Lactic acid excretion by *Streptococcus mutans*

Stuart G. Dashper and Eric C. Reynolds

Author for correspondence: Eric C. Reynolds. Tel: +61 3 341 0270. Fax: +61 3 341 0339.
e-mail: eric_reynolds.dental@muwaye.unimelb.edu.au

Biochemistry and
Molecular Biology Unit,
School of Dental Science,
Faculty of Medicine,
Dentistry and Health
Sciences, The University of
Melbourne, 711 Elizabeth
Street, Melbourne, Victoria
3000, Australia

Lactic acid is the major end-product of glycolysis by *Streptococcus mutans* under conditions of sugar excess or low environmental pH. However, the mechanism of lactic acid excretion by *S. mutans* is unknown. To characterize lactic acid efflux in *S. mutans* the transmembrane movement of radiolabelled lactate was monitored in de-energized cells. Lactate was found to equilibrate across the membrane in accordance with an artificially imposed transmembrane pH gradient (ΔpH). The imposition of a transmembrane electrical potential ($\Delta\psi$) upon de-energized cells did not cause an accumulation of lactate within the cell. The efflux of lactate from lactate-loaded, de-energized cells created a ΔpH , but did not create a $\Delta\psi$, indicating that lactate crosses the cell membrane in an electroneutral process, as lactic acid. ΔpH and $\Delta\psi$ were determined by the transmembrane equilibration of [^{14}C]benzoic acid and [^{14}C]tetraphenylphosphonium ion (TPP), respectively. The presence of a membrane carrier for lactic acid in *S. mutans* was suggested by counterflow. Enzymic determination of the intra- and extracellular lactate concentrations of *S. mutans* cells glycolysing at pH_o 6.8 and 5.5 showed that lactate distributed across the cell membrane in accordance with the equation $\Delta\text{pH} = \log[\text{lact}]_i/[\text{lact}]_o$. The addition of high extracellular concentrations of lactate to glycolysing *S. mutans* at acidic pH resulted in a fall in ΔpH and a subsequent decrease in glycolysis. The fall in ΔpH was attributed to the F₁F₀ ATPase being unable to raise the pH_i back to its initial level due to the build up of lactate anion within the cell creating a large $\Delta\psi$. The increase in $\Delta\psi$ resulted in the overall proton motive force remaining constant at about –110 mV. The results demonstrate that lactate is transported across the cell membrane of *S. mutans* as lactic acid in an electroneutral process that is independent of metabolic energy and as such has important bioenergetic implications for the cell.

Keywords: lactate, intracellular pH, dental caries, *Streptococcus mutans*, glycolysis

INTRODUCTION

Dental caries is initiated via the production of organic acids by certain plaque bacteria that lower the pH at the tooth surface and cause the demineralization of tooth enamel. *Streptococcus mutans* has been implicated as one of the major odontopathogens of human dental plaque due to its consistent clinical association with dental caries (Loesche, 1986). The pathogenicity of *S. mutans* relates to its ability to rapidly ferment sugar to lactic acid under the low pH conditions required to initiate caries (Loesche,

1986; Tanzer *et al.*, 1969). Geddes (1975) has demonstrated that the fall in plaque pH is due largely to lactic acid production by plaque bacteria.

S. mutans has no respiratory chain and produces ATP via glycolysis; under conditions of glucose excess lactate is the major end-product of this metabolism (Carlsson & Griffith, 1974; Yamada & Carlsson, 1975). Upon ingestion of food the level of sugar available to oral bacteria may increase from the normal resting level of 5–40 μM of salivary secretions (Kelsay *et al.*, 1972) to 5–40 mM (Carlsson, 1984). Under these conditions the *S. mutans* fermentation pattern will shift from the production of mixed acid end-products to lactic acid (Yamada & Carlsson, 1975). The enzymic mechanisms of this shift in fermentation patterns are well understood (Carlsson,

Abbreviations: ΔpH , transmembrane pH gradient; pH_i, intracellular pH; pH_o, extracellular pH; [lact]_i, intracellular lactate concentration; [lact]_o, extracellular lactate concentration; $\Delta\psi$, transmembrane electrical potential; pmf, proton motive force; TPP, tetraphenylphosphonium ion.

1984; Yamada, 1987); however, the fate of the intracellularly produced lactic acid has received little attention.

Konings and colleagues (Michels *et al.*, 1979; Otto *et al.*, 1980, 1982; ten Brink & Konings, 1980) have provided evidence for an 'energy recycling model' in *Lactococcus lactis* where the lactate ion, which is impermeable to the cell membrane, leaves the cell via a membrane carrier in symport with more than one proton. The energy recycling model is an extension of the chemiosmotic model presented by Mitchell (1963) and postulates that carrier-mediated excretion of end-products can lead to the generation of an electrochemical gradient across the cell membrane, thereby providing energy to the cell. Generation of an electrical potential can only be achieved if lactate leaves the cell with more than one proton.

