Effects of Potassium Ions on the Electrical and pH Gradients Across the Membrane of *Streptococcus lactis* Cells

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Bacteria transduce and conserve energy at the plasma membrane in the form of an electrochemical gradient of hydrogen ions (Δp). Energized cells of *Streptococcus lactis* accumulate K⁺ ions presumably in exchange for H⁺. We reasoned that if the movement of H⁺ is limited, then an increase in H⁺ efflux, effected by potassium transport inward, should result in changes in the steady-state Δp. We determined the electrical gradient (Δψ) from the fluorescence of a membrane potential-sensitive cyanine dye, and the chemical H⁺ gradient (ΔpH) from the distribution of a weak acid. The Δp was also determined independently from the accumulation levels of the non-metabolizable sugar thiomethyl-β-galactoside. KCl addition to cells fermenting glucose or arginine at pH 5 changed the Δp very little, but lowered the Δψ, while increasing the ΔpH. At pH 7, the ΔpH only increased slightly; thus, the decrease in Δψ, effected by addition of potassium ions, resulted in a lowered steady-state Δp. These effects were shown not to be due to swelling or shrinking of the cells. Thus, in these nongrowing cells, under conditions of energy utilization for the active transport of K⁺, the components of Δp can vary depending on the limitations on the net movement of protons.

Energy is required for the work entailed in the transport of solutes against electrochemical gradients. For there to be measurable expenditure of metabolic energy for active transport, at least two conditions have to be met. First, the solute must be actively transported under the specified incubation conditions. This is not necessarily the case for solutes such as metabolizable nutrients. For example, the active transport of lactose does not appear to be necessary, since mutants of *Escherichia coli* with lactose carriers defective in energy coupling can grow in media at lactose concentrations of 0.25 mM or more (11). Second, the total flux of solute must be large in order to consume a significant portion of the cells' available energy. Thus, the choice of cation transport for these studies is based on the assumption that massively transported solutes, such as K⁺, utilize more metabolic energy than solutes, such as amino acids, whose transport is expected to show lower flux rates.

The index of metabolic energy chosen for study is the protonmotive force (Δp), since bacteria transduce and conserve energy at the plasma membrane in the form of an electrochemical gradient of hydrogen ions, according to the chemiosmotic hypothesis of Mitchell (3, 14). This gradient (Δp), generated by the proton-translocating membrane-bound adenosine triphosphatase complex, consists of a membrane potential (Δψ) and a pH gradient (ΔpH) across the bacterial plasma membrane. These parameters bear the relationship, Δp = Δψ - 59 ΔpH, where ΔpH equals the pH_out (pH of the bulk medium) minus the pH_in (pH of the cytosol). The value 59 is a combination of constants for expression of ΔpH in millivolts at 25°C.

Fermenting conditions are required for K⁺ translocation across the streptococcal membrane (4); thus, under such conditions, the Δp is continuously replenished by adenosine triphosphatase-effected proton extrusion. However, if the movement of hydrogen ion is limited, then it is expected that coupling proton movements to those of other cations would result in an alteration of the proton electrochemical gradient. In this study, we report that the components of the electrochemical proton gradient across the plasma membrane of nongrowing, fermenting cells of *Streptococcus lactis* are altered by the active transport of potassium ions.

**MATERIALS AND METHODS**

Growth of cells. Cells of *S. lactis* ATCC 7962 were grown to exponential phase as described previously (8). After harvesting, the cells were washed and suspended in 0.1 M citrate-tris(hydroxymethyl)amino methane (Tris) base buffer, pH 5.0, or in 0.1 M 3-(N-morpholino) propanesulfonic acid (MOPS)-Tris buffer, pH 7.0. The cell density was routinely measured with a Klett-Summerson colorimeter and related to the dry weight of cells. One milliliter of cells
suspended in 0.1 M sodium phosphate buffer, pH 7, reading 100 Klett Units (no. 42 filter) was equivalent to 0.14 mg (dry weight) of cells.

A stock suspension of cells plus dye was prepared by mixing 20 ml of a cell density of 1.4 mg (dry weight) per ml and 100 μl of a 1 x 10^{-2} M solution of the dye, 1,1'-dipropyl-2,2'-thiodicarbocyanine, dissolved in 95% ethanol. This mixture was kept at 4°C until use. All experiments were carried out at 25°C.

