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Effect of Growth Rate and Glucose Concentration on the Biochemical Properties of *Streptococcus mutans* Ingbritt in Continuous Culture

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A comparison was made of the properties of *Streptococcus mutans* Ingbritt grown in continuous culture under conditions of excess glucose (nitrogen limitation) and limiting glucose at mean generation times of 1.7 to 14 h. Only low levels of glucoamylase-specific glycogen were formed in cells from either culture, and the total carbohydrate content of the cells under excess glucose was only at most 1.6-fold higher than in the glucose-limited culture. A negligible amount of cell-free polysaccharide was formed in either culture, although a significant level of glucosyltransferase activity was observed in both, with the highest activity at $D = 0.2$ and 0.4 h^{-1} with a glucose limitation. Other differences were observed. (i) Lactate was the main end product of the glucose-excess culture, whereas acetate, formate, and ethanol were the main products of the glucose-limited culture except at a mean generation time of 1.5, when lactate represented 30% of the products. (ii) The yield (in grams per mole of glucose) of the latter culture was 2.6- to 4.0-fold higher than the yield of the glucose-excess culture. (iii) Washed cells from the glucose-limited culture were much more acidogenic (1.7- to 6.2-fold) than the glucose-excess cells when incubated with glucose, sucrose, and fructose. Endogenous glycolytic activity by the latter cells was significant, being 31 to 92% of the exogenous glucose rate at the four dilution rates. (iv) Cells from the glucose-excess culture were more insensitive to fluoride than cells from the glucose-limited culture. The NaF 50% inhibition dose values for the effect of fluoride on the metabolism of glucose, sucrose, and fructose were calculated for the four dilution rates at four pH values. This analysis indicated that rapidly metabolizing cells were more sensitive to fluoride than cells that metabolized the sugars more slowly.

When studies of bacterial metabolism are undertaken, the growth of bacteria in continuous culture has distinct advantages over growth in batch systems (18, 30). The principal advantage is that the growth rate of the culture can be controlled by regulating the dilution rate provided that an essential nutrient is limiting growth. Control of the growth rate, which cannot be done readily in batch systems, is particularly important when one wishes to study the properties of microorganisms at the slow growth rates that these organisms often achieve in their natural ecosystems. It has, in fact, been shown that various cellular properties of bacteria change with changing growth rate (6, 25, 30).

We have used the technique of continuous culture to examine the biochemical properties of *Steptococcus mutans*, an organism known to be associated with some forms of dental caries (21, 23). One parameter varied in cultures of *S. mutans* has been the growth rate, since estimates have suggested that bacteria grow slowly in dental plaque. Gibbons (12) originally proposed a mean generation time (MGT) of 8 to 12 h for dental plaque, whereas subsequent estimates have ranged from 3 to 4 h for developing supragingival plaque (29) to 3 to 14 h for organisms colonizing tooth fissures (26). Recently (H. J. A. Beckers and J. S. van der Hoeven, Abstr. Annu. Meet. Eur. Org. Res. Caries, 1979, Abstr. no. 32, p. 85), in a study specifically designed to measure the initial growth rate and the colonization of oral bacteria in rat dental plaque, an MGT of 5 h was observed for the initial growth of *S. mutans* in rats fed sucrose.

In a previous study (10), we reported that the growth rate and the concentration of glucose in the growth medium had a significant effect on the activity of phosphoenolpyruvate phosphotransferase transport system in *S. mutans* strain Ingbritt. In could be shown that as the growth rate approached the maximum (i.e., MGT = 1.4

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h [9]), the activity of the glucose phosphotransferase system decreased to about 10% of the value obtained when the cells were growing at MGT = 14 h. Growth with excess glucose was also shown to repress the phosphotransferase system (10).

We now report the effect of growth rate on various other biochemical properties of cultures of *S. mutans* Ingbritt growing in a chemostat with either a nitrogen (glucose excess) or a glucose limitation. These limitations were used since there is evidence (11) suggesting that organisms in human plaque exist in an environment which ranges from glucose excess during diet intake to glucose limitation under fasting (no diet) conditions. It has been shown that the products of metabolism, glycolytic activity, endogenous metabolism, fluoride sensitivity, and extracellular glucosyltransferase activity in *S. mutans* all vary, depending on the growth rate and nutrient limitation.

MATERIALS AND METHODS

Organism. The *S. mutans* Ingbritt strain used in this study was kindly supplied by J. Sandham, Toronto. The methods of maintenance and purity control have been described previously (16).

