Carbohydrate Research, 107 (1982) 111–122
Elsevier Scientific Publishing Company, Amsterdam – Printed in The Netherlands

EFFECT OF VARIATION IN GROWTH CONDITIONS ON ENDO-DEXTRANASE PRODUCTION BY *Streptococcus mutans**

GWEN J. WALKER, MARGARET D. HARE, AND JILL G. MORREY-JONES
Institute of Dental Research, 2 Chalmers St., Sydney, N.S.W. 2010 (Australia)
(Received October 19th, 1981; accepted for publication, January 10th, 1982)

ABSTRACT

The effect of a variety of growth conditions on the dextranase activity of cell suspensions and cell-free filtrates of *Streptococcus mutans* strains in continuous culture has been studied. The strains produced little or no dextranase in the chemostat at low growth-rates, which were obtained with dilution rates of 0.05–0.20 h^{-1}, in glucose-limited and in glucose-sufficient (*N*-limited) media. Synthesis of dextranase was appreciable at dilution rates of 0.50–0.69 h^{-1}, and the lowering of the mean generation time from 1.4 to 1.0 h resulted in a 2-fold increase in activity. Dextranase activity was increased 1.5–2.0 fold when the medium contained an excess of D-glucose. Transient over-production of enzyme occurred during the change from a low growth-rate (0.075 h^{-1}) to a higher growth-rate (0.50 h^{-1}). This resulted in an 8-fold increase in activity over the final, steady-state value. The results indicate that *S. mutans* might produce no dextranase when growing slowly in dental plaque, but would promptly synthesize the enzyme in response to the periodic increased supply of nutrients.

INTRODUCTION

The release of endo-dextranase in batch cultures of *S. mutans* was reported to be maximal under conditions of rapid growth when D-glucose was present in excess[1]. In media supplemented with 2% of D-glucose, the enzyme was produced early in the exponential phase, and release of dextranase ended when the concentration of D-glucose had fallen to ~0.5%. It was not possible to distinguish between the relative importance of rapid growth and a sufficiency of glucose, because these two conditions occur together in batch culture, and slow growth could not readily be achieved in the presence of an excess of glucose. Furthermore, consistent values for dextranase activity were difficult to obtain when the concentration of D-glucose in the medium was lowered to give limiting values. It was therefore necessary to differentiate between the effects of growth rate and glucose concentration by growing *S.*

*Part V of the series Metabolism of the Polysaccharides of Human Dental Plaque. For Part IV, see ref. 1.

0008-6215/82/0000–0000/S 02.75,

mutans in a chemostat. By this means, the growth rate could be varied under conditions where glucose was either limiting or sufficient.

Detailed knowledge of the mechanisms governing the expression of extracellular enzymes is still sparse, and Glenn[2] suggested that, in view of the limitations of batch culture, a wider use of continuous-culture techniques would be of value for the study of exo-protein synthesis. We now report values for extracellular endodextranase activity released by several *S. mutans* strains grown under a range of standardized and reproducible conditions. As the chemostat provided growth conditions that were impossible to produce in batch culture, it was expected that the streptococci might display different characteristics from those expressed during batch growth. These studies would then provide new information on the environmental factors that might contribute to the formation and release of this extracellular enzyme.

METHODS

Organisms. — *Streptococcus mutans* strains AHT and BHT, Ingbritt, K1-R, OMZ176, and B13 were obtained from Dr. D. D. Zinner, Professor B. Krasse, Dr. R. J. Fitzgerald, Professor B. Guggenheim, and Dr. S. Edwardsson respectively.

Continuous culture was carried out at 37° in a 1-L fermentation vessel (Bioflo Model C30 New Brunswick Scientific Co. Inc., N.J., U.S.A.) containing 325 mL of medium[3] with the growth-limiting nutrient, usually D-glucose, added at a concentration of 0.5%. Anaerobic conditions were maintained with a gas mixture of nitrogen and carbon dioxide (19:1 v/v) sparged at 200 mL. min^{-1}. The pH was kept constant to within ± 0.05 pH unit by the automatic addition of 2M sodium hydroxide with pH-control equipment (Model pH-21) supplied by New Brunswick Scientific Co. Inc. The initial pH of the medium was 7.0, and for cultures grown at pH <7.0, the pH of the vessel contents was allowed to fall to the desired value by the natural production of acid by the bacteria. For pH values >7.0, the controller setting was increased gradually (0.5 unit per 3 h).