Centrifugation through silicone oil. This method of separating cells from medium consisted of layering a 1.0-ml portion of the reaction mixture over 0.5 ml of a mixture of silicone oils (25% Fluid 510, 50 centistokes, and 75% Fluid 550; Dow-Corning Corp., Midland, Mich.; vol/vol) in a 1.5-ml Microfuge tube and centrifuging for 2 min at 10,000 rpm in an Eppendorf Microfuge. The upper aqueous supernatant layer and most of the oil layer were removed with a Pasteur pipette. The bottom of the plastic tube containing the cell pellet was cut off and dropped into a vial for scintillation counting. The amount of trapped medium, although variable, was about 10-fold less than that obtained by membrane filtration (12).

Intracellular water spaces. Since Δp measurements were ultimately dependent on the distribution of solutes between the cells and the external medium, a trivial reason for any observed differences in solute accumulation could be osmotic swelling or shrinking of the cells. We, therefore, measured intracellular volume by methods described previously (12) under various incubation conditions. The total pellet water was determined gravimetrically. Cells plus indicator molecules were incubated for 2 or 15 min, followed by a 20-min centrifugation to separate cells from the medium. The cell water volume was determined from the total pellet water volume minus the volume calculated from the exclusion of two small, radioactively labeled molecules, [1^4C]sorbitol and [4^C]taurine. These, we infer, can penetrate up to the plasma membrane. Large non-electrolytes, such as [H]inulin and 20 [H]polyethylene glycol, apparently can penetrate only up to the cell wall (18). The difference in the exclusion volumes of the large and small molecules was thus designated as the intramural space (Table 1).

Alternatively, to detect the intramural volume, cells incubated for 2 to 4 min with compounds of low (e.g., taurine) and high (e.g., inulin) molecular weight were separated from the incubation medium by centrifugation through silicone oil, as above.

The following conditions of incubation gave values for cell water spaces that differed by less than one standard deviation of their means and are, thus, considered not significantly different: (i) 0.1 M Tris-citrate buffer (pH 5.0 or pH 5.5), 0.1 M Tris buffer (pH 7.0), 0.1 M 3-(N-morpholino)propanesulfonic acid-Tris buffer (pH 7.0); (ii) absence of energy source, glucose (20 to 80 mM), arginine (20 to 80 mM); (iii) NaCl (0 to 200 mM), KCl (0 to 200 mM), D-sorbitol (0 to 200 mM); and (iv) variation of osmolarity of the medium (measured with an osmometer) from 100 mOsm (buffer only) to 500 mOsm with added salt or D-sorbitol, singly or in combination. As there was no significant alteration in cell water volume, any changes in the cell-associated levels of solute were considered to be due to changes in solute concentration.

Measurement of ΔpH. The intracellular pH was determined as described previously (12), using the weak acids [1^4C]benzoic acid or [4^C]acetylsalicylic acid. Cells were separated from the medium by centrifugation through oil (as above), and the contaminating extracellular fluid volume trapped in the pellet was monitored with [H]inulin and corrected for the intramural volume.

Measurement of Δψ for calibration. In order to correlate the fluorescence of cell-dye mixtures with known Δψ values, we measured the fluorescence of preparations treated with valinomycin and incubated with K^+ at different concentrations (see below). The cells were separated from the medium by centrifugation through silicone oil. The potassium concentrations of lysed cells and of the supernatant fluids were determined by flame photometry. The cell membranes were disrupted by incubating the pellets with one drop of acetone overnight at room temperature. For membranes rendered permeable to K^+ ions by valinomycin, the Δψ (in mV) was calculated with the simplified Nerst equation,

\[ \Delta \psi = -59 \log \left( \frac{[K^+]_\text{in}}{[K^+]_\text{out}} \right) \]

where Δψ is the membrane potential in mV, [K^+]_in and [K^+]_out are the intracellular and extracellular K^+ concentrations, and 59 is a combination of constants for monovalent cations at 25°C.

Membrane potential. The membrane potential across the plasma membrane of S. lactis cells was followed by the changes in fluorescence intensity of the blue cyanine dye 1,1'-dipropyl-2,2'-thiodicarbocyanine iodide (hereafter referred to as "dye") (19). A fluorometer was assembled with a 630-nm filter for the activating light and a 670-nm filter for the emitted light.