Growth conditions. Cultures were grown in a Porton-type chemostat (19) at 37°C with a 500-ml working capacity; the pH was maintained at 6.5, and the gas phase was nitrogen plus 5% CO_2. The dilution rate was varied between 0.05 and 0.4 h^{-1}, which is equivalent to MGT values of 1.7 to 14 h. The cultures were allowed to reach equilibrium for at least 10 mean generations at each dilution rate before harvesting. The biochemical properties of cells grown under conditions of nitrogen limitation, where glucose was in excess, and in cultures where glucose was the limiting nutrient was determined. Both cultures were grown simultaneously in two chemostats in Carlsson's defined medium (M3) (1), modified as described previously (10). For the glucose-excess (nitrogen-limited) culture, the basic medium was modified to contain one-fifth the concentration of amino acids while glucose was added at 300 mM such that during growth asparagine and arginine were limiting growth. The glucose-limited culture was grown in the basal M3 medium modified to contain 25 mM glucose.

Washed-cell experiments. Cells were collected via the overflow from each chemostat into a container cooled to 0°C for various periods (usually overnight, but rarely 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 suspended in saline (0.98% NaCl in water) at a concentration of ≃30 mg (dry weight)/ml and stored in ice until used.

The rate of acid production from the anaerobic glycolysis of endogenous material, glucose, sucrose, and fructose by washed cells of *S. mutans* and the inhibition of acid production by fluoride were studied (i) under conditions of constant pH in a pH-stat or (ii) under conditions where the pH fall was monitored. In both cases, the reaction mixture consisted of washed cells (7 to 10 mg) suspended in 2.0 ml of saline and receiving 100 μl of the appropriate sugar solution (30 mg/ml of water). The cell suspension was mixed by a magnetic stirrer, and the suspension was kept anaerobic by the passage of a slow stream of nitrogen gas through the mixture. For the pH-stat experiments, the reaction mixture was made 10 mM with respect to phosphate buffer by the addition of 20 μl of a 1 M solution. The pH was kept constant at 7.0, 6.5, 6.0, or 5.5 with standardized 0.1 M NaOH, using a Radiometer pH-stat system as previously described (16). In the pH-fall experiments, the pH was monitored on a Vitraton-Lin-Log recorder (Fison Scientific Apparatus, Loughborough, England) for 20 min by the Radiometer system without the titrator (16).

In the pH-stat experiments, fluoride inhibition was studied by adding the requisite amount of 0.1 M NaF to the reaction mixture after the initial rate of alkali addition had been established (usually 4 to 8 min). Inhibition of the rate of acid production was followed in the same reaction mixture by the subsequent addition of NaF at suitable time intervals (ca. 4 min). Fluoride was added in pH-fall studies just before pH adjustment to 7.0.

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%.

Enzyme assays. Glucosyltransferase was assayed according to the method of Ellwood et al. (7). Invertase was measured as the release of glucose from sucrose in a reaction mixture containing culture filtrate and sucrose (50 mM) in a 1.0-ml volume at pH 6.5 and 37°C.

Analytical procedures. Amino acids, glucose, and metabolic end products present in the culture fluid were assayed after rapid filtration (<5 s) to remove cells as previously described (9). Total intra- and extracellular polysaccharides were assayed by the method of Dubois et al. (5). Glucoamylase-specific glycogen was assayed by the method of Hamilton (14), and cell wall carbohydrate was analyzed by the method of Ellwood et al. (7).

RESULTS

Effect of growth rate on cell yields and metabolic products. *S. mutans* Ingbritt was grown anaerobically under conditions of glucose excess (nitrogen limitation) and glucose limitation in two chemostats simultaneously at dilution rates of 0.05, 0.1, 0.2, and 0.4 h^{-1}. These rates correspond to MGT values of 14, 7, 3.5, and 1.7 h, respectively. Table 1 gives the yield and metabolic end products of the steady-state culture at each dilution rate for the glucose-limited culture. The yield of cells was relatively constant at different growth rates, whereas the

yield of cells relative to glucose utilized increased to $D = 0.2\ h^{-1}$ and then dropped at the highest growth rate. The major products of metabolism under glucose limitation were acetate, formate, and ethanol, whereas lactate was a minor product at $D = 0.05$ to $0.2\ h^{-1}$. Acetate production was maximum at $D = 0.05\ h^{-1}$ and declined at higher rates, whereas formate and ethanol production were maximum at $D = 0.1$ and $0.2\ h^{-1}$, respectively. Lactate increased to 30% of the end products at $D = 0.4\ h^{-1}$, confirming earlier observations (9).

