Acid Tolerance, Proton Permeabilities, and Membrane ATPases of Oral Streptococci

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Differences in acid tolerance among representative oral streptococci were found to be related more closely to the dynamic permeabilities of the bacteria to protons than to differences in the sensitivities of cell membranes to gross damage caused by environmental acidification. For *Streptococcus mutans* GS-5, *Streptococcus sanguis* NCTC 10904, and *Streptococcus salivarius* ATCC 13419, gross membrane damage, indicated by the release of magnesium from whole cells, occurred at pH values below about 4 and was rapid and extensive at pH values of about 3 or less. A more aciduric, lactic acid bacterium, *Lactobacillus casei* ATCC 4646, was more resistant to environmental acidification, and gross membrane damage was evident only at pH values below 3. Assessments of the movements of protons into *S. mutans* cells after an acid pulse at various pH values indicated that permeability to protons was minimal at a pH value of about 5, at which the average half time for pH equilibration across the cell membrane was about 12 min. The corresponding values for the less aciduric organism *S. sanguis* were pH 7 and 8.2 min, and the values for the intermediate organism *S. salivarius* were pH 6 and 6.6 min. The ATPase inhibitor dicyclohexylcarbodiimide acted to increase markedly the permeability of each organism to protons, and this action indicated that permeability involved not only the passive inflow of protons but also active outflow through the proton-translocating membrane ATPase. Membranes were isolated from each of the bacteria, and pH profiles for ATPase activities indicated pH optima of about 7.5, 7.0, 6.0, and 5.0 for *S. sanguis*, *S. salivarius*, *S. mutans*, and *L. casei*, respectively. Thus, the pH profiles for the enzymes reflected the acid tolerances of the bacteria and the permeabilities of whole cells to protons.

Studies of the physiology of oral streptococci have led to the view that the cell membrane plays major roles in acid-base regulation. These roles include the extrusion of protons out through the membrane and the exclusion of environmental protons. The net result, when the organisms are rapidly producing acid or when they are in acid environments, is that the cytoplasmic pH value is higher than that of the environment. This difference in pH values across the cell membrane is vital to the functioning of acid-sensitive systems such as the glycolytic system. The roles of the membrane in acid-base dynamics can be demonstrated readily by the use of agents, such as gramicidin, which increase the permeability of the membrane to protons and collapse the pH gradient across the membrane (4). Gramicidin greatly sensitizes glycolysis by intact cells or oral streptococci to acid and can shift the pH profile for glycolysis by as much as a full pH unit (1, 14).

Oral streptococci show a range of acid tolerance. Generally, *Streptococcus mutans* is the most aciduric and also the most cariogenic, whereas many strains of *Streptococcus sanguis* have relatively low acid tolerance. For example, the pH value at which glycolysis by *S. mutans* GS-5 is reduced to one-half of its maximal value in 100 mM potassium phosphate buffer with 1 mM $MgCl_2$ has been found to be approximately 5.0 (1). The corresponding value for *S. sanguis* NCTC 10904 is approximately 6.2. There appears to be only a very small difference in the inherent acid resistance of the glycolytic enzymes from the two species (3), and a major part of the difference seems to be related to the relative abilities of their membranes to maintain a transmembrane pH difference, which can be diminished by agents such as gramicidin. Glycolysis by cells of *Streptococ-*

cus salivarius* ATCC 13419 or ATCC 25975 is intermediate in acid tolerance, and the pH value for 50% inhibition has been found to be about 5.7. However, *S. salivarius* is able to grow in complex media at pH values as low as the minimum value for growth of *S. mutans*; *S. salivarius* therefore appears to be less aciduric in dense, glycolyzing suspensions but not in growing cultures.

Overall, it appears that the physiology of acid tolerance of oral streptococci is in large measure membrane physiology. In this paper, we present data from experiments designed to assess the permeabilities of cells to protons at various environmental pH values and to assess in a preliminary way the roles of membrane ATPases in these permeabilities.

MATERIALS AND METHODS

Bacteria and growth conditions. *S. mutans* GS-5, *S. sanguis* NCTC 10904, *S. salivarius* ATCC 13419, *S. faecalis* ATCC 9790, and *Lactobacillus casei* ATCC 4646 were maintained in the laboratory by weekly subculturing on tryptone-Marmite-glucose agar (9). Cells for proton permeability experiments were harvested from static cultures grown at 37°C in tryptone-Marmite-glucose broth until the phase of declining growth rate. Growth in the cultures was limited by acidification and could be increased by neutralizing part of the acid produced.