Sugar accumulation. The accumulation of [1^4C]methyl-β-thio-β-galactoside ([1^4C]TMG) was determined by incubating 0.1 ml of cell suspension, at 1.4 mg (dry weight)/ml plus 1.2 to 1.4 ml of the appropriate buffer with KCl as indicated, for 12 min at 23°C. Glucose was added to 60 mM; after 2 min, the reaction was started by adding [1^4C]TMG to 1.1 μM at 24 μCi/μmol. After 4 min, when steady state

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**Table 1. Intracellular spaces of S. lactis 7962 cells**

<table>
<thead>
<tr>
<th>Intracellular space</th>
<th>Volume (ml/g dry weight)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Taurine exclusion space</td>
<td>1.54 ± 0.035</td>
</tr>
<tr>
<td>Inulin exclusion space</td>
<td>2.64 ± 0.047</td>
</tr>
<tr>
<td>Intramural space</td>
<td>1.10</td>
</tr>
</tbody>
</table>

* The exclusion spaces for [1^4C]taurine and [H]inulin were determined as described in Methods. Each value is the average of 16 determinations with five batches of cells.

* ±, Standard error of the mean.
had been reached, 1.0-ml portions of the incubation mixture (total volume of 1.5 ml) were centrifuged through silicone oil, as described above. The bottoms of the centrifuge tubes and samples of the supernatant fluids were counted for radioactivity.

**Chemicals.** d-[1-3H]Sorbitol (14C)sorbitol), [1,2-14C]taurine (14C]taurine), [methoxy-14C]hemin, [7-14C]benzoic acid ([14C]benzoic acid), (carboxyl-14C)acetylsalicylic acid ([14C]acetylsalicylic acid), d-[U-14C]sorbitol ([14C]sorbitol), [1,2-14C]polyethylene glycol, [14C]methyl-β-thio-β-galactopyranoside ([14C]TMG), and [1,2-3H]polyethylene glycol were bought from New England Nuclear Corp., Boston, Mass. The radioactively labeled sugars were chromatographed before use (descending paper chromatography, Whatman no. 1 filter paper; isopropanol-water 3:1 (vol/vol)). 3-(N-morpholino) propanesulfonic acid was bought from Calbiochem, LaJolla, Calif.; Tris base and its hydrochloride (Tris-hydrochloride) came from Fisher Scientific Co.; valinomycin was purchased from Sigma Chemical Co., St. Louis, Mo.; 1,1′-dipropyl-2,2′-thiodicarbocyanine iodide was a generous gift from Alan Waggoner; all other reagents were of analytical grade and commercially available.

**RESULTS**

The basic experiment was to incubate *S. lac-tis* cells under two conditions: in buffer only and in buffer with K+ added. The cells in the medium containing this cation were expected to carry out the work of accumulating potassium ions in exchange for hydrogen ions. We postulated that if the movement of hydrogen ion is limited, then increasing the extrusion of hydrogen ions should result in a change of the components of the electrochemical gradient of protons. Therefore, each experiment consisted of: (i) calibrating the fluorescence intensity of cyanine dye plus cells with the ∆ψ calculated from the distribution of K+ of valinomycin-treated cells, (ii) measuring the fluorescence of cells incubated with glucose at varying KCl concentrations of the medium, and (iii) measuring the ∆pH in parallel reaction vessels. In addition, we determined the ∆p from the distribution of TMG between the cells and the medium.

**Calibration of fluorescence intensity.** The fluorescence intensity of the cell-plus-dye suspension in the absence of an energy source was dependent on a number of variables, including cell density, dye concentration, and cell-dye ratio. A decrease in fluorescence intensity denoted an increase in ∆ψ. The calibration of fluorescence intensity and membrane potential was achieved by a technique similar to that used with another cyanine dye (10, 12). We assumed that a transmembrane electrical potential affects dye fluorescence in the same way whether the potential is caused by proton extrusion, as in fermenting cells, or by the diffusion of potassium ions when it is added. Hence, we measured the fluorescence intensity of cells treated with valinomycin and incubated in media of varying K+ concentration (Fig. 1). In this representative run, as the [K+]out increased, the fluorescence level increased. Addition of high K+ to the medium brought the fluorescence of these suspensions to the same final level, whereas addition of equivalent amounts of NaCl had no effect (data not shown). The fluorescence changes are similar to those first observed with erythrocytes (7).

