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# Properties of *Streptococcus mutans* Ingbritt Growing on Limiting Sucrose in a Chemostat: Repression of the Phosphoenolpyruvate Phosphotransferase Transport System

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Growth of *Streptococcus mutans* Ingbritt on limiting sucrose in a chemostat at dilution rates of 0.05 to 0.4 $h^{-1}$ (mean generation time, 14 to 1.7 h) resulted in a heterofermentative pattern of metabolic end products. During fast growth, lactic acid was the major end product, whereas at slower growth rates, acetic and formic acids, as well as ethanol, increased to be major end products. The patterns obtained were similar to those seen with the same organism growing on glucose. The glycolytic rate by washed cells was maximum at the lowest dilution rates and decreased as the cells were made to grow faster. Transport of sucrose, glucose, and fructose via the phosphoenolpyruvate phosphotransferase system (PTS) was repressed during growth on sucrose after growth on glucose. Uptake rates suggested that sucrose was transported in the PTS as the intact disaccharide. Comparison of the rate of sugar uptake in the chemostat with the rate of PTS activity in the cells at each growth rate indicated that the PTS was capable of supporting growth only at a dilution rate of 0.05 $h^{-1}$. Growth on sucrose at faster growth rates required the activity of a second transport system, supporting our earlier observations with glucose that *S. mutans* contains at least two sugar transport systems.

Dental caries is caused by acid demineralization of dental enamel through the action of acidogenic bacteria degrading dietary sugar. The principal acidogenic bacteria associated in the carious process are streptococci and, in particular, *Streptococcus mutans*, which has been implicated in some forms of human dental caries (13, 16). Whereas oral streptococci are capable of degrading a variety of carbohydrates, their ability to metabolize sucrose is of particular interest (9, 17) since this disaccharide constitutes a significant portion of the diet in developed nations (1). Furthermore, early studies have established the cariogenicity of sucrose in animals monoinfected or super-infected with oral bacteria (8, 10).

We have undertaken the detailed study of the biochemical properties of *S. mutans* Ingbritt grown with glucose under a variety of conditions of growth rate, glucose concentration, and pH. Comparison of cultures grown on limiting and excess glucose concentrations indicated changes in the pattern of metabolic end product formation, cell yields, and fluoride sensitivity (12). Furthermore, these studies have demonstrated that the transport of glucose via the phosphoenolpyruvate (PEP) phosphotransferase system (PTS) is maximum under conditions of slow growth (mean generation time, 6 to 12 h) on limiting concentrations of glucose and was repressed by low pH, excess glucose levels, and growth rates approaching the maximum (7, 11). In fact, it could be shown that the glucose-PTS was sufficient to account for transport during growth in continuous culture only at mean generation times of longer than 4 h when growing with limiting glucose. A second transport system was shown to be necessary at faster growth rates and during growth with excess glucose (7).

Representatives of the major serotypes of *S. mutans* have been shown to contain sucrose-PTS activity (18, 20). Sucrose is transported by this system as the intake disaccharide and phosphorylated in the 6-position of the glucose moiety. Sucrose-phosphate is subsequently cleaved to fructose and glucose-6-phosphate. Recent batch culture studies with serotype *c* and *d/g* strains of *S. mutans* indicate that sucrose-PTS activity is repressed by both glucose and high concentrations of sucrose (19). The sucrose-PTS in the latter cultures was derepressed in the stationary phase, whereas increased sucrose-PTS activity was observed at low pH during slow growth on low (2 mM) concentrations of sucrose. Since *S. mutans* Ingbritt 1600 was reported to contain sucrose-PTS activity (18),

we were interested in determining the properties of our particular strain at various growth rates (mean generation time, 1.7 to 14 h) in continuous culture with a sucrose limitation. The results indicate that a change from growth on glucose to growth on sucrose resulted in repression of PTS activity for sucrose, glucose, and fructose. Furthermore, despite a sucrose limitation, sucrose-PTS activity in our strain was only capable of supporting growth in the chemostat at the lowest dilution rate corresponding to a mean generation time of 14 h. This confirms our earlier finding that the growth of *S. mutans* Ingbritt at the faster rates is mainly supported by a non-PTS transport system.