Procedures for assessment of acid damage. Cells were harvested by centrifugation from 1-liter cultures and washed once with cold deionized water. They were then suspended in 105 ml of deionized water. A 10-ml sample was taken for dry weight determination, and a 5-ml sample was taken for digestion in a Parr pressure bomb. Digestion was in a 6 N HCl solution at 100°C for 24 h. The digested samples were used to assay the total mineral contents of the cells by atomic absorption spectrophotometry (model 3030; The
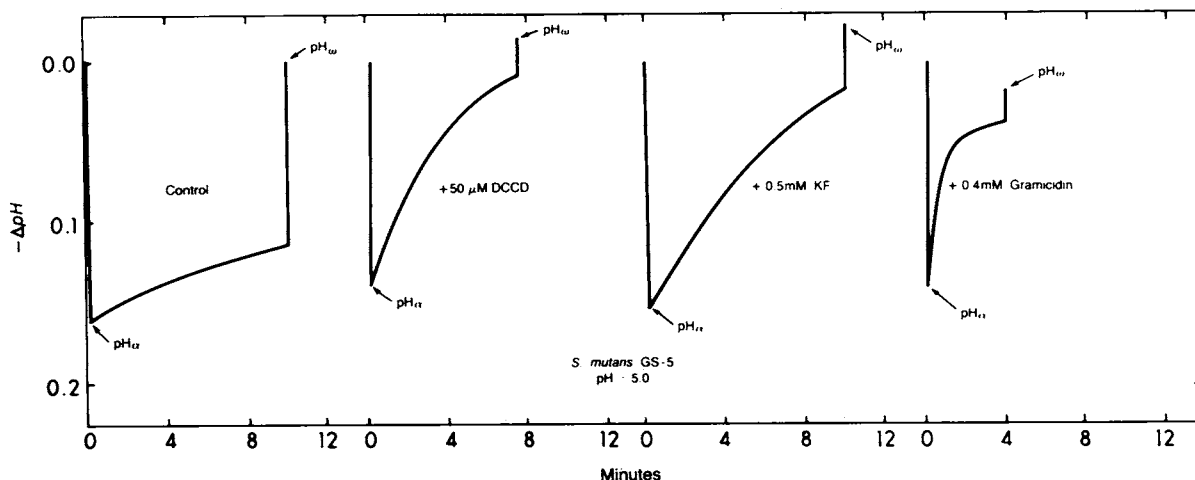
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FIG. 1. Proton uptake by *S. mutans* GS-5 cells suspended in a solution containing 150 mM KCl plus 1 mM MgCl₂ at an initial pH value of 5.0.

Perkin-Elmer Corp., Norwalk, Conn.). Samples (15 ml) of the cell suspension were placed in 50-ml plastic centrifuge tubes, and pH was adjusted to the desired values with concentrated HCl or NaOH. The pH values were monitored throughout the incubation period. At intervals, 1.0-ml samples were removed and centrifuged rapidly in an Eppendorf microfuge. The supernatant fluids were assayed for K and Mg.

Proton permeability determinations. Cells were harvested by centrifugation in the cold and washed once with a 5 mM MgCl₂ solution. They were suspended at a cell density of 5 mg (dry weight) of cells per ml in 20 mM potassium phosphate buffer (pH 7.2) with 1 mM MgCl₂ and 150 mM KCl and were incubated at 37°C partially to deplete endogenous catabolites. This incubation was 2 h for *S. mutans* but only 0.5 h for *S. sanguis*, *S. salivarius*, and *L. casei*. The cells were centrifuged again and suspended at a cell density of 20 mg (dry weight) of cells per ml in 150 mM KCl solution with 1 mM MgCl₂.

The suspensions were then titrated to the desired pH values with solutions containing 10 mM HCl or KOH plus 140 mM KCl or solutions containing 100 mM HCl or KOH plus 50 mM KCl. Titration was continued until the pH value stabilized, as indicated by no pH change for at least 2 min. Then, 2.5 ml of the suspension was placed in a 5-ml plastic beaker and stirred continuously. The pH of the suspension was monitored continuously with a combination glass electrode connected to a pH meter (model 45; Beckman Instruments, Inc., Fullerton, Calif.) connected to a chart recorder (model 1242; Soltec, Sun Valley, Calif.) set for full-scale expansion of 0.2 pH units. The pH value of the suspension was dropped by ca. 0.15 by adding a pulse of 10 mM HCl solution with 140 mM KCl from a micropipette.

The initial drop in the pH value of the suspension immediately after acid addition was reversed as protons flowed into the cytoplasm across the cell membrane. Recordings of increasing pH value, such as the ones shown in Fig. 1, were analyzed in terms of $t_{1/2}$ values for the movement of protons into the cells. The minimum pH value of the suspension after acid addition, pH_α, was estimated by extrapolation of the pH-versus-time curve. The final pH value, pH_ω, was assessed by adding 5% (vol/vol) butanol to the suspension at the end of the experiment to damage the cell membrane and allow for complete pH equilibration between the cells and

the suspending medium. The $t_{1/2}$ value is equal to the time required for the pH to reach a value halfway between pH_α and pH_ω.

