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Effects of Fluoride on Carbohydrate Metabolism by Washed Cells of *Streptococcus mutans* Grown at Various pH Values in a Chemostat

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Streptococcus mutans Ingbritt was grown anaerobically in a chemostat, at a rate (mean generation time, 13 h) similar to that in dental plaque, in a complex medium with excess glucose and at pH values of 6.5, 6.0, and 5.5. The yield of cells was constant at pH 6.5 and 6.0 (2.00 mg/ml) but fell to 1.25 at pH 5.5; $Y_{glucose}$ was relatively constant under all conditions. Lactic acid was the major end product. Amino acid analysis of the culture supernatants indicated that growth was probably limited by the availability of cysteine. Cells were harvested and monitored for their capacity to produce acid from endogenous polysaccharide and exogenous sugars in the presence and absence of NaF, as well as for their glucose phosphoenolpyruvate (PEP)-phosphotransferase activity. Surprisingly, cells grown at pH 5.5 possessed two to three times more glycolytic activity, as measured by the rate of acid production, than cells grown at pH 6.5 and 6.0 when incubated in a washed suspension at constant pH with a sugar source. Furthermore, the cells grown at pH 5.5 were about twice as resistant to the effect of NaF in reducing the rate of acid production in this system. Fluoride inhibition could be reversed by increasing the pH of the system. Cells grown at all three pH values showed significant acid production from endogenous reserves, despite the fact that the glucoamylase-specific glycogen content of the cells dropped from 33% of the total carbohydrate during pH 6.5 growth to only 3% after growth at pH 6.0 and 6.5. Incubation of washed cells for 18 h in phosphate buffer resulted in the loss of 62% of the total carbohydrate, indicating that nonglycogen cellular polysaccharide was metabolized. A comparison of the fluoride effect on endogenous and exogenous metabolism under pH fall conditions showed that, with pH 6.5- and 6.0-grown cells, the inhibitor was more effective in the presence of an exogenous carbon source than in its absence. This effect was not seen with pH 5.5-grown cells. The decreased sensitivity of the pH 5.5-grown cells to fluoride was probably associated with the decreased glucose PEP-phosphotransferase activity (11%) in these cells compared with the activity of those grown at pH 6.5. This evidence supports the hypothesis that *S. mutans* possesses at least two glucose transport systems, one of which is relatively fluoride insensitive.

Fluoride is known to have a significant inhibitory effect on acid production by dental plaque (4, 15, 16) and salivary sediment systems (19) that metabolize various carbohydrates. In detailed studies with oral streptococci, including *Streptococcus mutans* (see reference 10), it has been found that fluoride interferes with the glucose transport into these cells via the phosphoenolpyruvate (PEP)-dependent phosphotransferase (PTS) system, because PEP is formed by 2-phosphoglycerate by the action of enolase, which is fluoride sensitive. Thus, fluoride, in effect, reduces the amount of PEP available to drive the PTS and, in turn, decreases the amount of acid produced from glycolysis. In dental plaque, fluoride reduces acid production at the tooth surface by reducing the amount of glucose taken up by the plaque bacteria that possess the PTS system.

Most of the studies on glucose metabolism by strains of *S. mutans* have been carried out on batch cultures under conditions of glucose excess. This means that, unless the medium is heavily buffered, there is little control of the pH of growth. Furthermore, the growth rate of

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the organisms in such experiments is high during the exponential phase and is much higher than that thought to occur in dental plaque (8, 18).

This paper describes the results of experiments in which *S. mutans* Ingbritt was grown in a chemostat in a complex medium with excess glucose under conditions of controlled pH at a low growth rate similar to that thought to occur in vivo (mean generation time, 13 h). The effect of fluoride on the rate of acid production, the PEP-PTS activity, and the fermentation behavior was studied as a function of growth pH.

MATERIALS AND METHODS

Organism. *S. mutans* Ingbritt, isolated from human plaque, was supplied by W. H. Bowen and maintained in Trypticase-yeast extract-glucose broth. The purity of the chemostat cultures was checked daily by slide examination and by streaking on nutrient agar plates incubated aerobically and anaerobically in an atmosphere of nitrogen plus 5% CO_2. In addition, the purity of the test organism was checked initially and at intervals throughout the run by testing for its ability to agglutinate with type c antisera (supplied by G. Bowden, London Hospital). Because cells obtained directly from the chemostat frequently show variation in agglutination, the tests were always performed on cells growing on the nutrient agar plates used for routine purity checks.

Growth conditions. The culture was grown under conditions of glucose excess in a Porton-type chemostat (12) with a 500-ml working capacity, as previously described (6). The dilution rate was maintained at or near 0.05 h^{-1}, and the culture was allowed to reach equilibrium for at least 10 mean generation times (ca. 5 days) at each pH before harvesting.