By contrast, Table 1 shows similar data for the culture grown with an excess of glucose (nitrogen limitation). Although the yield of cells in this culture was relatively constant but somewhat lower, the yield of cells relative to glucose utilized was more than 2.6-fold lower than in the glucose-limited culture. Furthermore, lactate was the main product of metabolism, with the concentration decreasing with increasing dilution rate. Low concentrations of acetate, formate, and ethanol were detected at all dilution rates, except $D = 0.1\ h^{-1}$. Gas-liquid chromatographic analyses of both cultures demonstrated that no citric acid cyclic intermediates or volatile acids, other than those tested, with chain lengths C_2 to C_8 were present in the culture filtrates. The carbon recoveries based on the control glucose fermented ranged from 74 to 92% for the glucose-limited culture and from 60 to 65% for the glucose-excess culture.

Amino acid analysis. Amino acid analysis of the spent medium from the glucose-limited culture revealed that over the four dilution rates, asparagine, isoleucine, and arginine were utilized to the greatest extent by the cells (e.g., 36, 37, and 32% utilized, respectively). Significant trends between the dilution rates were not observed. With the glucose-excess (nitrogen-limited) culture, on the other hand, growth was limited by asparagine and arginine, since they were completely utilized. Amino acids showing in excess of 80% utilization at all dilution rates were: aspartate, glutamate, glycine, alanine, valine, leucine, and lysine. Since chemostat cultures of *S. mutans* Ingbritt growing in complex medium were limited by cysteine at $D = 0.05\ h^{-1}$ (16), it was interesting to note that utilization values for cysteine were 91, 94, 35, and 15% for dilution rates of 0.05, 0.1, 0.2 and $0.4\ h^{-1}$, respectively.

Total carbohydrate and glycogen. Considering the significant difference in the glucose concentrations in the two media (i.e., 300 mM with one-fifth the amino acid concentration versus 25 mM), one would expect a higher level of cellular polysaccharides (32) in the glucose-excess culture. However, there was relatively little difference in carbohydrate between the two cultures (Table 2), the maximum being a 1.6-fold-higher value for the glucose-excess culture at $D = 0.2\ h^{-1}$. The value at $D = 0.05\ h^{-1}$ for the glucose-excess culture was, however, twice that obtained for total carbohydrate for a similar culture of the organism grown in complex medium (16). Glucoamylase-specific glycogen was very low in both cultures, representing only 1.3 to 2.5% and 1.5 to 11.3% of the total carbohydrate in the glucose-limited and glucose-excess cultures, respectively. The highest value (27.4 μg/mg of cells) was obtained at $D = 0.05\ h^{-1}$ in the latter culture, which is very close to the value obtained in continuous culture with complex medium (16) and the value (29.2 μg/mg of cells) obtained with batch-grown washed cells of the organism incubated with excess glucose (14), assuming that 50% of the cell dry weight is protein (I. R. Hamilton, unpublished data).

Growth rate had little effect on the glycogen

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

Cell type and dilution rate (h^{-1})	Determination						
	Yield of cells (mg [dry wt]/ml)	Residual glucose (mg/ml)	$Y_{glucose}$ (g of cells/mol of glucose)	Metabolic products (mg/ml)			
				Lactic acid	Acetic acid	Formic acid	Ethanol
Glucose limited							
0.05	0.81	0	29.2	0.10	1.41	1.34	0.91
0.1	0.93	0	33.5	0.20	1.21	2.04	0.87
0.2	1.02	0	36.7	0.22	1.08	0.88	1.29
0.4	0.88	0	31.7	1.00	0.69	0.61	1.12
Glucose excess							
0.05	0.63	39.3	7.2	9.56	0.23	0.47	0
0.1	0.46	45.1	8.5	5.82	0	0	0
0.2	0.59	45.2	10.8	5.20	0.25	0.19	0.41
0.4	0.58	46.3	12.1	4.45	0.13	0.70	0

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

and carbohydrate fraction of the cells in the glucose-limited culture. The glycogen content of the glucose-excess cells declined as the dilution rate was increased, with the total carbohydrate increasing slightly (13%) between $D = 0.05$ and 0.4 h^{-1}.

Cells grown with a glucose excess at $D = 0.05$ h^{-1} were incubated at 37°C in 0.1 M phosphate buffer (pH 7.0) with 40 mM $MgCl_2$ to test for metabolic degradation. After a 10-h incubation period, 35% of the total carbohydrate (84 μg/mg [dry weight]) and 82% of the glycogen (22.5 μg/mg) were utilized by the cells. Lactate, acetate, and ethanol were the products of cellular polysaccharide degradation, confirming recent results (20).