The inoculum for the fermenter was grown for 16 h at 37° in the same medium supplemented with 0.2% (w/v) of D-glucose. When the organisms in the fermenter had utilized all of the glucose (batch-growth, stationary phase), a sample was withdrawn, and then pumping of fresh medium through the vessel was commenced. The overflow was collected in a refrigerated bath, and at least 5 culture volumes were allowed to flow before samples were taken for the analysis of a steady state (that is, after seven or more mean generation-times). In some experiments, samples were also taken during the transition periods in between steady states. Each dilution rate was maintained within a variation of ± 0.01 h^{-1}. The dry weight of the cells was determined on duplicate volumes (5 mL) of culture, by centrifuging and washing the cells, and drying them to constant weight at 90°. Utilization of glucose was determined with D-glucose oxidase reagent[4]. Daily checks on the culture in the vessel and on the collected culture-fluid included a Gram stain, and determination of pH and optical

density at 600 nm. Cells were also plated daily on sheep blood and *Mitis salivarius* agar containing 1% potassium tellurite.

Batch cultures were grown at 37° in the same medium, either in a Multigen Model F-1000 (1 L) fermenter (325 mL of culture) or in a Microferm 14-L fermenter (3 L of culture) with an automatic pH controller Model pH-131. All of the equipment was from New Brunswick Scientific Co. Inc. The cells were harvested when all of the sugar had been utilized.

Fractionation of cultures. — The culture fluid was cooled to 4° and centrifuged for 10 min at 12,000*g*. Portions (~20 mL) of the supernatant solution (42 mL) were dialysed against sodium citrate buffer (0.05M, pH 6) with two changes of buffer over a 20-h dialysis period. The cells were washed twice with the same buffer, and then resuspended in one-seventh of their original volume, namely, 6 mL of buffer.

Determination of dextranase activity. — Dextranase activity associated with the cell surface was determined in digests (4 mL) containing Sigma Type 2000 dextran (15 mg) and 2 mL of washed-cell suspension (equivalent to 14 mL of culture fluid), in 25mM sodium citrate buffer (pH 6.0). At intervals, portions (0.5 mL) were withdrawn, cooled in ice–water, and centrifuged at 12,000*g*. The supernatant solution was boiled (2 min, 100°) and then assayed for reducing power with neocuproine reagent[5], with D-glucose as the standard compound. Units of activity were adjusted to give the dextranase activity of cells in 1 mL of the original culture fluid. All enzyme assays were performed at 35° under toluene. The activity of the washed cells was not affected by the presence of toluene (0.2 mL) or by the addition of 5mM sodium fluoride.

Dextranase activity of the dialysed, cell-free filtrate was determined from the release of reducing sugars in a digest (1 mL) that contained Sigma Type 2000 dextran (15 mg) and an appropriate volume of enzyme, usually 0.5 mL, in 25mM sodium citrate buffer (pH 6.0). Samples were taken at intervals up to 24 h, and the reaction was stopped either by boiling or by addition of the copper reagent. One unit of activity is defined as the amount of enzyme that released 1 μmol of reducing power per min under the conditions of the assay.

RESULTS

The stability of dextranase in batch culture. — Inconsistent results[1] have been published for endo-dextranase activity in batch-culture filtrates of *S. mutans* grown in medium containing 0.5% of D-glucose. The widest variation between batches occurred with *S. mutans* strain OMZ176. The release of enzyme by strain OMZ176 was therefore determined at various stages during batch growth under three different sets of conditions. Two of the fermenters were fitted with pH controllers, and the organisms were grown at pH 6 and 7, respectively; the organisms grew without pH control in the third fermenter. The medium (305 mL) in each vessel was inoculated with cells (in 20 mL) transferred from a glucose-limited, continuous culture operating at a dilution rate of 0.3 h^{-1} and pH 6. The results (Fig. 1) clearly indicated the instability of strain OMZ176 dextranase in batch cultures, and exposed the difficulty