## MATERIALS AND METHODS

**Organism.** The *S. mutans* Ingbritt strain used in this study was kindly supplied by J. Sandham, Toronto, Ont., Canada. The methods of maintenance and purity control have been described previously (7).

**Growth conditions.** Cultures were grown as previously described (7) in a Porton-type chemostat with a 500-ml working capacity; pH was maintained at 6.5, and the gas phase was nitrogen plus 5% $CO_2$. The growth medium was that of Carlsson (M3) modified by increasing the concentrations of aspartic acid and cysteine to 400 mg/liter and those of leucine, isoleucine, and lysine to 200 mg/liter, while removing glucose and acetate (2). In addition, $MgSO_4 \cdot 7H_2O$ was increased to 400 mg/liter and $KH_2PO_4$ and $K_2HPO_4$ were decreased to 1.33 and 3.66 g/liter, respectively. To provide for conditions of limiting sugar, the sucrose concentration in the medium was 5 mg/ml. Growth of the culture was initiated on glucose (2 mg/ml) at a dilution rate ($D$) of 0.4 $h^{-1}$ (low PTS conditions) and then changed to sucrose-limited media at $D$ = 0.05 $h^{-1}$. The dilution rate was increased to 0.1, 0.2, and 0.4 $h^{-1}$ in stages, with the culture allowed to reach equilibrium for at least 10 generations at each dilution rate before harvesting. Analysis for total carbohydrate (5) was carried out on the spent medium at each dilution rate to ensure that the culture was sucrose limited.

**Washed-cell experiments.** Cells were collected for various periods via the overflow from the chemostat into a container cooled to 0°C (usually overnight, but never exceeding 16 h). They were then harvested by centrifugation (8,000 × $g$, 15 min) and washed once in potassium phosphate buffer (20 mM, pH 7.0). The cells were resuspended in saline (0.98% NaCl in water) at a concentration of ≈30 mg (dry weight)/ml and stored in ice until used (maximum time, 6 h). The rate of acid production from the anaerobic fermentation of sucrose, glucose, and fructose was studied with washed cells of *S. mutans* under conditions of constant pH in a pH stat, as previously described (12). Throughout this work, units of glycolytic activity are defined as nanomoles of metabolic acid neutralized per milligram (dry weight) of cells per minute. In all cases, at least two samples were removed at each dilution rate for analysis, with enough time between samples to reestablish steady-state conditions. The results of all tests and assays were always reproducible within 10%.

**Assay for PEP-PTS activity.** Sugar transport via the PEP-PTS system was assayed by the method of Kornberg and Reeves (15) with washed cells made permeable with toluene. Cell samples (50 ml) were removed directly from the chemostat, centrifuged at 10,000 × $g$ (15 min), washed by centrifugation, suspended in phosphate buffer (50 mM, pH 7.0) at a concentration of 4 to 10 mg (dry weight) of cells per ml, and mixed vigorously on a Vortex mixer with 0.01 volume of toluene for 60 s. The conditions of assay were as previously described (8). Net PTS activity was expressed as nanomoles of pyruvate formed per milligram (dry weight) of cells per minute.

**Analytical procedures.** Glucose and metabolic end products present in the culture fluid were assayed as previously described (11). Culture fluid was obtained by rapid filtration (<5 s) to remove cells. Amino acids were analyzed by the method of Ellwood et al. (6).

