

Effect of Growth Rate and pH on Intracellular Levels and Activities of the Components of the Phosphoenolpyruvate: Sugar Phosphotransferase System in *Streptococcus mutans* Ingbritt

CHRISTIAN VADEBONCOEUR,¹ SYLVIE ST. MARTIN,¹ DENIS BROCHU,¹ AND IAN R. HAMILTON^{2*}

Groupe de Recherche en Ecologie Buccale, Departement de Biochimie (Sciences et Genie) et Ecole de Medecine Dentaire, Universite Laval, Ste-Foy, Quebec G1K 7P4,¹ and Department of Oral Biology, University of Manitoba, Winnipeg, Manitoba R3E 0W2,² Canada

Received 5 September 1990/Accepted 5 December 1990

The growth of *Streptococcus mutans* Ingbritt in continuous culture at low pH or high growth rates repressed the biosynthesis of the components of the phosphoenolpyruvate:sugar phosphotransferase system (PTS). The cellular concentrations of the soluble components HPr, enzyme I (EI), and EIII for mannose (III^{man}) and EII activity for glucose, mannose, 2-deoxyglucose (2DG), and fructose were determined in membrane preparations from cells grown at pHs from 8.0 to 5.0 and at dilution (*D*) or growth rates from 0.1 to 1.0 h⁻¹. The cellular levels of HPr and EI varied less than threefold under all of the growth conditions tested. On the other hand, EII activity in membranes from cells grown at *D* = 0.1 h⁻¹ was repressed by growth at pHs below 8.0, with cells grown at pH 5.0 completely devoid of EII activity. In addition, cells grown at *D* = 0.5 and 1.0 h⁻¹ exhibited little PTS activity for glucose, mannose, and 2DG and twofold-lower activity for fructose. These activities were stimulated by the addition of a membrane-free cytoplasmic fraction, and this activating activity was shown to be due to the presence of III^{man}. Estimation of the cellular content of III^{man} indicated that the synthesis of this factor was repressed by growth above and below pH 7.0 and was particularly sensitive to growth at high rates. These results indicate that with *S. mutans* Ingbritt, both pH and growth rate regulate the genes for the synthesis of EIIs involved in the phosphorylation of glucose, mannose, 2DG, and fructose and the gene for the formation of III^{man}.

Sugar transport plays an important role in the metabolism of *Streptococcus mutans*, the principal etiological agent of dental caries (17). The major route of sugar transport by the organism is via the high-affinity phosphoenolpyruvate (PEP): sugar phosphotransferase system (PTS) (1, 3, 9, 13), a group translocation process which utilizes PEP as an energy source and substrate and catalyzes sequential phosphorylation of the general, non-sugar-specific proteins enzyme I (EI) and HPr, which are required for the uptake of all PTS sugars. In many cases, phospho-HPr, generated from phospho-EI, transfers the phosphoryl group directly to carbohydrate-specific, membrane-bound EII, which in turn phosphorylates the incoming sugar. In other cases, phospho-HPr transfers the phosphoryl group to a second sugar-specific protein called enzyme III, or simply III, prior to interaction with EII (6, 15, 16, 21, 23-26, 28).

Continuous-culture studies with *S. mutans* Ingbritt have indicated that the growth environment regulates glucose-PTS activity in a manner independent of the rate of glycolysis (8, 9). For example, early experiments with decrytified cells demonstrated that glucose-PTS activity was repressed by growth at low pH (11), at high dilution rates (*D*) or growth rates (5), with sucrose (4), and in the presence of excess glucose (5). More recently, EII activity for glucose and mannose, measured in isolated membrane preparations in the presence of excess EI and HPr, was shown to be repressed by as much as 62-fold under conditions of low pH, high growth rates, and excess glucose compared with optimal PTS conditions at neutral pH with low levels of glucose (27). Under the same conditions, the concentration of HPr

measured by immunoelectrophoresis varied only twofold, indicating that glucose-PTS activity in *S. mutans* Ingbritt is regulated at the level of the sugar-specific membrane component(s). Similar regulation of EII activity for glucose, mannose, and 2-deoxyglucose (2DG) was recently reported for a fresh strain of *S. mutans* growing in continuous culture (19), and, in addition, the cellular concentrations of EI, but not HPr, were shown to be influenced significantly by various environmental conditions.

We have recently studied in more detail substrate-dependent repression of the components of the glucose-PTS in *S. mutans* Ingbritt grown in continuous culture (12). Cells were grown to steady state at eight glucose concentrations between 2.6 and 304 mM, with growth initiated at the lowest glucose concentration to give glucose-limited conditions and increased progressively such that the culture became nitrogen limited or glucose excess. The results indicated that the specific EII activities for glucose (EII^{glc}), mannose (EII^{man}), 2DG (EII^{2DG}), and fructose (EII^{fru}) measured at each stage in isolated membrane preparations in the presence of excess concentrations of EI, HPr, and III^{man} were maximum at the lowest glucose concentration, and progressive repression (33-fold) was observed as the glucose level in the medium was increased, even when the culture was glucose limited. For example, significant repression (27-fold) of EII^{glc} was observed at 73 mM glucose when the culture was glucose limited. The cellular concentration of EI showed a similar profile of glucose repression but to a lesser extent (fourfold). The formation of HPr was less sensitive to the medium glucose level, showing only a fourfold repression as the culture approached conditions of glucose excess.

In this report, we have undertaken to examine in more detail the influence of growth pH and growth rate on the

* Corresponding author.

intracellular levels of HPr, EI, and III^{man} and on the activities of the membrane-bound, sugar-specific PTS components involved in the phosphorylation of glucose, mannose, 2DG, and fructose in cells of *S. mutans* Ingbritt growing in continuous culture with a glucose limitation. The results indicate that the cellular concentration of the general proteins HPr and EI were altered less than threefold by variations in growth pH from 8.0 to 5.0 or in *D* from 0.1 to 1.0 h⁻¹. The presence of III^{man} was necessary for the PEP-dependent phosphorylation of glucose, mannose, and 2DG but not of fructose, although III^{man} slightly stimulated this activity under certain conditions. Decreasing the growth pH to 5.0 and increasing the growth rate to 1.0 h⁻¹ repressed synthesis of EIIs as well as III^{man}.