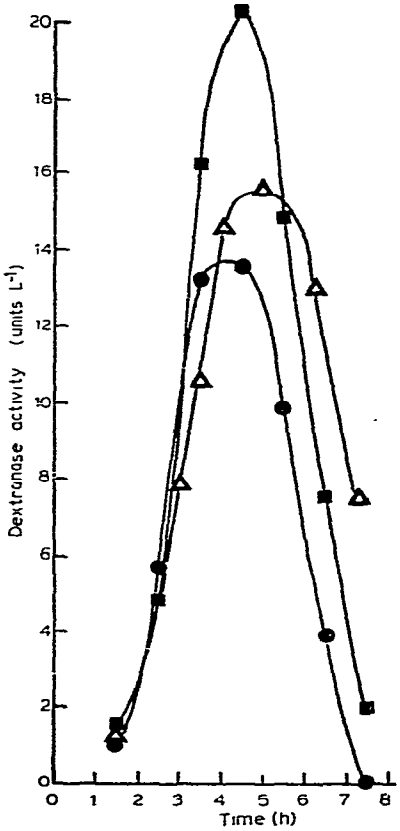

Fig. 1. Extracellular dextranase activity of *S. mutans* OMZ176 during growth in batch culture at pH 6 (△), pH 7 (■), and without control of pH (●). The medium was supplemented with D-glucose (2%).

of obtaining precise and reliable values for its activity. Any value recorded for extracellular dextranase activity of *S. mutans* OMZ176 would depend not only on the pH, but also on the length of time the culture had spent in the stationary phase. This contrasted with the dextranase of strain Ingbritt, which reached a maximum value of 16 units per L near the end of the exponential phase, and remained steady at that level for 16 h.

Dextranase activity of S. mutans strains grown in continuous culture. — Various strains of *S. mutans* were grown at pH 6 in the chemostat as described in Methods. The growth rate was controlled by adjusting the rate of supply of the medium, in which either D-glucose or D-fructose (0.5%) was growth limiting. Little or no dextranase activity was detected in culture filtrates from slowly growing cells, but, at a dilution rate of 0.55 h^{-1} the activity of dextranase approached that released in batch culture (Table I). Strain OMZ176 was exceptional in releasing the highest amount of dextranase in the chemostat while giving low and inconsistent values in batch cultures.

TABLE I

EXTRACELLULAR DEXTRANASE ACTIVITY IN CONTINUOUS CULTURE AND IN BATCH CULTURE

Strain	*Sugar*[a]	*Dextranase activity (units/L)*			
		Continuous culture			*Batch culture*
		Dilution rate (h^{-1})			*16 h*
		0.05	*0.55*	*0.69*	
AHT	Glc	0	0	0	0
BHT	Glc	0	0.09		0.08
K1-R	Glc	0	3.9	6.9	4.2–14
	Fru	0	3.8		6.8
OMZ176	Glc	0	5.6		0–2.0
B13	Glc	0	2.8		3.9
	Fru	0	3.0		3.5

[a]The concentration of sugar in the medium was 5 g/L.

It was possible to grow some strains at a dilution rate of 0.69 h^{-1} without wash-out, and increased amounts of enzyme were released at the higher growth-rate.

Effect of the sugar and its concentration. — Previous results from batch cultures[1] had shown that extracellular dextranase activity was in general 2-fold higher in medium supplemented with D-fructose (2%) than with D-glucose. In continuous

