INFECTION AND IMMUNITY, Nov. 1985, p. 378–381
0019-9567/85/110378-04$02.00/0

Vol. 50, No. 2

Regulation of Glycolytic Rate in *Streptococcus sanguis* Grown under Glucose-Limited and Glucose-Excess Conditions in a Chemostat

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The biochemical mechanisms of the acidogenic potential of *Streptococcus sanguis* ATCC 10556 grown in glucose-excess and glucose-limited continuous culture were studied. The rate of acid production during the glucose metabolism by the cells grown under glucose limitation (glucose-limited cells) was 2.1 to 2.6 times that by the cells grown in an excess of glucose (glucose-excess cells). When the glucose-limited cells were metabolizing glucose, intracellular concentrations of glucose 6-phosphate, fructose 6-phosphate, 3-phosphoglycerate, and pyruvate were higher, and that of glyceraldehyde 3-phosphate was lower, than those when the glucose-excess cells were metabolizing glucose. The levels of fructose 1,6-bisphosphate and dihydroxyacetone phosphate were not significantly different between these cells. The activities of glucose-phosphoenolpyruvate phosphotransferase system in decriptified cells and glyceraldehyde-3-phosphate dehydrogenase in cell-free extracts of the glucose-limited cells were higher than those in the glucose-excess cells. The activities of glucokinase, phosphoglycerate kinase, and pyruvate kinase in cell-free extracts of these cells were not different significantly. We conclude that the high glycolytic activity of the glucose-limited cells results from the increase in the synthesis of glucose-phosphoenolpyruvate phosphotransferase and glyceraldehyde-3-phosphate dehydrogenase.

Streptococci have been known as a major producer of acids in dental plaque (6, 7, 16), and the acid metabolites of microbial metabolism of sugars are responsible for the initiation and the development of dental caries. Thus the sugar metabolism of oral streptococci is of great interest in connection with dental caries.

Streptococci metabolize glucose through the Embden-Meyerhof glycolytic pathway (3, 23). They produce lactate as a main fermentation product when incubated with excess glucose (4, 22, 24). Inhibitory mechanisms of the glycolytic pathway of oral streptococci by some chemicals, such as fluoride and hypothiocyanite, have been studied (5, 8, 15, 20). The fluoride inhibition of enolase results in the inhibition of glucose transport by reducing the amount of phosphoenolpyruvate available for phosphorylation of the sugar (15). We previously reported that the glycolytic reaction catalyzed by glyceraldehyde-3-phosphate dehydrogenase was the primary inhibitory site of the glycolytic pathway by a product (hypothiocyanite) of the lactoperoxidase reaction (5).

Glycolytic activity of streptococci is also changed with parameters such as the glucose concentration or the pH of the growth medium (9, 10, 12, 19). It is known that the streptococcal cells grown under glucose limitation (glucose-limited cells) metabolize glucose much more rapidly than the cells grown in the presence of excess glucose (glucose-excess cells) (9, 12, 19).

Our aim was to elucidate the biochemical mechanisms which bring about the difference in acidogenic potential of the glucose-excess and glucose-limited cells of *Streptococcus sanguis*. The high glycolytic rate of the glucose-limited cells was suspected to stem from the high activities of glucose-phosphoenolpyruvate phosphotransferase (glucose-PTS) and glyceraldehyde-3-phosphate dehydrogenase.

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MATERIALS AND METHODS

Growth conditions. *S. sanguis* ATCC 10556 cells were grown at 35°C in a chemostat (1) with a 400 ml of working volume under glucose limitation and glucose excess. The pH was maintained at 6.5 by automatic titration of 2 N KOH. The gas phase of the culture was 95% N_2 and 5% CO_2. The dilution rate was 0.15 dilutions per h, and the cultures were allowed to reach equilibrium for at least 10 mean generations before the harvest. The medium for glucose-limited continuous culture contained glucose (2.7 g), NH_4HCO_3 (2 g), sodium L-glutamate (1 g), dried extract of yeast (Daigo Eiyokagaku Ltd., Osaka, Japan; 2 g), L-cysteine-HCl (0.1 g), $MgSO_4 \cdot 7H_2O$ (0.2 g), NaCl (0.01 g), $MnSO_4 \cdot 4H_2O$ (0.01 g), and $FeSO_4 \cdot 7H_2O$ (0.01 g) in 1 liter of 20 mM potassium phosphate buffer. The medium for glucose-excess continuous culture contained glucose (9.9 g), NH_4HCO_3 (2 g), sodium L-glutamate (0.3 g), dried extract of yeast (0.6 g), L-cysteine-HCl (0.1 g), $MgSO_4 \cdot 7H_2O$ (0.2 g), NaCl (0.01 g), $MnSO_4 \cdot 4H_2O$ (0.01 g), and $FeSO_4 \cdot 7H_2O$ (0.01 g) in 1 liter of 20 mM potassium phosphate buffer. Cultures were checked for purity by growing them aerobically and anaerobically on blood agar and mitis salivarius agar plates.