MATERIALS AND METHODS

Bacterial strains. The *S. mutans* Ingbritt strain used in this study was kindly supplied by J. Sandham, Toronto, Ontario; *S. mutans* DR0001 was obtained from the culture collection of the National Institute for Dental Research, Bethesda, Md.; and *Streptococcus salivarius* ATCC 25975 was isolated originally by one us (7). The methods of culture maintenance and purity control for the test strains have been described previously (5).

Growth conditions. Growth in continuous culture was carried out in LH 500 Series III chemostats (L. H. Engineering, Stokes Poges, Bucks, United Kingdom) with working volumes of 500 to 800 ml. The semidefined medium of Bowden et al. (2) was used, and the glucose concentration of the inflowing medium was 10 mM, making the cultures glucose limited. The pH in the chemostats varied between 5.0, 6.0, 7.0, and 8.0 according to the experiment and was maintained automatically by the addition of 2 M KOH. In addition, growth was carried out at *D* of 0.1, 0.5, and 1.0 h⁻¹ (*t_D* = 7, 1.4, and 0.7 h), and the gas phase was 5% CO₂ in nitrogen. Since glucose was limiting the growth of the organism in the chemostat, *D* is directly equivalent to the growth rate of the organism (20). Daily routine maintenance of the chemostats was as described previously (10). The cultures were grown under each condition for at least 10 mean cell residence times before steady state was considered to have been established and cells were collected for the appropriate assay. Cell biomass was determined with each cell preparation by filtering, drying, and weighing five samples on prewashed, preweighed 0.45- μ m-pore-size polycarbonate filters (Nucleopore Corp, Pleasanton, Calif.).

Assay of EI and HPr. The cellular concentrations of EI and HPr were determined by rocket immunoelectrophoresis with rabbit antibodies directed against the purified EI and HPr proteins isolated from *S. mutans* DR0001 by methods previously described (12). These antibodies react with the proteins from *S. mutans* Ingbritt (12, 27). Cellular concentrations of HPr were calculated on the basis of a molecular weight of 8,300, while calculations for EI were based on a weight of 67,600 (21).

Preparation of EII-containing membranes. Membranes were prepared from intact cells collected via the chemostat overflow onto ice for periods not exceeding 16 h during steady-state growth. The cells were washed twice in 10 mM potassium phosphate buffer (pH 7.5) containing 1 mM EDTA, 14 mM 2-mercaptoethanol, 0.1 mM phenylmethylsulfonyl fluoride, and 0.1 mM pepstatin A (PEMPP buffer) and frozen at -70°C until sufficient cells (3 to 5 g) had accumulated to prepare membranes. Cells (2 to 3 g) were ground in levigated alumina (3 g of alumina per g of wet cell

paste) for 20 min in a refrigerated mortar (-20°C), and 2 ml of PEMPP buffer was added for each g of cells (23). Alumina was removed by centrifugation at 2,400 \times *g* for 5 min, and the cells and cell debris from the supernatant were sedimented by a further centrifugation at 24,000 \times *g* for 20 min. The supernatant was collected (20,000 S fraction), and membrane fragments were sedimented at 110,000 \times *g* overnight. The resulting supernatant corresponded to the cellular extract, which was used to measure the intracellular concentration of HPr, EI, and III^{man}, while the resulting pellet was suspended in 50 mM PEMPP buffer (without pepstatin) containing 0.1 M KCl and centrifuged once more at 180,000 \times *g* for 4 h. The membranes were then suspended in the same buffer without KCl and frozen at -40°C.

Assay for EII activity. EII activity was assayed by measuring the PEP-dependent phosphorylation of ¹⁴C-labeled glucose, mannose, 2DG, and fructose. All assays were carried out in the presence of excess HPr and EI purified from *S. mutans* DR0001 as previously described (12). To ensure the saturation of EII-III pairs, membranes were titrated with HPr and EI for each set of conditions and for each sugar assayed. The amount of HPr required for saturation varied from 100 to 400 μ g, while that for EI was 3.0 μ g. To verify the stimulation of EII activity by III, the corresponding cellular extract, or a cellular extract obtained from batch-grown cells grown on 0.1% glucose, was added to the assay reaction following dialysis against PEMPP buffer and concentration by an Amicon minicon microsolite concentrator (Amicon, Danvers, Mass.). EII activity is expressed as nanomoles of phosphorylated substrate per milligram of membrane protein per minute.

Quantitation of III^{man} by Western blotting (immunoblotting). Cytoplasmic (5 to 20 μ g) and membrane (1.0 to 5.0 μ g) protein fractions prepared from cells of *S. mutans* Ingbritt grown under various test conditions in continuous culture were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis as described by Laemmli (14) on a 10% polyacrylamide resolving gel with a 4% polyacrylamide stacking gel. Following electrophoresis, the proteins were transferred electrophoretically to nitrocellulose paper (0.45- μ m pore size) by the method of Towbin et al. (22). The nitrocellulose sheets were then treated as previously described (1) for the immunodetection of III^{man} by using purified specific polyclonal antibodies obtained from New Zealand White female rabbits after immunization with III^{man} purified from *S. salivarius* ATCC 25975.

The integration volume of each band of III^{man} obtained was then measured using a model 620 Videodensitometer in association with the 2D Analyst II software, both from Bio-Rad (Mississauga, Ontario, Canada). For a given sample, the relation between the integration volume of the III^{man} band, expressed in square millimeters times optical density, and the amount of protein (and accordingly the amount of III^{man}) in that lane was shown to be linear for a range of protein concentrations used in the study. This was established with cytoplasmic and membrane preparations from cells of *S. mutans* Ingbritt grown in continuous culture at pH 7.0 and *D* = 0.1 h⁻¹. Using these values as 1.0, the relative amount of III^{man} in the different cytoplasmic and membrane samples could then be determined by interpolation on the standard linear plot.