The culture medium was as previously described (6) except that the concentration of Casamino Acids (Difco Laboratories, Detroit, Mich.) was reduced from 45 to 9 g liter, while the glucose concentration was increased from 1 to 5%, creating glucose excess. Amino acid analysis of the culture supernatant indicated that the medium was limiting in cysteine.

Washed-cell experiments. Cells were collected in a container cooled in ice for various periods (usually overnight). They were then harvested by centrifugation (8,000 × *g* for 15 min) and washed once in potassium phosphate buffer (20 mM, pH 7.0) by centrifugation. The cells were suspended in saline at a concentration of ca. 30 mg/ml and stored in ice until used.

Constant-pH experiments. The rate of acid production from the anaerobic glycolysis of glucose, sucrose, or fructose by washed cells of *S. mutans* and the inhibition of acid production by fluoride were studied at a constant pH.

Washed cells (7 to 10 mg) were suspended in a 2.0-ml volume of 20 mM potassium phosphate buffer. The sugar (100 μl of a 30-mg/ml solution in water) was added, and the pH was kept constant at the required value by adding 0.1 M NaOH with a Radiometer pH-stat system (model 26 pH meter, type SBR2c Titrigraph Titrator II, and Autoburette ABU 12). 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. The rate of alkali addition was recorded (usually for 4 to 8 min), and the inhibition of this rate by the addition of 0.1 M NaF was also followed at suitable time intervals (ca. 4 min).

pH-fall experiments. Cells (7 to 10 mg) were suspended in saline (2.0 ml of 0.98% NaCl in water) and stirred as described above. The pH was adjusted to slightly above 7.0, and then 100 μl of the 30-mg/ml sugar solution was added. The fall in pH was monitored on a Vitatron Lin-Log recorder (Fisons Scientific Apparatus, Loughborough, England) for 20 min by the Radiometer system without the titrator. Fluoride inhibition was studied by adding the requisite amount of 0.1 M NaF to the cells before the pH was adjusted to above 7.0. A series of pH fall curves was obtained for glucose, fructose, and sucrose and for different amounts of NaF.

Throughout this work, the glycolytic rate is defined as the rate of acid production by an anaerobic suspension of cells (2.0 ml) that were degrading either endogenous cellular material or exogenous carbohydrate, the rate being determined from the rate of addition of the standard NaOH to maintain a consant pH. Units of glycolytic activity are expressed as nanomoles of metabolic acid neutralized per milligram (dry weight) of cells per minute.

Assay for PEP-PTS activity. Sugar transport via the PEP-PTS system was assayed by the method of Kornberg and Reeves (17), with washed cells made permeable with toluene. Cell samples (50 ml) were removed 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 7 to 23 mg (dry weight) of cells per ml, and mixed vigorously on a Vortex mixer with 0.01 volume of toluene for 60 s. The assay mixture contained 50 mM phosphate buffer (pH 7.0), 10 mM $MgSO_4$, 2 mM PEP, 0.3 mM reduced nicotinamide adenine dinucleotide, 20 U of lactic dehydrogenase (beef heart, Sigma Chemical Co., London), and 0.5 to 1.2 mg (dry weight) of toluene-treated cells in a volume of 3.0 ml after the addition of the carbohydrate substrate (final concentration, 5.6 mM). Controls contained no carbohydrate. The rate of reduced nicotinamide adenine dinucleotide oxidation was monitored at 340 nm in a Pye Unicam double-beam recording spectrophotometer (SP 1800) with a thermostatically controlled cell compartment and an automatic sample changer. Net activity is expressed as nanomoles of pyruvate formed per milligram (dry weight) of cells per minute.

Analytical procedures: metabolic products. Culture filtrates were analyzed for volatile and nonvolatile acidic products of fermentation by gas chromatography (by means of Pye Unicam Series 104 gas chromatography coupled with a Hewlett-Packard HP 3380A reporting integrator). The following columns were employed: (i) glass (5 feet [ca. 1.5 m] by 1 mm) packed with Chromosorb 101, (ii) glass (5 feet [ca. 1.5 m] by 4 mm) packed with 10% polyethylene glycol adipate on Phasesep CL Aw 80–100 mesh, and (iii) glass (5 feet [ca. 1.5 m] by 4 mm) packed with Poropak Q. The carrier gas was O_2-free nitrogen at a rate of 10 ml/min for column i and ca. 40 ml/min for columns

ii and iii. Detection was by flame ionization for columns i and ii and by Katharometer for column iii. The temperature for column i was 110°C; it was programmed at 5°C/min to 200°C and held for 10 min. Column ii was at a temperature of 100°C for 4 min; it was then programmed at 3°C/min to 200°C and held for 15 min. Column iii was held at 150°C for 6 min and programmed at 12°C/min to 195°C.