ATPase isolation and assay. *S. mutans* GS-5 cells for membrane isolation were grown in brain heart infusion broth (BBL Microbiology Systems, Cockeysville, Md.) supplemented with 2% (wt/vol) glucose and 20 mM DL-threonine until the late exponential phase. The cells were washed twice with cold water and suspended in osmotic buffer (0.4 M sucrose plus 0.075 M Tris [pH 7.5] and 2.0 mM MgSO₄). The cells from 1 liter of culture were suspended in 40 ml of buffer, which was then transferred to 160 ml of buffer previously warmed to 37°C and containing 0.5 g of lysozyme and 2,000 U of mutanolysin (Sigma Chemical Co., St. Louis, Mo.). The suspension was then incubated for 2 h at 37°C with gentle shaking.

The resulting suspension was centrifuged at 9,000 × g for 20 min at 4°C. The pelleted cells were suspended in membrane buffer (50 mM Tris [pH 7.5] plus 10 mM MgSO₄), and NaCl was added to give a final concentration of 0.8 M. Shortly after the addition of the NaCl, the cells lysed, and the suspension became viscous. DNase and RNase (10 μg of each per ml) were added, and the suspension was incubated for 45 min at room temperature with gentle swirling. The inhibitors 6-aminohexanoate (40 mM) and p-aminobenzamide (6 mM) were added initially to reduce protease activity.

Membranes were pelleted by centrifugation at 35,000 × g for 30 min at 4°C and were washed twice with 30-ml portions of membrane buffer. They were stored in pellet form at −20°C. Before use, the pellets were thawed and suspended in membrane buffer, and the protein content of the suspension was assayed by the Lowry method (7).

Similar procedures were used for the isolation of membranes from cells of *S. sanguis* NCTC 10904, *S. salivarius* ATCC 13419, *S. faecalis* ATCC 9790, and *L. casei* ATCC 4646. These procedures were adapted from those of Scholler et al. (13). For *S. sanguis*, 0.75 g of lysozyme was used instead of 0.5 g and the final concentration of NaCl in the lysis medium was 1.0 M. *S. faecalis* cells were grown in tryptone-Marmite-glucose broth, and wall damage was produced with lysozyme (0.2 mg/ml) without mutanolysin. *L. casei* cells were grown in tryptone-Marmite-glucose broth plus 20 mM DL-threonine. Protoplasts were prepared with
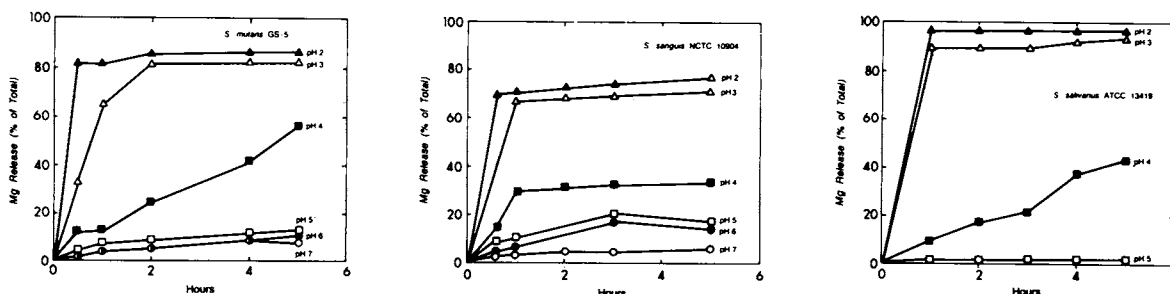
FIG. 2. Acid damage to membranes of *S. mutans* GS-5, *S. sanguis* NCTC 10904, and *S. salivarius* ATCC 13419, indicated by release of magnesium from cells at suspension pH values indicated. Total magnesium contents, determined by atomic absorption spectrophotometry of acid extracts of cells and expressed as micromoles per milligram (dry weight) of cells, were 0.17 for *S. mutans*, 0.13 for *S. sanguis*, and 0.17 for *S. salivarius*.

0.75 g of lysozyme for cells from 5 liters of culture and were lysed with 0.8 M NaCl solution.

ATPase activity was assessed in terms of the release of P_i from ATP and was expressed per unit of membrane protein. The buffer used was 50 mM Tris maleate with 10 mM $MgCl_2$. Phosphate was then assayed by a variant of the Fiske-SubbaRow method (15), and an enzyme unit was taken as that amount of enzyme that resulted in the release of 1 μmol of phosphate per ml per min per mg of membrane protein.

Chemicals. Materials for the phosphate assay were obtained from American Monitor Corp., Indianapolis, Ind. Dicyclohexylcarbodiimide (DCCD) was obtained from Calbiochem-Behring, La Jolla, Calif.

