

# $\beta$ -D-Phosphogalactoside Galactohydrolase of Lactic Streptococci<sup>1</sup>

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$\beta$ -D-Phosphogalactoside galactohydrolase ( $\beta$ -Pgal) was examined in a number of lactic streptococci by use of the chromogenic substrate *o*-nitrophenyl- $\beta$ -D-galactopyranoside-6-phosphate. Specific activity of  $\beta$ -Pgal ranged from 0.563 units/mg of protein in *Streptococcus lactis* UN, to 0.120 in *S. diacetylactis* 18-16. Essentially no  $\beta$ -D-galactoside galactohydrolase ( $\beta$ -gal) was found in these organisms when *o*-nitrophenyl- $\beta$ -D-galactopyranoside served as the chromogenic substrate. *S. lactis* 7962 was the one exception found. This organism contained rather high levels of  $\beta$ -gal, and very little  $\beta$ -Pgal could be detected.  $\beta$ -Pgal activity was examined in streptococci that differed widely in both their proteolytic ability and rates of lactic acid production during growth in milk. Differences in proteolytic ability did not influence  $\beta$ -Pgal synthesis; also, the rate of lactic acid production was independent of the level of  $\beta$ -Pgal present in the cell, since the rate of lactic acid production could be increased approximately fourfold without changing the amount of  $\beta$ -Pgal present in the cell. Various carbohydrates were tested as potential inducers of the enzyme. Although galactose, either as the free sugar or combined with glucose in lactose, was the only inducer, noninducing sugars such as mannose or glucose showed some ability to cause fluctuations in the basal level of  $\beta$ -Pgal. Cells growing in mannose or glucose exhibited about 30% of the maximal enzyme levels found in cells growing in lactose or galactose. No gratuitous inducers were found.

The initial metabolic steps in lactose utilization by the industrially important lactic streptococci have been under study in our laboratory. Previous reports have shown that the transport of lactose into the cell and subsequent hydrolysis of the sugar are mediated by a system quite similar to that found in *Staphylococcus aureus* (5-9, 11, 19).

The transport of lactose into the cell in *Streptococcus lactis* C2 is mediated by a phosphoenolpyruvate-dependent phosphotransferase system (16-18), similar to that first characterized by Roseman (13, 14) in *Escherichia coli*. The resultant lactose-phosphate is hydrolyzed by the enzyme  $\beta$ -D-phosphogalactoside galactohydrolase ( $\beta$ -Pgal) to yield, presumably, free glucose and galactose-6-phosphate. Galactose-6-phosphate has been shown to be the actual inducer of lactose utilization in *S. aureus*, but has not been conclusively shown to be the true inducer in the lactic streptococci. Also, the metabolic fate of galactose-6-phosphate is unknown (16, 19).  $\beta$ -Pgal, which is

present in all except one of the lactic streptococci so far examined, hydrolyzes *o*-nitrophenyl- $\beta$ -D-galactopyranoside-6-phosphate (ONPG-6-P) in cell-free extracts and toluene-acetone-treated cells, but cannot hydrolyze ONPG under similar conditions.  $\beta$ -Galactosidase ( $\beta$ -gal-EC 3.2.1.2.3,  $\beta$ -D-galactoside galactohydrolase), which hydrolyzes ONPG, has so far been demonstrated in only one lactic streptococcus, namely, *S. lactis* 7962 (1, 16), and the taxonomy of this organism is open to question (18).

The present study reports recent findings on the enzyme  $\beta$ -Pgal present in the majority of the lactic streptococci.

## MATERIALS AND METHODS

**Microorganisms and media.** All of the lactic streptococci used in this study were obtained from the stock culture collection maintained by the Department of Microbiology, Oregon State University. The cultures were regularly maintained in sterile 11% nonfat milk. Lactic broth (3) without Tween 80 and with lactose as the sole carbohydrate served as the growth medium for the routine assay of  $\beta$ -Pgal. The survey of organisms and the determination of opti-

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mal assay conditions were carried out in this broth. For induction experiments, lactic broth was prepared with a reduced level of yeast extract (1 g/liter) and without carbohydrate. After sterilization of the medium, a filter-sterilized solution of the carbohydrate to be tested was added to a final concentration ranging from 0.001 to 0.005 g/ml. In all experiments involving broth, the organism was transferred three times over 36 h at 32 C in the appropriate broth before the experiment was started.

