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Enhanced Transmembrane Proton Conductance in *Streptococcus mutans* GS-5 Due to Ionophores and Fluoride

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The uptake of protons by intact cells of *Streptococcus mutans* GS-5 was measured directly by assessing the pH increases in lightly buffered cell suspensions as protons moved across the plasma membrane after acid pulses. The barrier function of the cell membrane for protons was disrupted completely by 5% (vol/vol) butanol. This function was compromised severely by the ionophores valinomycin and gramicidin and by the uncoupler carbonylcyanide-*m*-chlorophenyl hydrazone. Proton conduction across the membrane was also enhanced by fluoride. It appeared that HF traversed the membrane and acted as a carrier. Once in the relatively alkaline cytoplasm, HF dissociated to yield F^- and H^+, which acted to acidify the cytoplasm and reduced the pH difference across the membrane.

Even though the anticaries activity of fluoride is well established and the use of fluoride in drinking water, in dentifrices, and in topical applications is widespread in developed countries, there is still much debate regarding the mechanisms by which fluoride reduces caries. One of the major proposed mechanisms involves incorporation of fluoride into fluoroapatite, with a resulting increase in the resistance of tooth enamel to acid solubilization. However, in the past few years there has been growing support for the hypothesis that at least part of the anticaries activity of fluoride is due to antimicrobial action. It has also become apparent that the physiological responses of bacterial cells to fluoride are complex. Fluoride can inhibit many enzymes, and it is felt that inhibition of enolase and the resulting secondary inhibition of the phosphotransferase system due to a reduced supply of phosphoenolpyruvate are particularly important in reducing acid production by bacteria in dental plaque (2). Recently, we have proposed (1) that fluoride may also act as a weak-acid, transmembrane proton conductor which mediates acidification of the cytoplasm of cells in media at low pH values. It is well known that bacteria such as *Streptococcus mutans* are more susceptible to fluoride at more acid pH values. It appears that part of this greater susceptibility is the result of increased fluoride uptake at low pH values (1a, 6). Fluoride is a weak acid with a pK_a of 3.5, and the protonated species HF can readily cross cell membranes. Bacterial cells are known to maintain a pH difference (ΔpH) across their cell membranes. Cells in acid media maintain a relatively alkaline cytoplasm by extruding protons, generally through the membrane adenosine triphosphatase complex, and by excluding reentry of extruded protons. Therefore, in an acidified suspension of *S. mutans* for example, external fluoride becomes protonated, and HF enters the cells. Once inside the cytoplasm, the HF should dissociate to yield F^- and H^+. The fluoride anion then inhibits enolase and other sensitive enzymes. The protons also are inhibitory because they acidify the cytoplasm, reduce the ΔpH across the cell membrane, and reduce the activities of acid-sensitive enzyme systems, such as glycolysis. The net result is that fluoride renders the cells acid sensitive. In essence then, fluoride has an activity similar to that described by Harold and Van Brunt (3) for ionophores.

In this paper, we present direct evidence that fluoride does enhance the movement of protons across *S. mutans* cell membranes and we compare the action of fluoride with that of the commonly used ionophores.

MATERIALS AND METHODS

Organism and growth conditions. *S. mutans* GS-5 was grown statically at 37°C in tryptone glucose Marmite broth (1a). Cells were harvested by centrifugation from early stationary-phase cultures which had final pH values of about 4.5.

Assay of transmembrane proton conduction. Cells were suspended at a density of 5 mg (dry weight) of cells per ml in a solution containing 20 mM potas-

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sium phosphate (pH 7.3), 1 mM $MgCl_2$, and 150 mM KCl and were incubated at 37°C for 2 h to deplete them of readily fermentable substrates. They were then centrifuged and suspended at a density of 20 mg/ml in a solution containing 150 mM KCl and 1 mM $MgCl_2$, which was adjusted to pH 6.5 with a solution containing 100 mM KOH and 50 mM KCl. Generally, the centrifugation and suspension steps were repeated to yield the final cell suspensions used for experiments.