In this investigation the process of lactate efflux from *S. mutans* was elucidated. The transmembrane movement of radiolabelled lactate under a variety of conditions and the measurement of lactate in glycolysing cells led to the conclusion that lactate leaves the cell as lactic acid in an electroneutral, carrier-mediated process that is energy independent. This has important consequences for the bioenergetics of the cell.

METHODS

Bacterial strain and culture conditions. *Streptococcus mutans* Ingbritt (Krasse, 1966; Linzer, 1976) was stored in 30% glycerol broth at -20°C . The strain was checked regularly according to the criteria of Hardie & Bowden (1976). Bacteria were grown as batch cultures at 37°C in TYE growth medium as described previously (Dashper & Reynolds, 1990, 1992). Cells were harvested at mid-exponential phase by centrifugation (1500 g, 15 min, 4°C).

Glycolytic activity. Cells were washed twice with fermentation minimal medium (FMM, Dashper & Reynolds, 1990) and resuspended in FMM to give a cell density of $1.25 \text{ mg dry wt cells ml}^{-1}$. Glycolytic activity was determined in a pH stat as described previously (Dashper & Reynolds, 1990). The cell suspension was maintained at the desired pH by the automatic addition of a volumetric standard titrant (0.1 M KOH). Glycolytic activity was expressed as nmol H^{+} neutralized ($\text{mg dry wt cells}^{-1} \text{ min}^{-1}$). The dry weight of cells was determined by filtering 1.0 ml samples of the cell suspension through dry, pre-weighed $0.2 \mu\text{m}$ polycarbonate filters supported on Whatman GF/A filters. The intracellular water volume was $1.69 \pm 0.07 \mu\text{l}$ ($\text{mg dry wt cells}^{-1}$) (Dashper & Reynolds, 1990).

Lactate transmembrane movement assay. Lactate transmembrane movement assays were conducted at 37°C in a pH stat. After the addition of [^{14}C]lactate [$0.025\text{--}1.000 \text{ Ci mol}^{-1}$ ($1 \text{ Ci} = 3.7 \times 10^{10} \text{ Bq}$)] under the described conditions (see below), samples (usually $2 \text{ mg dry wt cells}$) were centrifuged (15000 g , 15 s) through a silicon oil mixture (43:57, v/v, of 550 and 556 Dow Corning silicon fluids) with a specific gravity of 1.03 g ml^{-1} (Bakker & Harold, 1980). Extracellular lactate ($[\text{lact}]_o$) was quantified by determining the radioactivity in a $10 \mu\text{l}$ sample of the supernatant. Radioactivity was measured in a liquid scintillation counter after sample digestion in NCS and suspension in OCS (Amersham). Extracellular and total water volumes in the cell pellet were determined in parallel experiments using [^{14}C]polyethylene glycol ([^{14}C]PEG) and tritiated water, respectively. From these data the intracellular concentration of [^{14}C]lactate ($[\text{lact}]_i$) could be calculated.

Intracellular pH and transmembrane electrical potential.

Intracellular pH (pH_i) was determined by the distribution of the weak acid, [^{14}C]benzoic acid (120 Ci mol^{-1} , $2 \mu\text{M}$ final concentration) across the cell membrane as described previously (Dashper & Reynolds, 1990, 1992), except that cells were separated from the medium by centrifugation through silicon oil (see above) and not filtration. Cellular adsorption of [^{14}C]benzoic acid was determined using cells treated with $20 \mu\text{M}$ gramicidin.

The transmembrane electrical potential ($\Delta\psi$) was measured by the transmembrane equilibration of [^{14}C]tetraphenylphosphonium ion ([^{14}C]TPP; 20 Ci mol^{-1} , $5 \mu\text{M}$ final concentration). Centrifugation through silicon oil, as described above, was used to separate cells from the medium. The amount of [^{14}C]TPP adsorbed onto the cells was determined in the presence of 100 mM KCl and 100 mM NaCl (Dashper & Reynolds, 1990).

Imposed ΔpH in de-energized cells. After incubation in 50 mM K^{+} FMM ($\text{pH } 7.5$, 40 min), a *S. mutans* cell suspension was washed twice (to remove lactate) and resuspended in the same buffer to give a cell density of $5 \text{ mg dry wt ml}^{-1}$. Either [^{14}C]lactate (1.000 or $0.025 \text{ Ci mol}^{-1}$), to give a final concentration of 5 or 200 mM , or [^{14}C]benzoic acid (see above) was added to an aliquot of the cell suspension. After 2 min the pH was rapidly lowered to about 5.4 by the addition of 0.5 M HCl . Samples were centrifuged through silicon oil and $[\text{lact}]_o$, $[\text{lact}]_i$ and pH_i were determined as described above.