In the studies of acid production and proteolysis, the cells were grown in 11% nonfat milk which had been steamed for 30 min and cooled. Additives, when present, were added to the milk, before steaming, to a final concentration of 0.25%. The following additives were used: Stimilac (enzymatic digest of pancreas), Marschall Dairy Laboratory Inc., Madison, Wis.; and N-Z Amine type A (enzymatic digest of casein) and Edamine type S (enzymatic digest of lactalbumin), both obtained from Sheffield Chemical Co., Norwich, N.Y.

**Buffer solutions.** Cells were regularly washed, suspended, and assayed in 0.05 M sodium phosphate buffer at pH 7.0.

**Preparation of cell-free extracts.** An 18- to 24-h culture was used to inoculate the appropriate broth to the 1% level. Cells were grown for 6 to 7 h at 32 C, at which time they were centrifuged at  $3,000 \times g$  for 10 min at 2 C. The pellet was washed twice with the cold phosphate buffer and finally resuspended in 30 ml of buffer. This cell suspension was treated in a Raytheon sonic oscillator cell for 20 min at maximal settings. This mixture was then centrifuged at  $17,500 \times g$  for 15 min at 2 C. The clear supernatant fluid, which contained from 0.5 to 1.8 mg of total protein per ml, was assayed for enzyme activity.

**Preparation of toluene-acetone-treated cells.** Toluene-acetone (1:9)-treated cells were normally used when small numbers of cells were being assayed for enzyme activity. Cells were harvested, washed as above, and resuspended to a known optical density (420 nm); amounts of cells were expressed in terms of milligrams of cell dry weight. This insured that a standard amount of cells would be solvent-treated throughout the experiment. The procedure of Citti et al. (1) was followed when the cells were treated with toluene-acetone, but the cells were shaken vigorously for 10 min, instead of 5 min, after addition of the solvent. The resultant suspension was assayed for enzyme activity.

**$\beta$ -Galactosidase and  $\beta$ -phosphogalactosidase assays.** ONPG and ONPG-6-P were obtained from Sigma Chemical Co. A modification of the procedures for enzyme assays used by Citti et al. (1) and McKay et al. (18) was used. A solution containing either  $5.0 \times 10^{-3}$  M ONPG or ONPG-6-P was prepared in the phosphate buffer. The assay mixture contained 0.5 ml of either the cell-free extract or toluene-acetone-treated cells and 2.0 ml of the chromogenic substrate. Incubation proceeded at 37 C for 1 to 2 min in the case of the cell-free assay and for 10 min in the case of solvent-treated cells. The reaction was stopped by the addition of 2.5 ml of 0.5 M sodium carbonate. The release of *o*-nitrophenol (ONP) was measured colorimetrically at 420 nm and

milligrams of ONP released was determined from a standard curve. When the toluene-acetone assay was used, cells were removed by centrifugation before absorbancy was measured.

One unit of enzyme was equivalent to 1  $\mu$ mol of ONP liberated from ONPG or ONPG-6-P per min. Specific activity was expressed as units per milligram of protein (15) in the case of cell-free extracts and as units/0.1 mg of cell dry weight in the case of solvent-treated cells.

#### **Procedures for studies of cells grown in milk.**

A 1% inoculum from 18- to 24-h milk culture was grown in 200 ml of 11% nonfat milk for 13 h at 32 C. The milk had been previously steamed for 30 min. Cells were then harvested by the method described by Stadhouders et al. (20). The pellet was washed three times with the phosphate buffer and assayed for  $\beta$ -Pgal activity by the cell-free extract method described above. Titratable acidity was determined by titrating a 50-ml sample of the 13-h culture to the phenolphthalein end point (pH 8.3) by use of a Fisher Automatic Titrimeter. Results were expressed as percent titratable acidity. The method of Hull (10) was used to determine the degree of proteolysis that had occurred in the 13-h culture. Results were expressed as milligrams of tyrosine per milliliter of milk. *S. lactis* C2 was a rapid lactic acid producer, producing greater than 0.7% titratable acidity after 13 h in milk at 32 C; *S. cremoris* 175 was a slow acid producer, yielding less than 0.2% acid under the same conditions.