To 2.5-ml samples of rapidly stirred cell suspensions, 10-μl pulses of 10 mM HCl in 140 mM KCl were added. Changes in suspension pH values were monitored and recorded by using a combination pH electrode (Markson Scientific Corp.), a Radiometer model TTT2 pH meter, and an electronically modified Bausch & Lomb model VOM5 strip chart recorder. Fluoride was added to the cell suspensions from a pH-adjusted, concentrated stock solution. Uncouplers were added from methanolic solutions sufficiently concentrated so that the added methanol did not affect the cells.

When an acid pulse was added to a cell suspension, initially there was a rapid decrease in the pH due to acidification of the suspending medium and then a gradual increase in the pH as protons slowly moved across the cell membranes into the cytoplasm. This slow movement could be speeded up drastically by adding agents, such as butanol, which disrupted the barrier properties of the cell membranes. The data shown in Fig. 1 and 2 illustrate these phenomena. As Fig. 1 shows, sufficient acid was added to a typical cell suspension to decrease the suspension pH at zero time to approximately 5.2. The addition of 2% (vol/vol; final concentration) butanol resulted in an increase in the pH to approximately 5.4, whereas 5% (vol/vol; final concentration) butanol caused rapid equilibration to a pH of about 5.9. Further butanol addition did not result in more rapid proton movements. Therefore, 5% butanol was used routinely to determine pH^{ω}, the final pH after complete equilibration of protons between the interiors and the exteriors of the cells (4).

Figure 2 shows typical changes in pH values after acid pulses were added to cell suspensions which had initial pH values of about 6.6. The addition of acid resulted in a rapid decrease in the pH, which was followed by a gradual increase in the pH as protons

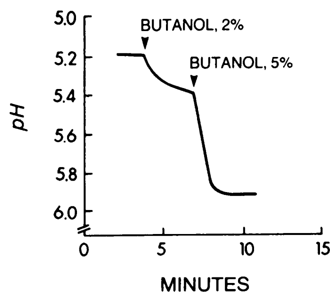


FIG. 1. *Disruption of the proton barrier of the cell membrane of S. mutans GS-5 by butanol. Cells were suspended as described in the text. The suspension was acidified to a pH of about 5.2 and then treated with the concentrations of butanol indicated.*

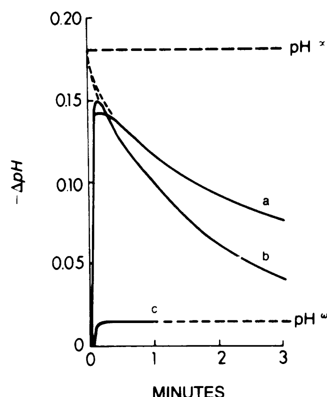


FIG. 2. *Typical time course for pH changes after an acid pulse at zero time without (curve a) or with (curve b) 0.1 mM KF or after treatment with 5% (vol/vol) butanol (curve c). As protons moved from the extracellular phase, the pH electrode registered an increase in pH. An extrapolated zero-time pH is indicated as pH^{α}, and an extrapolated equilibrium pH is indicated as pH^{ω}, which was the approximate final pH after butanol treatment.*

slowly entered the cells. pH^{ω} was the equilibrium pH obtained after long periods of time or after butanol addition. pH^{α} was the extrapolated value for maximal acidification of the external medium after acid addition. During the phase of exponential pH increases, there was a linear relationship between time and $-\ln(pH^{\omega} - pH)$. Using the procedures of Scholes and Mitchell (4), we estimated the half-time ($t_{1/2}$) for pH equilibration between cells and the suspending medium as the time required for the suspension pH to reach a value of $[pH^{\omega} + (pH^{\omega} - pH^{\alpha})/2]$. The data in Fig. 2 are typical and show that 0.1 mM KF increased the rate of equilibration; $t_{1/2}$ values were approximately 2.0 and 1.2 min for unfluoridated suspensions and suspensions treated with 0.1 mM KF, respectively.