Analysis of cell wall carbohydrate in both cultures revealed that the rhamnose/glucose ratio was stable between the two cultures and between the various dilution rates. For example, the ratios were 2.13 and 2.55 in cells from the glucose-excess culture, and 2.17 and 2.12 in cells from glucose-limited culture, for the $D = 0.05$ and 0.4 h^{-1} cells, respectively.

Glucosyltransferase activity. The synthesis of extracellular glucans by glucosyltransferases of *S. mutans* strains is thought to be an important factor in the cariogenicity of the organism (27). Growth of *S. mutans* Ingbritt under conditions of excess glucose was shown to repress the synthesis of extracellular glucosytransferase compared with the activity in the glucose-limited culture (Table 3). The difference was greatest at the lowest growth rate (10-fold) and declined such that at $D = 0.4$ h^{-1} the latter culture had only a 2.6-fold-greater activity than the glucose-excess culture. Maximum activity was observed at $D = 0.2$ h^{-1} in both cultures. Very little free polysaccharide was present in the medium in either culture, and there appeared to be little effect of growth rate. No invertase activity was observed in the culture fluid.

Carbohydrate metabolism. We previously demonstrated that cells of *S. mutans* Ingbritt grown at pH 5.5 in complex medium with excess glucose were more acidogenic than those grown at pH 6.5 and 6.0 (16). Table 4 demonstrates the effect of growth rate on the glycolytic activity of cells grown under glucose-limited and glucose-excess conditions when incubated as washed suspensions in the pH-stat with glucose, sucrose, fructose, and cells alone (endogenous). The glucose-limited cells had virtually no endogenous activity, whereas endogenous acid production by the glucose-excess cells was substantial, particularly with the $D = 0.2$ h^{-1} cells, where the endogenous rate approached that for glucose and sucrose. Apart from this, however, the glycolytic activity of the glucose-limited cells was much higher than that of the glucose-excess cells when incubated with 8.3 mM glucose (3.7- to 6.2-fold), 4.2 mM sucrose (2.9- to 4.4-fold), and 8.3 mM fructose (1.7- to 4.5-fold). In addition,

TABLE 2. *Glycogen and total carbohydrate content of cells of S. mutans Ingbritt grown under conditions of glucose excess and glucose limitation in a chemostat*

Cell type	Content (μg/mg [dry wt] of cells) at given dilution rate (h^{-1})							
	0.05		0.1		0.2		0.4	
	Glycogen	Total carbohydrate	Glycogen	Total carbohydrate	Glycogen	Total carbohydrate	Glycogen	Total carbohydrate
Glucose excess	27.4	241	8.1	257	7.4	259	4.0	273
Glucose limited	2.5	194	6.7	224	4.1	166	3.1	210

TABLE 3. *Extracellular glucosyltransferase and invertase activity in cultures of S. mutans Ingbritt grown under conditions of glucose excess and glucose limitation in a chemostat*

Cell type	Activity at given dilution rate (h^{-1})											
	0.05			0.1			0.2			0.4		
	Glucosyltransferase[a]	Invertase[b]	Free polysaccharide[c]	Glucosyltransferase	Invertase	Free polysaccharide	Glucosyltransferase	Invertase	Free polysaccharide	Glucosyltransferase	Invertase	Free polysaccharide
Glucose excess	26	0	36	13	0	31	250	0	11	225	0	9
Glucose limited	262	0	31	103	0	29	710	0	0	589	0	9

[a] Micrograms of polymer formed per hour per milliliter of culture filtrate.
[b] Micromoles of glucose produced per hour.
[c] Micrograms per milliliter of culture filtrate.

TABLE 4. *Rate of glycolysis by chemostat-grown cells of S. mutans Ingbritt catabolizing endogenous and exogenous carbon sources in a pH-stat at pH 6.5*

Cell type	Rate of glycolysis[a] at given dilution rate (h^{-1})															
	0.05				0.1				0.2				0.4			
	Endogenous	Glucose	Sucrose	Fructose	Endogenous	Glucose	Sucrose	Fructose	Endogenous	Glucose	Sucrose	Fructose	Endogenous	Glucose	Sucrose	Fructose
Glucose limited	3	244	233	189	4	290	268	145	4	259	216	140	3	163	149	85
Glucose excess	18	57	81	63	47	61	94	74	70	76	59	82	15	42	35	19

[a] Nanomoles of acid neutralized per milligram (dry weight) of cells per minute.

the glycolytic rate decreased with the glucose-limited cells as the dilution rate increased from $D = 0.1\ h^{-1}$ with glucose and sucrose and from $D = 0.05\ h^{-1}$ with fructose.