RESULTS

Gross membrane damage caused by acidification. To maintain a cytoplasmic pH value greater than that of the environment, cells must have a barrier to the influx of protons. This barrier is generally considered to be the lipoidal cell membrane. One possible basis for differences in acid tolerance among organisms could be differences in susceptibilities to gross membrane damage caused by acidification, which would then compromise the barrier functions of the membranes. However, the data presented in Fig. 2 indicate no such differences among *S. mutans* GS-5, *S. sanguis* NCTC 10904, and *S. salivarius* ATCC 13419. Gross damage to the membranes was assessed in terms of the release of Mg from cells during incubation at specific pH values at room temperature. The release of K was also assessed, but the values of Mg release were more indicative of major damage because K was leached from nonmetabolizing cells with time even at pH values close to neutrality, although the leaching was faster at extreme pH values. Mg was not lost from cells at pH values near neutrality (Fig. 2) but only after the pH value had been lowered below about 4. At pH values of 2 or 3, there was rapid and extensive release of Mg from all three organisms. Presumably, the cell membranes were damaged by acidification, and then Mg was displaced from sites within the cell and moved to the suspending medium. At these low pH values, the bacteria were killed, as indicated by the lack of growth after transfer to plates containing complex growth medium. Overall, there did not appear to be significant differences in the acid sensitivities of the membranes of the three test bacteria, at least not in terms of gross damage caused by acidification. However, the data do give a reasonable basis for interpreting the minimal pH value of about 4 for glycolysis by *S. mutans* cells.

Permeability to protons. Since it appeared that minimal pH values for functioning of oral streptococci were not deter-

mined by gross, acid-induced damage to membranes, we next considered the effects of acid environments on the abilities of the membranes of intact cells to exclude added protons from the cytoplasm, i.e., the permeabilities of the membranes to protons.

Typical pH traces obtained with suspensions of *S. mutans* GS-5 initially at a pH value of 5.0 are shown in Fig. 1. The drop in the pH value of the suspension immediately after acid addition was reversed as protons flowed into the cytoplasm across the cell membrane. pH_α is indicated, as well as pH_ω, after addition of butanol to damage the cell membrane. The $t_{1/2}$ value was the time required for the pH to reach a value halfway between pH_α and pH_ω. For the particular suspension used here, $t_{1/2}$ for the control was found by extrapolation of the pH-versus-time curve to be approximately 21.5 min. The addition of 50 μM DCCD to a comparison suspension resulted in a sharp reduction in $t_{1/2}$, in this instance to approximately 3 min. Thus, it appears that the type of permeability was dynamic, and inhibition of the proton-translocating activity of the membrane ATPase by DCCD resulted in a major reduction in the $t_{1/2}$ value.

Data on the effects of two other membrane-active agents, fluoride and gramicidin, on proton permeability are also presented in Fig. 1. The latter is known to produce channels in the cell membrane through which protons and other small ions can move. The result was a sharp increase in proton permeability and a reduction in $t_{1/2}$ to less than 1 min. As shown previously (4), fluoride also acts to increase the permeability of the membrane to protons, presumably because HF can act as a carrier of protons across the membrane, and in the presence of 0.5 mM fluoride, $t_{1/2}$ was reduced to 5.6 min. Presumably, mainly as a result of enhanced proton permeability, fluoride also acts to dissipate the proton motive force across the cell membrane (6).

Proton permeabilities can also be estimated from a knowledge of the buffering capacities of the suspending media and pH changes. For example, for the suspensions used to obtain the data in Fig. 1, it took 57.3 μl of a 10 mM HCl solution, added rapidly, to change the suspension pH value by 0.1. Thus, 573 nmol of acid was required to reduce the pH value by 0.1. This estimate is perhaps a slight overestimate of the true buffering capacity of the components of the suspension outside the cell protoplasts because even though the acid was added quickly and the resulting pH change was determined immediately after mixing, some protons may still have diffused into the cells. However, the numbers of protons should have been small relative to the total addition.

The initial change in pH value for the control was 0.008/min, and the cell density was 20 mg (dry weight) of cells per ml. Therefore, the apparent flow of protons into the

TABLE 1. Effect of membrane-active agents on proton permeabilities at various suspension pH values[a]

Organism	Suspension pH value	$t_{1/2}$ (min) for treatment:			
		Control	Fluoride	DCCD	Gramicidin
S. mutans GS-5	7.0	5.3	4.8	4.8	0.2
	6.0	6.4	2.1	2.6	0.3
	5.0	14.0	5.0	2.4	0.3
	4.5	3.0	0.3	0.6	0.4
S. sanguis NCTC 10904	7.0	9.0	6.5	4.8	2.2
	6.0	3.7	2.3	3.9	0.2
	5.0	3.0	0.8	2.3	<0.1
	4.5	0.5	0.5	0.5	<0.1
S. salivarius ATCC 13419	7.0	6.8	3.8	5.4	3.8
	6.0	7.6	5.0	1.0	0.2
	5.0	3.1	1.7	1.0	<0.1
	4.5	1.9	1.7	1.3	0.2

[a] Each of the experiments involved a single batch of cells for each of the organisms. Samples of the initial suspension of each organism were equilibrated at the indicated pH values. The test agents were added, and then an acid pulse was given. The final concentrations of the agents were 0.5 mM KF, 50 μM DCCD, and 0.4 mM gramicidin.

cell caused a pH change of 0.008/min × 5,730 nmol of acid per pH unit per 20 mg (dry weight) of cells, or 2.29 nmol/min per mg. Of course, this value is an underestimate of the gross flow of protons into the cells because the ATPases of the membrane would have acted to move inflowing protons back out.