Imposed $\Delta\psi$ in de-energized cells. A $\Delta\psi$ was generated in de-energized cells essentially as described by Otto *et al.* (1980). Briefly, cells were incubated (40 min) in 100 mM Tris/HCl buffer ($\text{pH } 7.0$) containing 200 mM KCl . After incubation the suspension was washed (to remove lactate) and resuspended in the same buffer to give a cell density of $90 \text{ mg dry wt ml}^{-1}$. Valinomycin was then added to the cell suspension to a final concentration of $20 \mu\text{M}$ and the cell suspension was incubated on ice for 40 min . Samples of the cell suspension were diluted 100-fold into 100 mM Tris/HCl buffer ($\text{pH } 7.0$) containing $10 \mu\text{M}$ valinomycin and 5 mM [^{14}C]lactate (1.0 Ci mol^{-1}) with 200 mM NaCl . Samples were centrifuged through silicon oil at specified time intervals and $[\text{lact}]_o$ and $[\text{lact}]_i$ were determined as described above. $\Delta\psi$ was determined using [^{14}C]TPP as described above.

Lactate efflux from de-energized, lactate-loaded cells. Cells were washed and resuspended in a 200 mM potassium phosphate buffer containing 200 mM potassium lactate ($\text{pH } 7.5$) to give a final cell density of $4 \text{ mg dry wt ml}^{-1}$. The cell concentrate was incubated at $\text{pH } 7.5$ and 37°C for 40 min , then divided into 1 ml aliquots, centrifuged (1500 g , 4°C , 15 min) and the supernatant carefully removed. The cell pellet was resuspended in 200 mM potassium phosphate buffer containing 200 mM KCl ($\text{pH } 7.5$) and either [^{14}C]benzoic acid or [^{14}C]TPP. Samples were centrifuged through silicon oil at indicated time intervals and ΔpH and $\Delta\psi$ determined as described above. A control where the cell pellet was resuspended into 200 mM potassium phosphate buffer containing 200 mM potassium lactate was conducted to account for non-specific binding of the radiolabel. This method of cell pellet resuspension gave a dilution factor of $91:1$ as determined in parallel experiments by [^{14}C]PEG.

Lactate counterflow. Counterflow experiments were conducted using a modification of the method of Maloney *et al.* (1975). The cell suspension was washed and resuspended in 50 mM potassium FMM containing 400 mM lactate and $20 \mu\text{M}$ gramicidin, and incubated at $\text{pH } 5.5$, 37°C for 30 min . The pH was then raised to 7.5 and the cells incubated for a further 10 min . After centrifugation, the supernatant was carefully removed by

suction and the tube wall wiped of liquid. To start the assay the cell pellet was rapidly resuspended in 100 mM Tris/HCl buffer pH 7.5 containing 20 μ M gramicidin and 1 mM [14 C]lactate (1 Ci mol $^{-1}$), to give a cell density of 5 mg dry wt ml $^{-1}$. Samples were centrifuged through silicon oil at indicated time intervals and [14 C] $_o$ and [14 C] $_i$ were determined as described above. An identical procedure was used for the control except that lactate was replaced with 400 mM KCl in the incubation step.

Enzymic lactate determination. Cells were washed and incubated in 50 mM K $^+$ FMM for 40 min to deplete endogenous energy sources, then washed and resuspended to give a cell density of 10 mg dry wt ml $^{-1}$ and stored on ice until used. To start the assay a glucose solution (final concentration 20 mM) was added to a sample of the cell suspension in the pH stat (pH 6.8 or 5.5). At indicated times samples were filtered through 0.2 μ m polycarbonate filters, on ice, without washing. The filters were immediately placed in 0.4 ml 5% (v/v) perchloric acid, vortexed and incubated overnight at 4 $^{\circ}$ C. A sample was removed and centrifuged to remove cell debris. Lactate was measured in the supernatant by a modification of the methods of Horn & Bruns (1956) and Cohen & Noell (1960). An aliquot of 100 μ l of neutralized sample (suitably diluted) was added to 340 μ l of reaction mixture. The reaction mixture contained, 3 mg β -NAD ml $^{-1}$, 200 units lactic dehydrogenase ml $^{-1}$ (EC 1.1.1.27, Sigma), 0.45 mM semicarbazide hydrochloride and 300 mM CHES buffer, pH 10.0. This was incubated at 37 $^{\circ}$ C for 2 h at which time the conversion of lactate to pyruvate was complete. Lactate concentration was determined spectrophotometrically at 340 nm by monitoring the conversion of NAD to NADH. The amount of extracellular water trapped on the filter was determined gravimetrically using duplicate samples. [14 C] $_i$ was calculated by subtracting [14 C] $_o$ from the total on the filter.

