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Polyol Metabolism by a Caries-Conducive *Streptococcus*: Purification and Properties of a Nicotinamide Adenine Dinucleotide-Dependent Mannitol-1-Phosphate Dehydrogenase

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The mannitol-1-phosphate dehydrogenase (M1PDH) (EC 1.1.1.17) from *Streptococcus mutans* strain FA-1 was purified to approximately a 425-fold increase in specific activity with a 29% recovery of total enzyme units, using a combination of (i) streptomycin sulfate and ammonium sulfate precipitation and (ii) diethylaminoethyl-cellulose (DE-52), agarose A 0.5M, and agarose-nicotinamide adenine dinucleotide (NAD) affinity column chromatography. Polyacrylamide gel electrophoresis of the purified enzyme preparation showed a single protein component that coincided with a band of M1PDH activity. The enzyme had a molecular weight of approximately 45,000 and was stable for long periods of time when stored at −80°C in the presence of β-mercaptoethanol. Its activity was not affected by mono- or divalent cations, and high concentrations of ethylenediaminetetraacetic acid were not inhibitory. The M1PDH catalyzed both the NAD-dependent oxidation of mannitol-1-phosphate and the reduced NAD (NADH)-dependent reduction of fructose-6-phosphate. The forward reaction was highly specific for mannitol-1-phosphate and NAD, whereas the reverse reaction was highly specific for NADH and fructose-6-phosphate. The K_m values for mannitol-1-phosphate and NAD were 0.15 and 0.066 mM, respectively, and the K_m values for fructose-6-phosphate and NADH were 1.66 and 0.016 mM, respectively. The forward and reverse reactions catalyzed by the M1PDH from *S. mutans* appeared to be under cellular control. Both adenosine 5′-triphosphate and fructose-6-phosphate were negative effectors of the forward reaction, whereas adenosine 5′-diphosphate served as a negative effector of the reverse reaction catalyzed by the enzyme.

Oral strains of *Streptococcus mutans* are causative agents of multisurface dental caries in rodents and humans (5, 10, 11, 15, 16, 18, 21, 22). A nutritional characteristic that serves to distinguish *S. mutans* from other streptococcal components of the oral microflora is their ability to utilize either mannitol or sorbitol as a primary energy source (8, 9, 11, 14). We have shown that mannitol and sorbitol are phosphorylated to mannitol-1-phosphate and sorbitol-6-phosphate, respectively, before their conversion to the glycolytic intermediate fructose-6-phosphate by the action of mannitol-1-phosphate dehydrogenase (M1PDH) (EC 1.1.1.17) or sorbitol-6-phosphate dehydrogenase (S6PDH) (4). The M1PDH and S6PDH are separate and distinct enzymes, both of which exhibit specificity for the coenzyme nicotinamide adenine dinucleotide (NAD) for catalytic activity. Since recent interest has focused upon the use of polyalcohols as dietary sucrose substitutes, we decided to investigate more thoroughly the metabolism of mannitol and sorbitol by oral strains of *S. mutans*. The purpose of this communication is to report the purification of M1PDH from *S. mutans* strain FA-1 and to describe some of its physical, kinetic, and regulatory properties.

MATERIALS AND METHODS

Chemicals. Mannitol-1-phosphate was purchased as the barium salt from Sigma Chemical Co., St. Louis, Mo., and the barium was removed before use by treatment with Sigma Dowex 50X as previously described (4). All enzyme assay components were also purchased from Sigma. Diethylaminoethyl (DEAE)-cellulose (DE-52) was purchased from Reeve Angel, Clifton, N.J.; agarose A 0.5M from Bio-Rad, Richmond, Calif.; and agarose-NAD (AG-NAD) from PBL Biochemicals, Milwaukee, Wis. All components of the *S. mutans* growth medium were purchased from Difco, Detroit, Mich.

Growth of organisms. *S. mutans* strain FA-1 was

grown in a complex medium containing 0.5% (wt/vol) tryptone, 0.5% (wt/vol) yeast extract, and 0.5% (wt/vol) dibasic potassium phosphate. The pH of the medium was adjusted to 6.8 with concentrated HCl before sterilization. The complex medium was supplemented with 0.5% (wt/vol) mannitol, which was sterilized separately and added aseptically. Cells were routinely grown in 4-liter cultures and were harvested after 16 h of incubation at 37°C.

Preparation of cell extracts. Cells from two 4-liter cultures were harvested by centrifugation and washed with 0.01 M potassium phosphate, pH 7.0, and then resuspended in 100 ml of 0.05 M tris(hydroxymethyl)aminomethane (Tris)-hydrochloride buffer, pH 7.5, which contained 0.005 M β-mercaptoethanol. Cell-free extracts were prepared by sonically treating the cell suspensions as described previously (1, 4).

Enzyme assay. M1PDH activity was assayed in both the forward and reverse directions by monitoring the oxidation or reduction of pyridine nucleotides at 340 nm, using a Gilford 300-N spectrophotometer equipped with a Sargent SRG recorder. The reactions were run at room temperature (approximately 25°C) and were initiated by the addition of enzyme. In all cases, 1 U of activity is the amount of enzyme required to oxidize 1 nmol of reduced pyridine nucleotide or to reduce 1 nmol of oxidized pyridine nucleotide per min.

Forward reaction. The NAD-dependent oxidation of mannitol-1-phosphate to fructose-6-phosphate was assayed by monitoring the mannitol-1-phosphate-dependent production of reduced NAD (NADH) from NAD at 340 nm. The assay contained the following components in a final volume of 1.0 ml: mannitol-1-phosphate, 1.0 nmol; NAD, 1.0 nmol; Tris-hydrochloride, pH 8.5, 100 nmol; and distilled water to volume.

Reverse reaction. The NADH-dependent conversion of fructose-6-phosphate to mannitol-1-phosphate was assayed by monitoring the fructose-6-phosphate-dependent oxidation of NADH at 340 nm. The assay contained the following components in a final volume of 1.0 ml: fructose-6-phosphate, 1.0 nmol; NADH, 0.1 nmol; potassium phosphate, pH 6.2, 100 nmol; and distilled water to volume.

