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Effect of Extracellular pH on Growth and Proton Motive Force of *Bacteroides succinogenes*, a Cellulolytic Ruminal Bacterium

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The utilization of cellulose or cellobiose by *Bacteroides succinogenes* S85 was severely inhibited at pH values of <5.7. Since low pH inhibited the utilization of both cellobiose and cellulose, changes in cellulase activity could not explain the effect. At an extracellular pH of 6.9, the pH gradient (ΔpH) across the cell membrane was only 0.07 U. As extracellular pH declined from 6.9 to 5.7, intracellular pH decreased to a smaller extent than extracellular pH and ΔpH increased. Below pH 5.7, there was a linear and nearly proportional decrease in intracellular pH. *B. succinogenes* took up the lipophilic cation tetraphenylphosphonium ion (TPP^+) in the presence of cellobiose, and uptake was sensitive to the ionophore valinomycin. As pH was decreased with phosphoric acid, the cells lost TPP^+ and electrical potential, ΔΨ, decreased. From extracellular pH 6.9 to 5.7, the decrease in ΔΨ was compensated for by an increase in ΔpH, and the proton motive force ranged from 152 to 158 mV. At a pH of <5.7, there was a large decrease in proton motive force, and this decrease corresponded to the inhibition of cellobiose utilization.

Bacteroides succinogenes is one of the predominant cellulolytic bacteria in the rumen (3) and lower gut of simple stomached animals (13, 16, 25). Ruminococci are also actively cellulolytic, but *B. succinogenes* S85 was the most active strain against crystalline types to cellulose (2, 6). All of the cellulolytic ruminal bacteria are sensitive to even modest declines in pH (21, 24), and low ruminal pH can be a critical factor limiting cellulose digestion under commercial feeding situations (17, 18). Many of the noncellulolytic ruminal bacteria are less sensitive to low pH than the cellulolytics (21), but little was known about the mechanism of pH as an inhibitor of cellulolytic ruminal bacteria. This paper describes the effect of extracellular pH on the cellulose digestion, growth, and proton motive force (PMF) of *B. succinogenes* S85.

MATERIALS AND METHODS

Cell growth. *B. succinogenes* S85 (3) was grown anaerobically in a medium containing salts (8), ammonia (10 mM), acetate (1 mM), C_4 and C_5 isoacids (1 mM each), Trypticase (1 g/liter; BBL Microbiology Systems, Cockeysville, Md.), yeast extract (0.5 g/liter), cysteine hydrochloride (0.6 g/liter), and either cellobiose or ball-milled Whatman no. 1 filter paper (ball milled for 2 days; approximately 1 g/liter). The cultures were incubated in tubes (18 by 150 mm) that were fitted with butyl rubber stoppers or 500-ml gas-washing bottles that were continuously purged with O_2-free CO_2.

B. succinogenes S85 produces large amounts of succinate, and this weak acid had little effect on the pH of the incubation media. pH was decreased by adding concentrated HCl directly to the incubation vessel (500 ml), and it was monitored continuously with a pH electrode. Cellulose and cellobiose utilizations were assayed by a phenol-sulfuric acid procedure (7). Preliminary experiments indicated that medium ingredients and cells gave little interference in this method.