RESULTS

Lactate transmembrane movement

A Δ pH was imposed on de-energized cells of *S. mutans* by rapidly lowering the pH $_o$ from 7.5 to 5.4. This produced a Δ pH (interior alkaline) of 1.05 units, equivalent to a proton motive force (pmf) of -64.6 mV (Fig. 1). In the presence of 5 mM lactate a rapid influx of lactate occurred when the Δ pH was imposed, leading to maximal in-

Fig. 1. Intracellular accumulation of [14 C]lactate ($\blacktriangle$) by de-energized cells of *S. mutans* in response to an imposed Δ pH ($\blacksquare$). The imposed Δ pH was produced by rapidly lowering the pH $_o$ with HCl after 2 min. The Δ pH was measured by the transmembrane equilibration of [14 C]benzoic acid.

Fig. 2. Generation of a Δ pH in de-energized, lactate-loaded cells of *S. mutans* by the efflux of lactic acid. Cells were loaded with 200 mM lactate prior to dilution in a lactate-free buffer at time zero.

tracellular accumulation ($[lact]_i:[lact]_o = 6.25:1$; Fig. 1). [14 C] $_i$ decreased as the imposed Δ pH decayed and after 10 min the [14 C] $_i:[lact]_o$ ratio had returned to about 1:1 by which time the Δ pH was zero. In the presence of 200 mM lactate little intracellular accumulation of lactate occurred as the rapid movement of lactic acid into the cell at this high [14 C] $_o$ caused the collapse of the imposed Δ pH.

The efflux of lactate from lactate-loaded, de-energized cells of *S. mutans* resuspended at pH 7.0 also resulted in the formation of a Δ pH (Fig. 2). However, no $\Delta\psi$ was formed under identical conditions of lactate efflux from lactate-loaded, de-energized cells.

To investigate whether a $\Delta\psi$ would drive lactate accumulation, K $^+$ -loaded, de-energized cells of *S. mutans* were treated with valinomycin, in a medium containing low potassium at pH 7.0. This treatment produced a $\Delta\psi$ of -62 mV as measured by [14 C]TPP transmembrane equilibration. The imposition of this $\Delta\psi$ resulted in no intracellular accumulation of lactate. This was confirmed by conducting a similar experiment also at pH 7.0 in which valinomycin-treated, de-energized cells suspended in a high potassium medium were diluted 100-fold in media with no potassium containing valinomycin. This treatment produced a $\Delta\psi$ of -120 mV and also resulted in no intracellular accumulation of lactate.

Lactate counterflow

The transient intracellular accumulation of extracellularly added [14 C]lactate within de-energized lactate-loaded cells of *S. mutans* demonstrated that counterflow did occur (Fig. 3), thereby suggesting the presence of a membrane carrier for lactic acid. The addition of [14 C]lactate to control cells that were not lactate-loaded resulted in only the rapid transmembrane equilibration of the radiolabel across the cell membrane (Fig. 3). The presence of a membrane carrier for lactic acid is consistent with the exceedingly rapid transmembrane equilibration of radiolabelled lactate in de-energized cells that occurred over a wide range of concentrations.

Fig. 3. Lactic acid counterflow in de-energized cells of *S. mutans*. Cells were incubated with either 400 mM potassium lactate (□) or 400 mM KCl (△) prior to centrifugation and suspension in buffer containing 5 μ M [14 C]lactate at time zero.

Effect of lactate on pH_i and glycolysis

In a dense suspension of *S. mutans* cells (about 10 mg dry wt cells ml⁻¹) glycolysing at pH_o 5.5, [lact]_i and [lact]_o were determined enzymically. Lactate rapidly built up within the cell over the first 3 min maintaining an intracellular to extracellular concentration ratio of about 24:1. This ratio then began to decrease such that at 6 min after glucose addition it had fallen to about 16:1 (Table 1). Over the same time period the Δ pH_i, as measured by the transmembrane equilibration of radiolabelled benzoic acid, fell from 1.32 to 1.18. These results correlate well with the predicted Δ pH assuming that lactate distributed across the cell membrane in accordance with the equation Δ pH = log[lact]_i/[lact]_o (Table 1). The Δ pH continued to fall such that at 25 min after glucose addition it was 0.91 and at 30 min it was 0.87. There was a consequent decrease in glycolytic activity over the same time period. In more dilute cell suspensions (1–2 mg dry wt cells ml⁻¹) of *S. mutans* glycolysing at pH_o 5.5 this fall in pH_i and decrease in glycolytic activity was not seen, as [lact]_o obviously did not increase as rapidly as in denser cell suspensions.