Preparation of cell suspensions. A culture was collected for 2 h via the overflow from a chemostat into a container cooled to 0°C. The rates of acid production of the cells collected at 0°C for 2 h and those harvested directly from the chemostat were equivalent. The collected cells were washed with 0.15 M KCl containing 5 mM $MgCl_2$ by centrifugation (14,000 × *g*, 7 min) and then suspended in 0.15 M KCl containing 5 mM $MgCl_2$.

Rate of acid production. The rate of acid production by the cells grown under glucose-limited and glucose-excess conditions was estimated by measurement of the titration volume of 20 mM KOH at pH 6.5 with a pH stat (model HSS-HTS-10A; Toa Co. Ltd. Tokyo, Japan). The reaction mixture contained 10.7 mg (dry weight) of cells, 2 mM potassium

TABLE 1. Rate of acid production by *S. sanguis* ATCC 10556

Time (min) after addition of glucose	Rate of acid production (nmol/min per mg [dry wt] of cells)[a]	
	Glucose-excess cells	Glucose-limited cells
2	152, 146	338, 314
4	127, 143	352, 348

[a] Values from two independent observations are shown.

phosphate buffer (pH 6.5), 20 mM glucose, 0.15 M KCl, and 5 mM $MgCl_2$ in a volume of 5.0 ml. The reaction was started by the addition of glucose and run at 35°C in air. The reaction mixture was agitated by a magnetic stirrer, the amount of KOH added was recorded continuously, and the glycolytic rates at 2 and 4 min after the addition of glucose were estimated.

Determination of glycolytic intermediates. The glycolyzing cells in the reaction mixture were filtered by the method of Carlsson et al. (5). At 2 and 4 min after the addition of glucose, the reaction mixture was filtered through a glass filter (GA-200; Toyo Roshi Co., Tokyo, Japan) and a membrane filter (pore size, 3 μm; Millipore Corp., Bedford, Mass.) to collect the cells. The cells on the filters were then immediately subjected to extraction with 2.0 ml of cold 0.6 N perchloric acid. The glycolytic intermediates in the cells were determined enzymatically as described previously (14).

Preparation of decriptified cells. For preparation of decriptified cells, the freshly harvested cells were washed twice with 0.05 M Tris hydrochloride buffer (pH 7.6) and suspended in the same buffer to 2 mg (dry weight) of cells per ml. The cells in the suspension were permeabilized by the addition of 0.01 volume of toluene-acetone (1:4), followed by vigorous agitation at room temperature for 2 min (21). The resultant decriptified cells were maintained at 0°C until used, but for less than 2.5 h.

Assay of glucose-PTS activity. The glucose-PTS activity of the decriptified cells was assayed spectrophotometrically with a double-wavelength spectrophotometer (model 557; Hitachi, Ltd., Tokyo, Japan) by following the reduction of NADP at 340 nm. The assay mixture contained (in 1.0 ml) 100 mM Tris hydrochloride buffer (pH 7.6), 10 mM $MgCl_2$, 1 mM NADP, 2 mM phosphoenolpyruvate, 0.1 mM glucose, 2.1 U of glucose 6-phosphate dehydrogenase, and 0.02 to 0.4 mg of decriptified cells.