PMF. Intracellular pH was measured by an acid distribution method that is based on the assumption that nondissociated forms of weak acids can diffuse freely through the cell membrane (20). Since the internal and external concentration equilibrate, distribution of the ionized form becomes a function of the pH gradient across the membrane. Samples (2 ml) of cells which had been provided with 6.0 g of cellobiose per liter were removed from the 500-ml incubation vessel during logarithmic growth (0.6 to 1.2 optical density [OD]) with a hypodermic syringe. Each sample was injected into a tube (13 by 100 mm) that had been filled with O_2-free CO_2 and capped with a butyl rubber stopper. The tubes contained [7-^{14}C]benzoate (1.00 μCi, 10 μCi/μmol); [U^{14}C] sucrose (1.00 μCi, 200 μCi/μmol), or tritiated water (1.00 mCi, 0.25 μCi/mg). All three isotopes were obtained from Dupont, New England Nuclear Corp., Boston, Mass. After 5 min of incubation at 39°C, 0.9-ml samples of culture were centrifuged through silicon oil (50:50 mixture of Dexter Hysol 550 and 560; Hysol Co., Olean, N.Y.) in a microcentrifuge (13,000 × *g*, 5 min; 1.5-ml tube). Tubes containing silicon oil (0.3 g) had been stored in a Coy anaerobic glove box for several days to remove O_2. After centrifugation, 50-μl samples of supernatant were removed for scintillation counting and the tube was frozen. After the liquid was solid, bottoms of the centrifuge tubes containing the pellets were removed with a pair of dog nail clippers, mixed to disperse the cells, and counted in a Packard Tri-Carb model B2450 scintillation counter (Packard Instrument Co., Inc., Rockville, Md.). The counting efficiency for ^{3}H was 55%, while that for ^{14}C was 94%. Intracellular space was calculated as the difference in specific activity between tritated water and [^{14}C]sucrose. This strain does not ferment sucrose and the intracellular volume was 5.9 μl/mg of protein. Intracellular pH was calculated from the Henderson-Hasselbach equation as described by Reibeling et al. (20). The chemical potential (ZΔpH) generated by the difference between intracellular and extracellular pH was calculated from the Nerst relationship, $[2.3(RT/F)] \times \Delta pH$, where RT/F was 62 mV (39°C).

The electrical potential (ΔΨ) was estimated from the uptake of the lipophilic, but positively charged ion tetraphenylphosphonium (TPP^+)-bromide, (Aldrich Chemical Co., Inc., Milwaukee, Wis.). TPP^+ uptake was measured with an electrode that was constructed from tetraphenylboron (Sigma Chemical Co., St. Louis, Mo.), polyvinylchloride, and dioctylphtalate (Aldrich), using tetrahydrofuran as a

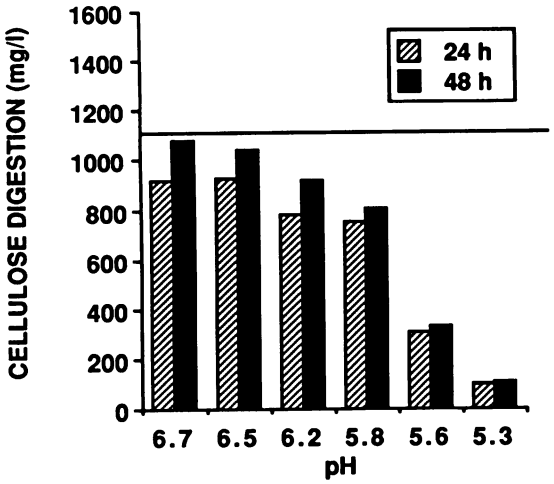

FIG. 1. Effect of pH on digestion of ball-mixed cellulose by *B. succinogenes* S85 at 24 and 48 h. The horizontal line indicates the initial concentration of cellulose. No change in pH was detected during the incubation period.