A further comparison between the two cultures can be seen in experiments where the pH of the washed suspension was allowed to fall in response to endogenous and exogenous metabolism. Figure 1 shows the pH response of cells from both cultures grown at $D = 0.4\ h^{-1}$. Again, except for endogenous metabolism, the pH minimum produced by the glucose-limited cells was lower. The rate and extent of the pH fall was influenced to a moderate degree by the dilution rate, although no pattern emerged. After a 15-min incubation period, the average pH minimum for the four dilution rates for the glucose-excess cells metabolizing 8.3 mM glucose was 4.59 (±0.09), whereas that for the same concentrations of glucose-limited cells under identical conditions was 4.08 (±0.16). The values for sucrose and fructose with the glucose-excess cells were 4.78 (±0.22) and 5.15 (±0.35), respectively, and similar values obtained with the glucose-limited cells were 4.19 (±0.23) and 4.51 (±0.24), respectively. Thus, the latter cells gave lower pH minima in the order of 0.51 (glucose), 0.59 (sucrose), and 0.64 (fructose) pH units.

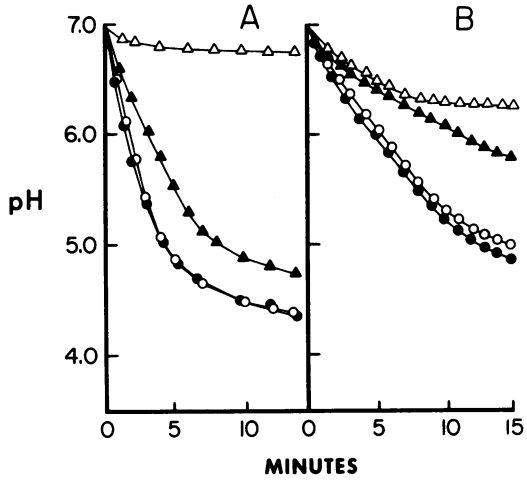

FIG. 1. *Acid production under pH-fall conditions by glucose-limited (A) and glucose-excess (nitrogen-limited) cells (B) grown at D = 0.4 h^{-1} and metabolizing glucose (●), sucrose (○), fructose (▲), and endogenous material (△).*

Fluoride inhibition. The ability of fluoride to inhibit carbohydrate metabolism by oral streptococci is well established, although a variety of factors are known to modify the inhibitory effect (15, 16, 22). Figure 2 is a representative example of the effect of fluoride, at concentrations of NaF between 0.1 and 5.0 mM, on the rate of glucose metabolism by $D = 0.1\ h^{-1}$ cells of both cultures. Glucose-limited cells reduced the pH to near 4.0 within 15 min, and this rapid fall was significantly inhibited by 0.1 mM NaF. The rate and extent of the pH fall were much less with the glucose-excess cells; however, the effect of fluoride was much less dramatic. Similar results were obtained with sucrose and fructose as carbon sources.

One drawback of pH-fall experiments in the study of fluoride inhibition is that as the pH declines, the cells become more sensitive to fluoride in what can be described as the pH effect (15). To obviate this problem, a series of pH-stat experiments was carried out with cells from all four dilution rates of both cultures and incubated with glucose, sucrose, and fructose under conditions where the pH was maintained at 7.0, 6.5, 6.0, and 5.5. The pH effect can be seen (Fig. 3) in an experiment where glucose-limited cells ($D = 0.05\ h^{-1}$) were incubated with sucrose at all four pH values. As the pH was reduced, the concentration of fluoride required to completely inhibit metabolism also declined.

Figure 4 demonstrates the inhibitory effect of NaF on the rate of glucose metabolism by cells maintained at pH 7.0 in the pH-stat. Whereas significant differences were observed in the rates of glycolysis for the glucose-limited cells between the four dilution rates at the lower (<5 mM) fluoride levels, less variation in activity

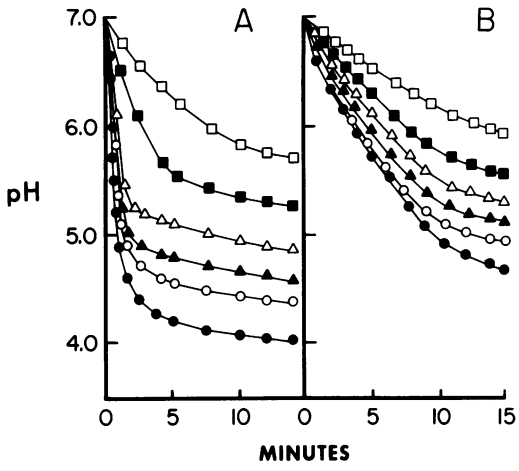

FIG. 2. *Effect of NaF on acid production under pH-fall conditions by glucose-limited (A) and glucose-excess (nitrogen-limited) (B) cells grown at D = 0.1 h^{-1} and metabolizing glucose. Final NaF concentrations (millimolar): 0 (●), 0.1 (○), 0.25 (▲), 0.5 (△), 2.5 (■), and 5 (□).*

