Vesicles prepared from *Streptococcus mutans* demonstrate the presence of a second glucose transport system

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***Streptococcus mutans*, an important aetiological agent of dental caries, is known to transport glucose via the phosphoenolpyruvate (PEP) phosphotransferase system (PTS). An alternative non-PTS glucose transport system in *S. mutans* Ingbritt was suggested by the increased ATP-dependent phosphorylation of glucose and the presence of higher cellular concentrations of free glucose in cells grown in continuous culture under PTS-repressed conditions compared to those resulting in optimal PTS activity. A method was developed for the preparation of membrane vesicles in order to study this system in the absence of PTS activity. These vesicles had very low activity of the cytoplasmic enzymes, glucokinase, pyruvate kinase and lactate dehydrogenase. This, coupled with the lack of glycolytic activity and the inability to transport glucose, suggested that the vesicles would also be deficient in PTS activity because of the absence of the general soluble PTS proteins, Enzyme I and HPr, required for the transport of all PTS sugars. Freeze-fracture electron microscopy and membrane H⁺-ATPase analysis indicated that over 90% of the vesicles had a right-side-out orientation. Vesicles from cells grown in continuous culture under PTS-dominant and PTS-repressed conditions both exhibited glucose counterflow. This indicates the presence of a constitutive non-PTS carrier in the organism capable of transporting glucose and utilizing ATP for glucose phosphorylation. Analysis of growth yields of cells grown under PTS-repressed and PTS-optimal conditions suggests that ATP, or an equivalent high energy molecule, must be involved in the actual transport process. This analysis is consistent with an ATP-binding protein model such as the *Msm* transport system reported by R. R. B. Russell and coworkers (*J Biol Chem* 267, 4631–4637), but it does not exclude the possibility of a separate permease for glucose.**

Keywords: *Streptococcus mutans*, glucose transport, PEP phosphotransferase system, membrane vesicles

INTRODUCTION

Streptococcus mutans is a major aetiological agent of dental caries, catabolizing dietary carbohydrates to acid end-products that, once excreted, can contribute to the demineralization of the tooth enamel (Hamada & Slade, 1980; van Houte, 1986). Results of early studies on sugar

transport by *S. mutans* suggested that the organism possesses at least two distinct systems for glucose uptake (Hamilton, 1984, 1987). The phosphoenolpyruvate (PEP):sugar phosphotransferase system (PTS) (Meadow *et al.*, 1990; Postma *et al.*, 1993) is a principal transport system in oral streptococci for glucose and a variety of sugars, including mannose, fructose, sucrose, lactose and maltose (Vadeboncoeur, 1984). In the PTS, phosphate from PEP is transferred via the general proteins HPr and Enzyme I (EI), through the sugar-specific, membrane-bound carrier, Enzyme II (EII), onto the incoming carbohydrate. In some cases, the latter phosphoryl transfer requires the participation of a sugar-specific Enzyme III,

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Abbreviations: PEP, phosphoenolpyruvate; PTS, phosphotransferase system.

or simply III, often in EII/III pairs (Meadow *et al.*, 1990; Postma *et al.*, 1993).

Early studies with toluene-permeabilized cells of *S. mutans* Ingbritt grown in continuous culture showed that the glucose-PTS was repressed under a variety of conditions including growth at low pH (Hamilton & Ellwood, 1978), at high growth rates and with excess glucose (Ellwood *et al.*, 1979), and following transition to growth on sucrose (Ellwood & Hamilton, 1982). Later studies demonstrated that the synthesis of the membrane-bound EII for glucose (EII^{glc}) and mannose (EII^{man}), but not the general proteins, was affected by environmental factors, particularly the glucose concentration and the pH of growth (Vadeboncoeur *et al.*, 1987, 1991; Hamilton *et al.*, 1989). Interestingly, the glucose uptake and glycolytic rates were not similarly inhibited, suggesting that during PTS repression *S. mutans* employed an alternative, non-PTS glucose transport system (Hamilton, 1987). An early model of glucose transport linked to proton-motive force in *S. mutans* was proposed by Hamilton & St Martin (1982), but subsequently questioned by studies using the glucose analogue 6-deoxyglucose (Dashper & Reynolds, 1990).

In order to examine glucose transport in intact cells, it is necessary to disrupt cellular metabolism and, as a consequence, it is then difficult to discriminate between glucose transport inhibition due to lack of a transporter or interference with an existing system that relies on active metabolism. A novel way to attack the problem is through the use of membrane vesicles. Kaback (1960) first reported that isolated membranes of *Escherichia coli* strain W could catalyse the uptake of proline. Subsequent studies showed that the membrane preparation contained plasma membrane vesicles devoid of cytoplasmic constituents that behaved as 'osmotically intact structures' retaining many transport functions of whole cells (Kaback, 1968, 1972, 1974). Vesicles of *S. mutans* afford several advantages over whole cells for the study of carbohydrate transport: (a) the PTS would be inactivated by the removal of the general proteins, HPr and EI, as well as the enzymes capable of generating PEP, (b) transport substrates would not be metabolized, and (c) the transport system could be studied in isolation without the use of inhibitors or substrate analogues.