Protein determination. Proteins were measured by the method of Lowry et al. (18) with bovine serum albumin as a standard. Prior to assay, membrane preparations were preincubated with 1 N NaOH (30 min, 37°C).

Chemicals. [U-¹⁴C]glucose, 2-deoxy-D-[U-¹⁴C]glucose,

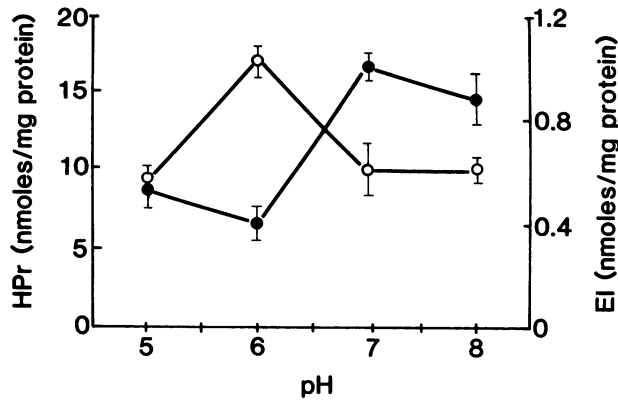


FIG. 1. Effect of growth pH on the cellular concentrations of HPr and EI in cells of *S. mutans* Ingbritt grown at $D = 0.1 \text{ h}^{-1}$ with a glucose limitation in continuous culture. Symbols: \circ , HPr; \bullet , EI.

and [^{14}C]mannose were from New England Nuclear (Montreal, Canada), while [^{14}C]fructose was obtained from ICN. Unlabeled sugar, phenylmethylsulfonyl fluoride, pepstatin A, and polyethylene glycol were from Sigma Chemical Co. (St. Louis, Mo.).

RESULTS

Effect of growth conditions on HPr and EI. Cells of *S. mutans* Ingbritt were grown in continuous culture with a glucose limitation under various conditions of pH and growth rate. As shown in Fig. 1, with the exception of pH 6.0, the cellular level of HPr was relatively constant in cells grown at $D = 0.1 \text{ h}^{-1}$ and pHs between 8.0 and 5.0, with an overall mean of $11.7 \pm 1.8 \text{ nmol/mg}$ of cellular protein. With respect to the concentration of EI, the levels at pHs 7.0 and 8.0 were approximately twice those in cells grown at pHs 6.0 and 5.0, with an overall mean value of $0.70 \pm 0.14 \text{ nmol/mg}$ of cellular protein. Thus, in overall terms, the levels of both general proteins were relatively constant in spite of the 3-U difference in growth pH.

Comparisons were also made of the cellular levels of HPr and EI when cells of *S. mutans* Ingbritt were grown at $D = 0.1, 0.5, \text{ and } 1.0 \text{ h}^{-1}$ and pHs 7.0 and 5.0. At pH 7.0 (Fig. 2A), the amount of HPr varied from 8.8 to 16.5 nmol/mg of protein, with the highest value at $D = 0.5 \text{ h}^{-1}$, while at pH 5.0, the cellular level increased almost twofold, from 9.5 to 17.4 nmol/mg of protein (Fig. 2B) when the growth rate was increased from 0.1 to 1.0 h^{-1} . Even with these differences between the two pHs, the overall means were similar, i.e., 11.8 ± 2.4 versus $13.7 \pm 2.3 \text{ nmol/mg}$ of protein for cells grown at pHs 7.0 and 5.0, respectively. Similarly, the values of EI varied from 0.38 to 0.62 nmol/mg at pH 5.0 and from 0.68 to 1.0 nmol/mg at pH 7.0. However, no trend in cellular level was observed at the three different growth rates. It can be noted, however, that the overall mean for cells grown at pH 7.0 ($0.84 \pm 0.09 \text{ nmol/mg}$ of protein) was significantly higher than that for cells grown at pH 5.0 ($0.51 \pm 0.07 \text{ nmol/mg}$ of protein).

Calculation of the mean value for the HPr concentration in cells grown at pHs 5.0 to 8.0 and at $D = 0.1, 0.5, \text{ and } 1.0 \text{ h}^{-1}$ gives a value of $13.0 \pm 1.3 \text{ nmol/mg}$ of protein, with a range from 8.8 to 17.4 nmol/mg of protein. The overall mean for the cellular level of EI was 0.67 nmol/mg of protein, with a range from 0.38 to 1.0 nmol/mg of protein. Consequently, while there were variations in the cellular levels of HPr and EI,

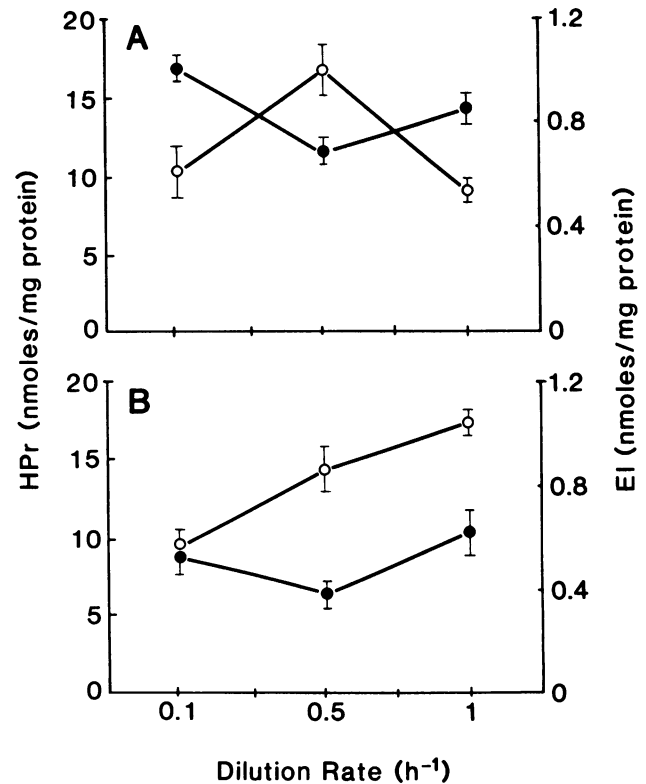


FIG. 2. Effect of dilution rate on the cellular concentrations of HPr and EI in cells of *S. mutans* Ingbritt grown in continuous culture at $D = 0.1 \text{ h}^{-1}$ with a glucose limitation at pHs 7.0 (A) and 5.0 (B). Symbols: \circ , HPr; \bullet , EI.