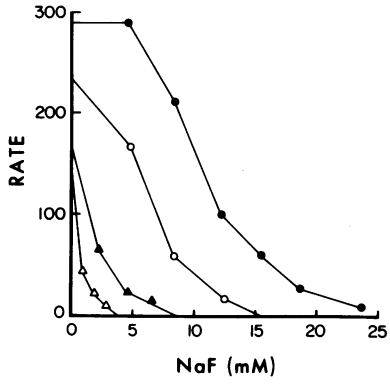

FIG. 3. *Effect of NaF on glycolytic activity of glucose-limited cells grown at D = 0.05 h^{-1} and incubated with sucrose in a pH-stat at pH 7.0 (●), 6.5 (○), 6.0 (▲), and 5.5 (△).*

was noted at the higher values. It was also noted that a low level of acid production persisted at NaF levels above 15 mM, particularly with the $D = 0.05\ h^{-1}$ cells. The effects of NaF on the glucose-excess cells was less dramatic because of the lower activity, but there was, nevertheless, a tendency to increased sensitivity at the lower dilution rates. Again, a similar pattern of activity was produced for cells degrading sucrose and fructose in the presence of increasing levels of NaF.

In an effort to consolidate the data from the various experiments, we calculated the concentration of NaF required to inhibit glycolysis by 50% (ID_{50}) for each dilution rate and at each pH endpoint during glucose metabolism. This ID_{50} value for each pH was then plotted against the dilution rate. A significantly lower level of NaF was required to inhibit metabolism of glucose-limited cells at pH 5.5 and 6.0 than at pH 6.5 and 7.0, with some tendency for a lower value at the lower dilution rate (Fig. 5). For the glucose-excess cells, on the other hand, the difference between the pH 6.0 and 6.5 values was much smaller for the $D = 0.05\ h^{-1}$ cells than for the cells at the other dilution rates. It was also apparent that the slower-growing cells required a lower ID_{50} value, particularly at pH 7.0 and 6.5. Similar findings were also obtained with sucrose and fructose.

One of the shortcomings of this analysis is that it does not take into consideration the inherent glycolytic activity of the cell in the absence of NaF. To eliminate differences in the activities without NaF, one can calculate the ratio of the ID_{50} value and the uninhibited glycolytic rate. Differences in this ratio indicate that there are inherent differences in fluoride sensitivity between the different samples. When these were calculated for the pH 6.5 and 7.0 rate values with the two cultures and plotted against

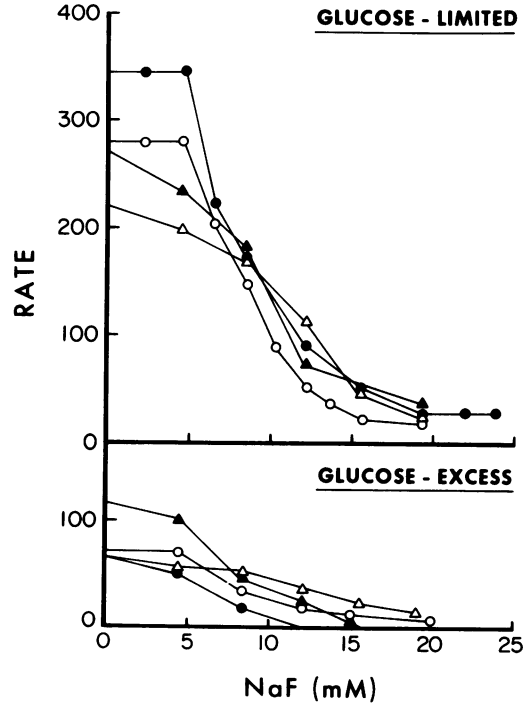

FIG. 4. *Effect of NaF on the glycolytic activity of glucose-limited and glucose-excess cells grown at D = 0.05 (●), 0.1 (○), 0.2 (▲), and 0.4 h^{-1} (△) and incubated in a pH-stat at pH 7.0.*

the glycolytic rate without fluoride (Fig. 6), the best-fit curves, obtained by computer analysis, were negative exponentials. This indicates that with rapidly metabolizing cells, less fluoride is required to inhibit glycolysis by 50% than with cells that metabolize glucose more slowly.