## RESULTS

**Effect of growth rate on cell yields and metabolic products.** The growth of *S. mutans* Ingbritt with limiting sucrose at $D$ = 0.05, 0.1, 0.2, and 0.4 $h^{-1}$, corresponding to mean generation times of 14, 7, 3.5, and 1.7 h, respectively, resulted in a heterofermentative pattern of metabolic end products (Table 1). These results are similar to those for the same organism growing with limited glucose (12) and other oral streptococci growing on glucose (3, 22). During slow growth ($D$ = 0.05 to 0.1 $h^{-1}$) lactic acid is only a minor end product of metabolism, whereas acetic and formic acids, as well as ethanol, are the major products. On the other hand, as the growth rate increased, the concentration of lactic acid increased until at $D$ = 0.4 $h^{-1}$ it accounted for 80% of the carbon fermented. End product recoveries ranged from 84 to 101%.

The yield of cells increased as the growth rate was increased, and this was also reflected in the molar growth yield (i.e., $Y_{glucose}$) such that the value at $D$ = 0.4 $h^{-1}$ was 45% higher than at $D$ = 0.05 $h^{-1}$. The major amino acids utilized during growth were arginine (90 to 95%), aspartic acid (53 to 59%), alanine (25 to 43%), glutamic acid (17 to 18%), and glycine (11 to 15%).

**Glycolytic activity.** Washed cells obtained from the chemostat at each steady-state growth rate were measured for their glycolytic activity in a pH stat at the pH of growth (6.5) with sucrose, glucose, and fructose. The glycolytic rate with sucrose and glucose was highest at the lowest dilution rates and decreased at rates above $D$ = 0.2 $h^{-1}$ (Fig. 1). Fructose metabolism by sucrose-grown cells, on the other hand, was 22 to 60% lower than that for the other sugars and increased slightly with the increased growth rate. The pattern of sucrose metabolism in this experiment is similar to that observed for metabolism of glucose-grown cells (7). The exception was for cells growing at $D$ = 0.04 $h^{-1}$ with

TABLE 1. Effect of dilution rate on growth parameters and metabolic end products of a culture of *S. mutans* Ingbritt grown under condition of sucrose limitation[a]

| $D$ ($h^{-1}$) | Determination | | | | | | |
|---|---|---|---|---|---|---|---|
| | Yield of cells (mg [dry wt]/ml) | Residual sucrose (mg/ml) | $Y_{sucrose}$ (g of cells/mol of sucrose) | Metabolic product (mg/ml) | | | |
| | | | | Lactic acid | Acetic acid | Formic acid | Ethanol |
| 0.05 | 0.73 | 0 | 52.5 | 0.25 | 0.89 | 1.85 | 1.85 |
| 0.1 | 0.81 | 0 | 58.3 | 0.46 | 0.87 | 1.02 | 1.71 |
| 0.2 | 0.86 | 0 | 61.9 | 2.18 | 0.49 | 0.99 | 1.33 |
| 0.4 | 1.06 | 0 | 76.3 | 3.19 | 0.39 | 0.17 | 0.89 |

[a] Values represent the average of at least two determinations.

sucrose, where the glycolytic rate exceeded that of cells grown on glucose.

**PEP-PTS activity.** From a comparison of the data in Table 1 and Fig. 1 with similar data in our earlier experiment (7, 12), it is clear that carbohydrate uptake and metabolism of sucrose-grown cells of *S. mutans* Ingbritt are not substantially different from those of glucose-grown cells. We therefore undertook to determine whether sucrose transport via the PTS was also the same. Initially the culture was grown on glucose as the limiting carbon source at $D = 0.4$ $h^{-1}$ (i.e., conditions repressing PTS activity) and then changed to the same medium with limiting sucrose as the carbon source at $D = 0.05$ $h^{-1}$. Previous data (7) had indicated that the PTS at this latter growth rate was capable of fully supporting growth when glucose was the limiting carbon source. Nevertheless, a change in the growth conditions from fast growth on limiting glucose to slow growth on sucrose resulted in repression of PTS activity for glucose and sucrose, but not for fructose (Table 2). Further growth on sucrose at $D = 0.1$, 0.2, and 0.4 $h^{-1}$ resulted in progressively lower levels of PTS activity for the three sugars, with very little difference between them (Fig. 2). This pattern is quite distinct from that seen for sucrose- and glucose-PTS activity with cells grown on glucose (broken lines, Fig. 2).