few trends were observed, and the differences were not profound. Furthermore, as will be seen later, although significant levels of HPr and EI were found during growth at pH 5.0, the cells grown at this pH were devoid of EII activity for glucose, mannose, 2DG, and fructose.

Effect of growth conditions on EII activity. EII activity for glucose, mannose, 2DG, and fructose was assayed in membrane preparations of *S. mutans* Ingbritt grown under the same conditions as those outlined for the estimation of HPr and EI. In all cases, washed membranes were assayed with an excess of HPr and EI in the presence and absence of 400 μg of crude membrane-free cytoplasmic extract to test for the presence of III. As seen in Fig. 3, EII activity in cells grown with limiting glucose at $D = 0.1 \text{ h}^{-1}$ and assayed in the absence of added cytoplasmic extract was directly related to the growth pH. Cells grown at pH 8.0 possessed the highest activity, while cells grown at pH 5.0 were completely devoid of EII activity for all four substrates with or without added soluble extract. In all other cases, activity was stimulated by the addition of crude cytoplasmic extract, indicating the involvement of a soluble component in EII activity. Control assays had indicated that the soluble fraction was free of membranes and that, therefore, the activating effect was due to a soluble component. EII activities for glucose (Fig. 3A), mannose (Fig. 3B), and 2DG (Fig. 3C) was stimulated almost 2-fold at pHs 7.0 and 8.0, while the activation at pH 6.0 was much more profound, i.e., 25-, 14-, and 10-fold for glucose, mannose, and 2DG, respectively. The stimulation of EII^{fru} by the cytoplasmic extract was only twofold at all pH values.

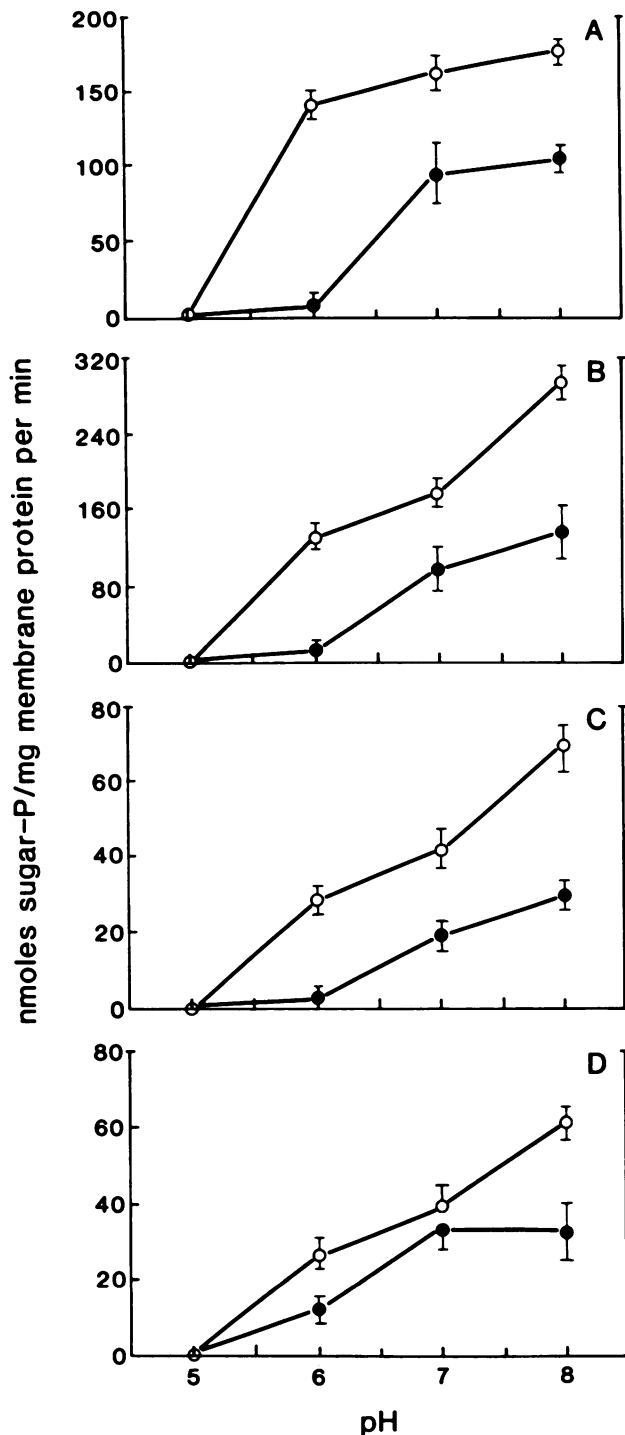


FIG. 3. Effect of growth pH on the specific activity of various EIIs in membrane preparations of *S. mutans* Ingbritt grown in continuous culture with a glucose limitation at $D = 0.1 \text{ h}^{-1}$ and assayed in the presence (○) and absence (●) of crude, membrane-free cytoplasmic extract obtained from batch-grown cells. EIIs assayed were glucose (A), mannose (B), 2DG (C), and fructose (D).