In this paper, we provide evidence for an ATP-dependent, non-PTS sugar transport system in *S. mutans* Ingbritt grown in continuous culture under conditions that repress PTS activity. For confirmation of such a non-PTS process, membrane vesicles were prepared with mutanolysin and lysozyme from cells grown under both PTS repressive and non-repressive conditions. Vesicles obtained by this method had a right-side-out orientation and were essentially free of cytoplasmic constituents permitting the study of non-PTS glucose transport in the absence of PTS activity. Glucose counterflow experiments conducted with such vesicles provide evidence for the presence in both cell types of a non-PTS carrier capable of transporting glucose. It is concluded that *S. mutans* possesses two glucose transport systems, the PEP-dependent PTS

and a second, constitutive process probably linked to the ATP-dependent phosphorylation of glucose.

METHODS

Bacterial strain and growth conditions. The *S. mutans* strain Ingbritt used in this study was kindly supplied by J. Sandham, Toronto, Canada. The culture was maintained and assessed for purity as described previously (Hamilton & Ellwood, 1978). Cultures were grown in chemostats at a dilution rate (*D*) of 0.1 h⁻¹ in semi-defined medium (Hamilton & Buckley, 1991) under the following conditions: condition A (5 mM glucose at pH 7.0), which results in optimal glucose-PTS activity (PTS-O cells), and condition B (50 mM glucose pH 5.5), which results in repression of the glucose-PTS (PTS-R cells) (Hamilton *et al.*, 1979, 1989; Vadeboncoeur *et al.*, 1987, 1991).

Phosphorylation of glucose by ATP and PEP. Cells of *S. mutans* Ingbritt growing under conditions A and B were harvested from the chemostat overflow over 3–16 h into a container cooled to 0 °C, centrifuged at 10 000 *g* for 10 min, washed twice and resuspended at 10 mg (dry wt) ml⁻¹ in 10 mM potassium phosphate buffer (pH 7.0) containing 14 mM 2-mercaptoethanol and 5 mM MgCl₂. Cells were permeabilized with toluene (Hamilton *et al.*, 1979) and assayed in the following reaction mixture (final concentrations): 50 mM potassium phosphate buffer (pH 7.0), 5 mM MgCl₂, 5 mM 2-mercaptoethanol, 10 mM NaF, 2 mM [¹⁴C]glucose (0.1 Ci mol⁻¹, 3.7 GBq mol⁻¹) 2 mM ATP or PEP and 250 µg (dry wt) cells in a 500 µl volume. Controls contained no phosphoryl donor. After 15 min incubation at 37 °C, the phosphorylated product was precipitated with 30 mM BaBr in 90% ethanol, collected on filters and the filters counted in Aquasol (NEN Research Products).

Determination of intracellular glucose and glucose 6-phosphate. Samples of steady-state cultures were rapidly drawn from the chemostat and filtered through 0.2 µm Uni-Pore Polycarbonate membrane filters (Bio-Rad) and immediately washed with 10 mM Tris/HCl buffer (pH 7.0). The filters were then immersed in 3.0 ml 0.5 M perchloric acid, incubated at room temperature for 30 min to extract the cells and then washed in 1.0 ml water. As a control for any residual medium in the filters, equivalent volumes of spent medium were passed through a duplicate set of filters and subjected to perchloric acid extraction. In all cases, the pooled extract and wash was titrated to pH 7.5 with 5 M K₂CO₃ following the addition of 100 µl 1 mM triethanolamine buffer (pH 7.5) and then centrifuged at 10 000 *g* for 10 min to remove the potassium perchlorate. The supernatant was then filtered through a 0.2 µm filter and immediately assayed for glucose and glucose 6-phosphate.

Glucose 6-phosphate was analysed by a modification of the glucose-6-phosphate dehydrogenase assay of Bergmeyer (1974). The reaction went to completion within 3 min, at which time known quantities of glucose 6-phosphate were added to samples to serve as internal standards and a second absorbance reading was made. Glucose was assayed with the glucose oxidase-peroxidase coupled reaction of Kingsley & Getchell (1960). A duplicate set of tubes was included in each assay to which known amounts of glucose were added as an internal standard.

