treatment and centrifugation. Samples of supernatant fluids in cellophane sacs were dialyzed to remove small molecules; the dialyzed solutions were dried (105 C for 24 hr), and the dried materials were weighed. Final dry weights were corrected by subtracting the dry weight of added lysozyme. It appeared from this analysis that 28.1% of cell dry weight was wall material. The dry weight of protoplasts, after fixation with 0.6 M formaldehyde and washing with water, was found to be equal to 70.6% of total cell dry weight. Thus, the fraction of the cell weight contributed by the cell wall appeared to be approximately 0.29. This value is slightly higher than the values of 0.20 to 0.25 given by Salton (21) and the value of 0.20 given by Weibull (28), but the disparity may be related to different growth conditions for the bacteria.

The dry weight to wet weight ratio (corrected for interstitial water) of the cells used for the work described here was 0.248 and their density was 1.06 g/ml. Hence, the cells contained about 0.264 g of dry matter per ml, of which approximately 0.077 g was wall material. Calculations based on the assumption that wall polymers have an average partial specific volume of 0.7 ml/g indicated that wall structural materials represent a relatively small fraction (0.05) of the wet cell volume.

The dextran-impermeable volume of bacterial cell walls is known to comprise both the volume of wall structural polymers and the volume of water within wall pores which is inaccessible to dextran molecules. The volume of wall water can be estimated from the cell volume accessible to radioactive sucrose, a compound which can enter wall pores but cannot cross the plasma membrane of nonrespiring cells (26). The sucrose space of nonplasmolyzed *B. megaterium* cells grown in this laboratory was reported previously (16) to be 0.36. Thus, the volume fraction of *B. megaterium* cells occupied by wall appears to be about 0.05 plus 0.36, or 0.41. This value is within the range of values, 0.40 to 0.47, calculated by Weibull (26) on the basis of direct microscopic measurements of live *B. megaterium* cells.

Titration procedures. Titrations were performed in two ways. For some titrations, no effort was made to keep ionic strength constant, and walls were suspended (10 mg of dried walls per ml) initially in distilled water. Successive additions of standardized solutions containing HCl or KOH were then made. The pH after each addition was measured with a Beckman E-2, blue-glass electrode. Separate samples were used for acid titration and for base titration. Degassed, CO₂-free solutions were used; for base titrations, a dish of concentrated NaOH was placed in the meter chamber to depress the CO₂ level in the air.

For other titrations, the ionic strength of the wall suspensions was held approximately constant by adding KCl to a series of suspensions. Measured volumes of water and HCl or KOH solutions were then added to each suspension. The required amount of KCl was calculated by assuming that only the anion of added acid or the cation of added base would contribute significantly to the final ionic strength of the suspensions because nearly all of the added hydronium or hydroxyl ions were bound by the walls. The pH, after addition of acid or base and mixing, was nearly constant for at least 10 min and titrations were reversible. The procedures described by Tanford (24) for assessing bound acid and base were followed.

Other procedures. Inorganic phosphate was measured by the method of Fiske and SubbaRow (9).

A Beckman model DU spectrophotometer was used to measure absorption of ultraviolet light by supernatant fluids.

RESULTS AND DISCUSSION

NaCl-induced volume changes of whole cells and isolated cell walls. When *B. megaterium* cells suspended in water were centrifuged and then resuspended in nonplasmolyzing NaCl solutions of

osmolality greater than about 0.001 osmolal, there was a marked decrease in their dextran-impermeable volumes (Fig. 1A). This contraction resulted in as much as a 26.5% decrease in cell volume, but did not appear to be primarily an osmotic response because it did not occur in sucrose solutions of equivalent osmolality. Cells did shrink when transferred to very concentrated sucrose solutions, but only in association with plasmolysis. Analyses of suspending-medium fluids for their content of inorganic phosphate or materials absorbing ultraviolet light (240 to 340 mμ wavelength) indicated only insignificant leakage of solutes from cells during plasmolysis. Protoplast retraction from the wall of the plasmolyzing cell seemed to relieve tension in the wall. But cells transferred from 0.1 osmolal NaCl solution to 8.0 osmolal NaCl solution grew larger, even though microscopic observations indicated that plasmolysis occurred after the transfer.

Cell shrinkage in NaCl solutions was also indicated by lower pellet weights of cells centrifuged from NaCl-containing suspensions compared with those centrifuged from distilled water suspensions. In these experiments NaCl promoted closer packing of the cells, and there was a loss of intercellular as well as intracellular water from the pellets. Hence, estimates of the extent of cell shrinkage based on changes in pellet weight were somewhat higher than the more reliable estimates based on changes in dextran-impermeable volume.

NaCl (but not sucrose) also induced contraction of isolated cell walls (Fig. 1B), and the range of effective concentrations for wall contraction was nearly identical to the range for cell contraction. Moreover, isolated walls expanded when the NaCl osmolality in the suspending medium was raised from about 0.1 osmolal to higher values.