Molecular-weight estimation. The molecular weight of the M1PDH from *S. mutans* strain FA-1 was estimated by using agarose A 0.5M molecular exclusion column chromatography. The following protein standards were used: bovine serum albumin (molecular weight, 68,000); ovalbumin (molecular weight, 45,000); α-chymotrypsinogen (molecular weight, 25,000); and ribonuclease A (molecular weight, 13,700). A 0.75-ml sample of each standard containing 10 mg of protein per ml was applied to an agarose A 0.5M column (1.9 by 60 cm) that had been equilibrated with 0.05 M Tris-hydrochloride, pH 7.5. The protein standards were eluted from the column with the equilibrating buffer by the upward-flow technique, and 2.0-ml fractions were collected. The elution volume of the standards was determined by monitoring the column effluent at 280 nm. A 0.75-ml sample of the M1PDH from *S. mutans* was then applied to the column and eluted in a similar manner. The elution volume of the enzyme was determined by assaying M1PDH activity as described above. The elution volume of the protein standards was plotted against the logarithm of their respective molecular weights. The molecular weight of the M1PDH was then estimated graphically.

Polyacrylamide gel electrophoresis. A sample of purified M1PDH from *S. mutans* strain FA-1 was applied to the top of 7.5% polyacrylamide gels prepared in glass tubes (6 by 100 mm) according to the pH 9.3 system of Davis (6) as described in the *Buchler Polyanalyst Manual*. After electrophoresis at 4°C for approximately 3 h, the gels were removed from the tubes and stained for either M1PDH activity or total protein. When stained for M1PDH activity, the gels were placed in a solution of the following composition: Tris-hydrochloride, pH 8.5, 100 mM; mannitol-1-phosphate, 2.0 mM; nitroblue tetrazolium chloride, 0.8 mM; and phenazine methosulfate, 0.15 mM. The gels were incubated in the staining solution at 25°C until colored activity bands developed and were stored in 7% acetic acid. When stained for total protein, the gels were placed in a 0.5% solution of Coomassie brilliant blue in 20% trichloroacetic acid for 30 min. The gels were then removed and destained in 10% trichloroacetic acid for 48 h.

Protein determination. Protein was measured by the method of Lowry et al. (19) or by the biuret method (13).

Purification of M1PDH. The source of the M1PDH was *S. mutans* strain FA-1. A cell extract was prepared in 200 ml of 0.05 M Tris-hydrochloride, pH 7.5, from the cells obtained from 16 liters of culture medium as described above. All purification procedures were carried out at 4°C, and all buffers contained 0.005 M β-mercaptoethanol.

Step I. Streptomycin sulfate precipitation. Twenty milliliters of a 10% streptomycin sulfate solution was added dropwise to 180 ml of cell-free extract prepared as described above. The solution was allowed to stand in an ice bucket for 30 min and was then centrifuged at 20,000 $\times$ *g* for 20 min. The pellet was discarded, and the supernatant was dialyzed overnight against 0.01 M Tris-hydrochloride, pH 7.5, and treated as described below.

Step II. Ammonium sulfate precipitation. Solid ammonium sulfate was added to the dialyzed supernatant from step I until 50% saturation was reached. The suspension was allowed to stand for 30 min, and then the precipitate was removed by centrifugation at 20,000 $\times$ *g* for 20 min and discarded. Solid ammonium sulfate was then added to the supernatant fluid until 70% saturation was attained. After the suspension was allowed to stand for 30 min, the precipitate was removed by centrifugation at 20,000 $\times$ *g* for 20 min and the supernatant was discarded. The precipitate was redissolved in 0.05 M Tris-hydrochloride, pH 7.5, and the volume was adjusted to 15 ml by addition of the same buffer. The sample was then dialyzed overnight against 6 liters of 0.01 M Tris-hydrochloride, pH 7.5.

Step III. DEAE-cellulose (DE-52) chromatography. The dialyzed preparation from step II was slowly pumped onto a DEAE-cellulose (DE-52) column (2.0 by 20 cm) that had been packed under

pressure and equilibrated with 0.05 M Tris-hydrochloride, pH 7.5. After the sample was applied to the column, the following eluting buffers were pumped through the column using a Buchler peristaltic pump at a flow rate of 160 ml/h: 0.05 M Tris-hydrochloride, pH 7.5, 200 ml; 0.05 M Tris-hydrochloride, pH 7.5, containing 0.10 M KCl, 200 ml; 0.05 M Tris-hydrochloride, pH 7.5, containing 0.25 M KCl, 200 ml; and 0.05 M Tris-hydrochloride, pH 7.5, containing 0.60 M KCl, 200 ml. Column fractions of 10 ml were collected and assayed for M1PDH activity as described above. Those fractions containing high enzyme activity were pooled and concentrated by ultrafiltration with an Amicon ultrafiltration cell. The concentrated enzyme preparation was then dialyzed overnight against 6 liters of 0.01 M Tris-hydrochloride, pH 7.5. An elution profile of M1PDH activity and protein is shown in Fig. 1A.

Step IV. DEAE-cellulose (DE-52) chromatography. The concentrated and dialyzed sample from step III was slowly pumped onto a DEAE-cellulose (DE-52) column (2.0 by 20 cm) that had been equilibrated as in step III. After the sample was applied to the column, the following eluting buffers were pumped through the column using a Buchler peristaltic pump at a flow rate of 160 ml/h: 0.05 M Tris-hydrochloride, pH 7.5, 200 ml; and a linear gradient of 0 to 0.70 M KCl in 0.05 M Tris-hydrochloride, pH 7.5, in a total volume of 450 ml. Column fractions of 10 ml were collected and assayed for M1PDH activity as described above. Those fractions containing high M1PDH activity were pooled and concentrated by ultrafiltration as described above. The concentrated enzyme preparation was then dialyzed overnight against 0.01 M Tris-hydrochloride, pH 7.5. An elution profile of enzyme activity and protein is shown in Fig. 1B.

Step V. Agarose gel permeation chromatography. The concentrated and dialyzed sample from step IV was slowly pumped onto an agarose A 0.5M column (1.9 by 90 cm) that had been equilibrated with 0.05 M Tris-hydrochloride, pH 7.5. M1PDH activity was eluted from the column with the equilibrating buffer by the upward-flow technique. Those fractions containing high M1PDH activity were pooled and concentrated by ultrafiltration as described above. An elution profile of enzyme activity and protein is shown in Fig. 1C.