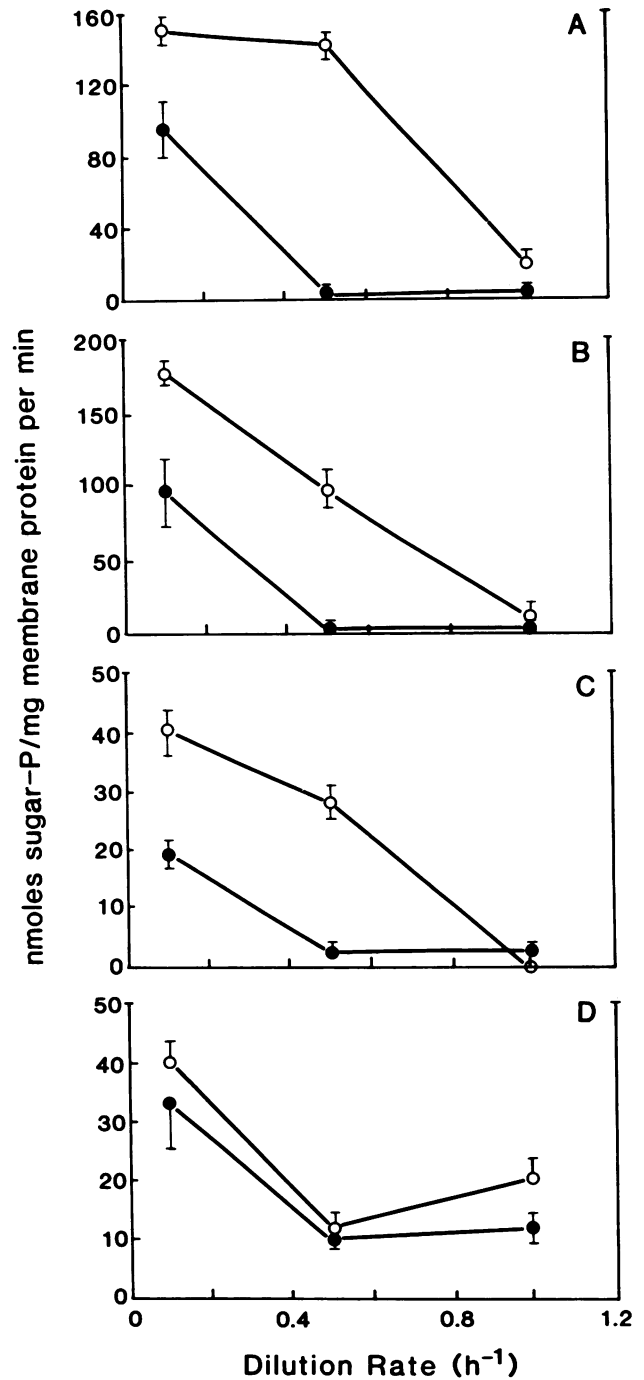


FIG. 4. Effect of growth rate on the specific activity of various EIIs in membrane preparations of *S. mutans* Ingbritt grown in continuous culture with a glucose limitation at pH 7.0 and assayed in the presence (○) and absence (●) of crude, membrane-free cytoplasmic extract obtained from batch-grown cells. EIIs assayed were glucose (A), mannose (B), 2DG (C), and fructose (D).

Increases in the D or growth rate from 0.1 to 1.0 h^{-1} repressed EII activities for glucose, mannose, and 2DG significantly in the absence of the crude cytoplasmic extract, such that negligible activities were observed at 0.5 and 1.0 h^{-1} (Fig. 4A through C). The addition of the soluble extract stimulated these activities almost twofold at $D = 0.1 \text{ h}^{-1}$.

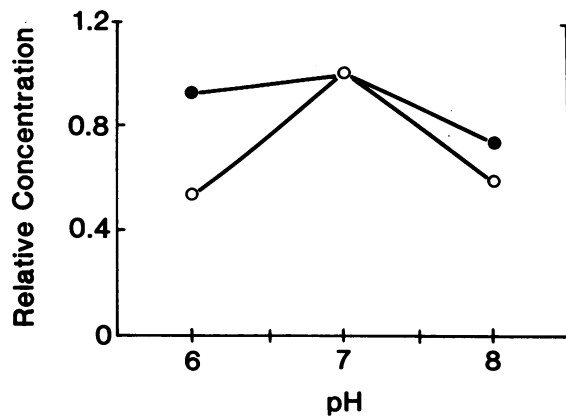


FIG. 5. Effect of growth pH on the relative concentration of III^{man} in membrane (●) and cytoplasmic (○) fractions of *S. mutans* Ingbritt grown at $D = 0.1 \text{ h}^{-1}$ with a glucose limitation.

However, at 0.5 h^{-1} , significantly higher activation was observed. The addition of extract to the membranes prepared from cells at $D = 0.5 \text{ h}^{-1}$ resulted in almost complete recovery of activity for EII^{glc} (i.e., 70-fold activation) compared with membranes incubated without extract. Although significant activation was observed with mannose (49-fold) and 2DG (14-fold), complete recovery of activity was not observed. In addition, while some small stimulation of activity was evident at $D = 1.0 \text{ h}^{-1}$ in the presence of added extract (except with 2DG), it was apparent that significant repression of EII activity had occurred at this growth rate. Figure 4D indicates that EII^{fru} activity was also repressed by high growth rates. However, the addition of the cytoplasmic extract resulted in minimal stimulation of activity.

Nature of the soluble factor. Previous research with the oral streptococci suggested that the soluble factor stimulating EII activity in the experiments whose results are shown in Fig. 3 and 4 may have been the soluble, sugar-specific enzyme III^{man} (1, 24). To test for this possibility, membranes from cells grown at pH 7.0 and $D = 0.1 \text{ h}^{-1}$ were preincubated for 30 min with purified EI and HPr, membrane-free cytoplasmic extract, and antibodies directed against III^{man} from *S. salivarius* ATCC 25975 prior to being assayed for EII^{man} by the addition of [^{14}C]mannose. In this assay, activity was completely inhibited, indicating that the essential component in the soluble extract was III^{man} . As a control, anti-EI prepared from *S. salivarius* was substituted for anti- III^{man} and gave similar results.

