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SHORT COMMUNICATIONS

PRODUCTION OF MANNITOL BY *STREPTOCOCCUS MUTANS*

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Summary—Mannitol was produced as an end product when resting cell suspensions of strains of *Streptococcus mutans* were given high levels of sucrose or glucose. The presence of mannitol in the supernatant from glucose incubated cells and the occasional detection of mannitol-1-phosphate suggest that mannitol was formed by a reduction of fructose-6-phosphate. This pathway would permit the regeneration of NAD under anaerobic conditions, thereby permitting maximal catabolism of hexoses via the glycolytic pathway.

Streptococcus mutans differs from other plaque viridans streptococci in its ability to ferment mannitol (M-OH) and sorbitol (S-OH) (Carlsson, 1968). These hexitols are phosphorylated at the carbon one and carbon six positions respectively, and, after oxidation by inducible enzymes, enter the glycolytic pathway as fructose-6-phosphate (F-6-P) (Brown and Wittenberger 1973). However, the ecological significance of hexitol fermentation by *Strep. mutans* is not understood. Mannitol-1-phosphate dehydrogenase, an enzyme involved in M-OH fermentation, is able to catalyze the reduction of F-6-P to mannitol-1-phosphate (M-1-P) (Brown and Wittenberger, 1973). This raises the possibility that M-OH could be formed as an end product of carbohydrate metabolism by a reversal of the fermentation pathway. Alternatively, M-OH could be formed by direct reduction of fructose. *Strep. mutans* produces fructose from sucrose by means of an invertase (Gibbons, 1970) and a glucosyl transferase (Schachtele, Loken and Knudson, 1972). As fructose can inhibit glucosyl transferase in *Strep. mutans* (Gibbons and Nygaard, 1968), there may be a need for *Strep. mutans* to remove fructose so that glucan synthesis can proceed optimally. These possibilities suggest that M-OH fermentation in *Strep. mutans* may be related to the production of this hexitol from other carbohydrates.

Strep. mutans strain 6715 (M-OH-positive) and strains 1068 and 31 (M-OH negative cultures obtained from Dr. I. Shklair) as well as recent isolates of *Streptococcus sanguis* (M-OH-negative) and *Lactobacillus casei* (M-OH-positive) were grown anaerobically under 85 per cent N_2, 10 per cent H_2 and 5 per cent CO_2) in trypticase soy broth (TSB) without dextrose and containing either 0.5 per cent (w/v) fructose, 0.5 per cent sucrose or 0.5 per cent M-OH. After 48 hr incubation at 37°C, the cultures were centrifuged and washed twice in 0.067 M phosphate buffer pH 7.2. Protein concentration was determined (Lowry *et al.*, 1952) and approximately equal amounts of bacteria, i.e. 0.55 to 0.70 mg protein, were transferred to 2 ml of the above buffer which contained one of the following substrates: 0.5, 1.0, 1.5, 2.0 or 2.5 per cent (w/v) sucrose, 1.0 per cent glucose or 1.0 per cent fructose. The atmosphere over the resting cell preparations was flushed with the gas, the tubes were stoppered and incubated at 37°C in a water bath. 0.5 ml samples were removed at 0, 30, and 60 min. These were centrifuged and the supernatants passed through a cation-exchange resin (BioRad AG 50 W) (Carlsson, 1973). The eluate was dried and silylated to produce volatile methyl ester-trimethylsilyl ether (Me-TMSi) derivatives of the hydroxy-compounds suitable for gas-liquid chromatographic analysis (GLC) (Sweeley *et al.*, 1963; Horning, Boucher and Moss, 1967). Me-TMSi derivatives of fructose, galactose, glucose, mannose, S-OH and M-OH were prepared. The mannitol-1-phosphate (M-1-P) standard was converted from the barium salt to the free acid and then silylated with N_1O-bis-trimethylsilyl-acetamide. The analysis of M-1-P presented some difficulties as at times the M-1-P standard was undetectable, a problem noted with GLC analysis of sugar phosphates (Sherman, Goodwin and Zinbo, 1971). Thus the occurrence of M-1-P could be under-estimated in our results. All GLC separations were isothermal at 200°C on a column of 3 per cent OV-17 on chromosorb G, 80/100 mesh (Varian) with helium as the carrier gas (26 ml/min). Experimental samples were compared to standards chromatographed the same day and each was expressed as retention time relative to α-D-glucose. The procedures used were able to differentiate fructose, galactose, M-OH, mannose, S-OH, α-D-glucose, β-D-glucose, and M-1-P (Table 1). The silylation procedure did not result in detectable isomerization of glucose to fructose or alditols as shown by the fact that glucose standards gave only detectable α and β peaks. The use of dimethylformamide, a solvent which reduces isomerization (Miettienen, Ahrens and Grundy, 1965), produced results comparable to those with pyridine.