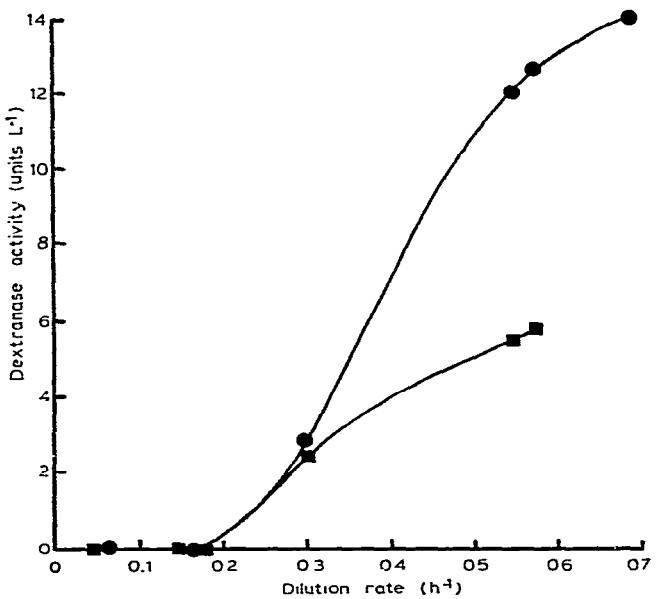

Fig. 2. Effect of glucose concentration in the medium on the extracellular dextranase activity of *S. mutans* OMZ176 in continuous culture at pH 6; ■, 0.5% of D-glucose; ●, 4% of D-glucose.

TABLE II

EFFECT OF THE NATURE OF THE LIMITING SUGAR ON THE EXTRACELLULAR ENDO-DEXTRANASE ACTIVITY OF *S. mutans* INGBRITT IN CONTINUOUS CULTURE[a] AT pH 6.0

Limiting substrate	*Concentration (g/L)*	*Dextranase activity (units/L)*	*(units/g)*
Glucose	2.5	0.07	0.10
Glucose	5.0	0.94	0.97
Fructose	2.5	0.15	0.25
Fructose	5.0	1.13	1.14
Sucrose	5.0	1.08	1.15

[a]The dilution rate was 0.50 h^{-1}.

culture, no significant difference in dextranase activity of strain Ingbritt occurred when D-fructose or sucrose replaced D-glucose as the limiting nutrient (Table II). Even at a high dilution rate (0.5 h^{-1}), dextranase activity of strain Ingbritt was extremely low (0.1–1.1 units/L) under all of the conditions shown in Table II. These values may be contrasted with those (16.5–23.5 units/L) found in batch cultures[1] in the fructose medium.

S. mutans OMZ176 was grown in the chemostat in medium containing an excess of D-glucose (4%). At high growth-rate, this resulted in a 2-fold increase in dextranase activity in the culture filtrate, relative to that in the glucose-limited medium (Fig. 2). The specific activity of dextranase (units per g of dry wt of cells) from strains OMZ176 and K1-R increased by 1.45- and 2-fold, respectively, at a dilution rate of 0.55 h^{-1}, when glucose-sufficient medium replaced glucose-limited medium. At the low growth-rates obtained when the dilution rate was in the range 0.05–0.175 h^{-1}, no release of dextranase was detected in either medium.

Dextranase activity of washed-cell suspensions. — A comparison between endo-dextranase activity of cell extracts and cell-free filtrates of three *S. mutans* strains[1] showed that the major portion of the enzyme (60–90%) in batch cultures was extracellular. The possibility that the distribution of enzyme between cells and filtrate might depend on the growth rate was examined in continuous culture. Cells of strains OMZ176 and K1-R obtained at a dilution rate of 0.05 h^{-1} did not hydrolyse dextran, and so the absence of dextranase from the culture filtrates could not be explained by the enzyme remaining in association with the cells. Cells of OMZ176 and K1-R harvested from cultures grown at a dilution rate of 0.55 h^{-1} accounted for 35 and 32%, respectively, of the total dextranase activity in the culture, and similar results were obtained in batch culture. The effect of mean generation-time of *S. mutans* BHT on the dextranase activity of cell suspensions is shown in Fig. 3. In general, dextranase activity could not be detected in cells or filtrate unless the dilution rate exceeded 0.2 h^{-1}; this corresponded to a mean generation-time of <3.5 h. However, the dextranase activity of strain Ingbritt remained low and varied little between D 0.05