A calibration run, such as that depicted in Fig. 1, was performed for each experiment. The fluorescence intensity was plotted versus the ∆ψ value calculated from the measured K+ concentration ratios in/out for every value of [K+]out by the Nernst equation (Fig. 2). There was a linear relationship between fluorescence intensity and ∆ψ from about 40 to about 120 mV. Above 120 mV the fluorescence intensity appeared to be constant with increasing ∆ψ. The reason for this is not clear. Two possibilities are: (i) at low [K+]out, the ∆ψ is underrated because the K+ ratio in/out is overrated. There could be a partial permeability barrier to K+ at the cell wall, so that the K+ concentration of greatest interest, namely that immediately outside the plasma membrane, is higher than that measured in the bulk medium. (ii) An alternative explanation is that at the high ∆ψ levels the limit of the “stacking,” and therefore fluorescence quenching, of dye molecules has been reached (28) due to insufficient dye present in the membrane. We were reluctant to increase the dye concentration because it could have had deleterious effects on membrane integrity (7). However, the presence of dye at the concentrations used did not affect the concentration ratio in/out of K+ or of TMG under various conditions, indicating that there were no noticeably deleterious effects on the membrane (data not shown). On the other hand, decreasing the cell concentration would have lowered the total K+ to levels below the sensitivity of the flame photometer.

**Effects of KCl on ∆ψ and ∆pH.** The fluorescence of a suspension of cells plus dye decreased when glucose, a source of fermentative adenosine 5′-triphosphate, was added to the cells (Fig. 3). A decrease in fluorescence was also observed when arginine rather than glucose was added (data not shown). A relatively greater decrease in fluorescence intensity was observed with cells incubated at extracellular pH 7 than at pH 5 (data not shown). However, the fluorescence values obtained at pH 7 could
**Fig. 1.** Effect of KCl on fluorescence of cells-dye mixtures treated with valinomycin. Stock mixtures of cells and dye (see Methods) were diluted 1:10 with 0.1 M citrate-Tris buffer, pH 5.0, and KCl was added to the concentrations indicated. At time A, 4 μl of 1 × 10⁻⁴ M valinomycin dissolved in 95% ethanol was added to 6.0 ml of reaction mixture. Three milliliters were placed in a fluorometer cuvette, and, at time B, two 1-ml portions were removed from the test tube and analyzed for K⁺. The Δψ was calculated as described in Methods. The volume of medium contaminating the pellet (measured in analogous centrifugations of cell suspensions containing [³⁵C]taurine) was variable, but sufficiently small (0.4 μl/pellet) so that it did not significantly affect the calculated K⁺ ratios in/out. The K⁺ concentrations indicated under the tracings refer to the measured K⁺ concentrations in the supernatant fluids of the centrifuged cell samples. At time C, KCl was added to a concentration of 130 mM. To construct a calibration curve, the fluorescence intensity at time B to C was related to the Δψ values calculated from the measured K⁺ ratios in/out; a representative curve is shown in Fig. 2.

**Fig. 2.** Relationship of fluorescence intensity and the Δψ calculated from K⁺ ratios in/out. These data are derived from experiments such as described in

not be calibrated by the present technique, since it appears that S. lactis cells, like E. coli vesicles (16) and valinomycin-treated E. coli cells (15), cannot maintain an internal pH greater than about pH 7.5. Thus the proportion of Δp in the form of ΔpH increases as the pHout is lowered while that in the form of Δψ decreases. Consequently, these experiments were carried out at pH 5.