(i) Volatile products. To analyze for volatile products, culture filtrates (0.25 ml) were acidified with 20 μl of 6 N H_2SO_4 just before injection on either column i or ii. The latter column was preferred because Chromosorb 101 soon produces ghost peaks after use, and lactic acid appears in an indistinguishable peak in the region of valeric and isovaleric acid. Quantitation was carried out by the HP 3380A Integrator programmed beforehand with authentic standards.

(ii) Nonvolatile acids. The analysis for nonvolatile acids was carried out as follows. Culture filtrates (0.5 ml) were dried in vacuo before heating in 2 N methanolic HCl (1.0 ml) in a sealed tube at 65°C for 2 h. The methyl esters were removed by ether extraction (3 × 3 vol) and dissolved in dichloromethane after removal of the ether in a stream of N_2. Portions of 1 to 5 μl were applied to column ii, and the concentration in the eluted peaks was determined by comparison with authentic standards.

(iii) Formic acid. The inability of flame ionization to detect formic acid necessitated the use of a Katharometer system. Samples (1.0 ml) were made alkaline (ca. pH 8.0) with NaOH, dried, redissoved in 0.5 N H_2SO_4 (0.1 ml), and injected in a 1- to 5-μl portion on column iii.

(iv) Lactic acid. Culture filtrates were analyzed for lactic acid by the method of Barber and Summerson (2).

Analytical procedures: carbohydrates. Residual glucose in the culture fluid was assayed as previously described (6); total carbohydrate was assayed by the method of Dubois et al. (5).

Glucoamylase-specific glycogen in cells was assayed by the method of Hamilton (9).

RESULTS

Effect of growth pH on cell yields and metabolic products. In the experiments reported here, *S. mutans* Ingbritt was grown anaerobically under conditions of glucose excess at a dilution rate of 0.05 h^{-1} and at pH values of 6.5, 6.0, and 5.5. Table 1 gives the yield and metabolic end products of the steady-state culture at each growth pH. The yield of cells was constant at pH 6.5 and 6.0 but fell sharply at pH 5.5, while the residual glucose increased progressively as the pH of growth was decreased. The $Y_{glucose}$, on the other hand, remained relatively constant at each pH value. Lactic acid was the major metabolic end product, with traces of succinic, formic, and acetic acids. Ethanol accumulation declined from pH 6.5 to 6.0, and no ethanol was detectable during growth at pH 5.5.

The utilization of amino acids by *S. mutans* during growth at the respective pH values is shown in Table 2. All of the amino acids except proline, valine, and tyrosine were utilized at pH 6.5; at pH 6.0, only valine and tyrosine were not utilized. Isoleucine was the only amino acid not utilized at pH 5.5. Few trends were observed in amino acid utilization from growth at the various pH values, except that the utilization of aspartic acid, serine, and glutamic acid declined with the decrease in growth pH. Cysteine was the major

TABLE 2. *Amino acid utilization by S. mutans grown in a modified Cybulska-Pabula medium in a chemostat at varying pH values*

Amino acid	Amt present in original medium (mM)	Utilization (%)[a]		
		6.5	6.0	5.5
Aspartate	3.7	57	34	17
Threonine	2.3	13	20	7
Serine	3.8	26	22	13
Glutamate	8.4	44	39	21
Proline	5.0	2	23	16
Glycine	2.3	21	33	30
Alanine	4.0	38	45	23
Valine	2.0	0	0	12
Methionine	1.4	64	29	46
Isoleucine	2.1	21	21	0
Leucine	4.4	31	35	9
Tyrosine	0.2	0	0	29
Phenylalanine	1.6	34	44	27
Histidine	0.9	20	28	6
Lysine	3.3	24	35	23
Arginine	1.1	18	36	9
Cysteine	0.16	94	94	94

[a] Values represent the mean of two determinations ±10%.

TABLE 1. *Effect of growth pH on the yield, residual glucose, and metabolic end products of an anaerobic chemostat culture of S. mutans Ingbritt*[a]

Growth pH	Yield of organism (mg [dry wt]/ml)	Residual glucose (mg/ml)	$Y_{glucose}$ (mg [dry wt]/mg of glucose)	Dilution rate (h^{-1})	Metabolic product				
					Lactic acid	Succinic acid	Formic acid	Acetic acid	Ethanol
6.5	2.00	9.0	0.049	0.054	24.7	tr	0.019	<0.1	0.68
6.0	2.00	15.9	0.059	0.054	24.7	tr	0.013	0	0.23
5.5	1.25	24.2	0.049	0.053	20.7	0.4	0	0	0

[a] Values represent the average of at least two collections made on separate days at each dilution rate.

amino acid utilized at all pH values, and methionine was also used extensively, suggesting that the limiting substrate could be the supply of cysteine.