Salt-induced dissociation of protons from isolated walls. The addition of NaCl to cell walls suspended in distilled water caused a rapid drop in suspension pH (Fig. 2) as well as contraction of the walls. NaBr, Na₂SO₄, KCl, LiCl, MgCl₂, and CaCl₂ also were found to be effective, and they all proved to be nearly equally potent based on their contributions to ionic strength of wall suspensions. Although the pH drop in these experiments was as great as 2.5 units, protons were released from less than 1% of the titratable wall groups. Salts also induced release of protons from whole cells suspended in water, but the maximal pH decrease was only 0.55 units in concentrated solutions.

Salt-induced pH changes were reported (23) for suspensions of insoluble proteins, and they were interpreted in terms of ion binding by the proteins. Cutinelli and Galdiero (7) showed by direct chemical analyses that isolated *Staphylococcus*

FIG. 1. Dextran-impermeable volumes of whole *Bacillus megaterium* KM cells (A) and isolated cell walls (B) suspended in NaCl or sucrose solutions. Values in Fig. 1A can be converted to milliliters of dextran-impermeable volume per gram of cell dry weight by multiplying them by 4.44; the conversion factor for changing values in Fig. 1B to milliliters of dextran-impermeable volume per gram of wall dry weight is 8.48. Suspending medium osmolalities shown are initial values; distilled water was considered to have zero osmolality. The plasmolysis threshold refers to the minimal sucrose osmolality inducing microscopically visible plasmolysis. Plasmolyzing osmolalities for NaCl solutions were slightly less than those for sucrose solutions.

aureus walls bind a variety of cations. Some of the proton loss from *B. megaterium* walls might be due to cation binding; but there is some question regarding the importance of ion binding because proton release depended more on environmental ionic strength than on the particular ions present. The charged state of wall groups that can be ionized was favored by increased ionic strength (Fig. 3B), and salts may have caused dissociation of wall carboxyl groups in the suspensions (pH 7.2 to 4.7) used here. Unfortunately, as Steinhart and Zaiser (23) pointed out, electrostatic factors for insoluble polyelectrolytes cannot be accurately calculated on the basis of current electrolyte theory.

In contrast to the salts previously mentioned, added sodium acetate and sodium citrate caused the pH of wall suspensions to rise, presumably because the organic anions were bound by the walls.

Contraction and expansion of isolated walls in relation to suspension pH. Figure 3A shows dextran-impermeable volumes in relation to suspension pH for isolated *B. megaterium* walls suspended in media of ionic strength of 0.02 and 0.4. Figure 3B points out the titration behavior of the walls in media of relatively low, varying ionic strength ranging from essentially 0 to 0.12 and in media of relatively high, constant ionic strength of 0.4. Ionic strength changes caused shifts in apparent dissociation constants of titratable groups but did not affect the total numbers of groups that could be titrated.

These curves can be interpreted at least qualitatively in terms of known chemical structure. Previous reports (6, 11) indicated that the cell wall of *B. megaterium* KM is made up of two polymers—first, a phosphomucopolysaccharide containing phosphate, glucose, *N*-acetylglucosamine, and glycerol, and second, a mucopeptide

or peptidoglycan containing *N*-acetylglucosamine, *N*-acetylmuramic acid, alanine, glutamic acid, and α, α' -diaminopimelic acid (DAP). These two polymers appear to be linked by covalent bonds in the intact wall. Bricas et al. (6) found that the predominant peptide structures in the peptidoglycan were -L-ala-D-glu-*meso*-DAP and -L-ala-D-glu-*meso*-DAP-D-ala. In addition, N-terminal group analyses indicated a low degree of peptide cross-linking with free amino groups present in about 70% of the DAP residues.

Thus, *B. megaterium* KM cell walls are large, amphoteric polyelectrolytes. The compactness of model polyampholytes, such as copolymers of methacrylic acid and diethylaminoethyl methacrylate, in aqueous solution was found (1, 2, 19) to be affected by addition of acid and base in such a way that the molecules are most compact when the solution pH approaches the polymer isoelectric pH. Evidently, electrostatic repulsion within these flexible molecules is minimal and electrostatic attraction is maximal when their net charge is minimal. Increased environmental ionic strength diminishes both attraction and repulsion within the polymers, and they can be made to expand or contract by adding salt to solutions under appropriate conditions.

Similar ionic effects occurred with insoluble bacterial cell walls. In media of relatively low ionic strength (0.02) and high pH (11.2), the walls were expanded polyanions with relatively large, dextran-impermeable volumes (Fig. 3A). In less alkaline solutions (pH 8 to 11.2), ϵ -amino groups of DAP residues became protonated and the walls contracted. Thus, conversion of amino groups

from the uncharged to the positively charged form apparently enhanced electrostatic attraction and diminished electrostatic repulsion within the walls. Transfer of walls to media of still lower pH resulted in greater contraction, and volume changes could be related at least qualitatively to titration behavior (Fig. 3B). Walls were maximally contracted in media of low ionic strength and a pH of about 4.0. Without detailed knowledge of structure, it is difficult to predict accurately the wall isoelectric pH from titration curves. Also, there is a problem in relating bulk suspension pH to pH at the wall surface. If all of the groups titrating at pH above 8 are amino groups (mainly ϵ -amino groups), and all groups titrating at lower pH are carboxyl or phosphate groups, then the pH for minimal wall charge should be less than 2.5, well below the pH for maximal contraction. But, if groups titrating at pH below 2.5 (mainly phosphate groups) are ignored, then the pH for minimal charge in the peptidoglycan portion of the wall should be about 4.1, a value close to the pH for maximal contraction.