RESULTS

Increased proton permeability due to ionophores. When a 10-μl pulse of 10 mM HCl in 140 mM KCl was added to a cell suspension of *S. mutans* GS-5, protons moved slowly across the cell membranes into the cytoplasm, and this process was accompanied by a slow increase in the pH of the suspension (characteristic $t_{1/2}$, about 2.5 min) (Fig. 3). However, when the uncoupler carbonyl cyanide-*m*-chlorophenyl hydrazone (CCCP) was added to a suspension just before the acid pulse, the $t_{1/2}$ for equilibration was reduced as a function of the uncoupler concentration (Fig. 3 and Table 1). The $t_{1/2}$ was reduced 75% by 1 μM CCCP, but an uncoupler concentration of 10 μM resulted in only an additional 10% decrease in the $t_{1/2}$.

Valinomycin was more effective than CCCP, and 10 μM valinomycin reduced the $t_{1/2}$ for proton equilibration to only about 5% of the control

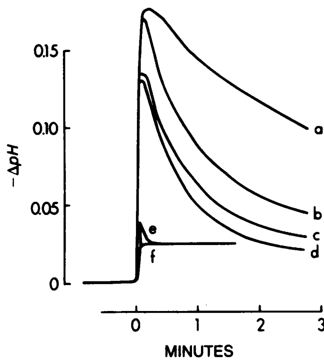


FIG. 3. *Reduction in the $t_{1/2}$ for equilibration of protons across S. mutans GS-5 cell membranes due to the uncoupler CCCP. The initial pH was 6.6. The uncoupler concentrations were 0, 1, 4, and 10 μM CCCP for curves a through d, respectively. Curves e and f show responses to combinations of 1 μM CCCP plus 1 μM valinomycin and 10 μM CCCP plus 1 μM valinomycin, respectively.*

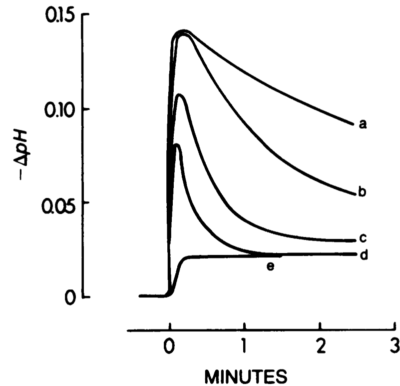


FIG. 4. *Reduction in the $t_{1/2}$ for equilibration of protons across S. mutans GS-5 cell membranes due to valinomycin. The initial pH was 6.6. Valinomycin concentrations were 0, 1, 4, and 10 μM for curves a through d, respectively. Curve e shows the results of proton equilibration in the presence of 10 μM valinomycin plus 1 μM CCCP.*

TABLE 1. *Reduction in $t_{1/2}$ values for proton equilibration between cells and suspending medium for S. mutans GS-5*

Agent	Concn (μM)	$t_{1/2}$ (min)	% of control value
CCCP	0	2.38	100
	1	0.59	25
	4	0.44	18
	10	0.38	16
CCCP + valinomycin (1 μM)	1	—[a]	~0
Gramicidin	0	2.38	100
	1	0.18	8
	10	—	~0
Valinomycin	0	2.63	100
	1	1.13	43
	4	0.38	14
	10	0.13	5
Valinomycin + CCCP (1 μM)	10	—	~0
Fluoride	0	2.40	100
	100	1.02	43
	400	0.69	29
	1,000	0.56	23
	4,000	0.25	10
Fluoride + valinomycin (1 μM)	4,000	0.19	8

[a] —, Equilibration occurred too rapidly for an accurate estimate of $t_{1/2}$.

value (Fig. 4). Valinomycin and CCCP acted together to reduce the $t_{1/2}$; for example, 1 μM CCCP plus 1 μM valinomycin reduced the $t_{1/2}$ to less than 30 s (Fig. 3 and Table 1). In effect, it appeared that movement of protons into cells was impeded by the potassium gradient between the cells and their environment. Valinomycin eliminated this gradient by forming channels for K^+ to pass out through the membrane. This loss of the K^+ gradient resulted in facilitated H^+ uptake by the cells.