Table 3 illustrates the amino acid pools in the cells grown at each of the pH values. Generally, as the pH for growth was lowered, the amino acid pool also decreased, with a notable decline in the amount of glutamate (69.4 versus 8.6 nmol/mg at pH 6.5 and 5.5, respectively).

Constant-pH experiments. The glycolytic activity of *S. mutans* Ingbritt grown at a constant slow rate (mean generation time, 13 h) at different pH values was examined at constant pH in a pH-stat. The glycolytic rate for cells grown at pH 5.5 and incubated with 8 mM glucose was two to three times higher than that for cells grown at pH 6.5 and 6.0, regardless of the end point pH of the reaction mixture (Fig. 1). As might be expected, the rate of acid production decreased as the end-point pH decreased.

Effect of exogneous carbon source on glycolytic rate. In pH-stat experiments similar to those described in the legend to Fig. 1, the cells grown in the chemostat with glucose as the carbon source were incubated with sucrose or fructose, as well as with glucose, to test the effect of the carbon source on the rate of acid production. The acid production rate of cells grown at pH 6.5 declined as the titration end point was lowered (Fig. 2). The rates for glucose and sucrose were similar; the rate for fructose was about half those values except at pH 5.5, when they were the same. A similar relationship among acid production rates for glucose, sucrose, and fructose was found for cells grown at pH 6.0 and 5.5.

TABLE 3. *Amino acid pools in cells of S. mutans grown in a modified Cybulska-Pabula medium in a chemostat at varying pH values*

Amino acid	Concn (nmol/mg [dry wt] of cells)		
	6.5	6.0	5.5
Aspartate	15.8	18.0	7.4
Threonine	8.1	28.0	2.5
Serine	15.8	10.8	4.9
Glutamate	69.4	50.4	8.6
Proline	6.3	7.2	3.7
Glycine	6.3	5.4	2.5
Alanine	15.8	14.4	7.4
Valine	3.2	3.6	2.5
Methionine	2.5	2.5	tr
Isoleucine	2.5	2.5	1.5
Leucine	6.3	7.2	2.5
Tyrosine	0	0	0
Phenylalanine	12.6	1.1	1.0
Histidine	1.9	1.4	1.2
Ornithine	tr	tr	tr
Lysine	15.8	18.0	9.9
Arginine	3.2	2.5	1.7
Cysteine	tr	tr	tr

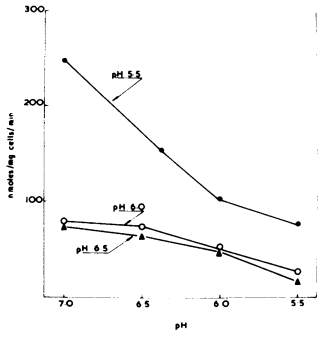

FIG. 1. *Glycolytic rate by washed cells of S. mutans Ingbritt, grown with excess glucose in a chemostat at pH 6.5, 6.0, and 5.5 and incubated with glucose in a pH-stat at various pH values.*

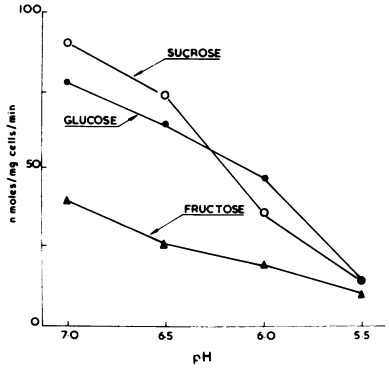

FIG. 2. *Effect of exogenous carbon source on the glycolytic rate of washed cells of S. mutans Ingbritt, grown with excess glucose in a chemostat at pH 6.5 and incubated with glucose, sucrose, or fructose in a pH-stat at various pH values.*

Effect of growth pH on fluoride sensitivity of glycolysis. In pH-stat experiments, it is possible to test the effect of metabolic inhibitors under conditions in which changes in pH due to metabolic activity are eliminated. These conditions are ideal for testing the fluoride sensitivity of cells grown and incubated at different pH values, because they permit a careful examination of the well-known fluoride-pH effect in which the sensitivity of cells to fluoride increases as the pH decreases (3). The effect of increasing NaF concentrations on the glycolytic rate of cells grown at pH 6.5, 6.0, and 5.5 and incubated with 8 mM glucose in the pH-stat at pH 7.0, 6.5, 6.0, and 5.5 was studied. The inhibitory effect of NaF increased significantly as the end-point pH was lowered from 7.0 to 5.5 (Fig. 3),