Step VI. Agarose-hexane NAD affinity chromatography. A 1.5-ml aliquot of the concentrated and dialyzed sample from step V was slowly pumped onto an agarose column (0.9 by 7.0 cm) that had been

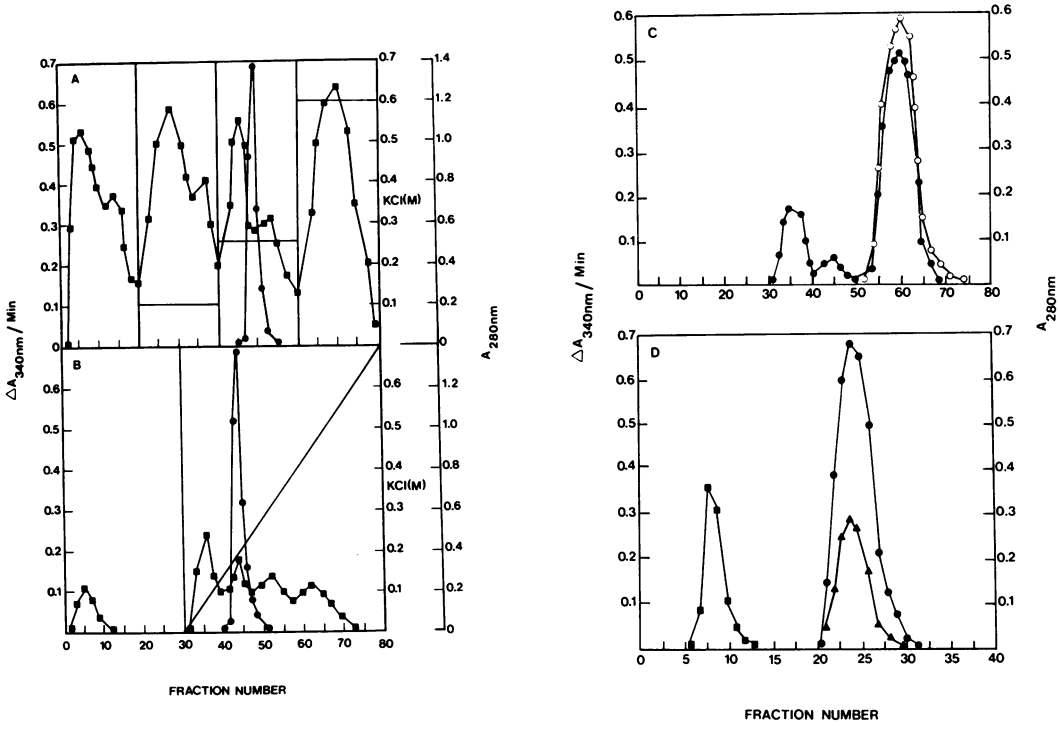

FIG. 1. *(A) Chromatography of fraction III (Table 1) on a DEAE-cellulose (DE-52) column. Symbols: M1PDH activity (●); absorbance at 280 nm (■). (B) Chromatography of fraction IV (Table 1) on a DEAE-cellulose (DE-52) column. Symbols: M1PHD activity (●); absorbance at 280 nm (■). (C) Chromatography of fraction V (Table 1) on an agarose A 0.5M column. Symbols: M1PDH activity (○); absorbance at 280 nm (●). (D) Chromatography of fraction VI (Table 1) on an agarose-NAD column. Symbols: M1PDH activity (●); absorbance at 280 nm (▲); ovalbumin (■). Effluent fractions were assayed for M1PDH activity as described in Materials and Methods.*

derivatized with hexane NAD and equilibrated with 0.05 M Tris-hydrochloride, pH 7.5, buffer. M1PDH activity was eluted from the column with the equilibrating buffer. Those fractions (1.5 ml) containing high M1PDH activity were pooled and frozen at −80°C in 0.5-ml aliquots. An elution profile of enzyme activity and a 1.5-ml ovalbumin standard containing 10 mg of protein per ml is shown in Fig. 1D. All protein in the sample from step V that was not found in the elution volume containing M1PDH activity was found in the elution volume containing the ovalbumin standard. The M1PDH preparation from step VI was stable indefinitely when stored at −80°C and showed no loss of activity when left in an ice bucket during the course of a day's experiments.

RESULTS

Purification of the M1PDH. A summary of the purification scheme discussed in Materials and Methods is shown in Table 1. The purification procedures resulted in approximately a 425-fold increase in M1PDH specific activity, with a 29% recovery of total units of enzyme activity. An examination of fraction VII by polyacrylamide discontinuous gel electrophoresis gave the results shown in Fig. 2. One major M1PDH activity band was observed that coincided with a single protein band when the gels were stained with Coomassie brilliant blue. The results obtained from the discontinuous electrophoresis studies reported here indicate that fraction VII (Table 1) is a highly purified if not homogeneous M1PDH preparation. All of the following studies were done using the fraction VII (Table 1) enzyme preparation.

Reversibility of the M1PDH. The purified M1PDH from *S. mutans* strain FA-1 readily catalyzed both the NAD-dependent oxidation of mannitol-1-phosphate and the NADH-dependent reduction of the glycolytic intermediate, fructose-6-phosphate. Consequently, the reactions catalyzed by this enzyme are reversible since the NAD-specific oxidation of mannitol-1-phosphate gave fructose-6-phosphate and NADH as reaction products, whereas the NADH-dependent reduction of fructose-6-phosphate yielded NAD and mannitol-1-phosphate.

The forward and reverse reaction rates for the M1PDH from *S. mutans* were dependent upon the pH of the assay system (Fig. 3). The rate of the forward reaction was maximum between pH 8.5 and 9.0, whereas the rate of the reverse reaction was maximum around pH 6.5. The reaction rates observed when the assays were run in phosphate buffer were always lower than the rates observed when the same reactions were run in Tris-hydrochloride at identical pH values.

Molecular-weight estimation of the M1PDH. The molecular weight of the M1PDH from *S. mutans* FA-1 was estimated by using agarose A 0.5M molecular exclusion chromatography. A graphical estimation of the molecular weight of the enzyme was derived from a standard curve obtained by plotting the logarithm of the molecular weight of a series of protein standards versus the elution volume of the standards as described in Materials and Methods and shown in Fig. 4. The molecular weight of the M1PDH was found to be approximately 45,000 as determined by this procedure.