GLC of supernatants from *Strep. mutans* 6715 cells grown in either fructose or sucrose broths and then incubated for 60 min in buffer with 1.5 per cent sucrose, contained five detectable peaks. These were identified by comparison to standards as fructose,

Table 1. Production of hexoses and hexitols by resting cell suspensions of various oral organisms grown in TSB with 0.5 per cent sucrose and then incubated for 60 min with 1.5 per cent sucrose

Standards	Range of retention times†	*Strep. mutans* 6715	*Strep. mutans* 1068,31	*Lact. casei*	*Strep. sanguis*
Galactose	0.606–0.619	—	—	—	—
Fructose	0.649–0.656	+	+	+	+
Mannose	0.654–0.661	—	—	—	—
Mannitol-1-phosphate	0.815–0.822	+(8)‡	—	—	—
Mannitol	0.851–0.866	+	—	+	—
Sorbitol	0.896–0.906	—	—	—	—
α-D-glucose	1.0	+	+	+	+
β-D-glucose	1.30–1.340	+	+	+	+
Unidentified	0.776–0.784	+			

† Relative to α-D-glucose.

‡ 8 of 34 separate determinations of those performed with strain 6715 were positive for M-1-P.

M-OH, α-D-glucose and β-D-glucose, with an unidentified peak between fructose and M-OH. The identification of the M-OH peak was confirmed by adding exogenous M-OH to the sample. This produced an enlarged peak with the same retention time as the M-OH peak and no additional peaks. Eight of 34 separate determinations using *Strep. mutans* 6715 had an additional peak which coincided with the retention time for M-1-P. M-OH or M-1-P peaks were not detected from resting cells incubated with less than 1.5 per cent sucrose. Cells incubated for 60 min in 1.0 per cent glucose produced peaks coincident with fructose, M-OH, α and β-D-glucose. *Strep. mutans* incubated for 60 min in 1.0 per cent fructose gave fructose as the only detectable monosaccharide in the supernatant.

The M-OH-negative *Strep. mutans* mutants 1068 and 31 produced detectable peaks coincident with fructose, α-D-glucose, and β-D-glucose when incubated with 1.5 per cent sucrose (Table 1). The quantity of carbohydrate end products detected from strain 1068 was approximately 20 per cent of that from strain 6715, but strain 31 produced amounts comparable to 6715. The mutant strains when incubated with 1.0 per cent glucose produced only α-D-glucose and β-D-glucose. *Strep. sanguis* produced no detectable M-OH, while *Lact. casei*, which ferments M-OH, produced detectable M-OH from 1.5 per cent sucrose (Table 1).

Mannitol was a minor end product in *Strep. mutans* strain 6715, accounting for approximately 0.3–1 per cent of the glucose or sucrose provided to the organisms: i.e. approximately 0.1–0.3 mg of M-OH was found in reaction mixtures containing 1.5 per cent sucrose. This M-OH could have arisen from: (1) direct reduction of fructose, derived from sucrose, as a result of invertase or glucosyltransferase activity and/or (2) the reduction of F-6-P to M-1-P followed by phosphate removal. The presence of M-OH in the supernatant incubated in glucose, and the occasional detection of M-1-P, suggest that reduction of F-6-P had occurred. As cells pre-grown in sucrose and fructose might not have optimal levels of the enzymes necessary for this reduction, the experiment was repeated using *Strep. mutans* cells pre-grown in M-OH. When sucrose and glucose were added to these resting cell suspensions, the amounts of M-OH formed were qualitatively similar to those produced by cells pre-grown in either fructose of sucrose. Thus, cultural conditions which should have elevated the levels of mannitol-1-phosphate dehydrogenase did not appreciably increase M-OH production from sucrose or glucose by resting cell preparations.