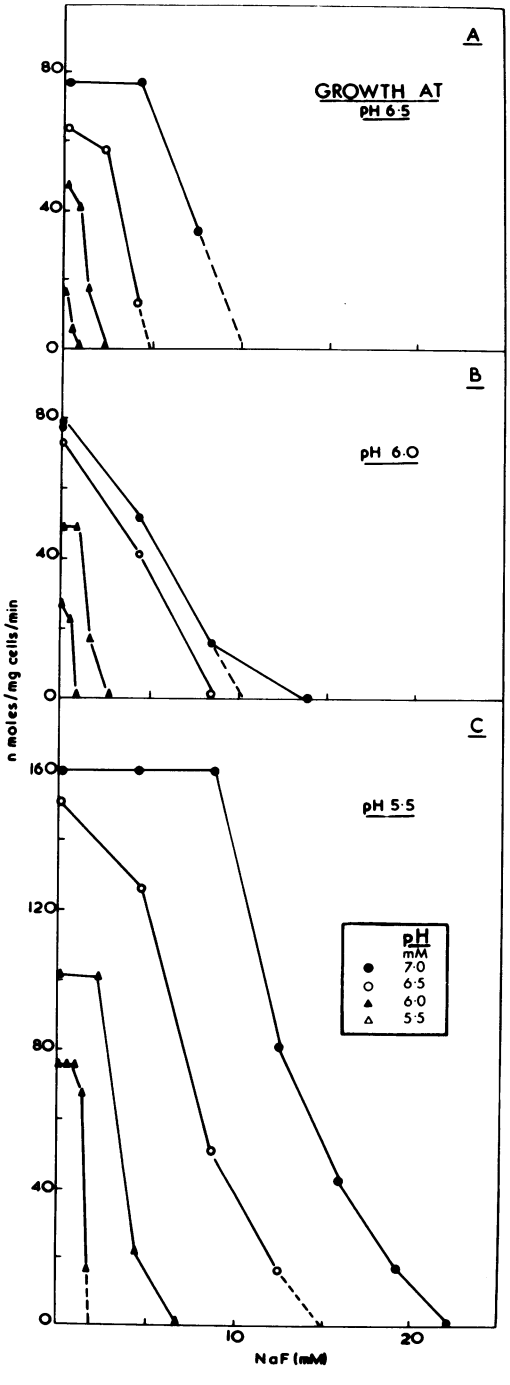

FIG. 3. *Effect of NaF on the glycolytic rate of washed cells of S. mutans Ingbritt, grown with excess glucose in a chemostat at pH 6.5, 6.0, and 5.5 and incubated with glucose in a pH-stat at varying pH values.*

confirming the fluoride-pH effect. However, the most significant observation was that the pH 5.5-grown cells produced acid at a rate twice that observed with the pH 6.5 and 6.0 cells and, in addition, required twice the concentration of NaF to inhibit carbohydrate metabolism completely. Similar observations were made with cells incubated with fructose and sucrose, although the decreased sensitivity of the pH 5.5-grown cells to fluoride was not as great (1.3-fold).

Reversal of inhibition. The pH-dependent inhibition of glycolysis by fluoride was reversed by altering the pH. Cells grown in the chemostat at pH 6.5 were incubated at pH 6.5 and were shown to degrade glucose at a rate of 62 nmol of acid per mg of cells per min (Table 4). The addition of 8.8 mM NaF inhibited acid production completely. However, when the titrator end point was readjusted to pH 7.0, the fluoride-containing cell suspension was shown to metabolize glucose at 74% of the rate of cells incubated at pH 7.0 in the absence of fluoride.

pH-fall experiments. To complement the pH-stat experiments, cell suspensions were given 8 mM glucose or fructose, or 4 mM sucrose, in the presence and absence of NaF under conditions in which the pH was allowed to fall in response to cellular acid production. Figure 4 illustrates the fall in pH resulting from the metabolism of glucose, sucrose, and fructose by pH 6.0-grown cells in the presence of NaF concentrations ranging from 0 to 4.6 mM. The pH minima in a 20-min incubation period for glucose and sucrose in the absence of NaF were 4.5 and 4.4, respectively, with the pH minimum for fructose somewhat higher at 4.7. NaF in a concentration as low as 0.24 mM had a significant inhibitory effect on the pH fall with all three sugars. Cells grown at pH 6.5 and 5.5 gave similar results.

TABLE 4. *Reversal of NaF inhibition of glycolysis by pH adjustment*[a]

Assay condition	Glycolytic rate[b]
1. pH 7.0 + 0 mM NaF	74
2. pH 6.5 + 0 mM NaF	62
3. pH 6.5 + 8.9 mM NaF	0
4. Cells in (3) adjusted to pH 7.0	55

[a] Cells grown at pH 6.5 in the chemostat were harvested, washed, and incubated in the pH-stat at pH 6.5 in the presence of absence of 8.8 mM NaF to establish the corresponding glycolytic rate. Then the pH of both flasks was adjusted to pH 7.0, and the glycolytic rate was determined.