The initial pH change in the companion suspension to which DCCD was added was 0.035/min. The estimated value for proton movement into the cells treated with DCCD was then 10.66 nmol/min per mg (dry weight) of cells; this is probably a better estimate of the gross permeability of the cell membrane to protons. Estimated proton movements for the cells treated with 0.5 mM KF or 0.4 mM gramicidin were 8.02 and 64.46 nmol/min per mg (dry weight) of cells, respectively.

In another set of experiments, the effects of environmental pH on increases in proton permeabilities caused by fluoride, DCCD, and gramicidin were determined (Table 1). As expected, the action of fluoride was markedly pH dependent, although even at a pH value of 7.0, 0.5 mM KF still had detectable activity in enhancing proton permeability. The action of gramicidin was much less pH dependent, and the antibiotic was fully active against S. mutans GS-5 even at a pH value of 7.0. For the other two bacteria, gramicidin was less effective at a pH value of 7.0 than at more acid pH values. The action of DCCD was also somewhat pH dependent. The most dramatic effects were associated with the higher $t_{1/2}$ values, especially for S. mutans. The failure of DCCD, even at the saturating levels used, to reduce $t_{1/2}$ to the low values seen with gramicidin is probably related to additional pathways for protons through the membranes of the test bacteria. Steps were taken in these experiments to minimize proton movements by mechanisms not involving membrane ATPases, e.g., in symport with lactate; nevertheless, DCCD-insensitive mechanisms did seem to contribute in at least minor ways to proton currents. Repeated experiments similar to the one from which the data in Table 1 were obtained supported this view.

The data presented in Fig. 3 show proton permeabilities, expressed in terms of $t_{1/2}$ values for pH equilibration, as a function of environmental pH value for the three oral streptococci. For these experiments, cells were allowed to come to pH equilibrium at the various pH values shown and were then pulsed with acid to decrease the pH value of the suspensions by approximately 0.15. Each of the organisms

showed a repeatable pattern of $t_{1/2}$ values. For S. mutans GS-5, $t_{1/2}$ was greatest at an environmental pH value of about 5.0 and dropped off at higher or lower pH values. In contrast, $t_{1/2}$ was greatest for S. salivarius ATCC 13419 at a pH value of about 5.5. Finally, $t_{1/2}$ was maximal for S. sanguis NCTC 10904 at a pH value of about 7.

The pH values for maximal $t_{1/2}$ or minimal permeability to protons can be related, at least roughly, to the acid tolerance of the bacteria. For example, the pH values at which glycolysis by nongrowing cells is 90% inhibited compared with the rates at a pH value of 7.0 were 3.7 for S. mutans, 4.8 for S. salivarius, and 4.9 for S. sanguis (1). Moreover, at a pH value of 5.0, glycolysis in S. mutans cells proceeded at about one-half the maximal rate, whereas for the other two bacteria, the rates were only about 10% of the maximal rates. Of course, other factors are involved in the responses of the glycolytic system of intact cells to environmental acidification, including mineral ions which affect acid toler-
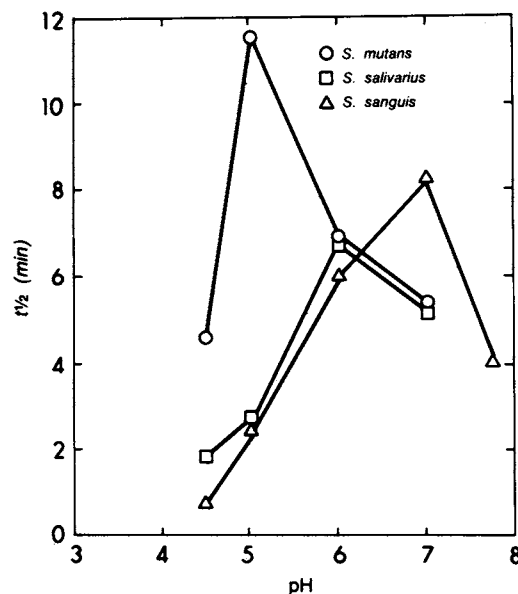