Preparation of membranes, vesicles and crude-cell extract. Membrane fragments of *S. mutans* Ingbritt were prepared with cells harvested from the chemostat overflow (Hamilton & Buckley, 1991). Vesicles were prepared with a combination of the membrane technique described above and the vesicle preparation procedure of Otto *et al.* (1984). Steady-state cells were collected from the chemostat, washed twice in cold water

and resuspended in 40 ml vesicle buffer [10 mM Tris/HCl (pH 7.5), 10 mM MgSO₄, 2 µM pepstatin and 14 mM mercaptoethanol]. The cell suspension was sonicated for 2 min with a Kontes sonifier (Kontes Scientific Glassware) at a power setting of 4 to disperse the cells before addition to 160 ml prewarmed osmotic buffer [75 mM Tris/HCl (pH 7.5), 2 mM MgSO₄, 40 mM amino-*n*-caproic acid, 6 mM *p*-aminobenzamide and 14 mM mercaptoethanol] containing 1.25 g lysozyme and 7500 U mutanolysin (Sigma). After a 3 h incubation at 37 °C, the resulting protoplasts were washed and resuspended in 40 ml vesicle buffer, and lysed by the addition of 10.8 ml saturated K₂SO₄. The preparation was treated with DNase and RNase (each 10 µg ml⁻¹) for 15 min with stirring at 37 °C, after which 9.0 ml 200 mM EDTA was added and the preparation incubated for an additional 15 min. The cell debris, including vesicles, was collected by centrifugation, washed once and resuspended in 60 ml vesicle buffer. The vesicles were purified by discontinuous density gradient centrifugation: 10 ml of the vesicle/membrane suspension was placed gently onto 10 ml 60% (w/v) sucrose overlaid with 15 ml 20% (w/v) sucrose and centrifuged at 67 500 *g* for 16 h in a Beckman SW 28 swinging-bucket rotor. The vesicles, collected at the interface between the two layers, were carefully removed, collected by centrifugation at 48 000 *g* for 30 min, washed once and resuspended in vesicle buffer. The final vesicle pellet was resuspended in buffer, distributed in 200 µl aliquots and stored at -70 °C until used.

Crude cell extract of *S. mutans* Ingbritt was prepared by a variation of the sonication method described by Khandelwal & Hamilton (1971). Cells grown in condition B (see above) were collected from the chemostat, washed in vesicle buffer and sonicated for 3 min at 0 °C in the presence of 5 µm diameter beads (cells-to-beads ratio of 3:1) in a Branson sonifier (Heat Systems Ultrasonics) followed by centrifugation (30 000 *g* for 15 min). The supernatant was then treated with RNase and DNase (each 10 µg ml⁻¹) for 30 min and dialysed (10 000 MW exclusion limit) overnight against 4 l vesicle buffer with one change of buffer.

Electron microscopy. To characterize the process of vesicle formation, samples were removed at different stages of vesicle preparation and fixed by the addition of 50% glutaraldehyde directly to the specimen in suspension (40 µl fixative per ml sample). They were post fixed with 1% osmium tetroxide and embedded in JemBed 812 (JB EM Services) (Yuan & Gulyas, 1981) and the specimens sectioned on an LKB Ultratome EIII with a diamond knife and the silver sections (50–60 nm) were collected on 300-mesh grids. The sections were stained with uranyl acetate (Stempek & Ward, 1964) and post-stained with lead citrate (Venable & Coggeshall, 1965). The sections were viewed with a Philips model 201 electron microscope at an acceleration voltage of 60 keV and images were recorded on Kodak positive reversal film 5302. The *S. mutans* vesicles were also examined by freeze-fracture electron microscopy to determine their orientation, in the laboratory of T. J. Beveridge, University of Guelph, using the techniques of Chapman & Staehelin (1986) and Martin & Beveridge (1986).

Enzyme assays. Vesicles, membranes and crude-cell extract prepared from cells grown under condition B were assayed for the presence of cytoplasmic and membrane-associated enzymes. Vesicles were sonicated for 1 min at 0 °C with a Branson sonifier prior to enzyme assays to liberate trapped cytoplasmic contents. Glucokinase was assayed by the method of Porter *et al.* (1980), while pyruvate kinase was assayed in the presence of the activator glucose 6-phosphate (Yamada & Carlsson, 1975). Lactate dehydrogenase was assayed in the presence of its activator, fructose 1,6-bisphosphate, and saturating amounts of pyruvate (Brown & Wittenberger, 1972). The membrane-

bound, proton-translocating ATPase (H⁺-ATPase) was assayed by the method of Bender *et al.* (1986) modified as described by Hamilton & Buckley (1991). Units are expressed as nmol P_i (mg protein)⁻¹ min⁻¹.

All enzymes were measured in vesicle and membrane preparations before and after sodium dodecyl sulfate treatment (SDS:protein ratio of 1:1) to solubilize bound protein. The protein content of the membranes, vesicles and crude cell extracts was determined by a modification of the Lowry technique as described by Peterson (1983) using bovine serum albumin as standard.

Measurement of glycolytic activity. Glycolytic activity of the vesicles prepared from cells grown under conditions A and B was assayed as described previously (Hamilton & St Martin, 1982), except that the vesicles were resuspended at 0.5 mg protein ml⁻¹ in vesicle buffer in a pH-stat at a final volume of 5.0 ml.