Preparation of cell-free extract. Cells for preparation of cell-free extract were harvested by centrifugation from cultures (120 ml) and washed twice with 0.04 M potassium phosphate buffer (pH 7.0) containing 5 mM $MgCl_2$. They were then suspended in 3 ml of 0.04 M potassium phosphate buffer (pH 7.0) containing 5 mM $MgCl_2$ and 5 mM dithiothreitol in an anaerobic glove box (90% N_2, 10% H_2) and disrupted by sonic oscillation for 15 min at 0°C (200 W, 9 kHz) under strictly anaerobic conditions (24). Cell-free extract was obtained after unbroken cells and cell debris had been removed by centrifugation at 19,000 × *g* for 30 min at 4°C. The cell-free extract was then dialyzed at 4°C against 0.04 M potassium phosphate buffer (pH 7.0) containing 5 mM $MgCl_2$. The protein concentration of the cell-free extract was measured by the biuret method (18).

Assay of enzyme activity in cell-free extracts. The activity of glucokinase was assayed by the method of Anderson and Kamel (2). The assay mixture contained 100 mM Tris hydrochloride buffer (pH 7.6), 10 mM $MgCl_2$, 1 mM NADP, 10 mM ATP, glucose-6-phosphate dehydrogenase (2.1 U/ml), cell-free extract, and 25 mM glucose. The activity of NAD-linked glyceraldehyde-3-phosphate dehydrogenase was assayed as described previously (5), and that of NADP-linked glyceraldehyde-3-phosphate dehydrogenase was assayed by the method of Yamada and Carlsson (23). The

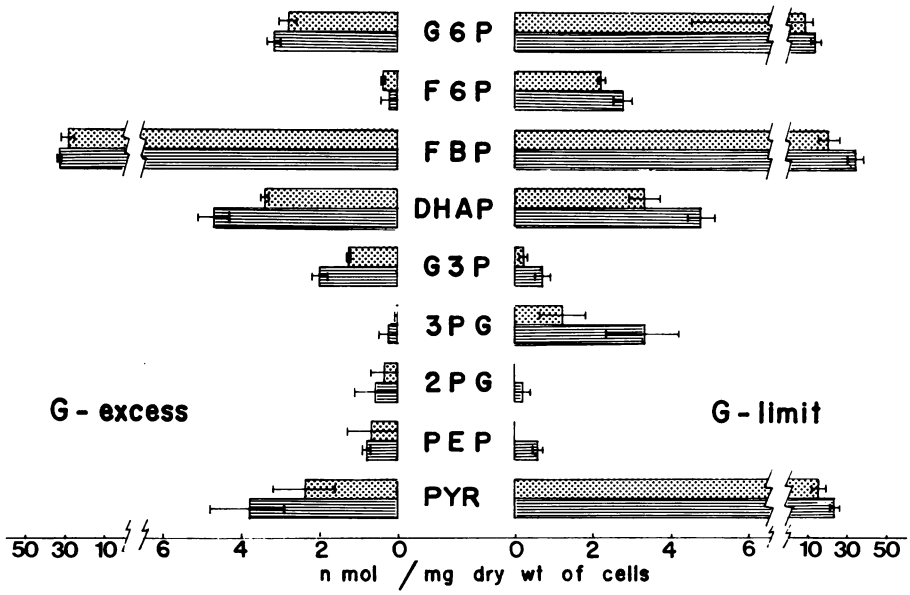

FIG. 1. Intracellular levels of glycolytic intermediates in the glycolyzing cells of *S. sanguis* grown under glucose limitation and glucose excess. Upper and lower bars show the values of the cells harvested 2 and 4 min after the addition of glucose, respectively. The values are expressed as the mean of two independent observations. Abbreviations: G6P, glucose 6-phosphate; F6P, fructose 6-phosphate; FBP, fructose 1,6-bisphosphate; DHAP, dihydroxyacetone phosphate; G3P, glyceraldehyde 3-phosphate; 3PG, 3-phosphoglycerate; 2PG, 2-phosphoglycerate; PEP, phosphoenolpyruvate; PYR, pyruvate.