Fig. 4. [lact]_i (▲), [lact]_o (●) and rate of acid production (—) in cells of *S. mutans* glycolysing at pH_o 6.8. Lactate was enzymically determined. Time zero refers to the addition of glucose. Data points represent the mean $\pm$ SD of three determinations.

In a dense suspension of *S. mutans* cells glycolysing at pH_o 6.8 similar results were obtained to those at pH_o 5.5 (Fig. 4). [lact]_i increased over the first 4–5 min of glycolysis to 95 mM. After this time the [lact]_i remained fairly constant whilst the ratio of [lact]_i to [lact]_o fell from 11.4 at 4 min to 3.9 at 10 min. The Δ pH of *S. mutans* cells was measured under similar experimental conditions and found to fall from 0.86 at 4 min to 0.50 at 10 min; it continued to fall as glycolysis progressed such that at 50 min it was 0.14. The fall in pH_i at this pH_o had little effect on glycolytic activity.

Effect of added extracellular lactate on pH_i and glycolysis

When 100 mM potassium lactate was added to *S. mutans* glycolysing at pH_o 5.5 there was an immediate decrease in pH_i and glycolytic activity. Based on the fall in pH_i, an inhibition of glycolytic activity of 38% was predicted from previous work (Dashper & Reynolds, 1992). The observed inhibition of 40% corresponds well with the predicted result, indicating that the decrease in glycolytic activity is due entirely to the fall in pH_i. There was a

Table 1. [lact]_i, [lact]_o and pH_i of *S. mutans* cells glycolysing at a pH_o of 5.5 in 50 mM K⁺ FMM

Time after glucose addition (min)	[lact] _i (mM)*	[lact] _o (mM)*	[lact] _i /[lact] _o	Predicted Δ pH†	Measured Δ pH‡ (mean $\pm$ SD, n = 4)
2	75.2	3.2	23.5	1.37	1.32 $\pm$ 0.03
3	114.3	4.8	23.8	1.37	—
4	133.9	6.7	19.9	1.30	1.22 $\pm$ 0.02
6	165.9	10.5	15.8	1.20	1.18 $\pm$ 0.02

* Lactate was measured enzymically.

† Based on the equation log[lact]_i/[lact]_o = Δ pH.

‡ Determined by the transmembrane equilibration of [14 C]benzoic acid.

Table 2. The effect of extracellular lactate on the transmembrane forces maintained by *S. mutans* cells glycolysing at a pH_o of 5.5 in 50 mM K⁺ FMM.

	ΔpH^*	$\Delta\psi$ (mV) [†]	pmf (mV) [‡]
Control (100 mM KCl)	1.31	−29.3	109.9
Lactate (100 mM potassium lactate)	0.63	−63.7	102.5

* Measured by the transmembrane equilibration of [¹⁴C]benzoic acid.

† Measured by the transmembrane equilibration of [¹⁴C]TPP.

‡ pmf = $\Delta\psi - Z\Delta\text{pH}$.

simultaneous increase in the $\Delta\psi$ upon the addition of lactate which maintained the pmf of these cells at approximately −105 mV (Table 2). The pmf values obtained in this study agree well with those found in earlier studies (Dashper & Reynolds, 1992).

DISCUSSION

Acidogenicity and acidurance have been proposed as the two major virulence traits of the odontopathogenic bacterium *S. mutans* (Loesche, 1986). Under conditions of sugar excess lactic acid is the major end-product of glycolysis by *S. mutans*. However the transmembrane movement of the intracellularly produced lactic acid and its effect on pH_i has received little attention.

Otto *et al.* (1980, 1982) have demonstrated that at neutral pH, lactate transmembrane movement in *Lactococcus lactis* subsp. *cremoris* is electrogenic, such that more than one proton is carried across the cell membrane in association with the lactate ion. This process has also been postulated to exist in *S. mutans* (Keevil *et al.*, 1984).

In this study when a ΔpH was imposed upon de-energized cells of *S. mutans*, lactate distributed across the cell membrane in accordance with the equation $\Delta\text{pH} = \log([\text{lact}]_i/[\text{lact}]_o)$. The efflux of lactate from lactate-loaded, de-energized cells produced a ΔpH but no $\Delta\psi$. This, and the failure of an imposed $\Delta\psi$ (produced by two distinct methods, giving values of −62 and −120 mV) to cause uptake of lactate into de-energized *S. mutans* cells at pH 7.0, demonstrates that the transmembrane movement of lactate in *S. mutans* is electroneutral, showing that the undissociated lactic acid is the species excreted. The relationship between $[\text{lact}]_i$ and $[\text{lact}]_o$ and ΔpH described in the above equation for de-energized cells was also found in glycolysing cells of *S. mutans*. The demonstration of transmembrane movement of lactic acid in de-energized (ATP-depleted) cells of *S. mutans* negates the necessity for metabolic energy in the translocation of lactate, as proposed recently by Distler *et al.* (1989).