FIG. 3. Proton permeabilities of S. mutans GS-5, S. sanguis NCTC 10904, and S. salivarius ATCC 13419 as a function of environmental pH value.
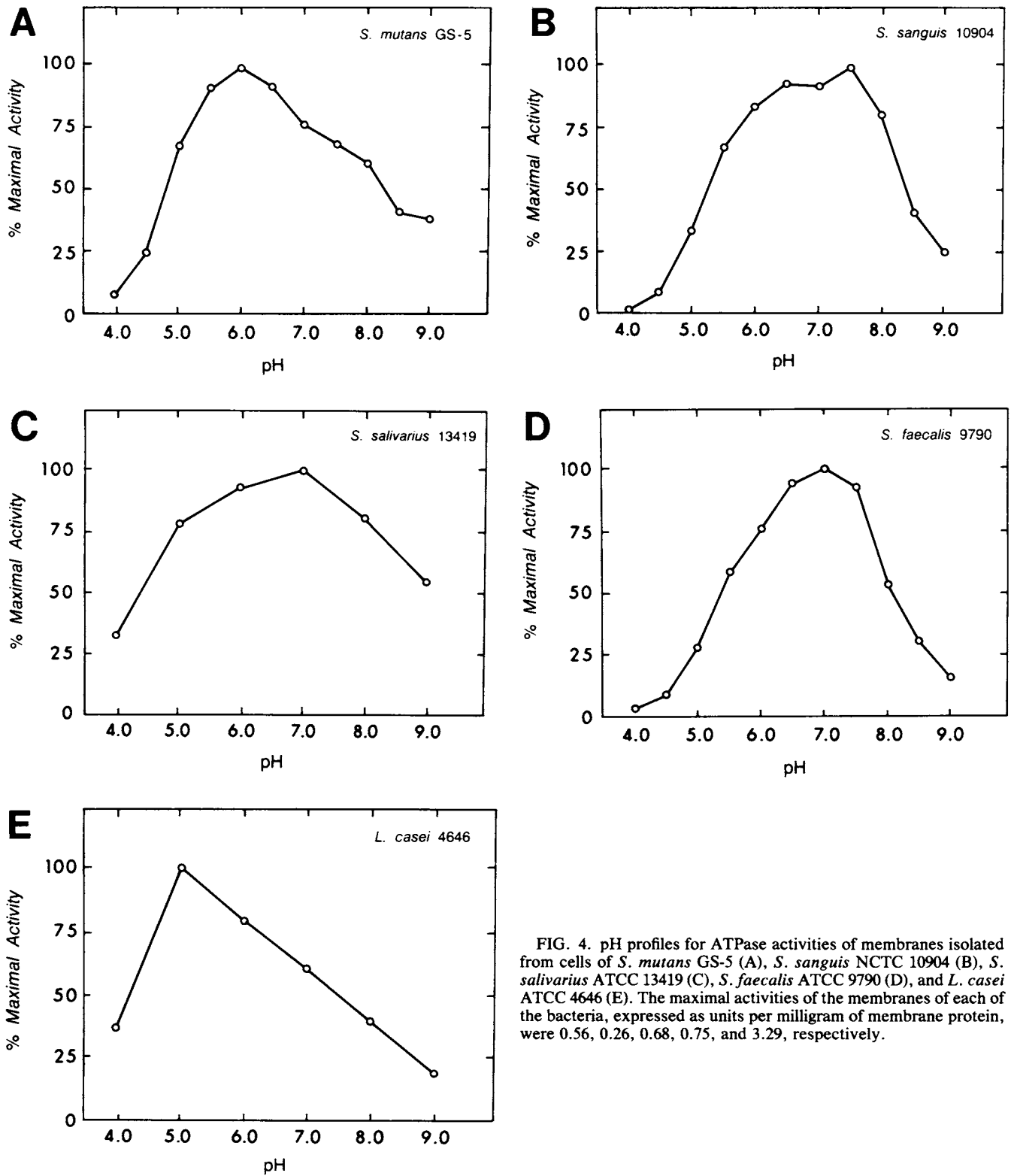
FIG. 4. pH profiles for ATPase activities of membranes isolated from cells of *S. mutans* GS-5 (A), *S. sanguis* NCTC 10904 (B), *S. salivarius* ATCC 13419 (C), *S. faecalis* ATCC 9790 (D), and *L. casei* ATCC 4646 (E). The maximal activities of the membranes of each of the bacteria, expressed as units per milligram of membrane protein, were 0.56, 0.26, 0.68, 0.75, and 3.29, respectively.
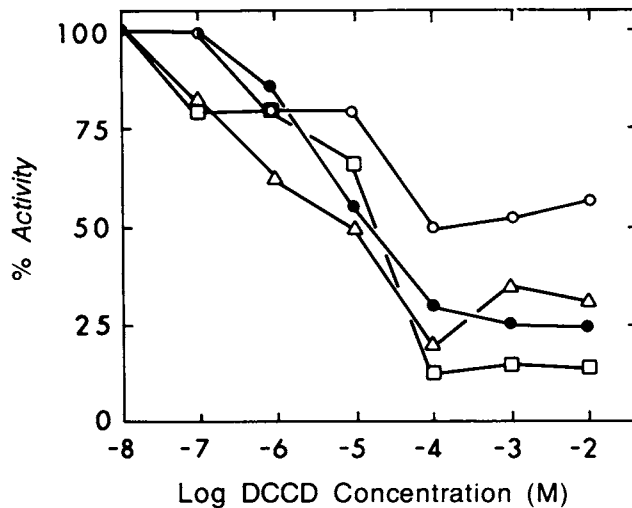
FIG. 5. Inhibition of membrane-associated ATPases of S. mutans GS-5 (O), S. sanguis NCTC 10904 (△), S. faecalis ATCC 9790 (□), and S. salivarius ATCC 13419 (●) by DCCD. The y axis indicates the percentage of maximal activity at the optimal pH value for each of the enzymes.

ance (10). However, there still appears to be a close relationship between proton permeabilities assessed by the methods described here and the acid tolerance of glycolysis by cells suspended in phosphate buffer with 10 mM MgCl₂.