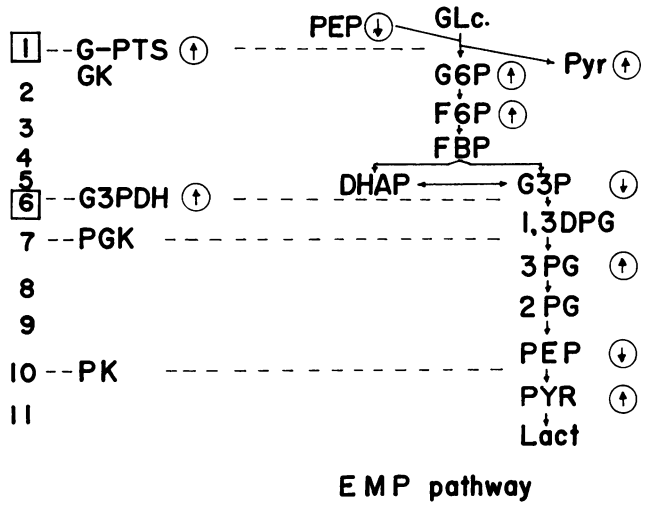

FIG. 2. Proposed scheme for the mechanism of acceleration of glycolysis in *S. sanguis* when the growing condition was changed from glucose excess to glucose limitation. Abbreviations are as in Fig. 1, plus the following: GLc, glucose; 1,3DPG, 1,3-diphosphoglycerate; Lact, lactate; G-PTS, glucose-PTS; GK, glucokinase; G3PDH, glyceraldehyde 3-phosphate dehydrogenase; PGK, phosphoglycerate kinase; PK, pyruvate kinase; EMP, Embden-Meyerhof-Parnas.

activity of phosphoglycerate kinase was assayed by a modification of the method of Krietsch and Bucher (17). The assay mixture contained 100 mM triethanolamine-HCl buffer (pH 7.6), 10 mM $MgCl_2$, 0.2 mM NADH, 5 mM ATP, glyceraldehyde-3-phosphate dehydrogenase (12 U/ml), triose phosphate isomerase (50 U/ml), glycerol-3-phosphate dehydrogenase (3.4 U/ml), 15 mM 3-phosphoglycerate, and cell-free extract. The activity of pyruvate kinase was assayed by the method of Abbe et al. (1). The assay mixture contained 100 mM Tris hydrochloride buffer (pH 7.6), 100 mM KCl, 10 mM $MgCl_2$, 0.17 mM NADH, 2 mM dithiothreitol, 2 mM phosphoenolpyruvate, 0.5 mM fructose 1,6-bisphosphate, lactate dehydrogenase (14 U/ml), 3 mM ADP, and cell-free extract.

Chemicals. Glucose-6-phosphate dehydrogenase (EC 1.1.1.49), glyceraldehyde-3-phosphate dehydrogenase (rabbit muscle, EC 1.2.1.12), triose phosphate isomerase (rabbit muscle, EC 5.3.1.1), glycerol-3-phosphate dehydrogenase (EC 1.1.1.8), and L-lactate dehydrogenase (EC 1.1.1.27) were obtained from Boehringer Mannheim GmbH, Mannheim, Federal Republic of Germany.

RESULTS

Rate of acid production. The rate of acid production by the glucose-limited cells was 2.1 to 2.6 times that by the glucose-excess cells (Table 1). The glycolytic rates of the glucose-limited and glucose-excess cells at 2 min after the addition of the sugar were similar to those at 4 min.

Intracellular concentrations of glycolytic intermediates. The levels of glucose 6-phosphate, fructose 6-phosphate, 3-phosphoglycerate, and pyruvate in the glucose-limited cells were higher than those in the glucose-excess cells (Fig. 1). On the other hand, the level of glyceraldehyde 3-phosphate was lower in the glucose-limited cells. The levels of fructose 1,6-bisphosphate and dihydroxyacetone phosphate were the same in these cells. The levels of most intermediates were higher at the time of 4 min than at the time of 2 min after addition of glucose.

Activity of glycolytic enzymes. The activities of glucose-PTS and NAD- and NADP-linked glyceraldehyde-3-phosphate dehydrogenase in the glucose-limited cells were higher than those in the glucose-excess cells, and the activities of glucokinase, phosphoglycerate kinase, and pyruvate kinase were not significantly different (Table 2).