Gramicidin forms channels through which H^+, K^+, and Na^+ can equilibrate across the membranes of streptococci (3). Gramicidin was very effective in reducing the $t_{1/2}$ for proton equilibration in *S. mutans* cells (Fig. 5 and Table 1); 10 μM gramicidin reduced the $t_{1/2}$ to less than 30 s.

Fluoride as a facilitator of proton uptake. The addition of fluoride to suspensions of *S. mutans* resulted in reductions in the $t_{1/2}$, and as little as 0.1 mM KF at a pH slightly below 6 reduced the $t_{1/2}$ to less than 50% of the control value (Fig. 6 and Table 1). Higher concentrations were more effective in reducing the $t_{1/2}$, but potency per mole of fluoride decreased with increasing concentration, as would be expected if the action of fluoride were due to its weak-acid properties. The addition of 1 μM valinomycin to 4 mM KF enhanced the reduction of $t_{1/2}$ values somewhat, but not greatly. The amounts of fluoride used did not have significant buffering effects on the suspensions, as indicated by the identical pH displacements with and without fluoride after acid pulses were added to butanol-treated cells. In fact, it appeared that the cells were the major source of buffering in the suspensions used.

Experiments with cell suspensions having different initial pH values indicated that fluoride was more effective in reducing the $t_{1/2}$ in suspensions having more acid pH values (Fig. 7 and Table 2). At pH 7, 0.1 mM fluoride had no effect on the $t_{1/2}$; at pH 6.4, the effect was an 18% reduction in the $t_{1/2}$, and at pH 6.0, the reduction

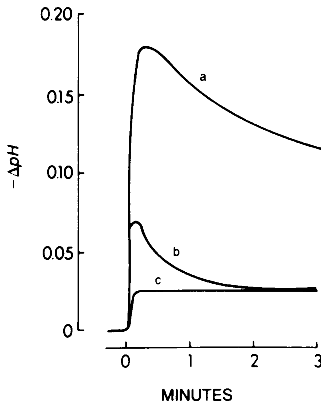


FIG. 5. *Reduction in the $t_{1/2}$ for equilibration of protons across S. mutans GS-5 cell membranes due to gramicidin. The initial pH was 6.6. Gramicidin concentrations were 0, 1, and 10 μM for curves a through c, respectively.*

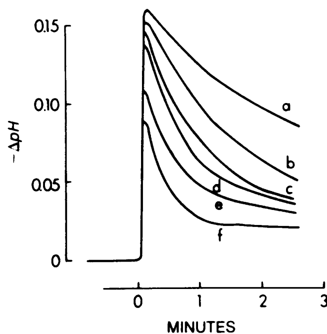
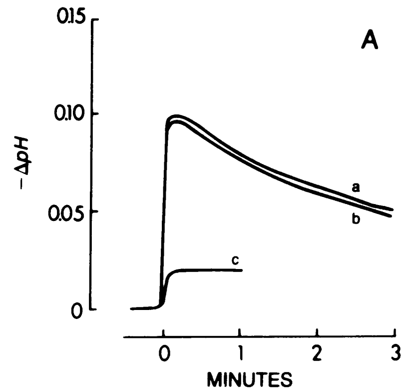
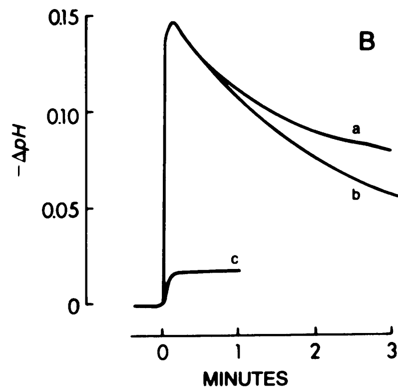


FIG. 6. *Reduction in the $t_{1/2}$ for equilibration of protons across S. mutans GS-5 cell membranes due to fluoride. The initial pH was 6.3. The cell suspensions were treated with 0, 0.1, 0.4, 1.0, and 4.0 mM KF and 4.0 mM KF plus 1 μM valinomycin (curves a through f, respectively).*

was 40%. Again, at all of these pH values fluoride had only insignificant buffering action.