The results of this study showing the transmembrane movement of lactic acid to be an electroneutral process in *S. mutans* are consistent with those of Harold & Levin (1974) and Kobayashi *et al.* (1982) who demonstrated the electroneutral transmembrane movement of lactic acid in *Enterococcus faecalis*, and Baronofsky *et al.* (1984) and Kell *et al.* (1981) who showed the electroneutral trans-

membrane movement of acetic acid in *Clostridium thermoaceticum* and *Clostridium pasteurianum*, respectively. Maloney (1983) also concluded that lactic acid transmembrane movement in *L. lactis* glycolysing at pH 5.0 was an electroneutral process. However, it has been proposed that some of these acidic end-products are able to passively diffuse across the cell membrane in the uncharged form (Baronofsky *et al.*, 1984; Kell *et al.*, 1981). Due to the high rate of lactic acid production during glycolysis by *S. mutans*, and in view of the polarity of the molecule, it is considered unlikely that the rapid translocation occurs via passive diffusion through the cell membrane (Gatje *et al.*, 1991). The existence of a carrier system for lactic acid in *S. mutans* was indicated in this study by counterflow in the presence of the ionophore gramicidin. This method has been used by other workers to show the existence of membrane carriers for a variety of substrates (Maloney *et al.*, 1975; Gatje *et al.*, 1991; Buckley & Hamilton, 1995).

The enzymic determination of $[\text{lact}]_i$ and $[\text{lact}]_o$ in a suspension of glycolysing *S. mutans* cells allowed the activity of the lactic acid carrier and its effect on pH_i to be elucidated in energized cells. When glucose was added to de-energized cells of *S. mutans*, lactate accumulated within the cell, indicating that the level of lactic acid was not high enough to allow the carrier to function at a sufficient rate to remove all of the lactate produced by glycolysis. However, as the level of intracellular lactate and lactic acid increased, so the transmembrane lactate chemical potential increased. As the level of intracellular lactate approached 100 mM the transmembrane lactate chemical potential approached −67 mV and the rate of lactic acid efflux approached the rate of proton extrusion by the cell.

The rate of proton extrusion from *S. mutans* was linear almost immediately after the addition of glucose to the cells. As the lactate concentration increased within the cell during this initial period, the extrusion of protons must be attributed to the activity of the F₁F₀-ATPase. When the $[\text{lact}]_i$ approached 100 mM, the rate of lactic acid efflux increased until all protons and lactate produced by glycolysis were translocated as lactic acid (Fig. 4). The F₁F₀-ATPase therefore appears necessary to establish the ΔpH and in so doing allows accumulation of lactate intracellularly, producing a transmembrane lactate gradient or driving force for lactic acid efflux through the carrier. The F₁F₀-ATPase will then only be necessary for

the maintenance of the ΔpH by extruding protons entering the cell in symport with pmf-linked substrates or by unspecified leakage across the cell membrane. This is very important from a bioenergetic point of view, as to extrude all of the protons produced by glycolysis via the F_1F_0 -ATPase would halve the net production of ATP produced per glucose molecule, assuming a stoichiometry of $2\text{H}^+/\text{ATP}$ (Mitchell & Koppenol, 1982).

This scheme of pH regulation associated with lactic acid efflux is supported by the fact that potassium accumulation is maximal over the first 4 min of glycolysis (Dashper & Reynolds, 1992), during which time lactate anion is accumulating within the cell. As the F_1F_0 -ATPase is extruding protons during this time and lactate anion is accumulating within the cell, the electrogenic uptake of potassium is necessary to dissipate the $\Delta\psi$ created by this process.

This metabolic-energy-independent, carrier-mediated transmembrane movement of lactic acid is an energy efficient way to remove the lactate and protons produced by glycolysis, unless lactate builds up in the extracellular environment. High $[\text{lact}]_0$ would result in lactic acid being unable to escape from the cell or at even higher concentrations extracellular lactic acid would move down its concentration gradient into the cell, via the carrier, where it would dissociate to lactate and protons, thereby lowering pH_i . Under these conditions the F_1F_0 -ATPase would begin to pump the protons out of the cell but would be unable to raise pH_i back to its initial level due to the large $\Delta\psi$. This $\Delta\psi$ is created by lactic acid movement into the cell, its dissociation and the extrusion of the protons with the resultant intracellular accumulation of negative charge in the form of lactate anions. The *S. mutans* F_1F_0 -ATPase is incapable of producing or maintaining a pmf of greater than -120 mV (Table 2; Dashper & Reynolds, 1993; Noji *et al.*, 1988). The results of this study are totally consistent with this proposal as the reduction in glycolysis caused by the addition of high $[\text{lact}]_0$ to *S. mutans* could be attributed to the fall in pH_i . There is no evidence from this study that the intracellular accumulation of lactate *per se* inhibits glycolysis. Also, as high concentrations of lactate did not result in a decrease in the pmf in glycolysing cells of *S. mutans*, lactate cannot be considered to act as an 'uncoupler' as has been proposed by Baronofsky *et al.* (1984) and Herrero *et al.* (1985).