[b] Expressed in nanomoles of acid neutralized per milligram (dry weight) of cells per minute.

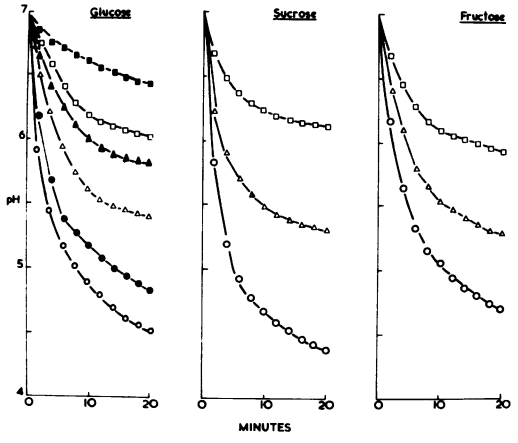

FIG. 4. *Effect of NaF on the pH fall of washed-cell suspensions of S. mutans Ingbritt, grown with excess glucose in a chemostat and incubated with glucose, sucrose, and fructose. NaF concentration (millimolar): 0* (○), *0.24* (●), *0.48* (△), *1.2* (▲), *2.4* (□), *and 4.6* (■).

Effects of NaF on endogenous and exogenous metabolism. The growth of *S. mutans* Ingbritt under conditions of glucose excess (Table 1) resulted in the formation of a considerable amount of cellular carbohydrate at all pH values of growth (Table 5). One third of this carbohydrate was found to be glucoamylase-specific glycogen during growth at pH 6.5; however, the cellular content of this material declined to only 3% of the total carbohydrate after growth at pH 6.0 and 5.5. Despite these low levels, however, much of the total carbohydrate was usable for energy, because incubation of washed cells in saline in the absence of an exogenous carbon source resulted in an appreciable fall in pH in a 20-min period (Fig. 5). Furthermore, incubation of washed cells in buffer for 18 h resulted in the loss of 62% of the total cellular carbohydrate, indicating that these cells can metabolize the non-glucoamylase-specific carbohydrate. The fall in pH during a 20-min incubation period decreased slightly as the pH for growth decreased (values of 5.32, 5.37, and 5.46 for pH 6.5-, 6.0-, and 5.5-grown cells, respectively).

Exogenous glucose metabolism with pH 6.5- and 6.0-grown cells gave the same pH minimum (4.48), whereas pH 5.5-grown cells had lower minima (between pH 4.09 and 4.24 for glucose, sucrose, and fructose for a 20-min incubation period). This confirms the more glycolytic nature of the pH 5.5-grown organisms (Fig. 1).

A comparison of the fluoride sensitivity of endogenous versus exogenous metabolism for the three cell types illustrates that endogenous metabolism for the pH 6.5 and 6.0 cells was more resistant to NaF than exogenous metabolism (Fig. 5), confirming earlier observations (10). It is significant that for these cells the presence of glucose and NaF in the reaction mixture prevented the degradation of the endogenous carbohydrate storage material, since the pH fall in the presence of glucose was much less than in its absence, with the same level of NaF. By comparison, pH 5.5-grown cells did not show this characteristic: On incubation with glucose and NaF, these cells gave a significantly greater pH fall than cells incubated with the same level of NaF but without glucose. A comparison of the effect of the same level of NaF on all three cell types confirms the earlier observations (Fig. 3) that pH 5.5-grown cells were significantly more resistant to NaF than the pH 6.5 and 6.0 cells.

PEP-PTS activity. Since fluoride is known to inhibit sugar transport via the PEP-PTS system in oral streptococci by means of its action on enolase (10), it was of interest to determine whether the relative fluoride resistance of the pH 5.5 cells was related to altered PTS activity in these cells. As shown in Table 6, glucose-PTS activity in the 5.5-grown cells was only 11% of that in pH 6.5-grown cells and 24% of that in pH 6.0-grown cells.

DISCUSSION

The main advantage of using a chemostat to study the physiology of bacteria is that one growth parameter can be varied while all the others are kept constant. A further benefit of this technique is that the effects of very slow growth can be investigated, permitting more meaningful comparisons to be made with conditions existing in natural environments.

In the experiments reported here, it was desirable to study the behavior of *S. mutans* when grown at slow rates in media containing an excess of glucose. Such conditions permit the organism to store the maximum amount of glycogen (12, 13), thus making possible the study of

TABLE 5. *Total carbohydrate and glucoamylase-specific glycogen in cells of S. mutans Ingbritt as a function of growth pH*

Growth pH	Total carbohydrate[a]	Glycogen[a]	Glycogen (% of total carbohydrate)
6.5	103	34	33
6.0	129	4	3
5.5	119	3	3

[a] Expressed in micrograms per milligram (dry weight) of cells.