Acid sensitization. One of the major physiological actions of agents that promote transmembrane proton transport should be to sensitize cells to acid conditions. The increases in acid sensitivity of *S. mutans* GS-5 caused by the agents which we used are shown in Fig. 8 and Table 3. As Fig. 8 shows, *S. mutans* GS-5 cells in control suspensions containing excess glucose were able to reduce the pH to a value of about 3.9 over a period of about 2 h. However, the addition of various test agents resulted in stoppage of glycolysis at pH values higher than 3.9, and there was a direct correlation between the concentration of each agent and the final pH of the suspension. In these suspensions, glycolysis was acid limited, and neutralization of the suspensions with KOH resulted in a new round of acid production.





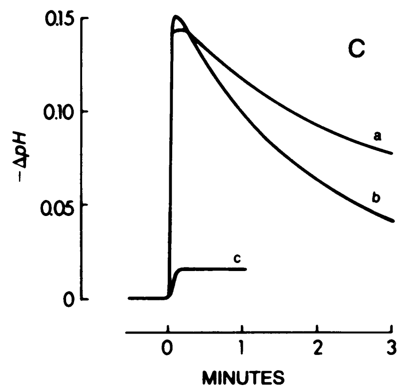


FIG. 7. *Effect of pH on fluoride-mediated, transmembrane proton conduction. The initial pH was 7.0 (A), 6.4 (B), or 6.0 (C). Curve a, Control suspension; curves b and c, suspensions treated with 0.1 mM KF and 5% (vol/vol) butanol, respectively. See Table 2 for $t_{1/2}$ values.*

From plots of the final pH versus the concen-

TABLE 2. *Effect of initial pH on fluoride-mediated changes in $t_{1/2}$ values*

Initial pH	$t_{1/2}$ of control (min)	Culture with 0.1 mM KF added	
		$t_{1/2}$ (min)	% of control
7.0	1.94	1.90	98
6.4	2.06	1.69	82
6.0	2.00	1.19	60

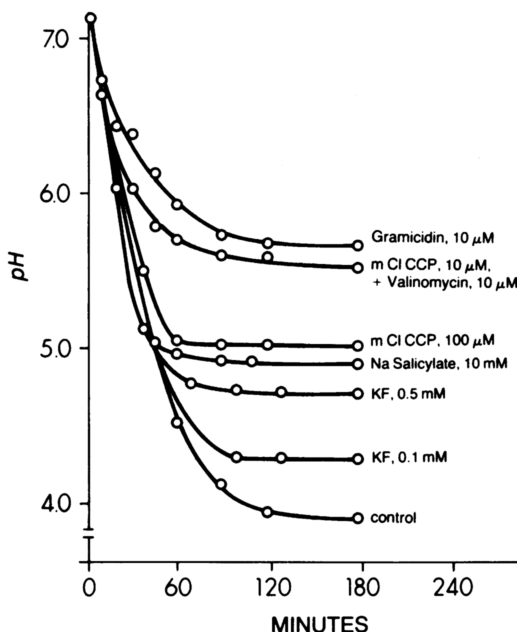


FIG. 8. *Effects of various agents which enhanced membrane proton conduction on the reduction of pH in suspensions of S. mutans GS-5 due to glycolysis. See Table 3, footnote a, for experimental details. mClCCP, CCCP.*

tration of agent used, we could estimate the concentrations required to sensitize the cells to acid sufficiently so that glycolysis was stopped at a pH of 5.0. These concentrations are shown in Table 3. Gramicidin was the most potent agent, as it was the most effective agent in reducing the $t_{1/2}$ for proton uptake (Table 1). The next most potent agent was CCCP, followed by valinomycin and finally by KF. Again, this pattern reflected the potency series for $t_{1/2}$ reduction. Data are also shown for hexachlorophene, which is a membrane-disrupting agent, and for sodium salicylate, which (like fluoride) is a weak acid. Salicylate was less potent than fluoride, probably because its larger molecular size resulted in slower movement across the membranes.