DISCUSSION

Washed cells of the type strain (ATCC 10556) of *S. sanguis* from the glucose-limited culture produced acid more rapidly than the glucose-excess cells when incubated with glucose (Table 1). These results are similar to those with cells of *S. mutans* (9, 12) and another strain (NCTC 7865) of *S. sanguis* (19). These results imply that the cells grown in deep layers of dental plaque, where sugar supply may be limited, have higher acidogenic potential than those grown in the surface layers, where more sugar can be available.

The observed difference in the levels of glycolytic intermediates in the glucose-limited and the glucose-excess cells (Fig. 1) suggested that the high glycolytic activity of the glucose-limited cells resulted from the high activity of glucokinase, glucose-PTS, glyceraldehyde-3-phosphate dehydrogenase, phosphoglycerate kinase, and pyruvate kinase in these cells (Fig. 2). Among them, however, only the activities of glucose-PTS and glyceraldehyde-3-phosphate dehydrogenase in the glucose-limited cells were significantly higher than those in the glucose-excess cells (Table 2).

It has also been shown that the glucose-limited cells of *S. mutans* (9) and *Actinomyces viscosus* (11) have greater glucose-PTS activity than the glucose-excess cells. The PTS of *S. mutans* is a rate-limiting step at slow dilution rates in a chemostat (9), and glyceraldehyde-3-phosphate dehydroge-

TABLE 2. Enzyme activity of glycolysis of *S. sanguis* ATCC 10556

Cells	Enzyme activity[a]					
	Glucose-PTS[b]	Glucokinase[c]	Glyceraldehyde-3-phosphate dehydrogenase		Phosphoglycerate kinase[d]	Pyruvate kinase[d]
			NAD[d]	NADP[e]		
Glucose excess	18, 22	0.73, 0.60	1.73, 1.78	0.90, 2.00	6.43, 6.32	1.49, 1.58
Glucose limited	250, 210	0.80, 0.99	3.90, 4.18	4.25, 4.05	6.88, 7.26	1.29, 1.50

[a] Values from two independent observations are shown.
[b] Activity expressed as micromoles of NADPH produced per minute per milligram (dry weight) of cells.
[c] Activity expressed as micromoles of NADPH produced per minute per milligram of protein.
[d] Activity expressed as micromoles of NADH consumed per minute per milligram of protein.
[e] Activity expressed as nanomoles of NADPH produced per minute per milligram of protein.

nase or phosphoglycerate kinase is limiting the glycolytic rate in *S. sanguis* (13).

These findings lead us to conclude that the higher glycolytic rate of the glucose-limited cells of *S. sanguis* results from the increase in the amount of glucose-PTS and glyceraldehyde-3-phosphate dehydrogenase in the cells (Fig. 2).

ACKNOWLEDGMENTS

This research was supported by Grants-in-Aid for Scientific Research (A) no. 59440077 (1984–1986) and (C) no. 59570782 (1984) and Grant-in-Aid for Developmental Scientific Research (2) no. 60870066 (1985–1987) from the Japanese Ministry of Education.