Glucose counterflow. Counterflow was used to test the ability of vesicles to carry out carrier-mediated facilitated diffusion of glucose. For 'glucose-loading', vesicles were incubated for 1 h at room temperature with 100 mM glucose in 10 mM Tris/HCl buffer (pH 7.5) containing 10 mM MgSO₄, 14 mM mercaptoethanol, 2 mM PMSF and 2 µM pepstatin. The vesicles were collected by centrifugation at 48 000 *g* for 10 min, the supernatant was decanted carefully and any residual fluid in the centrifuge tube removed with cotton swabs. Vesicles were resuspended in 10 ml of the same buffer containing 2 mM [¹⁴C]glucose (5000 d.p.m. nmol⁻¹) and 1 ml samples were collected periodically by centrifugation through silicone oil (1:1 mixture of Dow Corning fluid 550 and 556, specific gravity 1.0182 ± 0.00027) in a microfuge at 15 000 *g* for 3 min. The radioactivity of the vesicles in the pellet was then measured with Aquasol (NEN Canada) in a scintillation counter. Controls consisted of vesicles incubated without added glucose ('unloaded' vesicles). Counts derived in this process represented the total radioactivity associated with the pellet, including any glucose inside the vesicles and that trapped in the extracellular space. The internal radioactivity was calculated by subtracting the label in the external water volume of the sample from the total radioactivity in the vesicle pellet. The data were expressed as the ratio of the intracellular versus extracellular glucose concentration.

Water volume of whole cells and vesicles. The intracellular and extracellular water content of *S. mutans* Ingbritt cells was determined as previously described (Hamilton & Buckley, 1991). The total aqueous volume was determined with ³H₂O (2000 d.p.m. per ml final volume), while the extracellular water volume was measured with [³H]polyethylene glycol (1500 d.p.m. per ml final volume). After incubation with the labelled probe for 10 min, the cell pellets were collected by silicone centrifugation as described above and the radioactivity associated with the cells was measured. The intracellular water volume for *S. mutans* Ingbritt under the experimental conditions employed was 2.49 ± 0.06 µl (mg dry wt)⁻¹ at pH 7.0 and 2.52 ± 0.05 µl (mg dry wt)⁻¹ at pH 5.5, while the extracellular volumes were 0.33 ± 0.03 and 0.36 ± 0.02 µl (mg dry wt)⁻¹, respectively (means ± SE; *n* = 6).

The internal and external water volumes of the vesicles were determined as described above for whole cells, except that the suspensions were in vesicle buffer and centrifugation was through a 65:35 (v/v) silicone oil mixture of Dow Corning fluids 556 and 550 (specific gravity 0.9877 ± 0.00032), respectively. The internal vesicle water volume was 4.46 ± 1.50 µl (mg protein)⁻¹ and the external vesicle water volume was 4.98 ± 2.06 µl (mg protein)⁻¹.

Table 1. PEP- and ATP-dependent phosphorylation of glucose by permeabilized cells of *S. mutans* Ingbritt

Cells were grown in continuous culture at a dilution rate of 0.1 h^{-1} under conditions resulting in optimal (PTS-O) and repressed (PTS-R) PTS activity.

Cells	Glucose concn (mM)	Growth pH	EII ^{glc} activity*	Phosphorylation†		Ratio PEP/ATP‡
				Phosphoryl donor: PEP	ATP	
PTS-O	5	7.0	319§	74.4 ± 5.3	16.4 ± 2.3	4.54
PTS-R	50	5.5	4	6.9 ± 0.8	20.7 ± 3.1	0.33

* nmol (mg membrane protein)⁻¹ min⁻¹ in the presence of excess HPr, EI and III.

† nmol (mg dry wt cells)⁻¹ min⁻¹.

‡ Differences between PEP and ATP as substrates statistically significant at the 95 % level.

§ Adapted from Vadeboncoeur *et al.* (1989).

|| From Vadeboncoeur *et al.* (1987).

Table 2. Intracellular glucose and glucose 6-phosphate of *S. mutans* Ingbritt

Cells were grown in continuous culture at a dilution rate of 0.1 h^{-1} under conditions resulting in optimal (PTS-O) and repressed (PTS-R) PTS activity.

Cells	Medium glucose concn (mM)	Growth pH	Intracellular concn (μM)		Ratio G/G6P	Actual culture glucose (μM)	Ratio G _{in} /G _{out}
			G*	G6P*			
PTS-O	5	7.0	969 ± 125	836 ± 113	1.2	3 ± 1	323
PTS-R	50	5.5	1356 ± 128	264 ± 95	5.1	210 ± 90	6.5

* G, glucose; G6P, glucose 6-phosphate. Comparative values between the PTS-O and PTS-R cells are statistically significant at the 95 % level.

Chemicals. Radioactive glucose was purchased from ICN Radiochemicals. Amino-*n*-caproic acid, *p*-aminobenzamide, mercaptoethanol, lysozyme, mutanolysin, and the enzyme assay reagents were purchased from Sigma. All other reagents were of analytical grade.