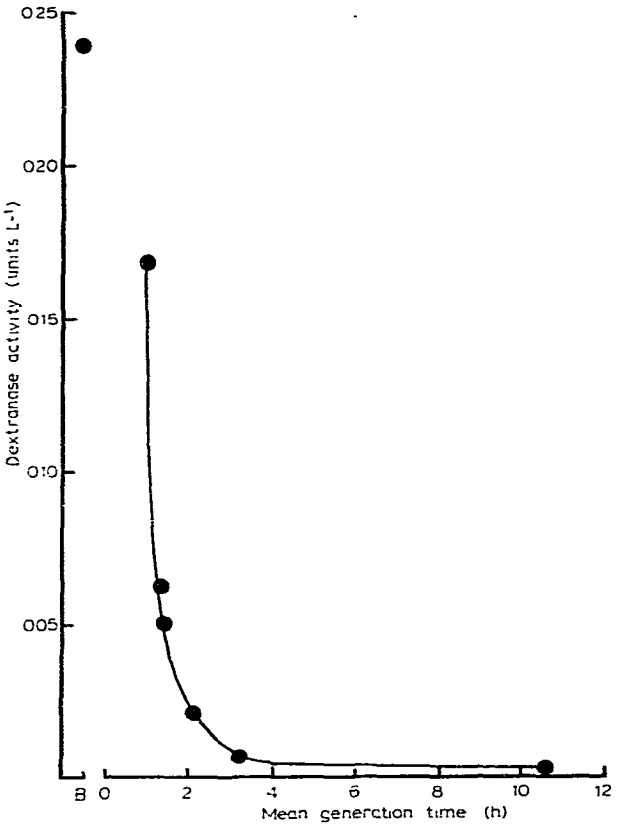

Fig. 3. Variation of dextranase activity of washed-cell suspensions with mean generation time. *S. mutans* BHT was grown at pH 6 in D-glucose-limited continuous culture.

to 0.40 h^{-1}; thereafter, the activity rose 16-fold as the dilution rate was increased to 0.69 h^{-1} (Fig. 4).

Transient production of dextranase between steady states. — The dextranase activity of *S. mutans* strain Ingbritt in a glucose-limited, continuous culture at pH 7 was 0.3 and 1.3 units/L when the dilution rate was 0.075 and 0.50 h^{-1}, respectively. In between these two steady-state conditions, the concentration of dextranase rose sharply, and reached over 9 units/L (12.4 units per g of dry wt) after only 1 culture volume had passed through the vessel at 0.5 h^{-1} (Fig. 5). Thereafter, the activity fell steadily to its final value. The same effect was observed at pH 6, but the maximum dextranase activity, which occurred after 1.5 culture-volumes had flowed at a dilution rate of 0.5 h^{-1}, was lower, being 5.3 units per g. The return from steady state at a dilution rate of 0.5 h^{-1} to that at 0.075 h^{-1} was uneventful, and dextranase activity fell steadily from 2.2 to 0.2 unit. No change in cell density occurred during these alterations in growth rate.

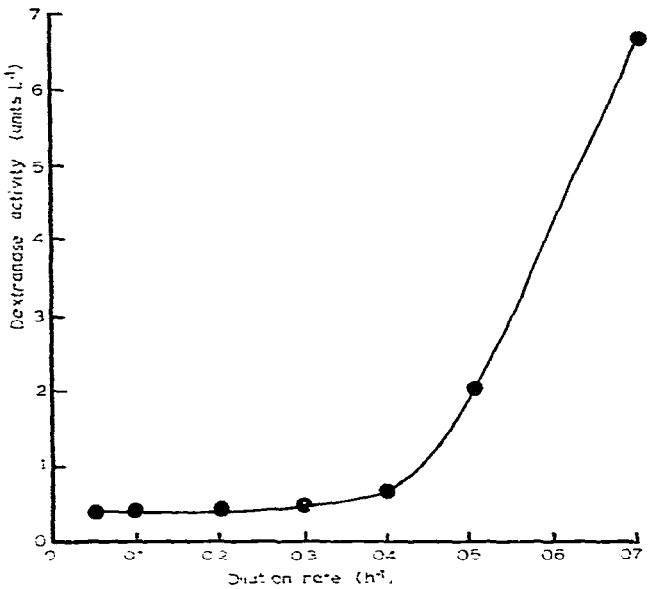

Fig. 4. Effect of growth rate on the release of dextranase by *S. mutans* Ingbritt in D-fructose-limited, continuous culture at pH 6.