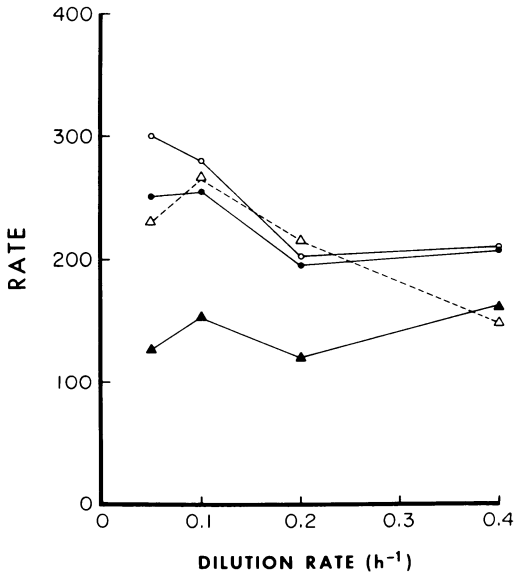


FIG. 1. Effect of exogenous carbon source on the glycolytic rate of washed cells of *S. mutans* Ingbritt grown at different growth rates under conditions of sucrose limitation at pH 6.5 and incubated with glucose (●), sucrose (○), and fructose (▲). Broken line (△) is sucrose from reference 7. Rate is given in nanomoles per milligram (dry weight) of cells per minute.

**Sucrose transport in the chemostat.** From the data in Table 1, it is possible to calculate actual sucrose uptake by the cells in the chemostat (7). When this is plotted (Fig. 3), the total transport activity is shown to increase with growth rate. By adding the sucrose-PTS activity data to this diagram, one can see that PTS activity with sucrose is adequate to account for total transport only at $D = 0.05$ $h^{-1}$. The data for glucose-PTS activity follows a similar course. As the growth rate is increased, therefore, it is apparent that higher levels of non-PTS system transport activity must be present to account for the actual growth of the cells in the chemostat. This clearly confirms our earlier observations with glucose-grown cells (7) and confirms the observations of Slee and Tanzer (18). Comparison of the actual acid produced in the chemostat with the theoretical acid formed as calculated from the sucrose consumed shows the curves to be coincident,

TABLE 2. PEP-PTS activity in cells of *S. mutans* Ingbritt after a change from rapid growth on glucose to slow growth on sucrose

| Carbon source | $D$ ($h^{-1}$) | PTS activity (nmol/mg [dry wt] of cells per min) | | |
|---|---|---|---|---|
| | | Glucose[a] | Sucrose | Fructose |
| Glucose | 0.4 | 62.9 | 45.3 | 28.2 |
| Sucrose | 0.05 | 37.0 | 24.6 | 32.4 |

[a] Substrate in PTS assay.

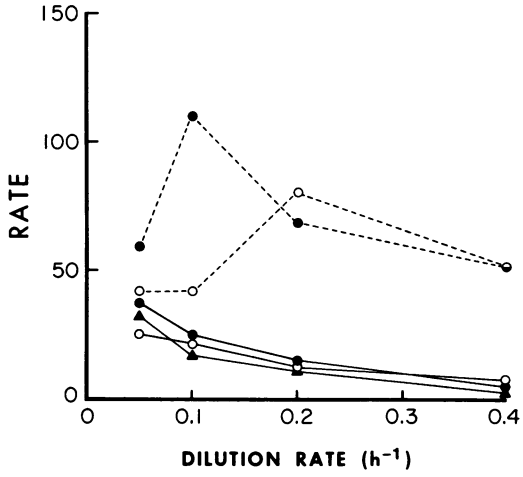


FIG. 2. PEP-PTS activity in cells of *S. mutans* Ingbritt grown under conditions of sucrose limitation at different growth rates in a chemostat. Substrate in the assay: glucose (●), sucrose (○), and fructose (▲). Broken lines are activities for glucose and sucrose in reference 7. Rate is given in nanomoles per milligram (dry weight) of cells per minute.

indicating that all of the substrate carbon is metabolized to metabolic end products.