Clearly, the decrease in sugar phosphorylation seen in Fig. 3 and 4 suggested repression of the genes for EII and possibly III^{man} . To test for this possibility, Western blotting was employed to determine the relative concentration of III^{man} in membrane and cytoplasmic preparations of cells of *S. mutans* Ingbritt grown at pHs 6.0 and 8.0 and at $D = 0.5$ and 1.0 h^{-1} and to compare this concentration with that of cells grown at pH 7.0 and $D = 0.1 \text{ h}^{-1}$. As seen in Fig. 5, cells grown under these latter conditions possessed the highest concentration of III^{man} in both the cytoplasmic and membrane fractions. Cytoplasmic III^{man} in cells grown at pHs 8.0 and 6.0 was 42 and 47% lower, respectively, than in cells grown at pH 7.0, while membrane III^{man} was 8 and 27% lower under the same conditions. Thus, the total reductions in the synthesis of III^{man} by cells at pHs 8.0 and 6.0 were 55 and 69%, respectively. However, only the reduction at pH 6.0 was coincident with the repression of EII^{man} (Fig. 3B).

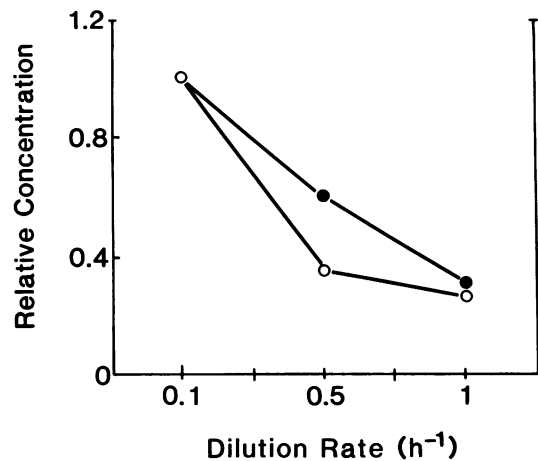


FIG. 6. Effect of growth rate on the relative concentration of III^{man} in membrane (●) and cytoplasmic (○) fractions of *S. mutans* Ingbritt grown at pH 7.0 with a glucose limitation.

The influence of growth rate on the formation of III^{man} was much more dramatic (Fig. 6) and coincided with the repression of EII^{man} (Fig. 4B). During growth of *S. mutans* Ingbritt at $D = 0.5$ and 1.0 h^{-1} , the respective cytoplasmic III^{man} levels were only 35 and 26% of that at $D = 0.1 \text{ h}^{-1}$, while the membrane III^{man} levels were 60 and 31%, respectively. Thus, the overall amounts of III^{man} at $D = 0.5$ and 1.0 h^{-1} were two- and fourfold lower, respectively, than the amounts of III^{man} synthesized in cells grown at $D = 0.1 \text{ h}^{-1}$. This repression did coincide with the EII repression observed in Fig. 4.

DISCUSSION

Earlier continuous-culture studies with *S. mutans* Ingbritt demonstrated the repression of overall glucose-PTS in de-cryptified cells by various environmental conditions without identifying the components of the system affected (4, 5, 8, 9, 11). More-recent studies with this and other oral streptococci have begun to provide information on the cellular concentration of HPr, EI, and various EIIs under changing growth conditions. The analysis of HPr and EI in cells was made possible by the isolation and purification of these proteins and the generation of antibodies against them, thereby permitting estimation by rocket immunoelectrophoresis. In studies with batch-grown cells of *S. mutans* ATCC 27352, Thibault and Vadeboncoeur (21) observed little variation in the cellular concentrations of HPr and EI at various stages of the growth cycle or with different substrates. The levels of HPr ranged from 2.8 (sorbitol) to 5.6 (mannitol) nmol/mg of protein, while EI varied from 0.6 to 0.9 nmol/mg of protein. Furthermore, comparisons with one other serotype d/g strain and four serotype c strains revealed similarities in HPr cellular levels, which ranged from 2.57 to 7.00 nmol/mg of protein, while the EI concentration in *S. mutans* 6715 was similar to that in *S. mutans* ATCC 27352 (1.17 versus 0.93 nmol/mg of protein).

More-recent continuous-culture studies have shown that HPr concentrations in *S. mutans* Ingbritt varied only twofold (5.5 to 11.4 nmol/mg) under conditions of glucose limitation and glucose excess (nitrogen limitation) at pHs 7.0 and 5.5 (27). Similar results were obtained with glucose-limited cells of *S. mutans* DR0001 and its PTS-defective mutant, strain

DR0001/6. In the same study, cells of *S. sobrinus* grown on limiting lactose showed remarkably little variation in the levels of HPr, EI, and III^{lac} or of EII^{glc} and EII^{fru} in spite of changes in pH from 7.0 to 5.0 and growth at between 0.1 and 0.8 h⁻¹. The similar study by Rodrigue et al. (19) with a fresh isolate of *S. mutans*, strain 123.1, also showed very little variation in the cellular concentration of HPr (5.1 to 7.7 nmol/mg) under conditions of glucose limitation and glucose excess at pHs of 7.0 and 5.5 and $D = 0.057$ and 0.4 h^{-1} . EI levels in the same cells were somewhat more variable, with fourfold differences occurring with changes in pH and dilution rate.

Although higher cellular concentrations of HPr were observed in this study than in previous studies, the changes in response to growth pH and growth rate were, nevertheless, relatively small in all cases. The most notable and surprising trend was a progressive increase in HPr levels as the growth rate or D was increased from 0.1 to 1.0 h^{-1} at pH 5.0, although the difference in the values was less than twofold (Fig. 2B). Similarly, variations in growth at pHs of 8.0 and 5.0 at $D = 0.1 \text{ h}^{-1}$ resulted only in differences between 9.5 ± 0.5 and $17.0 \pm 0.9 \text{ nmol/mg}$ (Fig. 1), and even under conditions of stress at $D = 1.0 \text{ h}^{-1}$ when the organism was near its maximal growth rate (9), the difference between pHs 5.0 and 7.0 was only twofold (17.4 ± 0.5 versus $8.8 \pm 0.2 \text{ nmol/mg}$, respectively). Interestingly, in spite of the high level of HPr at pH 5.0, the cells were devoid of membrane-bound EII activity for the four substrates.