In dense cell suspensions of *S. mutans*, glycolytic activity leads to a rapid increase in lactate concentration outside the cell. This high $[\text{lact}]_0$ causes a decrease in the ΔpH , that at acidic pH_0 results in inhibition of glycolysis. Therefore in dental plaque a build up of extracellular lactate may determine the final plaque pH after a sugar exposure. This may help explain the anticariogenic effect of added calcium lactate reported by Shrestha *et al.* (1982). Indeed, if the *S. mutans* lactic acid carrier resembles that found in *Lactobacillus helveticus* (Gatje *et al.*, 1991), as it appears to, then it will also allow equilibration of acetate and other carboxylic acids across the cell membrane, inhibiting glycolytic activity at acidic pH.

In conclusion lactate and protons generated as end-products of glycolysis in *S. mutans* are translocated across the cell membrane as lactic acid in a carrier-mediated electroneutral process. No input of metabolic energy is necessary for this efflux once a transmembrane lactate chemical potential has been established by the action of the F_1F_0 -ATPase establishing a ΔpH . As such this is of great bioenergetic significance to the cell.

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REFERENCES

- Bakker, E. P. & Harold, F. M. (1980). Energy coupling to potassium transport in *Streptococcus faecalis* (interplay of ATP and the proton motive force). *J Biol Chem* **255**, 433–440.
- Baronofsky, J. J., Schreurs, W. J. A. & Kashket, E. R. (1984). Uncoupling by acetic acid limits growth of and acetogenesis by *Clostridium thermoaceticum*. *Appl Environ Microbiol* **48**, 1134–1139.
- Buckley, N. D. & Hamilton, I. R. (1994). Vesicles prepared from *Streptococcus mutans* demonstrate the presence of a second glucose transport system. *Microbiology* **140**, 2639–2648.
- Carlsson, J. (1984). Regulation of sugar metabolism in relation to the feast and famine existence of plaque. In *Cariology Today. International Congress Zürich 1983*, pp. 205–211. Edited by B. Guggenheim. Basel: Karger.
- Carlsson, J. & Griffith, C. J. (1974). Fermentation products and bacterial yields in glucose-limited and nitrogen-limited cultures of streptococci. *Arch Oral Biol* **19**, 1105–1109.
- Cohen, L. H. & Noell, W. K. (1960). Glucose catabolism of rabbit retina before and after development of visual function. *J Neurochem* **5**, 253–276.
- Dashper, S. G. & Reynolds, E. C. (1990). Characterization of transmembrane movement of glucose and glucose analogs in *Streptococcus mutans* Ingbritt. *J Bacteriol* **172**, 556–563.
- Dashper, S. G. & Reynolds, E. C. (1992). pH regulation by *Streptococcus mutans*. *J Dent Res* **71**, 1159–1165.
- Dashper, S. G. & Reynolds, E. C. (1993). Branched-chain amino acid transport in *Streptococcus mutans* Ingbritt. *Oral Microbiol Immunol* **8**, 167–171.
- Distler, W., Kargermeier, A., Hickel, R. & Kroncke, A. (1989). Lactate influx and efflux in the '*Streptococcus mutans* group' and *Streptococcus sanguis*. *Caries Res* **23**, 252–255.
- Gatje, G., Muller, V. & Gottschalk, G. (1991). Lactic acid excretion via carrier-mediated diffusion in *Lactobacillus helveticus*. *Appl Microbiol Biotechnol* **34**, 778–782.
- Geddes, D. A. M. (1975). Acids produced by human dental plaque metabolism *in situ*. *Caries Res* **9**, 98–109.
- Hardie, J. M. & Bowden, G. H. (1976). The microbial flora of dental plaque, bacterial succession and isolation considerations. In *Microbial Aspects of Dental Caries, Vol. 3*, pp. 63–87. Edited by H. M. Stiles, W. J. Loesche & T. C. O'Brien. Washington, DC: Information Retrieval Inc.
- Harold, F. M. & Levin, E. (1974). Lactic acid translocation, terminal step in glycolysis by *Streptococcus faecalis*. *J Bacteriol* **117**, 1141–1148.
- Herrero, A. A., Gomez, R. F., Snedecor, B., Tolman, C. J. & Roberts, M. F. (1985). Growth inhibition of *Clostridium thermocellum* by carboxylic acids, a mechanism based on uncoupling by weak acids. *Appl Microbiol Biotechnol* **22**, 53–62.