Membrane ATPases. The effects of DCCD on proton uptake indicate a major role for membrane ATPases in proton permeability. Therefore, we isolated membranes from the three test streptococci and assessed the acid sensitivities of the ATPases associated with these membranes. For comparison, we also isolated membranes of S. faecalis ATCC 9790 because the proton-translocating membrane ATPase of this organism has been thoroughly studied. The data (Fig. 4) show clearly that the ATPase activity of S. mutans membranes is less sensitive to acid than is that of S. sanguis membranes. The optimal pH value for the S. mutans ATPase was approximately 6.0, whereas that for the S. sanguis enzyme was about 7.5. The optimal pH value for the S. faecalis enzyme was also close to 7.0, and the S. salivarius enzyme showed a similar optimum. At a pH value of 5.0, the S. mutans enzyme functioned at about 70% of its maximal rate, whereas the S. sanguis and S. faecalis enzymes functioned at only about 30% of the maximal rates. The ATPase from S. salivarius membranes was somewhat more acid resistant than the enzyme from S. mutans membranes and operated at a pH value of 5.0 at about 80% of the maximal rate. Thus, the S. salivarius enzyme had a pH optimum in the range of 7.0 to 7.5, like the S. sanguis and S. faecalis enzymes, but was least sensitive to acid in the pH range of 4.0 to 5.0.

Membranes were also isolated from L. casei ATCC 4646 because this lactic acid bacterium is even more aciduric than S. mutans. The membrane ATPase of L. casei had a low pH optimum of about 5.0, and activity declined at higher or lower pH values (Fig. 5); at a pH value of 4.0, the ATPase activity was still about 40% of that at pH 5. For some membrane preparations from L. casei, the pH optimum was somewhat above 5.0, but the enzymes still showed relatively high levels of activity at a pH value of 4.0. Determinations of the proton permeabilities of L. casei cells starved for 0.5 h

partially to deplete endogenous reserves indicated that the maximum $t_{1/2}$ was 24.4 min at a pH value of 4.0. It was reduced to 7.9 min at a pH value of 3.5 and to 14.9, 3.0, and 5.6 min at pH values of 5.0, 6.0, and 7.0, respectively.

The cell membrane of L. casei appeared to be well adapted for the aciduric nature of the organism, including its ability to carry out glycolysis at environmental pH values as low as 3.2. In experiments designed to assess gross membrane damage caused by environmental acidification, there was no release of Mg from L. casei cells at a pH value of 4.0 over 6 h. No release occurred at a pH value of 3.0 for about 4 h, but there was rapid release between 5 and 6 h. Mg was released completely over a 2-h period at a pH value of 2.0.

Data on the DCCD sensitivities of the streptococcal ATPases are presented in Fig. 6. Maximal inhibition occurred at DCCD levels of about 100 μM. However, inhibition was not total, especially for the S. mutans membranes, for which maximal inhibition was only about 50%. Supernatant fluids obtained by centrifuging the membrane suspensions were nearly devoid of ATPase activity. Thus, the lower levels of inhibition did not appear to be due to loss of attachment of the F_1 portions of the enzymes from the membranes, which would have freed the hydrolytic activity from obligatory coupling to proton translocation through the pore of the F_0 portion blocked specifically by DCCD. However, with each of the enzymes, DCCD was able to bring about substantial inhibition of the membrane-associated form and, presumably, a major part of the enhanced proton permeability of whole cells caused by DCCD is related to inhibition of the proton-translocating ATPase.

DISCUSSION

The overall view of the acid tolerance of oral streptococci developed in this paper is focused on the dynamic permeabilities of the cell membranes of the bacteria to protons. Differences in acid tolerance among the three prime test organisms clearly could not be interpreted in terms of differences in the barrier stabilities of the cell membranes in acidified suspensions. For each of the organisms, significant release of intracellular magnesium occurred only at pH values below about 4, and major, rapid release occurred only at pH values of about 3 or below. At these lower pH values, the cells were killed, presumably because of irreversible denaturation of membrane proteins. Thus, the behavior of the three oral streptococci was very similar to the behavior of S. faecalis ATCC 9790 in acidified suspensions described previously (9). The membranes of L. casei appeared to be somewhat more resistant to acid damage. Presumably, still more highly aciduric organisms must have membranes adapted to withstand the denaturing actions of acids at pH values of 2 or even lower. However, it seems that moderately aciduric bacteria, such as S. mutans GS-5, do not have membranes able to maintain barriers to small molecules at pH values much below 4.0. In dental plaque, minimal pH values are only about 4.0, or slightly lower (11).