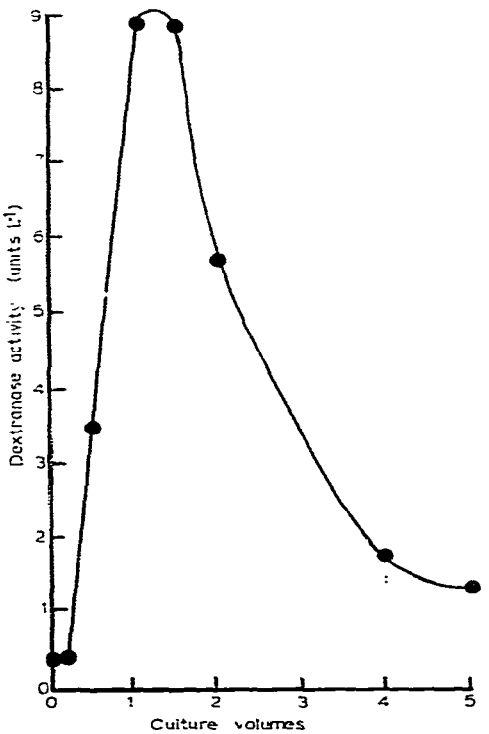

Fig. 5. Over-production of extracellular dextranase by *S. mutans* Ingbritt during transitional stages between two steady states. The initial value was for a dilution rate of 0.075 h^{-1}, and then the nutrient pump was set to give 0.50 h^{-1}.

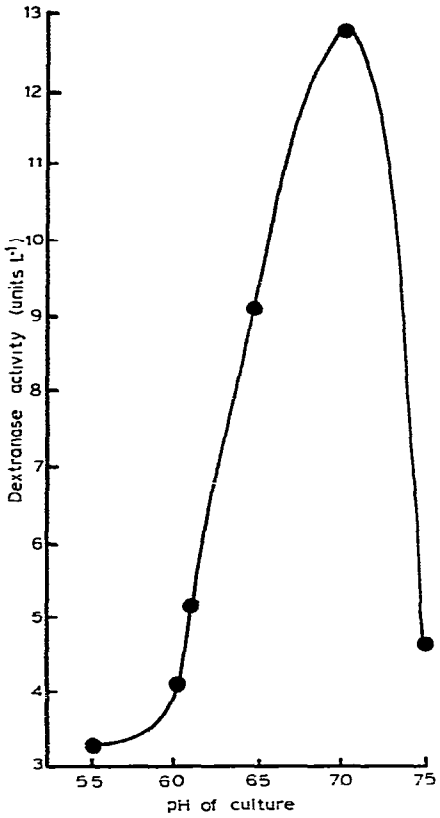

Fig. 6. Extracellular dextranase activity of *S. mutans* K1-R grown in continuous culture at different values of pH.

Effect of pH of growth on dextranase activity. — *S. mutans* K1-R was grown in the chemostat under glucose-limitation at a dilution rate of 0.56 h^{-1}, and at different values of pH (Fig. 6). Maximum activity was released during growth at pH 7, the specific activity being 2.5-fold higher than at pH 6.

DISCUSSION

Little information is available about the rates of many bacterial processes in natural environments. In such biological systems as the mouth, it is considered that the organisms grow at an average rate of ~2 generations per day. In dental plaque, the carbohydrate source is generally the growth-limiting substrate for streptococci[6], but as the microorganisms are periodically subject to wind-falls of food, an ability to adapt to rapid changes in their nutrient supply confers a biological advantage. A chemostat not only enables low growth-rates to be achieved in laboratory cultures, but also permits the effects of a controlled perturbation from one condition to another to be examined. We have studied the effect of growth conditions on dextranase

activity of several strains of *S. mutans* grown in a chemostat at different generation times and at defined values of pH.