Similarly, the alterations in cellular levels of EI as a function of growth pH and rate were small. The greatest difference was observed during growth at $D = 0.1 \text{ h}^{-1}$, and this represented less than a threefold difference in cellular concentration (0.40 ± 0.04 versus $1.00 \pm 0.00 \text{ nmol/mg}$), with no obvious trend. Perhaps the most notable observation concerning the cellular level of EI was the difference in the mean values for growth at pHs 5.0 and 7.0 as a function of growth rate (0.51 ± 0.07 versus $0.84 \pm 0.09 \text{ nmol/mg}$, respectively).

From the analysis given above, one must conclude that in spite of a 3-U change in growth pH and a 10-fold increase in the growth rate in this study, variation in the cellular concentrations of HPr and EI was less than 3-fold. This suggests a relatively minor role for the environment in the regulation of the genes for HPr and EI. Somewhat larger effects on the cellular levels of HPr and EI have been observed during concentration-dependent glucose repression of the glucose-PTS in *S. mutans* Ingbritt (12). In these experiments, both HPr and EI were repressed during growth at glucose concentrations above 2.6 mM; however, even at 304 mM, when the culture was growing in the presence of excess glucose, the repression of HPr and EI synthesis was only fourfold.

With the oral streptococci, the available evidence suggests that the most significant influence of the environment on the PEP PTS occurs via the regulation of the genes for the specific enzymes EII and III^{man}. Earlier studies with *S. mutans* Ingbritt (27) compared some effects of growth pH and growth rate on EII activity for various substrates employing purified HPr and EI. Growth of the organism at pH 5.5 and $D = 0.4 \text{ h}^{-1}$ resulted in 24- to 27-fold repression of the EII^{glc}, EII^{man}, and EII^{2DG} activity compared with activity at pH 7.0 and $D = 0.1 \text{ h}^{-1}$. Similar repression of EII activity was observed when the culture was shifted from a glucose limitation to a nitrogen limitation with excess glucose. Similar but somewhat lower repression was observed in a study with a chemostat-grown fresh isolate, *S. mutans*

123.1 that compared EII activity in cells growing at pH 7.0 ($D = 0.057 \text{ h}^{-1}$) and pH 5.5 ($D = 0.4 \text{ h}^{-1}$) (19). Severe glucose-dependent repression (27-fold) of EII^{glc} and EII^{man} was observed in a more comprehensive study employing cells grown at glucose concentrations of 2.6 to 304 mM (12). The type of repression seen with *S. mutans* Ingbritt and *S. mutans* 123.1 may not be universal, since EII activity for glucose, mannose, and 2DG increased with preparations from *S. mutans* DR0001 grown at pH 5.5 and $D = 0.2 \text{ h}^{-1}$ compared with cells grown at pH 7.0 and $D = 0.1 \text{ h}^{-1}$ (27).

The results of the present study indicate that EII^{fru} is regulated by a mechanism different from that controlling the activities of EII^{glc} and EII^{man}. While the pH effect (Fig. 3D) was similar to that observed with these EIIs, EII^{fru} activity was not repressed at $D = 1.0 \text{ h}^{-1}$ (Fig. 4D). Furthermore, the addition of the cytoplasmic fraction had only a modest activating effect on the enzyme, indicating that III^{man} is not a regulatory factor for EII^{fru} activity. Our earlier study (27) demonstrated lower repression of EII^{fru} activity by changes in the growth conditions than that of EII^{glc} and EII^{man}, and separate regulation for the fructose-PTS genes has also been suggested by the work of Gauthier and co-workers (6) and Liberman and Bleiweis (16). The lack of an effect by the cytoplasmic fraction on EII^{fru} may be explained by the presence in *S. mutans* of a specific constitutive EII^{fru} that functions without III (6).

In previous studies with *S. mutans* Ingbritt, the assumption was made that any III required for activity would be bound in the membrane preparation to the respective sugar-specific EII. When the EII assays were carried out with only purified HPr and EI, however, extensive repression of the EIIs for glucose, mannose, 2DG, and, to a lesser extent, fructose was observed when the cells were grown at pHs below 8.0 and at $D = >0.1 \text{ h}^{-1}$. In fact, cells grown at pH 5.0 and $D = 0.1 \text{ h}^{-1}$ (Fig. 3) and at $D = 0.5$ and 1.0 h^{-1} and pH 7.0 (Fig. 4) were essentially devoid of activity. However, the stimulation of EII activity by addition of the dialyzed, membrane-free cytoplasmic extract containing III^{man} indicates that, while repression was not completely ameliorated by the addition of the extract, the lack of EII activity when no extract was added was due to an inadequate supply of III^{man}. The lower relative concentration of III^{man} in cells grown at pH 6.0 (Fig. 5) and at $D = 1.0 \text{ h}^{-1}$ (Fig. 6) suggests that the alterations in growth pH and growth rate regulated the genes for the various EIIs as well as the gene for III^{man}.

ACKNOWLEDGMENTS

We thank Beverly Grimshire for her excellent technical assistance.

This work was supported by grant MA-10298 from the Medical Research Council of Canada.