LITERATURE CITED

1. **Abbe, K., S. Takahashi, and T. Yamada.** 1983. Purification and properties of pyruvate kinase from *Streptococcus sanguis* and activator specificity of pyruvate kinase from oral streptococci. Infect. Immun. **39**:1007–1014.
2. **Anderson, R. L., and M. Y. Kamel.** 1966. Glucokinase. II. *Aerobacter aerogenes*. Methods Enzymol. **9**:388–392.
3. **Brown, A. T., and C. L. Wittenberger.** 1971. The occurrence of multiple glyceraldehyde-3-phosphate dehydrogenases in cariogenic streptococci. Biochem. Biophys. Res. Commun. **43**:217–224.
4. **Carlsson, J., and C. J. Griffith.** 1974. Fermentation products and bacterial yields in glucose-limited and nitrogen-limited cultures of streptococci. Arch. Oral Biol. **19**:1105–1109.
5. **Carlsson, J., Y. Iwami, and T. Yamada.** 1983. Hydrogen peroxide excretion by oral streptococci and effect of lactoperoxidase-thiocyanate-hydrogen peroxide. Infect. Immun. **40**:70–80.
6. **Cole, J. A.** 1977. A biochemical approach to the control of dental caries. Biochem. Soc. Trans. **5**:1232–1239.
7. **Edwardsson, S.** 1968. Characteristics of caries-inducing human streptococci resembling *Streptococcus mutans*. Arch. Oral Biol. **13**:637–646.
8. **Eisenberg, A. D., G. R. Bender, and R. E. Marquis.** 1980. Reduction in the aciduric properties of the oral bacterium *Streptococcus mutans* GS-5 by fluoride. Arch. Oral Biol. **25**:133–135.
9. **Ellwood, D. C., P. J. Phipps, and I. R. Hamilton.** 1979. Effect of growth rate and glucose concentration on the activity of the phosphoenolpyruvate phosphotransferase system in *Streptococcus mutans* Ingbritt grown in continuous culture. Infect. Immun. **23**:224–231.
10. **Hamilton, I. R., and D. C. Ellwood.** 1978. Effects of fluoride on carbohydrate metabolism by washed cells of *Streptococcus mutans* grown at various pH values in a chemostat. Infect. Immun. **19**:434–442.
11. **Hamilton, I. R., and D. C. Ellwood.** 1983. Carbohydrate metabolism by *Actinomyces viscosus* growing in continuous culture. Infect. Immun. **42**:19–26.
12. **Hamilton, I. R., P. J. Phipps, and D. C. Ellwood.** 1979. Effect of growth rate and glucose concentration on the biochemical properties of *Streptococcus mutans* Ingbritt in continuous culture. Infect. Immun. **26**:861–869.
13. **Iwami, Y., and T. Yamada.** 1980. Rate-limiting steps of the glycolytic pathway in the oral bacteria *Streptococcus mutans* and *Streptococcus sanguis* and the influence of acidic pH on the glucose metabolism. Arch. Oral Biol. **25**:163–169.
14. **Iwami, Y., T. Yamada, and S. Araya.** 1975. Glycolytic intermediates in *Streptococcus mutans* PK 1. Arch. Oral Biol. **20**:695–697.
15. **Kanapka, J. A., and I. R. Hamilton.** 1971. Fluoride inhibition of enolase activity *in vivo* and its relationship to the inhibition of glucose-6-P formation in *Streptococcus salivarius*. Arch. Biochem. Biophys. **146**:167–174.
16. **Krasse, B., H. V. Jordan, S. Edwardsson, I. Svensson, and L. Trell.** 1968. The occurrence of certain "caries-inducing" streptococci in human dental plaque material. Arch. Oral Biol. **13**:911–918.
17. **Krietsch, W. K. G., and T. Bucher.** 1970. 3-Phosphoglycerate kinase from rabbit skeletal muscle and yeast. Eur. J. Biochem. **17**:568–580.
18. **Layne, E.** 1957. Spectrophotometric and turbidimetric methods for measuring proteins. Methods Enzymol. **3**:447–454.
19. **Marsh, P. D., M. I. Williamson, C. W. Keevil, A. S. McDermid, and D. C. Ellwood.** 1982. Influence of sodium and potassium ions on acid production by washed cells of *Streptococcus mutans* Ingbritt and *Streptococcus sanguis* NCTC 7865 grown in a chemostat. Infect. Immun. **36**:476–483.
20. **Mickelson, M. N.** 1966. Effect of lactoperoxidase and thiocyanate on the growth of *Streptococcus pyogenes* and *Streptococcus agalactiae* in a chemically defined culture medium. J. Gen. Microbiol. **43**:31–43.
21. **Slee, A. M., and J. M. Tanzer.** 1979. Phosphoenolpyruvate-dependent sucrose phosphotransferase activity in *Streptococcus mutans* NCTC 10449. Infect. Immun. **24**:821–828.
22. **Tanzer, J. M., M. I. Krichevsky, and P. H. Keyes.** 1969. The metabolic fate of glucose catabolized by a washed stationary phase caries-conducive streptococcus. Caries Res. **3**:167–177.
23. **Yamada, T., and J. Carlsson.** 1973. Phosphoenolpyruvate carboxylase and ammonium metabolism in oral streptococci. Arch. Oral Biol. **18**:799–812.
24. **Yamada, T., and J. Carlsson.** 1975. Regulation of lactate dehydrogenase and change of fermentation products in streptococci. J. Bacteriol. **124**:55–61.