solvent (10). The electrode was filled with 1 mM TPP^+ and mounted in a vessel (16 by 100 mm) that was surrounded by circulating water (39°C). A combination pH electrode provided the reference and a means of monitoring changes in pH. The vessel was purged with N_2 and stirred with a glass stir bar. A 400-ml culture (2.0 g of cellobiose per liter) was inoculated, and after 16 h of incubation the OD was 1.7 (Gilford 260 spectrophotometer [Gilford Instrument Laboratories, Inc., Oberlin, Ohio]; 1-cm cuvettes). The culture was then provided with another 1.0 g of cellobiose per liter. Two hours later the OD had increased to 2.0, and the culture was harvested anaerobically (CO_2 headspace; 5,000 × *g*, 15°C, 10 min). The supernatant was discarded and the pellet was washed once in anaerobic buffer (50 mM K_2HPO_4, 5 mM cysteine hydrochloride, pH 6.5). The pellet was suspended in a total of 8 ml of buffer and transferred to a tube that was capped with a butyl rubber stopper. The TPP^+ uptake experiments were typically conducted with 4.3 ml of buffer, 0.1 ml of TPP^+ (125 μM), 0.1 ml of cellobiose (20%, wt/vol), and 0.5 ml of the cell suspension (3.5 mg [dry weight]/ml, final concentration). pH was increased or decreased with 1 M phosphoric acid or 2.5 M NaOH (3 to 8 μl per addition), respectively. Valinomycin (0.05 ml, 500 μg/ml) was dissolved in 95% ethanol. The TPP^+ standard curve was plotted as log TPP^+ versus deflection, and the plot was linear from 1 to 10 μM. $\Delta\Psi$ was calculated from the Nernst relationship, $-2.3(RT/F) \times (\log [TPP^+]\text{intracellular}/[TPP^+]\text{extracellular})$. $\Delta\Psi$ was only calculated from the portion of uptake that was valinomycin sensitive. Additional uptake was assumed to be nonspecific binding. The total PMF was calculated as $PMF = \Delta\Psi - Z\Delta pH$.

RESULTS

When *B. succinogenes* S85 was grown with ball-milled cellulose, the digestion of cellulose was dependent on the pH of the incubation medium. At pH 6.2 or greater, >80% of the cellulose was degraded during the first 24 h of incubation, and by 48 h >90% had been degraded (Fig. 1). At pH 6.2 and below there was a significant drop in cellulose digestion, and this decrease was most dramatic at pH values of <5.8. When the pH was either 6.2 or 5.8, there was some additional cellulose digestion in the second 24 h, but at pH 5.6 or 5.3 there was little change.

To ascertain the minimum pH allowing growth, S85 was grown on cellobiose (1 g/liter) and the pH of the medium was decreased (approximately 0.20 U every 30 min) by the addition of concentrated HCl (Fig. 2). These experiments allowed us to monitor the effect of pH on an actively growing culture rather than the initiation of growth at a different pH. Growth occurred logarithmically at a rate of 0.24 h^{-1} and was not affected until pH was <5.7. At pH 5.65 there was a marked decrease in growth rate, and no growth was observed after 1 h at pH 5.3. Logarithmic growth was accompanied by a logarithmic decrease in cellobiose, and cellobiose utilization ceased soon after the organism stopped growing. These latter results indicated that the pH affected uptake and metabolism, not the efficiency of growth.

When intracellular pH was estimated by an acid distribution method, the pH gradient across the cell membrane at pH 6.9 was only 0.07 U (Fig. 3). As extracellular pH was decreased, intracellular pH also declined, but the relationship was not linear. Between extracellular pH 6.3 and 5.7, there were only small declines in intracellular pH, and during this phase ΔpH increased. Below extracellular pH 5.7 there was once again a large and linear decrease in intracellular pH.

When cells were added to anaerobic potassium phosphate buffer (pH 6.5) containing approximately 5.8 μM TPP^+ and 5 mM cellobiose, there was a rapid and immediate decline in the extracellular concentration of TPP^+ (Fig. 4, top). At 4 μM TPP^+ there was a lag in the uptake, and after this time uptake proceeded at a slower and decelerating rate. The extracellular TPP^+ concentration stabilized at approximately 1.8 μM, and 5 μg of valinomycin per ml caused an immediate increase in the extracellular TPP^+ concentration. The TPP^+ concentration in the presence of valinomycin (3.8