RESULTS

Phosphorylation of glucose by ATP and PEP

Previous research indicated that repression of the glucose-PTS in *S. mutans* Ingbritt was associated with an increase in glucokinase activity (Hamilton, 1984). Consequently, a non-PTS system might be a carrier-mediated active-transport process with the incoming glucose molecule phosphorylated internally by glucokinase and ATP. Thus, phosphorylation of glucose by PEP would be a measure of PTS activity, while ATP-dependent phosphorylation could reflect a non-PTS transport system that generates free intracellular glucose that can be phosphorylated by glucokinase. To test for this, PEP and ATP-dependent phosphorylation of [¹⁴C]glucose was measured with permeabilized cells of *S. mutans* Ingbritt grown in continuous culture ($D = 0.1 \text{ h}^{-1}$) under conditions op-

timal for the PTS (5 mM glucose, pH 7.0) (PTS-O cells) and those repressing PTS activity (50 mM glucose, pH 5.5) (PTS-R cells).

As shown in Table 1, PEP-dependent phosphorylation of glucose by PTS-O cells was almost fivefold higher than that by ATP, confirming that the glucose-PTS is the primary mode of glucose transport in these cells. On the other hand, PTS-R cells growing with higher glucose and at a lower pH in condition B exhibited a 90 % decrease in PEP phosphorylation and a 20 % increase in ATP phosphorylation. This change, indicated by the decrease in the PEP/ATP ratio from 4.54 to 0.33, indicates the increased importance of ATP in glucose phosphorylation as the glucose-PTS is repressed. These results support previous suggestions (Hamilton, 1984, 1987) that at least two distinct glucose uptake systems are present in *S. mutans* Ingbritt: one is the PTS and the second involves the intracellular phosphorylation of glucose by ATP.

This hypothesis was tested by analysing the intracellular steady-state levels of glucose and glucose 6-phosphate during growth in continuous culture under the two conditions. The glucose-PTS would result in the ap-

Fig. 1. Electron micrographs of *S. mutans* Ingbritt. (a–d) Negative stain, (e–f) freeze-fracture. (a) Intact cells, (b) protoplasts, (c) vesicles, (d) vesicles at higher magnification, (e) intact cell, (f) vesicles. Bars, 0.1 μm (a, b, d, e, f), 0.5 μm (c).

pearance of glucose 6-phosphate in the cell, while non-PTS glucose transport would generate free intracellular glucose. As seen in Table 2, PTS-R cells possessed

significantly higher free intracellular glucose than PTS-O cells. This supports the contention that the non-PTS system results in the accumulation of free intracellular

Fig. 2. Glucose counterflow by vesicles of *Streptococcus mutans* Ingbritt grown under (a) PTS-optimal (PTS-O) and (b) PTS-repressed (PTS-R) conditions in continuous culture. ●, Glucose-loaded vesicles; □, glucose-unloaded vesicles.

glucose. The intracellular glucose 6-phosphate concentration in the PTS-R cells was also lower, giving a glucose/glucose 6-phosphate ratio fourfold higher than the PTS-O cells. An analysis of the actual glucose concentration in the chemostat culture fluid, as opposed to the incoming medium, indicated that the PTS-O culture was glucose limited (3 μM), while the PTS-R culture contained a very low level of glucose (210 μM). The ratio of the intracellular to extracellular glucose ($G_{\text{in}}/G_{\text{out}}$) was 323 and 6.5, respectively, in the PTS-O and PTS-R cultures.

Vesicles of *S. mutans* Ingbritt

In order to study the non-PTS transport in the absence of PTS transport activity, vesicles of *S. mutans* Ingbritt were prepared by mutanolysin/lysozyme treatment and potassium sulfate lysis. Electron microscopy was used to evaluate the purity and orientation of the vesicle preparations. Mutanolysin/lysozyme treatment completely removed the cell wall and the cytoplasmic contents were clearly visible, indicating protoplast formation (Fig. 1b). The vesicles obtained following potassium sulfate lysis were intact and of high quality, as evidenced by the lack of contaminating cytoplasmic debris (Fig. 1c, d).

Freeze-fracture of *S. mutans* Ingbritt whole cells (Fig. 1e) resulted in the removal of the cell wall and outer layer of the membrane, revealing the hydrophobic, intramembranous region with clustered particles. Other whole-cell preparations had intramembranous particles at the midline along the poles exposed in dividing cells (data not shown). More than 90% of vesicles examined by this technique had the same orientation as the whole cells as identified by the aggregation of particles on the inner surface of the membrane (Fig. 1f).

Table 3. Activity of the cytoplasmic enzymes glucokinase, pyruvate kinase and lactate dehydrogenase in vesicles of *S. mutans* Ingbritt compared with that in cell-free extract and membrane preparations

Activities are given as nmol product formed (mg protein)⁻¹ min⁻¹. ND, Not detected.