Substrate specificity of the M1PDH. The forward reaction catalyzed by the M1PDH from *S. mutans* was specific for mannitol-1-phosphate. A number of hexose phosphates, hexoses, polyols, pentose phosphates, and pentoses were unable to substitute for mannitol-1-phosphate when included at equimolar concentrations in the M1PDH assay system (Table 2). It is also significant that sorbitol-6-phosphate showed only 3% of the activity observed with

TABLE 1. *Summary of the purification scheme for M1PDH from Streptococcus mutans* [a]

Fraction	Total enzyme U	Percent recovery	Total protein (mg)	Sp act (U/mg)
I. Crude cell extract	6,328		6,750	0.94
II. Streptomycin sulfate precipitation	5,948	94	6,250	0.96
III. 50–70% ammonium sulfate precipitation	5,671	90	2,200	2.58
IV. DEAE-cellulose chromatography	4,538	72	305	14.8
V. DEAE-cellulose chromatography	2,991	47	19	157
VI. Agarose A 0.5M chromatography	2,344	37	6.25	375
VII. AGNAD affinity chromatography	1,860	29	4.37	427

[a] The fractions are described in Materials and Methods, as is the M1PDH assay. M1PDH activity was measured by monitoring the fructose-6-phosphate-dependent oxidation of NADH at 340 nm. AGNAD, Agarose-NAD.

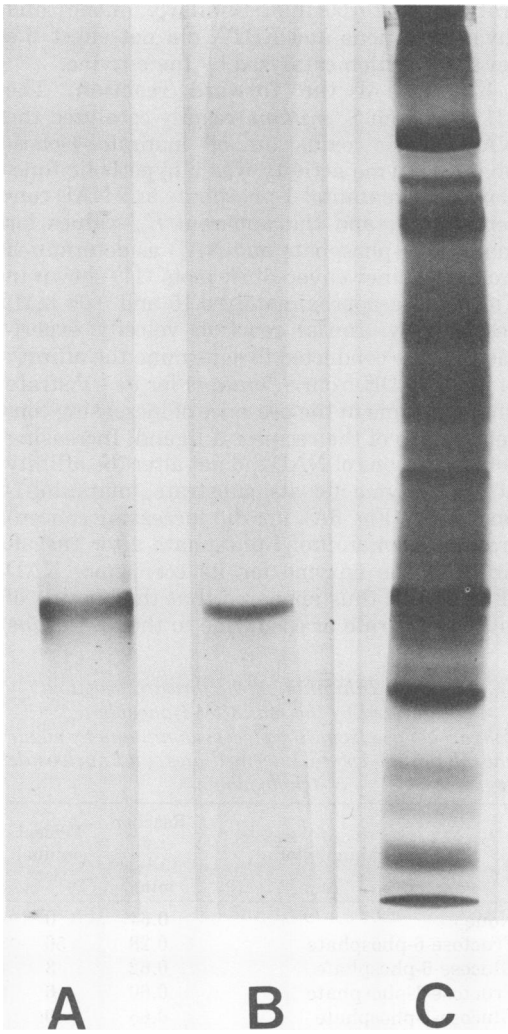

FIG. 2. *Polyacrylamide discontinuous electrophoresis of the M1PDH from Streptococcus mutans FA-1. The electrophoresis and staining procedures were the same as described in Materials and Methods. Tube A contained 20 μg of fraction VII (Table 1) enzyme and was stained for M1PDH activity. Tube B contained 20 μg of fraction VII enzyme (Table 1) and was stained for total protein. Tube C contained 500 μg of fraction I enzyme (Table 1) and was also stained for total protein.*

mannitol-1-phosphate when tested as a potential substrate in the M1PDH assay mixture. Thus, the forward reaction catalyzed by the M1PDH from *S. mutans* showed a high specificity for mannitol-1-phosphate. Similarly, the reverse reaction catalyzed by the enzyme was specific for its substrate fructose-6-phosphate (Table 2).

Coenzyme specificity of the M1PDH. In addition to being specific for mannitol-1-phosphate, the forward reaction catalyzed by the M1PDH from *S. mutans* exhibited specificity for the coenzyme, NAD. No M1PDH activity was observed when equimolar concentrations of NADP were substituted for NAD in the standard assay. Similarly, the reverse reaction catalyzed by the M1PDH from *S. mutans* was specific for the reduced pyridine nucleotide, NADH.

Effect of metals and chelating agents. The forward reaction catalyzed by the M1PDH from *S. mutans* was not dependent upon nor was its activity stimulated by K^+, Na^+, Mg^{2+}, Mn^{2+}, Ca^{2+}, or Zn^{2+} in a concentration range from 0.1 to 3 mM. Similar results were obtained with the chloride or the sulfate salts of these cations. The lack of an appreciable metal effect on enzyme activity was the same before and after extensive dialysis of the highly purified M1PDH preparation. In addition to the lack of stimulation by a number of mono- and divalent cations, ethylenediaminetetraacetic acid (EDTA) had no effect on enzyme activity when included in the incubation mixture at concen-

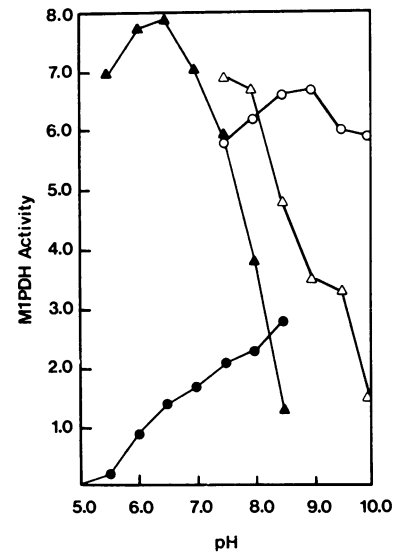

FIG. 3. *Effect of pH on the forward and reverse reactions catalyzed by the M1PDH from Streptococcus mutans FA-1. The forward and reverse reactions for the M1PDH were assayed as described in Materials and Methods except that the pH of the Tris-hydrochloride and potassium phosphate buffers were varied as shown above. In all cases the final concentration of buffer in the assay system was 100 mM. Symbols: M1PDH forward reaction in potassium phosphate buffer (●); M1PDH forward reaction in Tris-hydrochloride buffer (○); M1PDH reverse reaction in potassium phosphate buffer (▲); M1PDH reverse reaction in Tris-hydrochloride buffer (△). The reactions were initiated by the addition of 0.28 μg of fraction VII (Table 1) enzyme.*