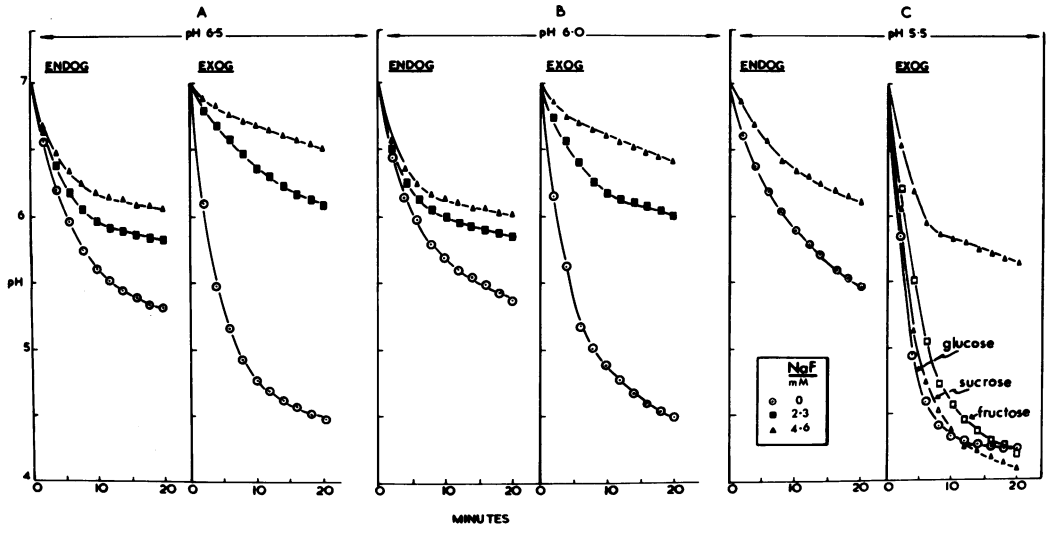

FIG. 5. *Effect of NaF on the pH fall of washed-cell suspensions of S. mutans Ingbritt, grown with excess glucose in a chemostat at pH 6.5 (A), 6.0 (B), and 5.5 (C) and incubated with (exogenous) and without (endogenous) glucose. NaF concentration (millimolar): 0 (⊙), 2.3 (■), and 4.6 (▲).*

TABLE 6. *Glucose PEP-phosphotransferase activity in toluene-treated cells of S. mutans Ingbritt grown in the chemostat at varying pH*

Growth pH	Activity[a]
6.5	6.47
6.0	2.92
5.5	0.70

[a] Expressed in nanomoles per milligram (dry weight) of cells per minute.

the rate of acid production from both endogenous and exogenous carbon sources in the presence and absence of NaF. It was also of interest to compare in this way the effect of the pH on growth under the same conditions of slow growth. This kind of experience would not be possible with the use of conventional batch methods.

The growth rate chosen was equivalent to a mean generation time of 13 h (dilution rate, 0.05 h^{-1}) because it has been suggested (8, 18) that the mean generation time in plaque is one to two generations per day. The pH values for growth were chosen because saliva is about pH 6.5, and cells could not be grown at a pH lower than 5.5. As stated earlier, it was desirable to have a glucose excess medium. This condition was chosen on the basis of previous work that showed that glucose was limiting in cultures of *S. mutans* growing in a complex medium with an initial glucose concentration of 1%. Hence the complex medium was cut to one-fifth the concentration used previously, and the glucose concentration was increased to 5%.

When growth is carried out in such media in the chemostat, it is difficult to be precise about which nutrient is limiting. However, the analysis of the amino acids utilized during growth (Table 2) shows that cysteine was probably limiting and that methionine was used to a considerable extent. It is interesting that the sulfur amino acids are in low concentration in dental plaque (1) and that they may be growth limiting in that environment.

The amino acid pools are also interesting in this regard, in that cells grown at all pH values had only traces of cysteine in their pools. Further, methionine was found in the pools of cells grown at pH 6.5 and 6.0, but only traces were present in the pool of cells grown at pH 5.5; again, this suggests that sulfur amino acids were the limiting nutrient. The level of pool amino acids fell as the pH of growth was lowered. This was particularly true for glutamate; these results are in agreement with the results of Griffith and Melville (8), who studied the effect of growth pH on the amino acid pools of *S. mutans* grown in a chemically defined medium in a chemostat.