## DISCUSSION

The growth of *S. mutans* Ingbritt in a chemostat with a sucrose limitation resulted in yields and end products not unlike those obtained by growing the same cells with limiting glucose (12). The yield of cells was similar for all four growth rates, and the molar growth yield was approximately twice that for glucose, as one would expect. Furthermore, although quantitatively different, the general pattern of the metabolic end products was the same for the two carbon sources. Lactate was a minor product for slowly growing cells, increasing until it was the major metabolic product at $D = 0.4\ h^{-1}$. Conversely, acetic and formic acids were major components during slow growth ($D = 0.05\ h^{-1}$) and decreased to minor components at $D = 0.04\ h^{-1}$. Only with ethanol was the pattern different between the sucrose- and glucose-grown cells. With the latter cells, ethanol production was relatively constant over the growth range $D = 0.05\ h^{-1}$ at $1.05 \pm 0.18$ mg/ml. With sucrose as the carbon source, on the other hand, the concentration decreased steadily with increasing growth rate such that at $D = 0.1\ h^{-1}$ the ethanol concentration was only half that at $D = 0.05\ h^{-1}$ (Table 1).

Table 1 also reveals increasingly higher molar growth yields as the dilution rate was increased from 0.05 to 0.4 $h^{-1}$ (i.e., 52.5 to 76.3 g of cells per mol of sucrose), indicating more efficient utilization of the carbon source by the faster-growing cells. Since the more slowly growing cells were more heterofermentative, one would have expected more available energy (3) and, consequently, a higher yield at the slower rates than that observed with the homofermentative, faster-growing cells. The reason for these results is unknown; however, a similar finding was obtained in our previous study with *S. mutans* growing on both glucose and nitrogen limitations under the same conditions, and Jacques et al. (14) obtained similar results with the same organism in a recent study in Australia. Furthermore, similar findings have been obtained with other bacteria (4, 21).

Although the increased formation of lactate at the high growth rates can be explained on the basis of the regulation of lactic dehydrogenase and pyruvate formate-lyase by high cellular levels of certain glycolytic intermediates (3, 22), the relationship of the growth rate to the growth yield is less clear. Figure 3 clearly shows that sucrose was converted to metabolic end products stoichiometrically, indicating that the growth yield was not a function of channelling sucrose carbon for biosynthesis. One possible explanation would be the requirement of the more slowly growing cells for more maintenance energy than cells growing at the higher rates. An

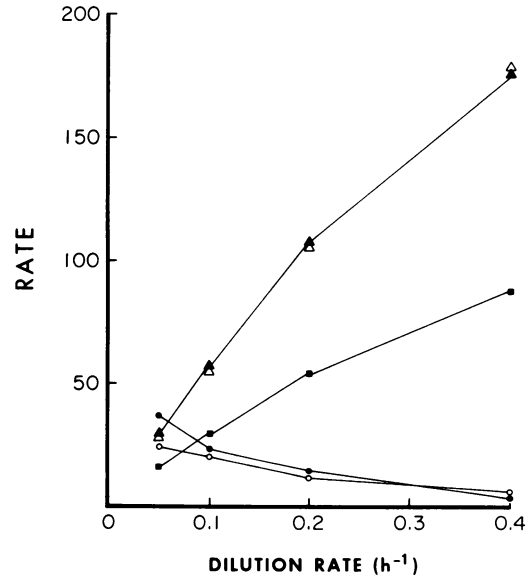


FIG. 3. Comparison of the rate of glucose (●) and sucrose (○) transport via the PTS system with the rate of sucrose uptake (■) and acid formation (▲) by cells of *S. mutans* Ingbritt growing sucrose limited in a chemostat. Theoretical rate of acid production (△). Rate is given in nanomoles per milligram (dry weight) of cells per minute.

alternate explanation would have the growth of the slower-growing cells limited by a second unknown nutrient essential for growth.