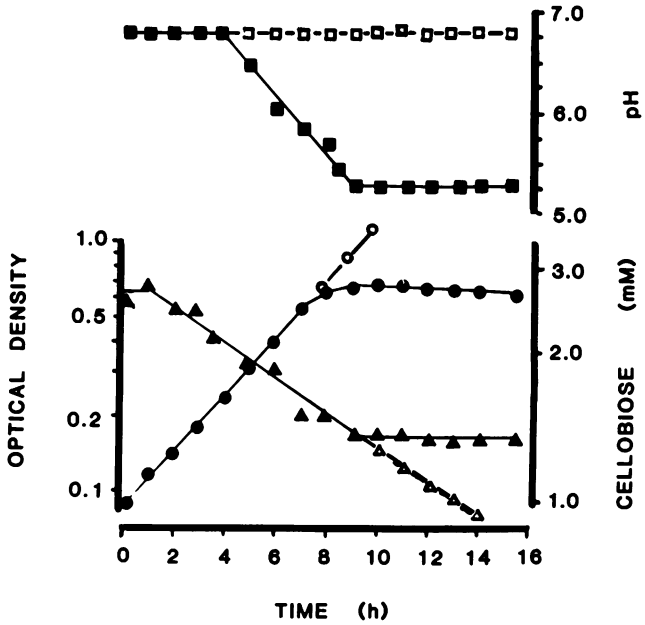

FIG. 2. Effect of decreasing pH (■) on OD (●) and utilization of cellobiose (▲) by *B. succinogenes* S85. (OD and cellobiose utilization are both shown on $\log_{10}$ scale.) Control incubations are shown as open (□, ○, △) symbols.

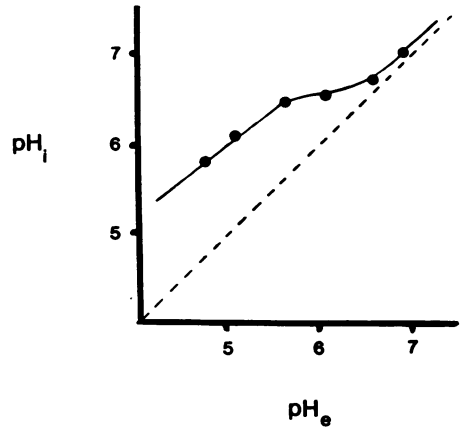

FIG. 3. Relationship between extracellular pH (pH_e) and intracellular pH (pH_i) for *B. succinogenes* S85.

μM) was surprisingly close to the value observed after the initial phase of TPP^+ uptake. Uptake by valinomycin-treated, deenergized cells was assumed to be nonspecific binding of the TPP^+. $\Delta\Psi$ values were corrected for this nonspecific binding.

When phosphoric acid was added to cells which had taken up TPP^+, there was a stepwise decrease in pH and a loss of TPP^+ from the cells (Fig. 4, bottom). At pH 4.9, the TPP^+ concentration was approximately the same as that observed for valinomycin-treated cells. When NaOH was added to the incubation mixture, some TPP^+ was again taken up by the cells. Increases in pH, however, never restored maximum TPP^+ uptake, even if the initial drop in pH was less dramatic. NaCl (20 μmol/ml) had no effect on TPP^+ uptake.

The total PMF, $\Delta\Psi$, and $Z\Delta pH$ are shown in Fig. 5. As pH decreased from 7.0 to 5.7, there was an increase in the $Z\Delta pH$ but a decline in TPP^+ uptake and hence $\Delta\Psi$. Until pH 5.7 the decline in $\Delta\Psi$ was compensated for by the increase in $Z\Delta pH$, and PMF remained relatively constant. At extracellular pH values below 5.7, there was no further increase in $Z\Delta pH$ and $\Delta\Psi$ continued to drop. At pH 5.4, PMF dropped below 150 mV, and this decrease corresponded to the inhibition of growth and cellobiose uptake (Fig. 2).

DISCUSSION

According to the chemiosmotic theory of Mitchell (15), most bacteria translocate protons outwardly through the cell membrane to establish a PMF which is composed of chemical ($Z\Delta pH$) and electrical ($\Delta\Psi$) gradients. The PMF of bacteria generally ranges from 140 to 160 mV, but values from 50 (respiring alkalaphiles) to 270 (respiring acidophiles) mV have been reported (11). Energy stored in the PMF can be used for a variety of functions, including active transport and motility.