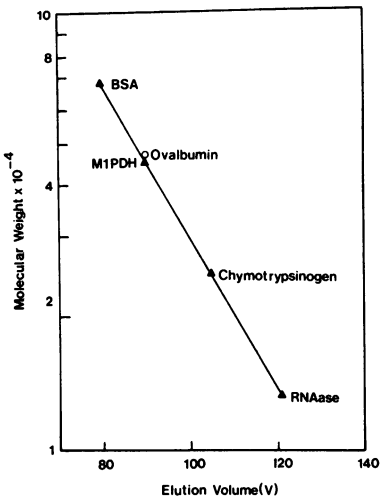

FIG. 4. *Molecular weight of the M1PDH from Streptococcus mutans FA-1. M1PDH activity was assayed as described in Materials and Methods. The elution volume of each of the protein standards was determined by monitoring their elution at 280 nm. The M1PDH sample used for this determination contained 10 μg of fraction VII enzyme (Table 1). BSA, Bovine serum albumin; RNase, ribonuclease.*

TABLE 2. *Substrate specificity of the M1PDH from Streptococcus mutans*[a]

Substrate	Relative activity	
	Forward reaction	Reverse reaction
Mannitol-1-phosphate	100	
Fructose-6-phosphate		100
Sorbitol-6-phosphate	0	0
Glucose-6-phosphate	0	0
Fructose-1-phosphate	0	0
Glucose-1-phosphate	0	0
Fructose-1,6-diphosphate	0	0
Glucose	0	0
Fructose	0	0
Mannose	0	0
Galactose	0	0
Mannitol	0	0
Sorbitol	0	0
Dulcitol	0	0
Ribitol	0	0
Ribose	0	0
Ribose-5-phosphate	0	0
Xylulose-5-phosphate	0	0

[a] The forward and reverse reactions catalyzed by the M1PDH from *S. mutans* were assayed as described in Materials and Methods except that the potential substrates listed above were substituted for mannitol-1-phosphate and fructose-6-phosphate at a final concentration in the reaction mixture of 2 mM. The reactions were initiated by the addition of 0.25 μg of fraction VII enzyme (Table 1).

trations up to 50 mM. Similarly, mono- and divalent cations and EDTA did not effect the reverse reaction catalyzed by the enzyme.

Kinetics of the forward reaction. The M1PDH from *S. mutans* readily catalyzed the NAD-specific oxidation of mannitol-1-phosphate. Enzyme activity was a hyperbolic function of the mannitol-1-phosphate and NAD concentrations, and the apparent K_m values for mannitol-1-phosphate and NAD as determined from the Lineweaver-Burk plots (17) shown in Fig. 5 were approximately 0.15 and 0.06 mM, respectively. Initial reaction velocity experiments were conducted to determine the affinity of the M1PDH from *S. mutans* for its substrate and coenzyme in the presence of increasing concentrations of the reciprocal ligand. Increasing concentrations of NAD did not alter the affinity of the enzyme for its substrate, mannitol-1-phosphate (Fig. 5A), nor did increasing concentrations of mannitol-1-phosphate alter the affinity of the enzyme for its coenzyme, NAD (Fig. 5B). It thus appeared that the binding of either substrate or coenzyme to the *S. mutans*

TABLE 3. *Inhibition of the forward reaction catalyzed by the mannitol-1-phosphate dehydrogenase from Streptococcus mutans by sugar phosphates, glycolytic intermediates, and nucleoside 5'-triphosphates*[a]

Potential inhibitor	Reaction rate (ΔA_{340}/min)[b]	Percent inhibition
None	0.64	0
Fructose-6-phosphate	0.28	56
Glucose-6-phosphate	0.62	3
Fructose-1-phosphate	0.60	6
Glucose-1-phosphate	0.66	0
Fructose-1,6-diphosphate	0.56	12
Glyceraldehyde-3-phosphate	0.62	3
3-Phosphoglycerate	0.60	6
2-Phosphoglycerate	0.64	0
Phosphoenolpyruvate	0.55	14
Pyruvate	0.64	0
Adenosine 5'-triphosphate	0.30	53
Guanosine 5'-triphosphate	0.57	11
Cytidine 5'-triphosphate	0.58	9
Thymidine 5'-triphosphate	0.60	6
Uridine 5'-triphosphate	0.64	0
Inosine 5'-triphosphate	0.60	6
Adenosine 5'-diphosphate	0.48	20

[a] The forward reaction was assayed as described in Materials and Methods except that the potential effectors were included in the reaction mixture at a final concentration of 3 mM. The reaction was initiated with 0.21 μg of fraction VII (Table 1) enzyme.

[b] A_{340}, Absorbance at 340 nm.

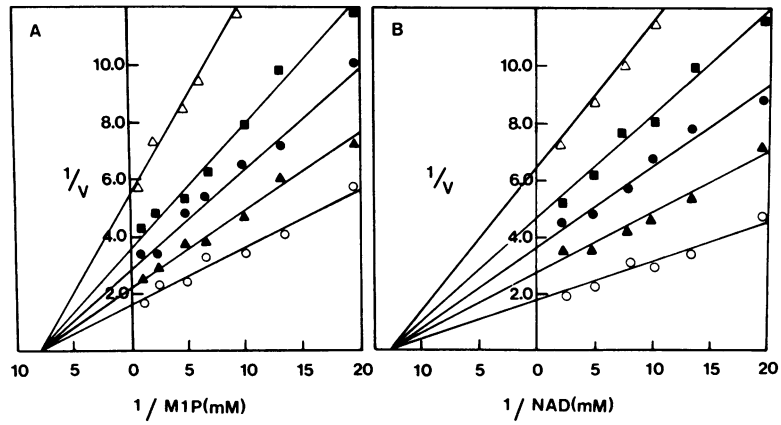

FIG. 5. *(A) Effect of NAD on the interaction of mannitol-1-phosphate (M1P) with the M1PDH from Streptococcus mutans. The forward reaction was assayed as described in Materials and Methods except that the concentration of mannitol-1-phosphate was varied as indicated at different concentrations of NAD. Each assay contained 0.24 μg of fraction VII enzyme (Table 1). The following symbols indicate the NAD concentrations used: 1.0 mM (○); 0.15 mM (▲); 0.075 mM (●); 0.050 mM (■); 0.025 mM (△). (B) Effect of mannitol-1-phosphate on the interaction of NAD with the M1PDH from S. mutans. The forward reaction was assayed as described in Materials and Methods except that the NAD concentration was varied as indicated at different concentrations of mannitol-1-phosphate. Each assay contained 0.24 μg of fraction VII enzyme (Table 1). The following symbols indicate the mannitol-1-phosphate concentrations used: 1.0 mM (○); 0.25 mM (▲); 0.15 mM (●); 0.10 mM (■); 0.05 mM (△).*