The most striking observation to emerge from the study was the inability of all strains except Ingbritt to produce dextranase at low growth-rates (Table I). At dilution rates in the range 0.05–0.20 h^{-1}, strains of serotype *d* produced no dextranase in glucose-limited or glucose-sufficient medium. Dextranase could readily be estimated in all strains except AHT at 0.5 h^{-1} (Table I, Figs. 2–4), but the activity reached the values recorded in batch culture only at high dilution-rates closer to 0.7 h^{-1}. At high growth-rates, more enzyme was released in glucose-sufficient than in glucose-limited medium. Thus the rate of growth was the major factor governing dextranase production, and such other factors as glucose sufficiency, were of secondary importance. This is in agreement with our earlier study[1] of batch cultures, where the release of dextranase began early in the exponential phase of growth, and ended before all the sugar had been consumed. The use of a chemostat has provided more reliable results for dextranase activity of *S. mutans* strain OMZ176, and successive samples from steady-state cultures gave constant values. The reason for the high decay rate in batch culture (Fig. 1) is not known: other extracellular enzymes, such as D-fructanase, in the same culture remained stable for 16 h in the stationary phase.

Changes in steady-state levels of enzyme activity as the dilution rate is changed are more common than constant levels, and the relationship may be proportional, inverse, or show one, or even two, points of inflexion, usually maxima[7]. Some of the effects may be explained in terms of induction, repression, or combinations of these[8], and an inverse relationship is obtained with enzymes that are synthesized mainly during the stationary phase[9]. Most enzymes show increases or decreases in activity that are linear, and the pattern found for *S. mutans* dextranase is uncommon, as it combines a constant relationship close to, or at, zero activity at low dilution rates, with a sudden increase in activity at high dilution rates.

A somewhat similar growth-rate dependency was reported for dextransucrase[10] in chemostat cultures of *Leuconostoc mesenteroides* NRRL B-512F. No enzyme could be detected in sucrose-limited cultures at low dilution rates ($<0.2\ h^{-1}$), but thereafter, dextransucrase activity in the culture filtrate increased with dilution rate to a maximum at 0.53 h^{-1}. Because sucrose was not limiting above a dilution rate of 0.35 h^{-1}, it was concluded that an increase in enzyme activity occurred only after a critical concentration of inducer (sucrose) had been achieved. A similar explanation would not account for the pattern of growth-rate dependency shown by *S. mutans* dextranase. Glucose was not detected at high dilution-rate in glucose-limited cultures. Moreover, the presence of an excess of glucose in the medium did not materially alter the kinetic pattern. D-Glucose was therefore not an inducer of dextranase.

When an increase in the supply of glucose permitted a higher growth rate, then an increased release of dextranase resulted. This effect was demonstrated during transition stages when the dilution rate was increased from 0.075–0.50 h^{-1} (Fig. 5). After only 0.125 culture volume had flowed at the higher dilution rate, the release of a burst of dextranase began. Within one culture volume of receiving the surge of

nutrients, dextranase productivity had increased 200-fold with no detectable change in cell concentration. *S. mutans* cells were thus responding almost immediately to the new condition by forming the equipment for synthesizing dextranase at a rate not at all characteristic for a steady state at the new growth-rate. The over-synthesis was most probably a response to the acceleration in growth rate, and when approximately one culture volume had been replaced, the growth rate had accommodated to the dilution-rate change, and the accumulated enzyme began to wash out.

Transient over-production of enzymes in response to more favourable conditions has been recorded previously for streptococci. A change in constituents of the medium resulted in a 4-fold over-production of dextransucrase by *S. sanguis*[11], and perturbation of *S. mutans* from steady state, glucose-limited growth by a pulse of glucose was characterized by an immediate increase (~8-fold) in the specific productivity of lactic acid, leading to transient accumulation[12]. Adjustment of a *S. mutans* Ingbritt culture[13] to the pH giving maximum production of glucosyltransferase activity (6.5) led to increased levels of enzyme activity during the transition from higher or lower values of pH. In all these cases, changes in cell density were slight.