When cells were placed in media of relatively high KCl concentration, the level to which the fluorescence dropped after addition of glucose was higher than without KCl in the medium (Fig. 3). This higher fluorescence intensity was thought to reflect a smaller Δψ. The more KCl present, the higher were the fluorescence intensities observed after glucose addition. The
FIG. 3. Effect of KCl on fluorescence intensity of fermenting cells: three representative tracings of chart recordings. Stock cells and dye (see Methods) were diluted 1:10 with 0.1 M citrate-Tris buffer, pH 5.0, and KCl was added to the concentrations indicated under each tracing. The reaction mixtures were added to the cuvettes at the time indicated by CELLS; glucose was added at time GLUC. The values corresponding to the fluorescence intensity at 2 min after glucose addition were read off a calibration curve such as that shown in Fig. 2.

corresponding values of Δψ were determined from the calibration curve. From the data presented in Fig. 4 we see that addition of 10 mM KCl or more to cells fermenting glucose at pH\textsubscript{out} 5 alters the relative values for Δψ and ΔpH. With KCl concentrations of about 10 mM, the Δp increased slightly from about 155 to about 170 mV, because of an increase in internal pH; thus, the increase in ΔpH offset the decrease in Δψ. Addition of 100 mM K\textsuperscript{+} increased the ΔpH from 1 to 2 U, whereas the Δψ decreased from 95 to about 40 mV.

Effects of KCl on TMG accumulation. The Δψ could not be determined by the dye technique at K\textsuperscript{+} concentrations greater than 100 mM because the fluorescence intensity reached levels above those of the calibration curve. Thus, the accumulation levels of TMG, which have been shown to correlate directly with Δp (9, 10), were used to measure the Δp at higher KCl concentrations and at higher medium pH (Table 2).

When cells fermented glucose at pH 5, the Δp showed little change on addition of 50 mM KCl. KCl addition up to 200 mM showed little further effect on Δp or ΔpH (data not shown). Since the ΔpH increased, the calculated Δψ value decreased under these conditions. The value for Δp in the absence of added salt was
TABLE 2. Effects of KCl on TMG accumulation and \( \Delta\text{pH} \)

<table>
<thead>
<tr>
<th>Energy source</th>
<th>pH_m</th>
<th>With 50 mM KCl</th>
<th>None</th>
<th>With 50 mM KCI</th>
<th>None</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose</td>
<td>5</td>
<td>144.9</td>
<td>139.2</td>
<td>85.4</td>
<td>115.8</td>
</tr>
<tr>
<td>Glucose</td>
<td>7</td>
<td>137.9</td>
<td>98.1</td>
<td>21.1</td>
<td>32.9</td>
</tr>
<tr>
<td>Arginine</td>
<td>5</td>
<td>96.1</td>
<td>114.5</td>
<td>63.3</td>
<td>118.5</td>
</tr>
<tr>
<td>Arginine</td>
<td>7</td>
<td>127.5</td>
<td>103.7</td>
<td>0</td>
<td>15.1</td>
</tr>
</tbody>
</table>

* The accumulation of TMG and the \( \Delta\text{pH} \) were measured as described in Methods. The values are expressed in equivalent units. Each value is the average of 9 to 16 determinations, using three to five batches of cells. The standard errors of the means (SEM) were about 2.5% of the average values, except for 59\( \Delta\text{pH} \) values lower than 33 mV, where the SEM's were 15% of the means. All the values obtained with KCl present were significantly different from those in the absence of KCl (\( P < 0.02 \) by Student's t test), except for TMG accumulation by glucose-energized cells at pH 5.

145 mV by the sugar distribution method. This value agrees fairly well with that calculated from the dye method (Fig. 4), 155 mV.

When the experiment was repeated at pH 7, the \( \Delta p \) decreased upon K+ addition, and the \( \Delta\text{pH} \) increased slightly. The calculated \( \Delta\psi \) thus decreased with K+ addition, as it had at pH 5. Increasing the KCl concentration of the medium had little further effect (data not shown).

Similar effects were found for cells fermenting arginine instead of glucose; hence, an effect of K+ on glycolysis per se was not the primary reason for any observed changes in \( \Delta p \). At pH 5, arginine-energized cells showed a lower \( \Delta p \) than glucose-energized cells. The \( \Delta p \) rose on K+ addition because of an increase of 1 U in the \( \Delta\text{pH} \). Arginine-energized cells incubated at pH 7 had no \( \Delta\text{pH} \) in the absence of K+. When this cation was added, a small \( \Delta\text{pH} \) was observed. Addition of K+ resulted in a decrease of \( \Delta p \). Again, increasing the KCl concentration from 50 to 200 mM had little further effect on the \( \Delta p \) and the \( \Delta\text{pH} \) of arginine-energized cells, either at pH 5 or at pH 7 (data not shown).