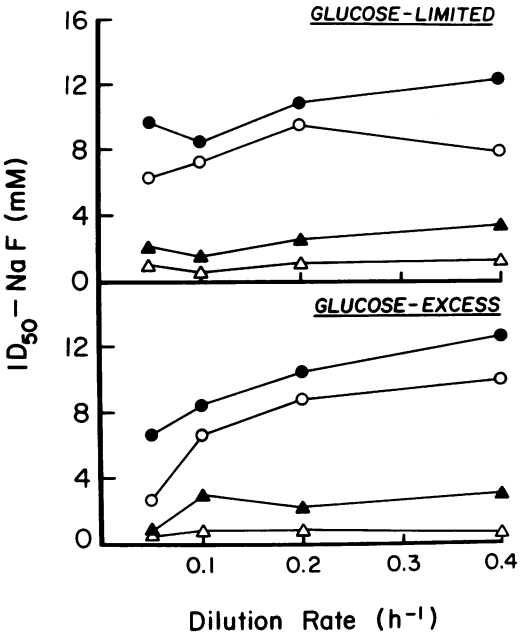

FIG. 5. *Relationship between the dilution rate and the concentration of NaF required to inhibit glycolysis by glucose-limited cells and glucose-excess cells by 50% (ID_{50}-NaF). Cells were incubated in the pH-stat at pH 7.0 (●), 6.5 (○), 6.0 (▲), and 5.5 (△).*

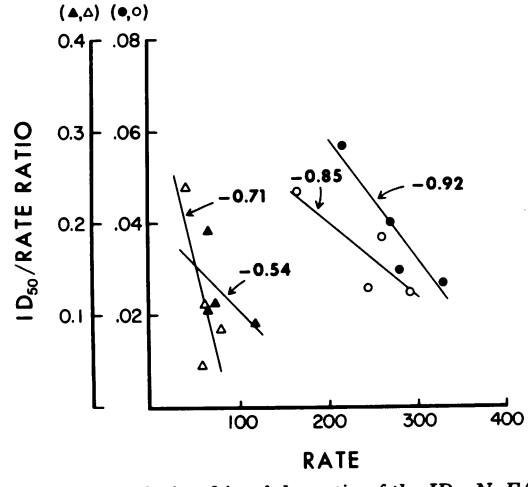

FIG. 6. *Relationship of the ratio of the ID_{50}-NaF/uninhibited glycolytic rate and the uninhibited glycolytic rate for glucose-limited cells (●, ○) and glucose-excess cells (▲, △) incubated at pH 7.0 (solid symbols) and pH 6.5 (open symbols). Value associated with each line is the correlation coefficient obtained by computer analysis.*

DISCUSSION

The results from this and other continuous-culture studies (3, 25) demonstrate the metabolic versatility of *S. mutans* not apparent from previous studies with batch-grown cells. These latter studies (4, 28) concluded that the organism was homofermentative, and this has led to the suggestion that under in vivo conditions lactate is the major metabolic end product of its metabolism. However, analysis of the metabolic products of a dental plaque of germfree rats monoassociated with *S. mutans* showed that acetate and ethanol were present in addition to lactate (31). Before this, Carlsson and Griffith (3) had demonstrated that *S. mutans* under glucose-limited conditions in a chemostat at $D = 0.125\ h^{-1}$ produced ethanol, acetate, and formate, whereas nitrogen-limited cells produced mainly lactate.

Similar products were obtained in the present study, but the concentration of these products varied, depending upon the dilution rate. For example, with the glucose-limited culture, the concentrations of lactate and ethanol increased with increasing growth rate, whereas the concentrations of acetate and formate decreased (Table 1). Lactate production also declined in the glucose-excess (nitrogen-limited) culture as the growth rate increased. Less glucose was catabolized with increasing growth rate. Mikx and van der Hoeven (25) have shown that the growth rate can influence the concentrations of lactate formed from glucose under conditions of glucose limitation depending on the concentration of glucose. In a study with *S. mutans* $C_{67\text{-}1}$ grown in a chemostat with 5 and 50 mM glucose, they demonstrated that whereas glucose was completely utilized (i.e., limiting) in both cases, lactate increased significantly with increasing growth rate with 50 mM glucose, but only very slowly with 5 mM glucose in the medium. Acetate and ethanol decreased in both cultures with increasing growth rate.