REFERENCES

1. Bourassa, S., L. Gauthier, R. Giguere, and C. Vadeboncoeur. 1990. A III^{man} protein is involved in the transport of glucose, mannose and fructose by oral streptococci. *Oral Microbiol. Immunol.* 5:288-297.
2. Bowden, G. H., J. M. Hardie, and E. D. Fillery. 1976. Antigens from *Actinomyces* species and their value in identification. *J. Dent. Res.* 55 (Special Issue A):A192-A204.
3. Calmes, R. 1978. Involvement of phosphoenolpyruvate in the catabolism of caries-conducive disaccharides by *Streptococcus mutans*: lactose transport. *Infect. Immun.* 19:934-942.
4. Ellwood, D. C., and I. R. Hamilton. 1982. Properties of *Streptococcus mutans* Ingbritt growing in limiting sucrose in a chemostat: repression of the phosphoenolpyruvate phosphotransferase transport system. *Infect. Immun.* 36:576-581.

5. 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.
6. Gauthier, L., D. Mayrand, and C. Vadeboncoeur. 1984. Isolation of a novel protein involved in the transport of fructose by an inducible phosphoenolpyruvate fructose phosphotransferase system in *Streptococcus mutans*. *J. Bacteriol.* **160**:755-763.
7. Hamilton, I. R. 1968. Synthesis and degradation of intracellular polyglucose in *Streptococcus salivarius*. *Can. J. Microbiol.* **14**:65-77.
8. Hamilton, I. R. 1984. The oral 'pathogen', *Streptococcus mutans*, possesses two glucose transport systems, p. 55-71. In A. C. R. Dean, D. C. Ellwood, and C. G. T. Evans (ed.), *Continuous culture 8: biotechnology, medicine and the environment*. Ellis Horwood, Chichester, England.
9. Hamilton, I. R. 1987. Effect of changing environment on sugar transport and metabolism by oral bacteria, p. 94-133. In J. Reizer and A. Peterkofsky (ed.), *Sugar transport and metabolism by gram-positive bacteria*. Ellis Horwood, Chichester, England.
10. Hamilton, I. R., and G. H. Bowden. 1982. Response of freshly isolated strains of *Streptococcus mutans* and *Streptococcus mitior* to change in pH in the presence and absence of fluoride during growth in continuous culture. *Infect. Immun.* **36**:255-262.
11. 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.
12. Hamilton, I. R., L. Gauthier, B. Desjardins, and C. Vadeboncoeur. 1989. Concentration-dependent repression of the soluble and membrane components of the phosphoenolpyruvate:sugar phosphotransferase system of *Streptococcus mutans* by glucose. *J. Bacteriol.* **171**:2942-2948.
13. Hamilton, I. R., and G. C. Y. Lo. 1978. Co-induction of β -galactosidase and the lactose-P-enolpyruvate phosphotransferase system in *Streptococcus salivarius* and *Streptococcus mutans*. *J. Bacteriol.* **136**:900-905.
14. Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature (London)* **227**:680-685.
15. Liberman, E. S., and A. S. Bleiweis. 1984. Role of the phosphoenolpyruvate-dependent glucose phosphotransferase system of *Streptococcus mutans* GS-5 in the regulation of lactose uptake. *Infect. Immun.* **43**:536-542.
16. Liberman, E. S., and A. S. Bleiweis. 1984. Transport of glucose and mannose by a common phosphoenolpyruvate-dependent hexose-phosphotransferase system in *Streptococcus mutans* GS-5. *Infect. Immun.* **43**:1106-1109.
17. Loesche, W. J. 1986. Role of *Streptococcus mutans* in human dental decay. *Microbiol. Rev.* **50**:353-380.
18. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193**:265-275.
19. Rodrigue, L., L. Lacoste, L. Trahan, and C. Vadeboncoeur. 1988. Effect of nutritional constraints on the biosynthesis of the components of the phosphoenolpyruvate:sugar phosphotransferase system in a fresh isolate of *Streptococcus mutans*. *Infect. Immun.* **56**:518-522.
20. Tempest, D. W. 1970. The place of continuous culture in microbiological research. *Adv. Microbiol. Phys.* **4**:223-249.
21. Thibault, L., and C. Vadeboncoeur. 1985. Phosphoenolpyruvate-sugar phosphotransferase system of *Streptococcus mutans*: purification of HPr and enzyme I and determination of their intracellular concentrations by rocket immunoelectrophoresis. *Infect. Immun.* **50**:817-825.
22. Towbin, H., T. Staehelin, and J. Gordon. 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proc. Natl. Acad. Sci. USA* **76**:4350-4354.
23. Vadeboncoeur, C. 1984. Structure and properties of the phosphoenolpyruvate:glucose phosphotransferase system of oral streptococci. *Can. J. Microbiol.* **30**:495-502.
24. Vadeboncoeur, C., and L. Gauthier. 1987. The phosphoenolpyruvate:sugar phosphotransferase system in *Streptococcus salivarius*. Identification of a III^{man} protein. *Can. J. Microbiol.* **33**:118-122.
25. Vadeboncoeur, C., and M. Proulx. 1984. Lactose transport in *Streptococcus mutans*: isolation and characterization of factor III^{lac}, a specific component of the phosphoenolpyruvate-lactose phosphotransferase system. *Infect. Immun.* **46**:213-219.
26. Vadeboncoeur, C., M. Proulx, and L. Trahan. 1983. Purification of the HPr and enzyme I-like proteins from the oral bacterium *Streptococcus salivarius*. Biochemical and immunochemical properties. *Can. J. Microbiol.* **29**:1694-1705.
27. Vadeboncoeur, C., L. Thibault, S. Neron, H. Halvorson, and I. R. Hamilton. 1987. Effect of growth conditions on levels of components of the phosphoenolpyruvate:sugar phosphotransferase system in *Streptococcus mutans* and *Streptococcus sobrinus* grown in continuous culture. *J. Bacteriol.* **169**:5686-5691.
28. Vadeboncoeur, C., and L. Trahan. 1982. Glucose transport in *Streptococcus salivarius*. Evidence for the presence of a distinct phosphoenolpyruvate:glucose phosphotransferase system which catalyses the phosphorylation of α -methylglucoside. *Can. J. Microbiol.* **28**:190-199.