Sample	Glucokinase	Pyruvate kinase	Lactate dehydrogenase
Cell-free extract	269 $\pm$ 20	75 $\pm$ 3	185 $\pm$ 16
Vesicles	14 $\pm$ 3	9 $\pm$ 1	13 $\pm$ 5
Membrane fraction	ND	12	7

Biochemical properties of vesicles

Cytoplasmic contamination of the vesicles was evaluated by comparing the levels of glucokinase, pyruvate kinase and lactate dehydrogenase in membranes, vesicles and crude-cell extracts (Table 3). The activity of the three enzymes in vesicles relative to that in crude-cell extracts was only 0.05, 0.12 and 0.07, respectively, and represented activity typical of membrane preparations from the same cells. Thus, potassium sulfate lysis rendered the vesicles essentially free of cytoplasmic enzymes, a fact confirmed by the lack of glycolytic activity by vesicles incubated with glucose in a pH-stat (data not shown).

The sidedness of the vesicles was tested by assaying for H⁺-ATPase, a membrane-bound enzyme whose catalytic portion faces the cytoplasm in intact cells (Futai & Kanazawa, 1983). Right-side-out vesicles will not display enzyme activity since ATP cannot traverse the membrane, whereas activity will be detected with inside-out vesicles since the catalytic portion of the H⁺-ATPase will be exposed to the exterior. Normal intact vesicles possessed no H⁺-ATPase activity, indicating that they were of the same orientation as whole cells. As a control, vesicles were disrupted by treatment with SDS (SDS:protein ratio 1:1) and again assayed for H⁺-ATPase activity. Significant H⁺-ATPase activity [21.7 nmol phosphate (mg protein)⁻¹ min⁻¹] was detected, indicating that the SDS treatment resulted in exposure of the catalytic portion of the H⁺-ATPase to ATP and confirming the right-side-out nature of the vesicles. Furthermore, H⁺-ATPase activity in membranes similarly treated with SDS was reduced fourfold, from 45.5 nmol phosphate released min⁻¹ (mg protein)⁻¹ to 11.6 nmol phosphate released min⁻¹ (mg protein)⁻¹, indicating that the vesicles contained significant activity.

Counterflow

Counterflow experiments were performed on vesicles prepared from cells of *S. mutans* Ingbritt grown under conditions A and B to test for the presence of a glucose carrier. Counterflow predicts that when the vesicles are loaded with unlabelled glucose and then exposed to labelled glucose, entry through a glucose-specific carrier

will be unaltered, but its exit will be inhibited as it competes with unlabelled substrate for the carrier inside the cell. Thus, if a carrier is present in the vesicle membrane, transient accumulation of glucose will be observed, whereas glucose will not accumulate in the absence of a transport carrier, i.e. simple diffusion. As seen in Fig. 2(a) 'glucose-loaded' vesicles of *S. mutans* Ingbritt prepared from PTS-R cells showed transient glucose accumulation, while no accumulation was observed with 'unloaded' vesicles, demonstrating the presence of a carrier capable of transporting glucose. Similar results were obtained with five different vesicle preparations and the accumulation was independent of pH. Interestingly, 'glucose-loaded' vesicles prepared from PTS-O cells containing high PTS activity also demonstrated counterflow (Fig. 2b).

DISCUSSION

The method devised for vesicle preparation with *S. mutans* Ingbritt reported here, as with other vesicle methods (Kaback, 1972), consisted of two stages: protoplast formation followed by lysis and re-formation of the membrane as vesicles. However, unlike previous reports (Eisenberg & Lillmars, 1975; Chassey & Giuffrida, 1980; Siegel *et al.*, 1981; Schöller *et al.*, 1983), protoplasts generated by lysozyme and mutanolysin in our system showed little lysis in the absence of osmotic stabilizers. Goodman *et al.* (1981) reported that significant cell wall loss could occur without acquisition of osmotic sensitivity by protoplasts of *S. mutans* and suggested that strategic cell wall remnants could protect the cell. Nevertheless, electron micrographs of our preparations generated without osmotic stabilization (Fig. 1b) indicated complete loss of cell wall material after mutanolysin and lysozyme treatment, and the vesicles obtained after potassium sulfate lysis of the protoplasts and sucrose density gradient centrifugation were homogeneous, with little contaminating membrane debris.

The vesicles obtained were suitable for transport carrier studies in that they were intact, and possessed the same membrane sidedness as whole cells as shown by freeze-fracture electron microscopy (Fig. 1e) and the absence of the membrane H^+ -ATPase activity. Furthermore, the vesicles were essentially devoid of the cytoplasmic enzymes glucokinase, pyruvate kinase and lactate dehydrogenase (Table 3), and therefore would lack the soluble, general PTS proteins, EI and HPr, required for the transport of all PTS sugars. As expected, the vesicles exhibited no glycolytic activity, and therefore would be unable to generate ATP and PEP to support active transport even from endogenous energy reserves. This is supported by the observation that unloaded cells in the counterflow experiment did not take up labelled glucose (Fig. 2).