**Induction of  $\beta$ -Pgal.** The broth with the reduced level of yeast extract (1 g/liter), containing the filter-sterilized carbohydrate, was used for examination of the inducing ability of a number of carbohydrates. Glucose, mannose, lactose, and galactose were examined along with methyl- $\beta$ -D-thiogalactopyranoside (TMG), isopropyl- $\beta$ -D-thiogalactopyranoside (IPTG), and ONPG. The procedure of Citti et al. (1) was used with the following modifications. Cells of *S. cremoris* HP were grown for either 3 or 12 h in 300 ml of the low-level (1 g/liter) yeast extract broth containing 0.002 g of mannose/ml. At the appropriate time, cells were harvested, washed once, and resuspended in 4 to 6 ml of the sterile phosphate buffer. This suspension served as inoculum for a series of flasks containing 50 ml of the low-level yeast extract broth with the appropriate carbohydrate to be tested. Flasks were incubated for 5 h at 32 C, with 10-ml samples being removed at hourly intervals and prepared for the toluene-acetone enzyme assay as previously described. The point at which the cells were harvested and shifted to the 50 ml of broth was designated as time zero. In some experiments, cells grown for either 3 or 12 h in 300 ml of broth containing 0.002 g of lactose or galactose per ml served as the washed and resuspended inoculum of time zero.

## **RESULTS**

### **Enzyme activity of lactose-grown cells.**

The specific activity of both  $\beta$ -Pgal and  $\beta$ -gal in cell-free extracts and in toluene-acetone-

treated cells is given in Table 1. The only lactic strain in which  $\beta$ -gal activity was detected was *S. lactis* 7962. This organism also exhibited a low level of  $\beta$ -Pgal activity, which could be the result of a low level of ONPG in the ONPG-6-PO<sub>4</sub>. The rest of the organisms examined contained various levels of  $\beta$ -Pgal, but essentially no  $\beta$ -gal could be detected by the assays used.

**Optimal enzyme assay conditions.** Toluene-acetone-treated cells of a 7-h-old culture of *S. cremoris* HP were used to determine optimal temperature and pH conditions for the assay of  $\beta$ -Pgal. Figure 1 shows the effect on enzyme activity of different assay incubation temperatures. Specific activity of  $\beta$ -Pgal increased with increasing temperature up to about 37 C and decreased sharply at temperatures above 37 C. Figure 2 shows the response of enzyme activity to the pH of the assay solution. Optimal pH was about 7.0 with a sharp decrease in activity above and below pH 7.0.

**Enzyme activity of cells grown in milk.**  $\beta$ -Pgal activity, proteolysis, and titratable acidity were examined in a fast lactic acid producer, *S. lactis* C2, and a slow lactic acid producer, *S. cremoris* 175 (Table 2). In the case of *S. lactis* C2, the amount of lactic acid produced and the specific activity of  $\beta$ -Pgal in plain milk did not change significantly when

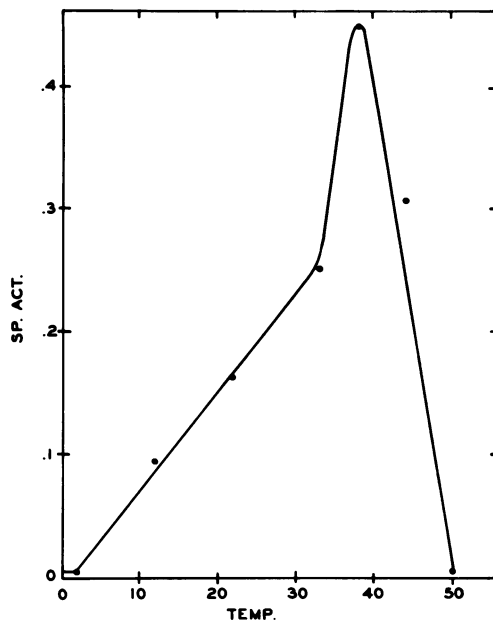


FIG. 1. Effect of temperature on specific activity (units/0.1 mg of cell dry weight) of  $\beta$ -D-phosphogalactoside galactohydrolase in toluene-acetone (1:9)-treated cells of *Streptococcus cremoris* HP.

TABLE 1.  $\beta$ -Galactosidase and  $\beta$ -D-phosphogalactoside galactohydrolase activities found in lactic streptococci as measured with the substrates ONPG and ONPG-6-P, respectively

Organism	Specific activity			
	Cell-free <sup>a</sup>		Solvent-treated cells <sup>b</sup>	
	ONPG-6-P	ONPG	ONPG-6-P	ONPG
<i>Streptococcus lactis</i>				
C 2 .....	367	<1.0	300	<1.0
UN .....	563	<1.0	—	—
7962 .....	14	259	132	216
<i>S. cremoris</i>				
HP .....	263	<1.0	450	<1.0
163 .....	256	<1.0	—	—
459 .....	242	<1.0	—	—
<i>S. diacetylactis</i>				
18-16 .....	120	<1.0	443	<1.0
DRC-1 .....	151	<1.0	—	—
3D-1 .....	135	<1.0	—	—

<sup>a</sup> Specific activity of cell-free extracts is expressed as units per milligram of protein. All values should be multiplied by 10<sup>3</sup>.