Strep. mutans apparently produces small amounts of M-OH as an end-product at high levels of sucrose or glucose by reduction of F-6-P. A similar pathway for removing F-6-P occurs in *Escherichia coli*, where M-1-P accumulates in cells cultured anaerobically in the presence of glucose (Helle and Klungsoyr, 1962).

This pathway in *E. coli* is important for the oxidation of NADH under conditions of high glucose concentration.

If a similar pathway exists in *Strep. mutans*, then the regeneration of NAD under anaerobic conditions might permit maximal uptake of hexoses during periods of high sugar availability. The significance of M-OH fermentation in *Strep. mutans* and possibly *Lact. casei* would then be the formation of M-OH under conditions of sugar excess and the subsequent reutilization of M-OH when the sugar is depleted. This hypothesis might explain why some mutants of *Strep. mutans* which are weak M-OH fermenters have reduced ability to use sucrose, i.e., certain polysaccharide defective mutants (Johnson, Bozzola and Schechmeister, 1974). Additional studies involving cell-free preparations and enzymes purification are necessary to define more precisely the parameters of M-OH production by *Strep. mutans*.

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REFERENCES

Brown A. T. and Wittenberger C. L. 1973. Mannitol and sorbitol catabolism in *Streptococcus mutans*. *Archs oral Biol.* **18**, 117–126.

Carlsson J. 1968. A numerical taxonomic study of human oral streptococci. *Odont. Revy* **19**, 137–160.

Carlsson J. 1973. Simplified gas chromatographic procedure for identification of bacterial metabolic products. *Appl. Microbiol.* **25**, 287–289.

Gibbons R. J. 1970. Presence of an invertase-like enzyme and a sucrose permeation system in strains of *Streptococcus mutans*. *Caries Res.* **6**, 122–131.

Gibbons R. J. and Nygaard M. 1968. Synthesis of insoluble dextran and its significance in the formation of gelatinous deposits by plaque-forming streptococci. *Archs oral Biol.* **13**, 1249–1262.

Helle K. B. and Klungsoyr L. 1962. Mannitol-1-phosphate formation in *Escherichia coli*. *Biochem. biophys. Acta.* **65**, 461–471.

Horning M. G., Boucher E. A. and Moss A. M. 1967. The study of urinary acids and related compounds by gas phase analytical methods. *J. Gas Chromat.* **5**, 297–302.

Johnson M., Bozzola J. J. and Schechmeister I. L. 1974. Morphological study of *Streptococcus mutans* and two extracellular polysaccharide mutants. *J. Bact.* **118**, 304–311.

Lowry O. J., Rosebrough N. J., Farr A. L. and Randall R. J. 1951. Protein measurement with the folin phenol reagent. *J. biol. Chem.* **193**, 265–275.

Miettienen T. A., Ahrens E. H., Jr. and Grundy S. M. 1965. Quantitative isolation and gas–liquid chromatographic analysis of total dietary and fecal neutral steroids. *J. Lipid Res.* **6**, 411–424.

Schachtele C. F., Loken A. E. and Knudson D. J. 1972. Preferential utilization of the glucosyl moiety of sucrose by a cariogenic strain of *Streptococcus mutans*. *Infect. Immun.* **5**, 531–536.

Sherman W. R., Goodwin S. L. and Zinbo M. 1971. The GC of some completely trimethylsilylated inositol and other sugar phosphates. *J. Chrom. Sci.* **9**, 363–367.

Sweeley C. C., Bentley R., Makita M. and Wells W. W. 1963. Gas–liquid chromatography of trimethylsilyl derivatives of sugars and related substances *J. Am. chem. Soc.* **85**, 2497–2507.