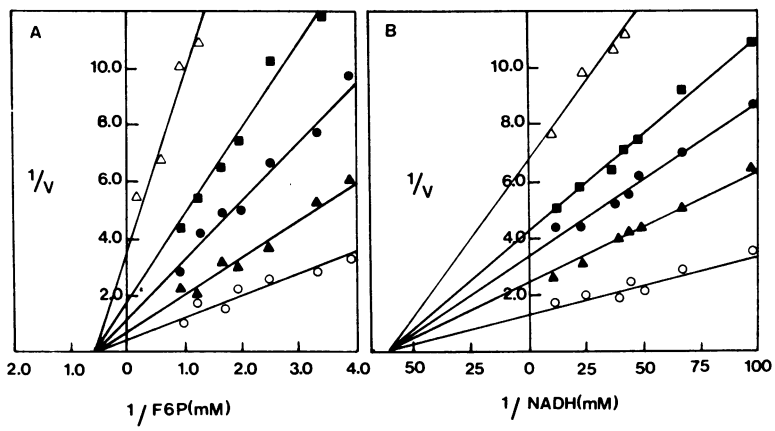

FIG. 6. *(A) Effect of NADH on the interaction of fructose-6-phosphate (F6P) with the M1PDH from Streptococcus mutans. The reverse reaction was assayed as described in Materials and Methods except that the concentration of fructose-6-phosphate was varied as indicated in the presence of different concentrations of NADH. The reactions were initiated with 0.27 μg of fraction VII enzyme (Table 1). The following symbols indicate the NADH concentrations used: 0.1 mM (○); 0.05 mM (▲); 0.025 mM (●); 0.015 mM (■); 0.01 mM (△). (B) Effect of fructose-6-phosphate on the interaction of NADH with the M1PDH from S. mutans. The reverse reaction was assayed as described in Materials and Methods except that the NADH concentrations were varied as indicated at different levels of fructose-6-phosphate. The reactions were initiated with 0.27 μg of fraction VII enzyme (Table 1). The following symbols indicate the fructose-6-phosphate concentrations used: 5.0 mM (○); 2.5 mM (▲); 1.5 mM (●); 1.0 mM (■); 0.5 mM (△).*

M1PDH was independent of the concentration of the other ligand.

Kinetics of the reverse reaction. The rate of the reverse reaction catalyzed by the M1PDH from *S. mutans* was a hyperbolic function of both the NADH and fructose-6-phosphate concentration (Fig. 6). The apparent K_m value for the coenzyme, NADH, was approximately 0.016 mM, whereas the K_m value for the substrate, fructose-6-phosphate, was approximately 1.66

mM as estimated from the Lineweaver-Burk plots shown in Fig. 6. The K_m value for NADH was independent of the fructose-6-phosphate concentration, and likewise the K_m value for fructose-6-phosphate was independent of the NADH concentration (Fig. 6). Consequently, the K_m values for the coenzyme and substrate of the reverse reaction catalyzed by the M1PDH from *S. mutans*, like those of the forward reaction, were independent of the concentration of the other ligand.

Regulation of the forward reaction. A number of mono-, di-, and trinucleotides were examined for their ability to modulate the NAD-specific oxidation of mannitol-1-phosphate catalyzed by the M1PDH from *S. mutans*. The forward reaction was strongly inhibited by adenosine 5'-triphosphate (ATP) (Table 3), whereas other nucleoside 5'-triphosphates had little or no effect on enzyme activity. In addition, adenosine 5'-diphosphate (ADP) was not nearly as effective an inhibitor of the forward reaction as was ATP. The K_i for ATP under the standard assay conditions was approximately 2.2 mM as estimated from Dixon plots (7) of the ATP saturation data (Fig. 7A, B), and 100% inhibition of enzyme activity could be obtained at high concentrations of the nucleoside 5'-triphosphate.

The substrate of the forward reaction, mannitol-1-phosphate, had no effect on the apparent K_i for ATP (Fig. 7A), but increasing concentrations of NAD significantly raised the K_i for this negative effector (Fig. 7B). It thus appears that the inhibition exerted on the M1PDH from *S. mutans* by ATP is noncompetitive with respect to mannitol-1-phosphate but competitive with respect to NAD.

A number of sugar phosphates and glycolytic intermediates were also examined for their ability to modulate the forward reaction catalyzed by the M1PDH from *S. mutans* (Table 3). The only compound tested that had a significant effect on the forward reaction was fructose-6-phosphate. Other sugar phosphates or glycolytic intermediates tested had little or no effect on enzyme activity. The K_i value for fructose-6-phosphate under standard assay conditions was approximately 3.33 mM as estimated from Dixon plots of the fructose-6-phosphate saturation data (Fig. 7C, D), and high concentrations of this glycolytic intermediate were able to inhibit the forward reaction 100%.

The apparent K_i for fructose-6-phosphate was not dependent upon the NAD concentration in the reaction mixture (Fig. 7D). In contrast, increasing concentrations of mannitol-1-phosphate raised the apparent K_i for fructose-6-phosphate (Fig. 7C). Consequently, the interaction of fructose-6-phosphate with the M1PDH from *S. mutans*, in contrast to the interaction of ATP with the enzyme, appeared to be competitive with respect to mannitol-1-phosphate but noncompetitive with respect to NAD.

Regulation of the reverse reaction. A number of mono-, di-, and trinucleotides were tested for their ability to serve as negative effectors for the reverse reaction catalyzed by the M1PDH from *S. mutans*. Nucleoside 5'-diphosphates were the most effective inhibitors of the reverse reaction catalyzed by the enzyme, and specificity studies showed that ADP was the most potent inhibitor of M1PDH activity (Table 4). This is in contrast to what was observed with the forward reaction catalyzed by the enzyme that was most susceptible to inhibition by ATP. In contrast to the forward reaction catalyzed by the enzyme, no sugar phosphate or glycolytic intermediate tested exerted an appreciable effect on the reverse reaction. The apparent K_i for ADP calculated from Dixon plots of the ADP saturation data under standard assay conditions was 2.0 mM (Fig. 8), and the substrate of the reverse reaction, fructose-6-phosphate, did not alter the K_i for ADP (Fig. 8A). In contrast, however, the K_i for ADP was inversely proportional to the concentration of NADH in the reaction mixture (Fig. 8B). It thus appears that the interaction of ADP with the M1PDH from *S. mutans* is competitive with respect to its coenzyme, NADH, but noncompetitive with respect to its substrate, fructose-6-phosphate.