The yield of cells relative to glucose utilized for the glucose-limited culture was similar to that obtained by Carlsson and Griffith (3) and was 2.6- to 4.0-fold higher than that obtained for the glucose-excess culture. The recovery of metabolic products from the glucose-excess culture was somewhat lower than recoveries obtained in the other studies (3, 25), even considering the amount of total carbohydrate in the cells (Table 2) and assuming that it all originated with glucose. No citric acid cycle intermediates or C_2–C_8 volatile acids, other than those detected, were observed during gas-liquid chromatography

analysis. Carbon dioxide may have been one product of metabolism not determined, but since CO_2 (5%) was present continuously in the gas phase, metabolic CO_2 evolution could not be determined. Probably the most efficient way to ensure complete recoveries would be to carry out runs with uniformly labeled glucose in a chemostat with a small working volume.

Such a technique would also provide useful information for the distribution of glucose into endogenous polysaccharide storage material. The majority of the intracellular polysaccharide in *S. mutans* Ingbritt was not attacked by glucoamylase (Table 2) and is therefore not typical glycogen, thus confirming previous research (14, 16). Glycogen standards in the assays gave greater than 90% recovery, indicating that the assay itself is reliable. Since part of the nonglycogen polysaccharide is readily degraded to acid end products (Fig. 1; 5), it is evident that *S. mutans* Ingbritt, and probably other *S. mutans* strains, possesses another storage polymer that can be utilized as an energy reserve. The composition and structure of this material are unknown, but preliminary experiments have demonstrated that glucoamylase-hydrolyzed, ethanol-precipitable carbohydrate from glucose-excess cells contains rhamnose in addition to glucose (Hamilton and Ellwood, unpublished data). Since rhamnose is a cell wall constituent of *S. mutans* (17), and since cell wall thickening occurs with the organism under conditions of amino acid deprivation (24), it is possible that a polysaccharide cell wall precursor may be synthesized and stored in cells in granules (24) in association with glycogen. During nitrogen-limiting conditions with excess glucose, the polymer is synthesized, but would be capable of being metabolized for energy when the exogenous carbon source is depleted. However, this does not preclude the possibility that other energy storage compounds are present in the cells.

The only other study of glucosyltransferase (dextransucrase) synthesis by oral streptococci in continuous culture was reported by Carlsson and Elander (2). These workers demonstrated that glycosyltransferase synthesis by *S. sanguis* strain 804 was at a maximum in complex medium at low dilution rates ($<0.1\ h^{-1}$) and declined with increasing dilution rate. Activity of the enzyme was, however, low in defined medium. These workers suggested that glucosyltransferase in this *S. sanguis* strain might only be synthesized under conditions where an excess of nutrients is available. Ellwood and Hunter (8) have also reported that maximum glucosyltransferase activity appears in slowly growing cells ($D = 0.05\ h^{-1}$) of *S. mutans* Ingbritt growing with a glucose limitation in complex medium. The results from the present study differ in that substantial enzyme activity was available in defined medium under both glucose-limited and glucose-excess conditions (Table 3). In fact, the highest activity in both cultures appeared at the higher dilution rates ($D = 0.2$ to $0.4\ h^{-1}$) and maximum activity was with a glucose-limiting culture ($D = 0.2\ h^{-1}$). The reason for the differences between this and the previous study with *S. mutans* Ingbritt is unknown but may be related to the composition of the medium.

The comparison between the two *S. mutans* cultures with respect to fluoride sensitivity has outlined several interesting features. For example, although glucose-limited cells are sensitive to fluoride, they nevertheless continue to metabolize carbohydrate at a slow rate in the presence of fluoride concentration above 15 mM (Fig. 3). Since these cells possess very little endogenous metabolism, which is known to be less sensitive to fluoride (13), this residual fluoride-insensitive metabolism may be a unique feature of glucose-limited cells.

Another feature confirmed in these experiments is the inverse relationship between fluoride concentration and the rate of glycolysis. Previous studies with oral streptococci, including *S. mutans*, have demonstrated that cells metabolizing either endogenous polysaccharide (22) or exogenous carbohydrate (15) at a slow rate are more insensitive to fluoride than cells with rapid glycolytic activity. This phenomenon has also been demonstrated in this study by comparisons between the two cultures and between fast and slow cells within a culture. Figure 2 illustrates the comparison between the glucose-limited and the glucose-excess cultures, where it can be seen that whereas the latter cells produce acid at a slower rate, low levels of NaF have less effect on them than on the more acidogenic glucose-limited cells. A more complete analysis of this is made in Fig. 6, which plots the ratio of the NaF ID_{50}/glycolytic rate versus the inherent glycolytic rate of cells in the absence of NaF in both cultures at the four dilution rates at pH 6.5 and 7.0. This indicates clearly that less fluoride is required to bring about 50% inhibition of glycolysis with rapidly metabolizing cells than with cells with slower activity.

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