TABLE 3. *Acid sensitization of glycolysis in S. mutans by membrane-active agents*

Agent	Concn required to sensitize so that the final pH of the glycolyzing suspension was 5.0 instead of 3.9 (mM)[a]
Gramicidin	0.003
Hexachlorophene	0.021
CCCP	0.100
KF	1.1
Sodium salicylate	11.0

[a] Cells from an early stationary-phase culture were harvested by centrifugation and were washed once with 20 mM potassium phosphate buffer (pH 7.3) containing 1 mM $MgCl_2$. They were then suspended in 20 mM potassium phosphate buffer (pH 7.3) containing 1 mM $MgCl_2$ and 0.5% (wt/vol) glucose. Changes in pH were monitored with a Radiometer pH meter.

DISCUSSION

Previous studies had suggested that fluoride may act as a transmembrane proton conductor, and the data presented here show in a direct and unequivocal way that fluoride is indeed a proton conductor or, at least, that it increases membrane permeability to protons. The effectiveness of fluoride is clearly pH dependent, and presumably, it is the HF conjugate which moves readily across membranes to bring protons into cells. The enhanced uptake of fluoride at low pH values has been demonstrated previously with both dilute and thick cell suspensions (1a, 6). Also, it appears from the data presented above that the acid sensitization of *S. mutans* by ionophores reported previously (1) can be related to increased proton conduction across cell membranes. The ionophores are much more potent per mole than fluoride is, and, in fact, it seems that the actions of the ionophores are basically different from the action of fluoride. We think that the ionophores act by forming channels in the membrane through which ions can pass, whereas fluoride seems to act as a transmembrane carrier of protons. The carrier activity of fluoride depends on the ΔpH across the membrane. Fluoride should accumulate on the more alkaline, cytoplasmic side of the membrane. However, HF should then dissociate and thereby reduce the ΔpH. When the ΔpH is reduced to zero, there should no longer be any net movement of fluoride into the cells. Presumably, this dependence of uptake on the ΔpH is responsible for the greater catalytic potency per mole of fluoride at low concentrations than at high concentrations (e.g., 0.1 versus 4.0 mM). Since the major factor in fluoride uptake and proton con-

duction seems to be the ΔpH rather than the membrane potential, it is reasonable that valinomycin does not potentiate fluoride action greatly. However, valinomycin does seem to act in concert with fluoride, as would any other ionophore which enhances proton uptake. Indeed, any agents which cause membrane damage, including detergents, should act in concert with fluoride.

The results of our work lead to a more complicated view of fluoride action on cariogenic streptococci than that previously held. Fluoride can inhibit enolase and then, as a result of a restricted supply of phosphoenolpyruvate, secondarily inhibit the phosphotransferase system for sugar uptake. The net result is a reduction in glycolytic activity and acid production. Our data indicate that fluoride also interferes with the regulation of the internal pH. Acidification of the cytoplasm could have major inhibitory effects on glycolysis. Muscle cells are one of the best-studied examples in this regard, and presumably, the glycolytic system in streptococci has the same acid sensitivity that the muscle system has. One of the most acid-sensitive enzymes in the glycolytic system is phosphofructokinase. At cytoplasmic levels of adenosine triphosphate, the optimal pH of phosphofructokinase is about 7.5, and activity drops off sharply below pH 7 so that this enzyme is completely inactive at pH 6.5 (5). There is a strong possibility that the acidification of the cytoplasm of *S. mutans* cells in acid environments due to proton conduction by fluoride is a major cause of inhibition of glycolysis and acid production. In fact, it is possible that the inhibition of these processes contributes to the anticaries action of fluoride.

ACKNOWLEDGMENTS

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