The surprising feature of the counterflow experiment was the transient accumulation of labelled glucose by the glucose-loaded vesicles of *S. mutans* Ingbritt grown under both the PTS optimal conditions and the PTS repressed conditions. Transient accumulation indicates the presence

of a carrier that can recognize glucose and since this occurred with vesicles from both cell types, it is apparent that the carrier is constitutive. It could be argued that counterflow was due to an uncoupled EII^{glc} of the PTS. This is unlikely for several reasons. Studies with mutants of *Salmonella typhimurium* defective in HPr and EI lost all ability to grow on PTS sugars unless they possessed alternative non-PTS mechanisms of transport (Postma & Stock, 1980). In addition, other mutants of *E. coli* and *S. typhimurium* have been reported that contain EII enzymes able to mediate the facilitated diffusion of substrates, but in all cases, the mutations occur in the genes coding for the EII (Ruijter *et al.*, 1992). Robillard (1987) carried out extensive studies on wild-type EII for mannitol in *E. coli* both in vesicles and proteoliposomes. These workers have reported that some facilitated diffusion can be observed, but at rates too low to sustain the organisms. Finally, *S. mutans* Ingbritt regulates the glucose-PTS at the genetic level through repression (80-fold) of the EII enzyme under growth conditions similar to those used in this study (Hamilton *et al.*, 1989; Vadeboncoeur *et al.*, 1987; Table 1). Thus, it appears highly unlikely that the carrier mediating transport is a membrane-associated EII enzyme. Whether the carrier is specific for glucose alone remains to be determined.

Dasher & Reynolds (1990) have proposed that simple or passive diffusion accounts for non-PTS glucose transport in de-energized cells of *S. mutans*. On teleological grounds, relevant carbohydrate transport by passive diffusion in bacteria is questionable. As stated by Saier (1985), 'as transmembrane permeation represents the first step in the catabolism of exogenous sugars, one would expect the process to be subject to stringent regulatory control'. This would exclude carbohydrate transport by simple diffusion, since the only regulation of such transport would be through induction and repression of pore expression. Saccharolytic oral bacteria are exposed to large, rapid carbohydrate fluxes in the oral cavity (Carlsson, 1986) and it is difficult to envisage how this process would allow for the stringent control suggested by Saier. Experimentally, it has been recognized that passive diffusion of carbohydrates is not a relevant physiological event (Roseman, 1969; Kaback, 1972; Postma & Stock, 1980; Saier, 1985; Robillard, 1987), with the exception of the glycerol permease which mediates pore-type diffusion in several bacteria, but not streptococci (Saier, 1985). In addition, simple diffusion does not display counterflow, and therefore would not account for the results obtained in this study.

Based on previous data (Hamilton, 1987), a non-PTS glucose carrier in the membrane of *S. mutans* cells would function at high glucose concentrations, at low pH and at high growth rates, i.e. conditions of glucose-PTS repression. Such a carrier might function to allow facilitated diffusion of glucose into the cell, particularly at high exogenous glucose levels, without any cost to the cell. Indeed, Russell (1990) has reported the presence of a glucose permease in *S. bovis*, the level of which was directly proportional to the external sugar concentration with saturation at 3.6 mM glucose. Facilitated diffusion

Table 4. Observed and predicted growth yields of *S. mutans* Ingbritt growing in continuous culture at dilution rates from 0.05 to 0.4 h⁻¹ (pH 6.5) with limiting and excess glucoseAdapted from Hamilton *et al.* (1978) (Table 1).

Medium glucose	Predominant metabolism	Observed yield*	Predicted yield*	Observed ATP yield†
Limiting	Heterofermentation	32.8 ± 3.2	31.5	3.12
Excess	Homofermentation	9.7 ± 2.2	10.5	0.92

* Yield = g dry wt cells (mol glucose)⁻¹.† Yield = mol ATP (mol glucose)⁻¹ (assuming 10.5 g cell wt per mol ATP).

would appear not to account for the results with intact cells of *S. mutans* Ingbritt since the glucose concentration gradient across the membrane during growth (G_{in}/G_{out} 323 to 6.5; Table 2) indicates the requirement for active transport.

The existence of a non-PTS glucose transport system in the oral streptococci is supported by recent results showing glucose uptake in strains with altered PTS activity. In our laboratory a mutant of *S. mutans* BM-71 was generated possessing a truncated Enzyme I, lacking 150 amino acids at the C-terminal end. The EI in this strain cannot be phosphorylated by ³²PEP and yet can metabolize glucose (unpublished results). A similar observation has been made with an EI-deficient mutant of *S. salivarius* (C. Vadeboncoeur, personal communication).

Facilitated diffusion is usually seen with 'uncoupled' active transporters, that is, active transporters in de-energized cells, such as the *lac* permease that mediates the active transport of lactose in association with proton motive force (Maloney *et al.*, 1975). In the absence of an electrochemical gradient, the permease mediates the equilibration of the sugar across the membrane. The counterflow detected in cells of *S. mutans* Ingbritt (Fig. 2) could reflect the presence of an 'uncoupled' active transporter. The original model of non-PTS glucose transport in *S. mutans* was a proton-motive force coupled process that was based on the sensitivity of glucose transport to agents known to perturb the proton electrochemical gradients (Hamilton & St Martin, 1982). Experiments performed by Dashper & Reynolds (1990), and confirmed by us (unpublished results), appeared to negate this possibility by demonstrating no uptake of the glucose analogue 6-deoxyglucose in response to applied proton and electrical gradients. In addition, the transmembrane gradients of *S. mutans* during growth under conditions typical of dental plaque are probably too low to sustain active transport of glucose (Hamilton, 1990), although leucine transport appears to be linked to proton-motive force (Dashper & Reynolds, 1993).