TABLE 4. *Inhibition of the reverse reaction catalyzed by the M1PDH from Streptococcus mutans by nucleoside 5'-diphosphates*[a]

Nucleoside 5'-diphosphate	Reaction rate (ΔA_{340}/min)[b]	Percent inhibition
None	0.72	0
Adenosine	0.38	48
Guanosine	0.65	10
Cytidine	0.60	17
Thymidine	0.66	8
Uridine	0.60	16

[a] The reverse reaction was assayed as described in Materials and Methods except that the potential inhibitors were included in the reaction mixture at a final concentration of 3 mM. The reactions were initiated by the addition of 0.20 μg of fraction VII (Table 1) enzyme.

[b] See Table 3.

DISCUSSION

The M1PDH from *S. mutans* strain FA-1 obtained by the purification scheme described here consisted of a highly purified enzyme preparation as determined by polyacrylamide dis-

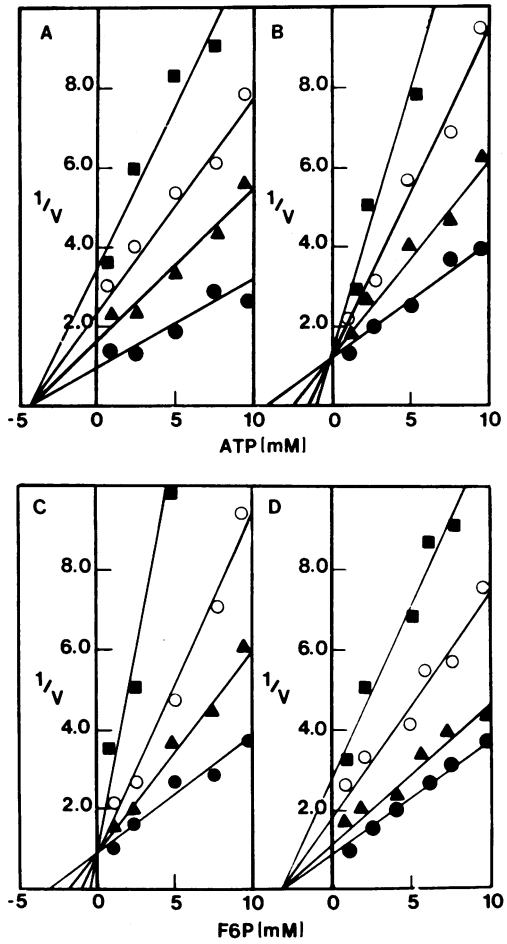

FIG. 7. *(A) Effect of mannitol-1-phosphate on the interaction of ATP with the M1PDH from Streptococcus mutans. The forward reaction catalyzed by the M1PDH was assayed as described in Materials and Methods except that the concentrations of ATP were varied as indicated in the presence of different mannitol-1-phosphate concentrations. All reactions were initiated with 0.22 μg of fraction VII enzyme (Table 1). The following symbols indicate the mannitol-1-phosphate concentrations used: 1.0 mM (●); 0.50 mM (▲); 0.20 mM (○); 0.10 mM (■). (B) Effect of NAD on the interaction of ATP with the M1PDH from S. mutans. The forward reaction was assayed as described in Materials and Methods except that the ATP concentration was varied at different levels of NAD. The reactions were initiated by the addition of 0.22 μg of fraction VII enzyme (Table 1). The following symbols indicate the NAD concentrations used: 1.0 mM (●); 0.20 mM (▲); 0.10 mM (○); 0.05 mM (■). (C) The effect of mannitol-1-phosphate on the interaction of fructose-6-phosphate (F6P) with the M1PDH from S. mutans. The forward reaction was assayed as described in Materials and Methods except that the fructose-6-phosphate concentration was varied as indicated in the presence of different levels of mannitol-1-phosphate. The reactions were initiated by the addition of 0.22 μg of fraction VII enzyme (Table 1). The following symbols give the mannitol-1-phosphate concentrations used: 1.0 mM (●); 0.50 mM (▲); 0.20 mM (○); 0.10 mM (■). (D) The effect of NAD on the interaction of fructose-6-phosphate with the M1PDH from S. mutans. The forward reaction was assayed as described in Materials and Methods except that the concentration of fructose-6-phosphate was varied at different levels of NAD. The reactions were initiated by the addition of 0.22 μg of fraction VII enzyme (Table 1). The following symbols show the NAD concentrations used: 1.0 mM (●); 0.20 mM (▲); 0.10 mM (○); 0.05 mM (■).*

continuous gel electrophoresis. The enzyme was purified to approximately a 425-fold increase in specific activity with a 29% recovery of total enzyme units. Polyacrylamide discontinuous gel electrophoresis of the purified enzyme preparation showed a single protein band which coincided with a band of M1PDH activity. In addition, the enzyme was stable over extended periods of time when stored at −80°C

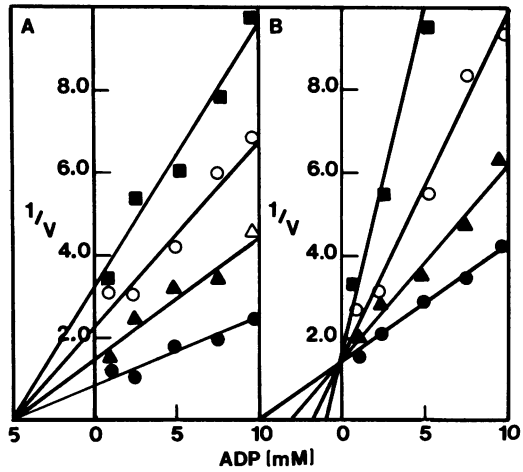

FIG. 8. *(A) Effect of fructose-6-phosphate on the interaction of ADP with the M1PDH from Streptococcus mutans. The reverse reaction was assayed as described in Materials and Methods except that the ADP concentration was varied as indicated in the presence of different levels of fructose-6-phosphate. All reactions were initiated by the addition of 0.25 μg of fraction VII enzyme (Table 1). The following symbols indicate the fructose-6-phosphate concentrations used: 5.0 mM (●); 2.5 mM (▲); 1.0 mM (○); 0.5 mM (■). (B) The effect of NADH on the interaction of ADP with the M1PDH from S. mutans. The reverse reaction was assayed as described in Materials and Methods except that the ADP concentration was varied as indicated in the presence of different levels of NADH. All assays were initiated by the addition of 0.25 μg of fraction VII enzyme (Table 1). The following symbols indicate the NADH concentrations used: 0.1 mM (●); 0.05 mM (▲); 0.025 mM (○); 0.01 mM (■).*

in the presence of β-mercaptoethanol and was not inhibited by EDTA, nor was its activity stimulated by monovalent or divalent cations. The M1PDH is a relatively small enzyme having a molecular weight of approximately 45,000. However, the enzyme readily catalyzed both the NAD-specific oxidation of mannitol-1-phosphate and the NADH-dependent reduction of the glycolytic intermediate, fructose-6-phosphate.