The rate of decay of extracellular dextranase in the chemostat is unknown, but it is probably exponential[14] and certain to be pH-dependent. Thus the variation in yield of dextranase with pH of growth (Fig. 6) is dependent on stability as well as on production. The ratio between the yield of dextranase during growth at pH 7 relative to pH 6 for the K1-R culture in steady state at high dilution rate was the same (2.4) as that for the transient dextranase peak produced by Ingbritt in an unsteady state. Values for the relationship between the pH of growth and the yield of dextranase were less consistent in batch cultures of strain OMZ176. Although the overall production of enzyme was best at pH 7 (Fig. 1), the enzyme was more stable at pH 6. Guggenheim and Burckhardt[15] grew *S. mutans* OMZ176 in batch culture at pH 6.8, but carried out steps for the purification of the dextranase at pH values of 6 and 5.5. The enzyme was totally unstable[1] in batch-culture filtrates when the pH fell below 5 because of the natural production of acid by the microorganisms (Fig. 1). However, the pH for optimal activity of purified dextranase was[15] in the range 4.5–5.0 for *S. mutans* strains OMZ176 and was[16] pH 5.5 for strain K1-R.

Although the environment in a chemostat is far less complex than that of dental plaque, the results of controlled experiments in the chemostat indicate the *S. mutans* would produce little or no dextranase when growing slowly in plaque. They also indicate that the organisms could promptly realise their potential to produce dextranase in response to a sudden, increased supply of nutrients. The amount of dextranase released would be sufficient to hydrolyse the (1→6)-linked sequences in the α-D-glucans synthesized from sucrose by the α-D-glucosyltransferases of oral streptococci.

ACKNOWLEDGMENTS

This investigation was supported by grants from the National Health and Medical Research Council of Australia, and the Australian Research Grants Committee.

REFERENCES

1 G. J. WALKER, A. PULKOWNIK, AND J. G. MORREY-JONES, *J. Gen. Microbiol.*, 127 (1981) 201–208.
2 A. R. GLENN, *Annu. Rev. Microbiol.*, 30 (1976) 41–62.
3 J. VAN HOUTE AND C. A. SAXTON, *Caries Res.*, 5 (1971) 30–43.
4 A. DAHLQVIST, *Biochem. J.*, 80 (1961) 547–551.
5 S. DYGERT, L. H. LI, D. FLORIDA, AND J. A. THOMA, *Anal. Biochem.*, 13 (1965) 367–374.
6 J. S. VAN DER HOEVEN, *Arch. Oral Biol.*, 21 (1976) 431–433.
7 A. C. R. DEAN, *J. Appl. Chem. Biotechnol.*, 22 (1972) 245–259.
8 K. TODA, *Biotechnol. Bioeng.*, 18 (1976) 1117–1124.
9 F. G. PRIEST, *Bacteriol. Rev.*, 41 (1977) 711–753.
10 G. R. LAWFORD, A. KLIGERMAN, T. WILLIAMS, AND H. G. LAWFORD, *Biotechnol. Bioeng.*, 21 (1979) 1121–1131.
11 J. CARLSSON AND B. ELANDER, *Caries Res.*, 7 (1973) 89–101.
12 C. J. COONEY, J. LEUNG, AND A. J. SINSKEY, in H. M. STILES, W. J. LOESCHE, AND T. C. O'BRIEN (Eds.), *Proc. Microbial Aspects of Dental Caries*, Sp. Suppl., *Microbiol. Abstr.*, Information Retrieval Inc., Washington D.C., 1976, pp. 799–807.
13 D. C. ELLWOOD AND J. R. HUNTER, in A. C. R. DEAN, D. C. ELLWOOD, C. G. T. EVANS, AND J. MELLING (Eds.), *Continuous Culture: Applications and New Fields*, Ellis Horwood, Chichester, 1976, pp. 270–272.
14 G. EAVES, J. GREENMAN, AND K. T. HOLLAND, *FEMS Microbiol. Lett.*, 6 (1979) 333–336.
15 B. GUGGENHEIM AND J. J. BURCKHARDT, *Helv. Odont. Acta*, 18 (1974) 101–113
16 A. PULKOWNIK AND G. J. WALKER, *Carbohydr. Res.*, 54 (1977) 237–251.