DISCUSSION

It should be emphasized that the present report deals with steady-state measurements, not unidirectional fluxes. The rates at which H+ and K+ ions traverse the cell membrane in either direction under various conditions of incubation await measurement.

The \( \Delta p \) measured in the absence of added KCl was about 155 mV by the dye technique and 145 mV by the accumulation of \( \beta \)-galactoside. The mean is similar to the protonmotive force estimated for E. coli membrane vesicles (16), illuminated Halobacterium halobium cells (13), and respiring Staphylococcus aureus cells (1), but is about 40 to 80 mV lower than the values reported for glycolyzing S. faecalis cells (5, 6), respiring E. coli cells (1), and illuminated H. halobium vesicles (17). The \( \Delta p \) value previously reported for S. lactis cells (10) was much lower, about 80 mV; under those conditions, the medium contained 0.3 M NaCl, which can dissipate \( \Delta p \) (unpublished observations).

The \( \Delta p \) values obtained by two independent methods agreed reasonably well. The effects of K+ addition were similar with glucose- or arginine-energized cells: the \( \Delta\psi \) decreased on KCl addition, whereas the cellular pH rose. When the medium pH was 7, the intracellular pH rose only slightly, by about 0.2 U. Thus, at pH 7 the \( \Delta p \) was dissipated by addition of KCl, with both arginine- and glucose-energized cells, whereas at pH 5, glucose-energized cells showed no change in \( \Delta p \). However, arginine-energized cells showed an increase in this value, because the pH_m rose by about 1 U.

The results reported here can be interpreted as follows: K+ ions are exchanged for H+, in analogy to the systems in S. faecalis cells (4, 5, 20) and E. coli cells (3). The inward movement of K+ results in a decrease of the \( \Delta\psi \). The exchange of K+ for H+ occurs to an extent greater than the cell's internal buffering capacity, since the pH_m increases. It should be pointed out that a quantitative relationship between the change in \( \Delta\text{pH} \) and in \( \Delta\psi \) cannot be determined without measuring the buffering power of the cytosol; thus, the stoichiometry of a K+/H+ antiporter cannot be determined from these experiments. When the external pH is 5, the increase in \( \Delta\text{pH} \) effected by K+ transport compensates for the decrease in \( \Delta\psi \); thus, the \( \Delta p \) is poised at a fairly constant level (glucose-energized cells) or increased (arginine-energized cells). When the external pH is 7, a sufficiently large \( \Delta\text{pH} \) can not be effected by K+ addition, presumably because the cytosol buffers at about pH 7.5. Therefore the decrease in \( \Delta\psi \) without a compensatory increase in \( \Delta\text{pH} \) results in a lowered noise of the \( \Delta p \). Compensation for the dissipation of \( \Delta p \) would not be expected from the activity of the membrane adenine triphosphatase complex, because this activity results in the outward movement of protons (3), which would tend to increase the \( \Delta\text{pH} \) rather than decrease it. Under the conditions that obtained in these experiments, the cells are amply provided with a metabolizable energy source, either glucose or arginine. The cells maintain intracellular K+ concentrations in excess of 300 mM under all the conditions of incubation listed in Table 2 (data not shown). Hence, the energy coupling is sufficient to maintain K+ ratios in/out as high.
as about 10,000. As Harold and Altendorf have pointed out (4), such K⁺ ratios exceed the usual Δp values (here, 150 mV), which would not be expected of an electroneutral K⁺/H⁺ antiporter. One possibility is that (a portion of) K⁺ accumulation is energized directly by phosphate bond energy, as in E. coli cells (2). Consonant with this possibility is the observation that the exchange of K⁺ across the membrane of S. faecalis cells requires energy (4).

In the present report, then, the K⁺-effect changes in Δp, ΔpH, and Δp observed under the various conditions of incubation would not be due to inadequate rates of energy supply in the form of adenosine 5'-triphosphate or of activity of the membrane adenosine triphosphatase, but rather on the limitations imposed on the net movement of H⁺ by the buffering properties of the cytosol and by the permeability of the plasma membrane to ions.

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LITERATURE CITED