The data on the proton permeabilities of the test bacteria at pH values in the range of 4.5 to 7.0 and on the acid sensitivities of the membrane ATPases offer a more reasonable basis for interpreting differences in acid tolerance, especially in view of current information on the general acid-base physiology of bacteria (2). Moreover, the data indicate clearly than an important concept in considering proton currents across the cell membrane is that of dynamic permeability. Even though steps were taken in our experiments to reduce endogenous metabolism and not to supply

rapidly metabolized substrates such as glucose, which could have produced lactate-coupled proton flows, the organisms were still able to regulate the flow of protons. The experimental conditions in our study were designed to at least approach those which occur in dental plaque during a period of starvation, when the bacteria would be expected to have reduced capacities for acid-base regulation. In addition, relatively dense suspensions of cells were used. Even after mild starvation of the sort we imposed, plaque bacteria, including S. mutans GS-5, retain a moderately high level of ATP (5), and this ATP could be used for acid-base regulation when the organisms are subjected to an acid pulse. The effects of DCCD on proton permeabilities indicate that membrane ATPases play important roles in dynamic permeability. For example, $t_{1/2}$ for S. mutans GS-5 at a pH value of 5 was reduced from about 14 min to only about 2 min by 50 μM DCCD. The effects of DCCD were less dramatic in other situations, especially at higher pH values. However, DCCD did increase the permeability of each organism to protons at each pH value, except for S. sanguis at a pH value of 4.5 (Table 1).

Previously, Maloney (8) had studied in some detail proton conductances of S. lactis cells. Remarkably, with the experimental procedures used, he found a constant proton conductance over the pH range of about 5.0 to 8.5 of 14 nmol/min per mg (dry weight) of cells per pH unit. The conductance increased at lower pH values. If our values are converted to the same units, the proton conductance of S. mutans GS-5 at a pH value of 5, for example, would be essentially the same, ca. 14 nmol/min per mg (dry weight) of cells per pH unit. We assumed that the initial pH drop across the cell membrane after acid pulsing was that shown in Fig. 1. The cells used had been allowed to come to pH equilibrium with the suspending medium before pulsing. However, true pH equilibrium does not occur for metabolizing cells, as indicated, for example, by a rise in the pH value of the suspension after butanol addition. In essence, the ATPase of the membrane is not idle when the cells are still able to metabolize. In fact, our results indicate that the procedure suggested initially by Scholes and Mitchell (12) for assessing pH_{ω} by extrapolation is not valid. Moreover, this lack of equilibrium affects the estimated pH difference across the cell membrane after an acid pulse.

Even though proton flows across the cell membrane of S. mutans at a pH value of 5.0 appeared to be the same as those for S. lactis, the $t_{1/2}$ values for pH equilibration were much longer, partly because of differences in estimated pH_{ω} values. The $t_{1/2}$ values for pH equilibration for S. lactis reported by Maloney were relatively short, e.g., ca. 0.7 min at a pH value of 7.9, 0.8 min at pH 6.8, and 0.4 min at pH 5.3, with pH_{ω} estimated by the extrapolation method. Previously, we had estimated shorter $t_{1/2}$ values of about 2 min for S. mutans GS-5 (4), but these values were for cells at pH values between 6 and 7. Even if the average $t_{1/2}$ of about 14 min determined in this study is considered, the conclusion reached is that the cell membrane is not totally impermeable to protons. In fact, it appears that the cells must work constantly to move protons out of the cytoplasm to maintain a cytoplasmic pH higher than the external value.

The pH profiles for the ATPase activities of the test bacteria give a reasonable basis for interpreting in part the differences in pH profiles for proton permeabilities and differences in acid tolerance. There is, of course, a need to appreciate that proton currents through the membrane are complicated and that we are here considering rather specific experimental conditions involving starved cells. Moreover,

we considered only specific strains of the test species. However, it still seems reasonable to conclude that membrane ATPases play major roles in the acid tolerance of the bacteria, especially in view of the effects of DCCD on proton permeability. In the work with isolated ATPases, we purposely used enzymes which were still bound to membranes because this state is closest to the native state in the cell. Moreover, when the enzyme is attached to the membrane, ATP hydrolysis is obligatorily coupled to proton movement through the F_0 pore in the membrane.

Overall, the data presented in this paper indicate that the membrane ATPases are major engines of acid tolerance for oral streptococci. Moreover, the central role of the cell membrane in acid tolerance is emphasized. It can then be considered that the membrane plays a critical role in the cariogenicity of the bacteria because the extent of the acid challenge to the teeth from plaque acid can be related to the acid tolerance of bacteria such as S. mutans, which can carry out glycolysis at pH values as low as 4.0. The pertinent cell function for cariogenicity is glycolysis, which for S. mutans is more aciduric than is growth. Thus, the bacteria have evolved a mechanism, or set of mechanisms, to protect the acid-sensitive glycolytic enzymes in the cytoplasm by moving protons out through the cell membrane, especially through the proton-translocating ATPase. It may be wondered why acid-resistant glycolytic enzymes did not evolve, but perhaps they would not be of advantage to the bacteria at higher pH values.

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