The cell density of the organism in the chemostat fell at pH 5.5, but the $Y_{glucose}$ was roughly constant, as was the glucose uptake rate observed in the chemostat. The utilization of glucose fell as the pH of growth decreased, but the major product of fermentation was lactic acid. This shows that fermentation was proceeding by the homolactic mechanisms (presumably the fructose 1,6-diphosphate control of lactate dehydrogenase) known to occur in *S. mutans* (21).

However, the recovery of products based on

the glucose metabolized was only 62% at pH 6.5 but increased to 73 and 82% at pH 6.0 and pH 5.5, respectively. Gas-liquid chromatography analysis demonstrated that, other than succinic, acetic, and formic acids, no citric acid cycle intermediate or volatile acid with a chain length C_2 to C_8 was present in the culture filtrate. The reason for this low recovery is unknown, but it should be remembered that cells were storing intracellular polysaccharide under these conditions (Table 5). The acid production rates of cells taken from the chemostat and measured in the pH-stat with an exogenous sugar source in a buffer suspension were similar for cells grown at pH 6.5 and 6.0. This was true for all the sugars tested; sucrose and glucose gave similar results, but fructose gave only about half the rate of the other sugars when incubated at pH 7.0. This rate difference declined as the pH of incubation decreased, so that at pH 5.5 all three sugars gave the same rate of acid production.

The most significant result of this study is the observation that the acid production rate of cells grown at pH 5.5 and incubated with exogenous glucose at pH 7.0 was approximately twice the rate of cells grown at pH 6.5 and 6.0 when these cells were incubated with glucose in the same way. This was also true when sucrose and fructose were used as the sugar sources. Thus, cells grown at pH 5.5 were much more acidogenic in this test system, which correlates with the more acidogenic nature of the organism in the chemostat itself (see above).

In contrast to these results, the activity of the glucose PEP-PTS transport system in the organism grown at pH 5.5 was only 11% of that in cells grown at pH 6.5. This latter result relates to the fluoride sensitivity of the organism, since cells grown at pH 5.5 were almost twice as resistant to fluoride as those grown at pH 6.5 in both the constant-pH and the pH-fall experiments. It is known that fluoride acts by inhibiting enolase, preventing the formation of PEP, so that the transport of glucose by the PTS system is inhibited (10).

Calculations of the rate of glucose uptake by the cells growing in the chemostat (11) always gave values that were higher than the rates obtained in the PEP-PTS assays for glucose uptake. This suggests either that the assay system used for the PEP-PTS does not give a true reflection of activity or that a second uptake system for glucose is being deployed by these bacteria. The assay system used for the PEP-PTS determinations was the toluene treatment of cells; it has been found that this method gave the highest values with *S. mutans* grown under conditions of glucose excess or glucose limitation (14). The activity of the PEP-PTS system of glucose-limited cells was slightly higher than the activity published for *S. mutans* grown in batch conditions and assayed by the radioactive technique with labeled glucose (20). Thus, it appears that the assay system can give reliable results with this organism. The trend of lower values for the glucose PEP-PTS system for cells grown at lower pH values is in line with the increasing resistance to inhibition of glycolysis by NaF of the cells grown at pH 5.5. A second transport system for *S. mutans* has already been postulated for *S. mutans*, because chemostat-grown cells of the organism, when grown at a high rate under conditions of glucose limitation, possessed only 10% of the PTS activity of slow-growing cells (14). Thus, slow growth at low pH with gluocse excess and fast growth under conditions of glucose limitation both repress PTS activity in *S. mutans*.

The pH fall experiments were in line with the pH-stat results. The fall in pH due to acid production from endogenous carbohydrates was slightly greater for cells grown at the higher pH, although the amount of intracellular carbohydrate present in the cells seemed to peak in cells grown at pH 6.0 (but the variation was not great). As expected, the provision of an exogenous carbohydrate source gave a much more rapid fall in pH. Sucrose gave the greatest fall, followed by glucose and fructose for cells grown at pH 6.5. However, in cells grown at pH 5.5, the fall in pH with different sugars was not markedly different. Low levels of fluoride caused quite marked inhibition of pH fall. A very interesting feature was that, at high fluoride levels, the fall in pH in the presence of exogenous sugar was less than the fall in pH in the presence of the endogenous carbon source. It would seem that at these high fluoride levels the exogenous sugar prevents the utilization of stored carbohydrate.

The results of this study illustrate why selected plaque sites on the tooth surface give rise to carious lesions despite the application of inhibiting levels of fluoride. Clearly the selection of bacteria capable of growing at low pH should occur in sites such as pits and fissures after the addition of sugar. The resultant population should, if it possesses characteristics similar to those of *S. mutans* Ingbritt, be capable of significant acid production and be relatively impervious to fluoride. This again emphasizes the necessity in caries prophylaxis of ensuring that pH values in plaque do not fall below 6.0 for prolonged periods of time.

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