<sup>b</sup> Specific activity of solvent-treated cells is expressed as units per 0.1 mg of cell dry weight. All values should be multiplied by 10<sup>3</sup>.

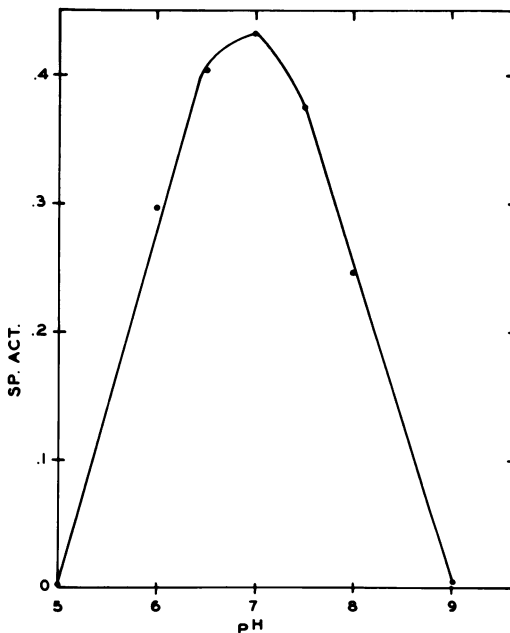


FIG. 2. Effect of pH on specific activity (units/0.1 mg of cell dry weight) of  $\beta$ -D-phosphogalactoside galactohydrolase in toluene-acetone (1:9)-treated cells of *Streptococcus cremoris* HP, suspended in 0.05 M sodium phosphate buffer at the appropriate pH.

protein hydrolysates were added to the milk. There also was a considerable amount of tyrosine liberated under all conditions, as a result of the proteolytic activity of the culture. The slow lactic acid producer *S. cremoris* 175 exhibited a different pattern. In plain milk, very little acid was produced. Upon addition of protein hydrolysates, however, the amount of acid produced was increased approximately fourfold.  $\beta$ -Pgal activity remained about the same whether or not protein hydrolysates were present. The amount of free tyrosine remained low in all cases.

**Enzyme induction.** Figure 3 plots the response of  $\beta$ -Pgal when a 3-h-old culture of *S. cremoris* HP was shifted at time zero to broth containing glucose, mannose, lactose, or galactose (0.002 g/ml). Doubling time of the organism was 60 min in glucose, mannose, and lactose broth, and 120 min in galactose broth.

The initial rate of  $\beta$ -Pgal synthesis was maximal in cells shifted to lactose. The highest level of enzyme activity reached, however, was not maintained but decreased with time. The rate of  $\beta$ -Pgal induction of galactose-grown cells lagged behind that of lactose-grown cells but eventually reached approximately the same level. The maximal level of enzyme attained in galactose-grown cells also started to decline with the 6-h sample (not shown on Fig. 3). Cells shifted to either mannose or glucose showed no ability to induce enzyme and gave similar curves.

The relatively high level of enzyme activity of the 3-h-old mannose-grown cells when shifted at time zero was thought possibly to be interfering with the true nature of  $\beta$ -Pgal induction during the first few hours after shift. Since this basal level did decrease with time, in cells shifted to glucose or mannose, it was

TABLE 2. Effect of added protein hydrolysate products (0.25%) on acid production, proteolysis, and  $\beta$ -D-phosphogalactoside galactohydrolase ( $\beta$ -Pgal) activity in a fast (*S. lactis* C2) and a slow (*S. cremoris* 175) lactic streptococcus

Medium	Titratable acid (net %)		Tyrosine (net $\mu$ g/ml)		$\beta$ -Pgal (specific activity)	
	<i>S. lactis</i> C2	<i>S. cremoris</i> 175	<i>S. lactis</i> C2	<i>S. cremoris</i> 175	<i>S. lactis</i> C2	<i>S. cremoris</i> 175
Nonfat milk (NFM), 11% .....	0.74	0.18	28	0.0	$68 \times 10^3$	$93 \times 10^3$
NFM + Stimilac .....	0.78	0.65	28	6.0	$71 \times 10^3$	$85 \times 10^3$
NFM + NZ Amine .....	0.74	0.72	62	12.0	$65 \times 10^3$	$81 \times 10^3$
NFM + Edamin .....	0.74	0.61	32	0.0	$65 \times 10^3$	$85 \times 10^3$