B. succinogenes is a nonmotile organism that does not have a phosphotransferase system of glucose transport (14). Franklund and Glass noted that glucose uptake was sensitive to uncouplers and stimulated by sodium, but not potassium (5). Since glucose uptake was sodium dependent, transport may be driven by sodium rather than protons. The use of a sodium gradient may explain how the organism was able to grow when a ΔpH was sometimes <0.1 (Fig. 3).

Most bacteria maintain a relatively neutral intracellular pH and control is generally achieved by the activity of ion transport systems which facilitate the entry or exit of protons (1). With *B. succinogenes*, decreases in extracellular pH were associated with a decline in intracellular pH, but intracellular pH did not drop below 6.5 until extracellular pH was <6.0 (Fig. 3). Further decreases in extracellular pH, however, were associated with a proportional decrease in intracellular pH. One might argue that the inhibition of growth (Fig. 2) resulted from a decrease in intracellular pH, but similar experiments with *Bacteroides ruminicola* (data not shown) showed that this organism was able to grow with

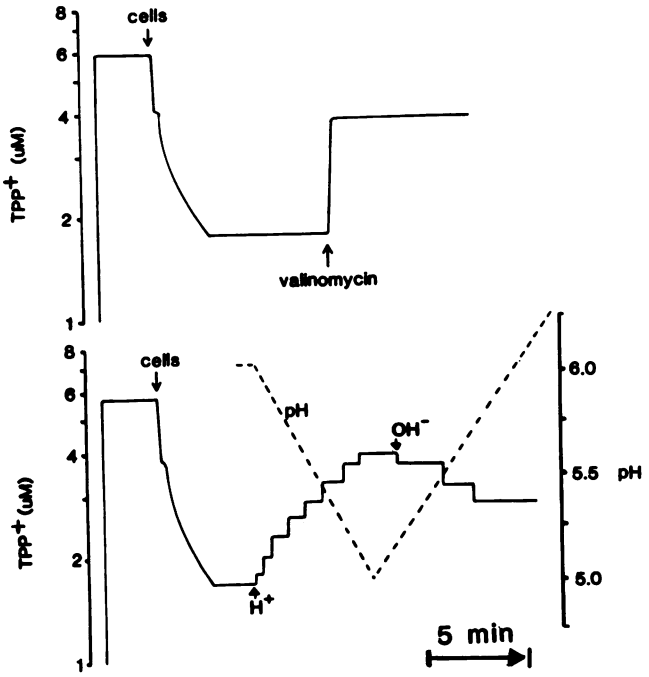

FIG. 4. Uptake of TPP^+ by *B. succinogenes* S85 under anaerobic conditions. The effect of 5 μg (5 μM) of valinomycin per ml (a) or H_3PO_4 and NaOH addition (b) on TPP^+ uptake and pH are shown.

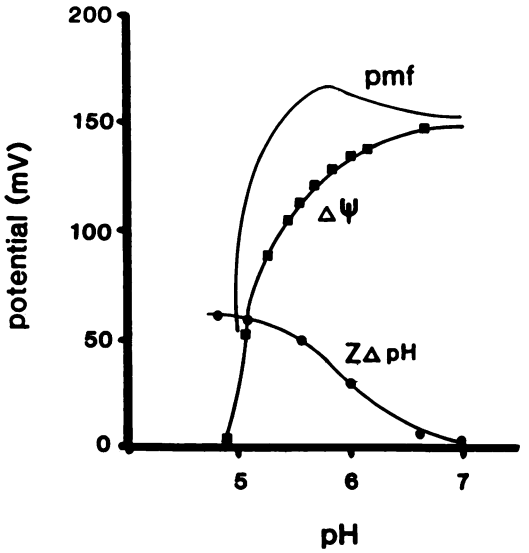

FIG. 5. Relationship between extracellular pH and the chemical ($Z\Delta pH$, ●) and electrical ($\Delta\Psi$, ■) gradients. PMF was calculated from PMF = $\Delta\Psi - Z\Delta pH$.

an extracellular pH of 5.0 and an intracellular pH of 5.5. Since both organisms make the same fermentation products (succinate, acetate, and formate) (9), it is uncertain whether a decline in intracellular pH per se caused the inhibition of growth observed in Fig. 2.