Walls transferred to media with a pH of less than 4 acted as polycations, and protonation of negatively charged groups caused them to expand.

Figure 3A also shows that pH-dependent expansion and contraction of isolated walls were greatly diminished in media of relatively high ionic strength (0.4). Therefore, electrostatic attraction appeared to be dominant in highly contracted walls, whereas electrostatic repulsion was dominant in highly expanded walls.

The effect of ionic strength on ionization is seen in the titration curves (Fig. 3B); the charged forms of fixed groups that can be ionized in the walls were favored by increased environmental ionic strength. Amino groups were more easily protonated and carboxyl groups were less easily protonated in media of higher ionic strength. The titration behavior of *B. megaterium* KM walls described here is in accordance with the chemical analyses given by Bricas et al. (6).

Relationship of wall contraction to cell contraction. Whole cell contraction and wall contraction appeared to be closely related because the ranges of effective salt concentrations for the two processes were nearly identical. With the value of 0.41 for the fraction of cell volume occupied by wall (see Materials and Methods), it is possible to relate, at least roughly, the extent of wall contraction to the extent of cell contraction. For example, isolated walls contracted about 50% when transferred from water to 0.186 osmolal (0.1 M) NaCl solution (Fig. 1B). If the walls of whole cells were to behave in the same way, the cell dextran-

FIG. 2. Salt-induced release of protons from isolated, *Bacillus megaterium* cell walls. A single curve was drawn to fit pH values for suspensions containing 4.1 mg of walls (dry weight) per ml and NaCl (○), LiCl (□), Na_2SO_4 (●), or CaCl_2 (■). The dextran-impermeable volumes (x) are the ones presented in Fig. 1B for isolated walls suspended in unbuffered NaCl solutions. Arrows indicate the axes associated with each curve.

FIG. 3. Compactness of isolated *Bacillus megaterium* KM walls related to suspension pH. (A) Dextran-impermeable volumes of walls suspended in 0.02 M (○) or 0.4 M (□) NaCl solutions; plotted values can be converted to milliliters of dextran-impermeable volume per gram of wall dry weight by multiplying them by 8.06. (B) Titration curves for cell walls suspended initially in distilled water (○) (μ , the environmental ionic strength, increased from essentially 0 to a maximum of 0.12 during the titration) or in 0.4 M KCl solution (□) (μ was held constant at 0.4); the zero point on the ordinate was arbitrarily chosen on the basis of the suspension pH before addition of acid or base.

impermeable volume should decrease from 0.85 to 0.68 because of wall shrinkage after a similar transfer. The measured change from 0.85 to 0.63 (Fig. 1A) indicated that there might also have been compression of the underlying protoplast or that the isolated walls behaved differently than the walls of whole cells. Actually, the wall of a whole cell should show less tendency to contract because of mechanical tension in the structure due to cell turgor pressure. Protoplast compression in a nonplasmolyzed turgid cell would be accompanied by compression or convolution of the protoplast membrane, which would result in decreased contact area between wall and membrane. Other structures within the cell may also be affected by salts; salt-induced changes in the appearance of bacterial nucleoids have been described previously (29).

Environmental pH and ionic strength seem to be important factors affecting wall tone in bacterial cells; the results presented in this paper strongly suggest that the primary process in salt-induced contraction of nonplasmolyzed *B. megaterium* cells is electrostatic wall contraction

rather than any osmotic response of the cells. Also cells and walls in well-buffered suspensions showed diminished sensitivity to the action of salts.

Durham et al. (8) microscopically observed shrinkage of whole *B. cereus* cells exposed to vancomycin. They considered that this shrinkage was due to elastic contraction of the wall after loss of cell turgor. Thus, the antibiotic-induced process appeared to be more closely related to the type of contraction which occurs during plasmolysis in concentrated sucrose solutions rather than to salt-induced contraction.

Isolated cell walls of *Micrococcus lysodeikticus* also were found (E. L. Carstensen and R. E. Marquis, unpublished data) to contract when transferred from water to NaCl solutions. Indeed, all microbial cell walls containing amphoteric peptidoglycans probably exhibit this effect to some degree. The compactness of other flexible, amphoteric cell wall polymers, e.g., proteins, may also be affected by changes in environmental pH and ionic strength. Also, a number of wall polyelectrolytes, e.g., teichoic and techuronic acids,

are highly electronegative, and electrostatic repulsions may affect their compactness. Certainly, the effects of salts on bacterial structure are complex, but these effects must be considered in interpretations of the physiological action of salts on bacteria.

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