- Horn, H. D. & Bruns, F. H. (1956). Quantitative bestimmung von L(+)-milchsaure mit milchsauredehydrogenase. *Biochim Biophys Acta* **21**, 378–380.
- Keevil, C. W., Williamson, M. I., Marsh, P. D. & Ellwood, D. C. (1984). Evidence that glucose and sucrose uptake in oral streptococcal bacteria involves independent phosphotransferase and proton-motive force-mediated mechanisms. *Arch Oral Biol* **29**, 871–878.
- Kell, C. W., Peck, M. W., Rodger, G. & Morris, J. G. (1981). On the permeability to weak acids and bases of the cytoplasmic membranes of *Clostridium pasteurianum*. *Biochem Biophys Res Commun* **99**, 81–88.
- Kelsay, J. L., McCague, K. E. & Holden, J. M. (1972). Variations in flow rate, metabolites and enzyme activities of fasting human parotid saliva. *Arch Oral Biol* **17**, 304–320.
- Kobayashi, H., Murakami, N. & Unemoto, T. (1982). Regulation of the cytoplasmic pH in *Streptococcus faecalis*. *J Biol Chem* **257**, 13246–13252.
- Krasse, B. (1966). Human streptococci and experimental caries in hamsters. *Arch Oral Biol* **11**, 429–436.
- Linzer, R. (1976). Serotype polysaccharide antigens of *Streptococcus mutans* composition and serological cross reactions. In *Immunological Aspects of Dental Caries*, pp. 91–99. Edited by W. H. Bowen, R. J. Genco & T. C. O'Brien. Washington, DC: Information Retrieval Inc.
- Loesche, W. J. (1986). Role of *Streptococcus mutans* in human dental decay. *Microbiol Rev* **50**, 353–380.
- Maloney, P. C. (1983). Relationship between phosphorylation potential and electrochemical H⁺ gradient during glycolysis in *Streptococcus lactis*. *J Bacteriol* **153**, 1461–1470.
- Maloney, P. C., Kashket, E. R. & Wilson, T. R. (1975). Methods for studying transport in bacteria. *Methods Membr Biol* **5**, 1–50.
- Michels, P. A. M., Michels, J. P. J., Boonstra, J. & Konings, W. N. (1979). Generation of electrochemical proton gradient in bacteria by excretion of metabolic end-products. *FEMS Microbiol Lett* **5**, 357–362.
- Mitchell, P. (1963). Molecule, group and electron translocation through natural membranes. *Biochem Soc Symp* **22**, 142–168.
- Mitchell, P. & Koppenol, W. H. (1982). Chemiosmotic ATPase mechanisms. *Ann NY Acad Sci* **402**, 584–601.
- Noji, S., Sato, Y. & Taniguchi, S. (1988). Effect of intracellular pH and potassium ions on a primary transport system for glutamate/aspartate in *Streptococcus mutans*. *Eur J Biochem* **175**, 491–495.
- Otto, R., Sonnenberg, A. S. M., Veldkamp, H. & Konings, W. N. (1980). Generation of an electrochemical proton gradient in *Streptococcus cremoris* by lactate efflux. *Proc Natl Acad Sci USA* **77**, 5502–5506.
- Otto, R., Lageveen, R. G., Veldkamp, H. & Konings, W. N. (1982). Lactate efflux-induced electrical potential in membrane vesicles of *Streptococcus cremoris*. *J Bacteriol* **149**, 733–738.
- Shrestha, B. M., Mundorff, S. A. & Bibby, B. G. (1982). Preliminary studies on calcium lactate as an anticaries food additive. *Caries Res* **16**, 12–17.
- Tanzer, J. M., Krichevsky, M. I. & Keyes, P. H. (1969). The metabolic fate of glucose catabolized by a washed stationary phase caries-conductive *Streptococcus*. *Caries Res* **3**, 167–177.
- ten Brink, B. & Konings, W. N. (1980). Generation of an electrochemical proton gradient by lactate efflux in membrane vesicles of *Escherichia coli*. *Eur J Biochem* **111**, 59–66.
- Yamada, T. (1987). Regulation of glycolysis in streptococci. In *Sugar Transport and Metabolism on Gram-positive Bacteria*, pp. 66–93. Edited by J. Reizer & A. Peterofsky. Chichester: Ellis Horwood Ltd.
- Yamada, T. & Carlsson, J. (1975). Regulation of lactate dehydrogenase and change of fermentation products in Streptococci. *J Bacteriol* **124**, 55–61.

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