The reversible nature of the reactions catalyzed by the M1PDH from *S. mutans* may be of important physiological significance for these organisms. The forward reaction provides them with a mechanism for the entry of mannitol-1-phosphate into the glycolytic sequence at the level of fructose-6-phosphate, where it can serve as a precursor for ATP production, which supports the life functions of these organisms. On the other hand, the potential importance of the reverse reaction is different. In the presence of fructose-6-phosphate, the reverse reaction would provide these organisms with an alternative to the NADH-specific lactate dehydrogenase (2) for regenerating NAD so that glycolysis and energy production may proceed.

The NADH-dependent reduction of fructose-6-phosphate catalyzed by the M1PDH from *S. mutans* may have another important physiological role in addition to regenerating NAD so that glycolysis and energy production can proceed. This role may be in relationship to glucan synthesis from sucrose by these organisms. The significance of this lies in the fact that glucans are primary determinants for the adherence of *S. mutans* to the tooth surface and consequently play an important role in the formation of dental plaque (12). Since free fructose is an inhibitor of sucrose-dependent glucan synthesis by *S. mutans* (12) and is also a product produced during glucan synthesis from sucrose by these organisms, the reverse reaction catalyzed by the M1PDH may provide them with one step in a metabolic sequence in addition to glycolysis which would serve to remove fructose from the extracellular environment through its sequential conversion to fructose-6-phosphate, mannitol-1-phosphate, and mannitol. Several lines of evidence tend to support this hypothesis. First, we have found that broken-cell suspensions of *S. mutans* strain FA-1, which contain M1PDH activity, also have a phosphatase activity that liberates inorganic phosphate from mannitol-1-phosphate, giving free mannitol as the reaction product (A. T. Brown, unpublished data). Of even greater significance is the observation that mannitol has been found in the culture supernatant after exposure of *S. mutans* cells to levels of sucrose sufficient to lead to glucan synthesis (K. S. Kornman and W. J. Loesche, J. Dent. Res. 54:634, 1975). Consequently, the importance of the reverse reaction catalyzed by the M1PDH from *S. mutans* in removing an inhibitor of glucan synthesis from the extracellular environment cannot be overlooked.

The forward reaction catalyzed by the M1PDH from *S. mutans* plays a key role in the entry of mannitol carbon into the glycolytic sequence, whereas the reverse reaction catalyzed by this enzyme appears to play a major role in mannitol production. The flow of carbon between mannitol catabolism and mannitol production by *S. mutans* appears to be under cellular control. The forward reaction catalyzed by the enzyme appears to be under negative control by ATP and fructose-6-phosphate, whereas the reverse reaction appears to be under negative control by ADP. Control of the forward and reverse reactions by the specific metabolites mentioned above occurs at the concentrations of these metabolites that are found in lactic acid bacteria (20).

The sensitivity of the forward reaction to inhibition by ATP is of interest because this might provide a physiological mechanism by which *S. mutans* can restrict the input of mannitol carbon into the glycolytic pathway at the level of fructose-6-phosphate when the intracellular pool levels of ATP are high. This process would effectively conserve the expenditure of exogenous mannitol carbon. Negative control of oxidation of sugar phosphates by ATP has been documented in a number of microorganisms, including oral streptococcal species. For instance, an inducible NAD-specific 6-phosphogluconate dehydrogenase is specifically inhibited by ATP (3), as is the glucose-6-phosphate dehydrogenase from this organism (A. T. Brown, unpublished data). ATP also has other roles in regulating the flow of exogenous carbon into and through the glycolytic sequence in *S. mutans*. For example, ATP is a potent inhibitor of the enzyme lactate dehydrogenase, which catalyzes the terminal step in this pathway (2), and this nucleoside triphosphate also functions as a negative effector for phosphofructokinase in these organisms (R. B. Bridges, unpublished data). A second regulatory parameter may be operative on the forward reaction catalyzed by the M1PDH from *S. mutans* when the fructose-6-phosphate pool levels are elevated. The oxidation of mannitol-1-phosphate, which leads to the accumulation of fructose-6-phosphate, may be decreased through inhibition of the enzyme by fructose-6-phosphate. Inhibition of the NAD-dependent oxidation of mannitol-1-phosphate by fructose-6-phosphate would serve

to make the NADH-specific reduction of fructose-6-phosphate an essentially irreversible process at high intracellular pool levels of this glycolytic intermediate.

The inhibition exerted on the reverse reaction catalyzed by the M1PDH from *S. mutans* by ADP may also be of physiological significance. When the intracellular levels of ATP are low, ADP will serve to direct the flow of fructose-6-phosphate through the glycolytic sequence by preventing its alterative conversion to mannitol-1-phosphate through inhibition of the reverse reaction catalyzed by the M1PDH. The flow of fructose-6-phosphate through the glycolytic sequence will result in increased ATP production, which will serve to fulfill the biosynthetic needs of the cell.

In conclusion, the ability of *S. mutans* cells to utilize mannitol as a primary energy source may provide them with a competitive advantage in the oral environment due to the recent widespread use of polyols such as mannitol as artificial sweeteners. In addition to the dietary intake of this polyol, the production of mannitol from sucrose by *S. mutans* may be yet another source of this polyol in dental plaque. The very fact that *S. mutans* is one of the few oral microorganisms capable of utilizing mannitol as a primary energy source means that the mannitol produced from exogenous sucrose may be utilized by these organisms when the extracellular sucrose levels are depleted. Central to both the utilization and production of mannitol by *S. mutans* are the reversible reactions catalyzed by the M1PDH.

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