Some form of high-energy phosphate-bond linked transport process may function as the non-PTS glucose transport in *S. mutans*. Examples of this type of transport include the Na⁺-ATPase of *Enterococcus faecalis*, which

accumulates Na⁺ in the cell at the expense of ATP (Kobayashi *et al.*, 1978), and transporters for various amino acids in *E. faecalis* and *Lactococcus cremoris* mediating the uptake of L-glutamate, L-aspartate and L-asparagine (Konings *et al.*, 1987). It has also been suggested that in *S. mutans*, glucose uptake could occur via the binding-protein-dependent sugar uptake system recently reported by Russell *et al.* (1992). This multiple sugar system (*Msm*) is encoded on a 12 kb multi-gene region of the *S. mutans* chromosome and is primarily responsible for the transport of raffinose, melibiose and isomaltosaccharides. One essential gene in this operon is *msmK*, which codes for an ATP-binding protein, the first such protein identified in Gram-positive bacteria. A recent report (Tao *et al.*, 1993) indicated that unlabelled glucose could effectively disrupt transport of radiolabelled melibiose by *S. mutans*, suggesting the possibility of glucose transport by the *Msm* system.

Whether the transport carrier observed in this study is a component of the *Msm* system or a separate glucose-specific system is unknown and certainly warrants further investigation. Glucose transport via both systems would require intracellular phosphorylation of glucose by glucokinase and ATP or an equivalent process. Glucokinase is a constitutive enzyme in *S. mutans* Ingbritt; however, its activity is modulated in an inverse relationship with the glucose-PTS (Hamilton, 1984). This is confirmed by the increase in the ATP-dependent phosphorylation of glucose under PTS-repressed conditions (Table 1). Thus, despite variations in growth conditions, the cells are capable of the intracellular conversion of glucose to intermediates of the glycolytic pathway.

What is more problematic is the nature of the transport process itself. Analysis of growth yields of cells grown under PTS-repressed and PTS-optimal conditions suggests that ATP or an equivalent high-energy molecule must be involved in the transport process. This analysis compares the yield coefficients [g (dry wt cells) mol glucose⁻¹] for cells of *S. mutans* Ingbritt grown anaerobically in continuous culture under conditions where the glucose-PTS dominates (limiting glucose) and where the PTS is repressed (excess glucose) (Hamilton, 1987). Since *S. mutans* is hetero- and homofermentative with limiting

and excess glucose, respectively (Carlsson & Griffith, 1974), cell yields can reflect ATP yields, with a value of $10.5 \text{ g bacteria (mol ATP)}^{-1}$ generally being accepted for most bacteria regardless of the substrate source (Gottschalk, 1978). As shown in Table 4 with data generated in a previous chemostat study with *S. mutans* Ingbritt (Hamilton *et al.*, 1979), PTS-dominant cells yielded $32.8 \text{ g dry wt cells (mol glucose)}^{-1}$ or $3 \text{ mol ATP (mol glucose)}^{-1}$ for cells grown with limiting glucose at dilution rates 0.05 to 0.4 at pH 6.5. This is consistent with the expected ATP yield from cells utilizing the PTS sugar transport system and recovering an extra ATP during acetate formation via acetyl kinase. On the other hand, PTS-repressed cells, grown with excess glucose under the same conditions, yielded $9.7 \text{ g (mol glucose)}^{-1}$ or $1 \text{ mol ATP (mol glucose)}^{-1}$. This would be in agreement with a phosphate-bond-driven transport in which one ATP is used to bring glucose into the cell and lactate is the sole acid end-product. This analysis is consistent with an ATP-binding protein model such as the *Msm* system, but it does not exclude the possibility of a separate permease for glucose.

From the ecological perspective of *S. mutans* in dental plaque, the PTS would function as the high-affinity system most of the time since normal plaque has a very low carbohydrate content. However, with frequent dietary sugar consumption, which promotes the formation of dental caries, the combination of the high sugar concentration and the resultant low pH would favour the proposed phosphate-bond-driven transport system. Thus, this system would be the dominant glucose transport system under conditions promoting the pathogenicity of *S. mutans* in dental plaque.

ACKNOWLEDGEMENTS

This work was supported by the Medical Research Council of Canada Grant MT-3546 to I.R.H. During a portion of this research, N.D.B. was the recipient of a Manitoba Health Research Council Studentship. We thank Dr T. J. Beveridge, University of Guelph, for his valuable assistance with respect to the electron microscopy of our preparations and Elke Stanik for her assistance with vesicle counterflow experiments.

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Received 4 February 1994; revised 22 April 1994; accepted 16 May 1994.