The glycolytic rates for washed cells grown in the chemostat with sucrose were also similar to those grown with glucose (12) when incubated with glucose and sucrose in a pH stat. With both sugars, maximum glycolytic activity occurred with cells obtained from either $D = 0.05$ or 0.1 $h^{-1}$, with the rate decreasing at higher dilution rates. However, despite similarities in the overall glycolytic activity and end product profiles of cells grown on glucose and sucrose, substantial differences can be seen in the levels of the enzymes of the PTS transport system (Fig. 2). It is apparent that limiting concentrations of sucrose in the growth medium repressed PTS activity for sucrose, glucose, and, to a lesser extent, fructose. A somewhat similar finding was observed in batch experiments when *S. mutans* NCTC 10449 (19) was grown in the presence of both sucrose and glucose. In batch experiments with *S. mutans* NCTC 10449 grown in the presence of 5 mM sucrose, it was found that at the end of logarithmic growth, induction of a specific sucrose-PTS occurred. This suggests that induction occurred when the sucrose level fell to a very low level indeed. In the experiments reported here, it was found that sucrose at 14 mM in the growth medium led to sucrose limitation. However, in our experiments the induction of a specific sucrose-PTS did not take place. This suggests that the level of sucrose which was limiting in the chemostat was not sufficiently low enough to induce a specific PTS uptake system. An alternative explanation is, of course, that there are strain differences between these two types of *S. mutans* or between the batch and continuous systems.

It should be noted that the experimental protocol used could not distinguish between authentic sucrose-PTS activity and PTS activity for the constituent monosaccharides after cleavage of the disaccharide. We have determined that cells contain more than adequate levels of invertase activity to account for cleavage of the sucrose glycosidic bond. However, since the PTS activity with sucrose was almost identical to that for glucose alone and since the assay measured total PTS activity regardless of the number of sugars present, one must conclude that sucrose was transported intact via a separate enzyme II. Cleavage to glucose plus fructose before transport should have given values greater than that for sucrose alone.

The most pertinent finding relative to sugar transport is seen in Fig. 3, which compares the rate of transport in the chemostat with the actual PTS activity at each dilution rate. Only at the lowest growth rate was sucrose-PTS activity adequate to account for the growth of the organism in the chemostat. From this comparison, it is apparent that the organism possesses at least one additional non-PTS sugar transport system to account for the increased transport requirements as the dilution rate was increased above 0.05 $h^{-1}$. This picture is almost identical to that seen with glucose (7), except that with the latter sugar the "threshold" for PTS activity was slightly higher ($D = 0.17$ $h^{-1}$) due to the increased PTS activity. Further confirmation of the existence of an additional transport activity was observed earlier during experiments measuring the effect of growth at low pH on PTS activity (11). Low growth pH (e.g., 5.5) resulted in repression of glucose-PTS activity while increasing overall glycolytic activity far beyond that capable of being supported by the PTS system.

The repression of the PTS system by various growth conditions with glucose (7, 11) and sucrose (Fig. 2) without significant effect on growth yields suggests that the non-PTS transport activity may be constitutive and functioning simultaneously with the PTS system. The evidence presented here and elsewhere (7, 11, 12a) indicates that the PTS system is the high-affinity system, whereas the non-PTS transport system is a low-affinity system less sensitive to the inhibiting effects of fluoride.

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