B. succinogenes cells readily took up TPP^+, and this uptake was dependent on the presence of cellobiose. When the ionophore valinomycin was added, TPP^+ was released from the cells. Chen and Wolin (4) reported that this strain was resistant to the ionophores monensin and lasalocid, but growth was not initiated for at least 72 h. The long lag time meant that the organism was initially sensitive to ionophores and that ionophore-resistant forms eventually grew. The organisms used in this experiment were not grown in the presence of ionophores and were sensitive to valinomycin.

$\Delta\Psi$ can be calculated by the Nernst equation from the uptake of lipophilic cations such as TPP^+, if the cation "behaves ideally and becomes distributed between the interior of cells and the external aqueous phase" (26). Probes are, however, nonspecifically bound to anionic groups at the interior and exterior cell surfaces (11). This nonspecific binding overestimates uptake and inflates the $\Delta\Psi$. The ionophore valinomycin is often used to deenergize cells, and it is generally assumed that uptake in the presence of valinomycin is due to nonspecific binding, an amount which can be subtracted (19). Recently, Wetzstein et al. measured the membrane potential of *Bacteroides amylophilus* (26), and this species was highly resistant to monensin, valinomycin, or valinomycin plus nigericin. Since ionophores could not be used to de-energize the cells, other means of estimating nonspecific binding were needed. Using spheroplasts and the modified Nerst equation of Lolkema et al. (12), they estimated a $\Delta\Psi$ of approximately 140 mV, a value in close agreement to ours.

As intracellular pH was decreased by additions of acid, there was a decrease in the amount of TPP^+ retained by the cells. By pH 4.9, the extracellular TPP^+ approached the value observed with valinomycin-treated cells (Fig. 4, bottom). When corrections were made for nonspecific binding, the $\Delta\Psi$ was 144 mV at pH 6.1 and essentially 0 mV at pH 4.9 (Fig. 5). The difference between the collapse of the $\Delta\Psi$ (144 mV) and the increase in proton concentration (67 mV) due to acid addition was 77 mV, and this value is in reasonable agreement with the increase in the $Z\Delta pH$ (62 mV). The PMF was >150 mV if the pH was 5.4 or greater, but at lower pH the PMF decreased rapidly. Since transport of sugar appears to be driven by active transport (5), the PMF at a pH of <5.4 was probably insufficient to take up cellobiose (Fig. 2).

The $\Delta\Psi$ collapsed after acid addition (Fig. 5), and this would be consistent with an intracellular accumulation of cations. Since $Z\Delta pH$ increased, net proton influx alone may not explain the decrease in $\Delta\Psi$. Franklund and Glass (5) indicated that glucose uptake in this organism was sodium dependent. Some sodium-proton antiporters have been demonstrated in bacteria (23). An influx of sodium could have been responsible for the decrease in $\Delta\Psi$. In our experiments, the cells were incubated in potassium phosphate buffer, but added sodium chloride (20 μmol/ml) had little effect on TPP^+ uptake. The absence of a sodium requirement may be related to the cell-washing procedure. The cells were only washed once in potassium phosphate buffer and the pellet was large enough to retain a considerable amount of sodium. Repeated washings of this organism resulted in irreversible loss of capacity for sugar uptake (5).

The cellulolytic ruminal bacteria have evolved together for millions of years, and under natural feeding situations in which forages are the primary component of the diet, the pH is rarely <6.0. Only under modern feeding conditions in which large amounts of starch are added to the diet does pH decline significantly. It is apparent that the cellulolytic bacteria have not yet evolved mechanisms to deal with low pH. Genetic engineering provides a means of accelerating evolution, but little is known about the genetics of proton flux in bacteria (1). Current efforts at developing acid-resistant, cellulolytic, ruminal bacteria make use of non-cellulolytic but acid-resistant ruminal bacteria as recipients for acid-resistant cellulase genes (22).

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