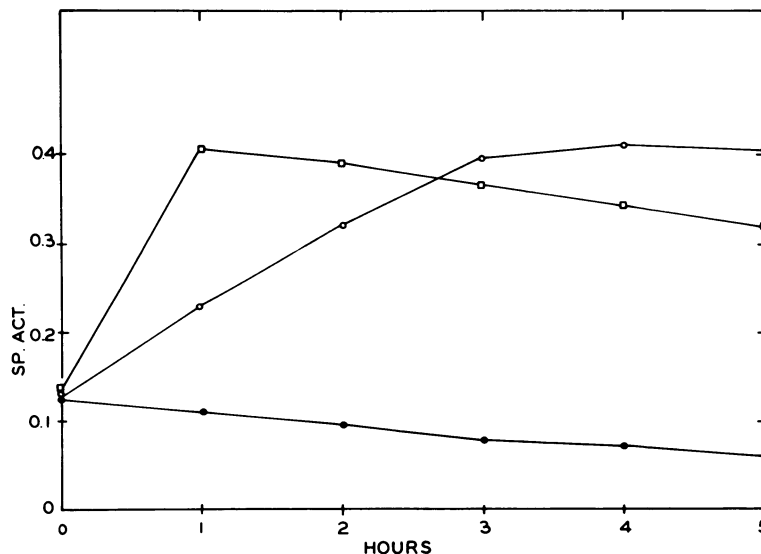


FIG. 3. Effect of shifting 3-h-old mannose-grown cells to glucose or mannose (●), galactose (○), or lactose (□) on the specific activity (units/0.1 mg of cell dry weight) of  $\beta$ -D-phosphogalactoside galactohydrolase in *Streptococcus cremoris* HP.

decided to follow induction in cells shifted at 12-h instead of 3-h.

Figure 4 plots the response of  $\beta$ -Pgal levels of 12-h-old cells shifted at time zero from mannose to glucose, mannose, lactose, and galactose. The initial level at time zero of  $\beta$ -Pgal was observed to be significantly lower than that observed in the 3-h shift cells. Again, lactose induced at a maximal rate and the highest level of enzyme reached was not maintained. Induction of  $\beta$ -Pgal, when cells were shifted to galactose, could not be distinguished from an increase that also occurred in cells shifted to either mannose or glucose during the first 2 h of sampling. This increase was reflected in the relatively high initial (time zero) enzyme level found in Fig. 3. The maximal level of enzyme obtained with the galactose-grown cells again decreased at times greater than 6 h. The initial increase in enzyme level exhibited by cells grown on sugars presumed to be noninducers was also observed when 12-h mannose-grown cells were shifted to broth containing no carbohydrate, TMG, IPTG, or ONPG, although no cell growth was observed. This type of response was taken to be the normal basal levels of  $\beta$ -Pgal in cells grown in a noninducing medium.

Figure 5 shows the influence on  $\beta$ -Pgal levels in 12-h cells shifted from galactose to lactose or galactose. The pattern of enzyme induction was the same whether the growth carbon source, before shift, was lactose or galactose.

Cells shifted to lactose started to induce  $\beta$ -Pgal with little, if any, lag period. Cells shifted to galactose did not show any increase in enzyme levels during the first 2 h after shift. The maximal level of enzyme found in the cells grown in noninducing carbohydrates was never observed to be higher than the lowest level of enzyme found when the cells were grown in lactose or galactose. Enzyme levels of cells grown on lactose or galactose fluctuated between specific activities ranging from 0.16 to 0.40 over a 12-h period, and specific activity of  $\beta$ -Pgal in glucose- or mannose-grown cells ranged from 0.01 to 0.12 over the same period (Fig. 5).

The rate of induction of  $\beta$ -Pgal when 12-h mannose-grown cells were shifted to lactose was independent of the concentration of the sugar (0.002 to 0.005 g/ml) and yielded essentially the same curve as shown in Fig. 6. Increasing concentrations of galactose over the same range, while not changing the curve over the first 2 h after shift, increased the rate of enzyme induction and the maximal level of enzyme during the next few hours of growth (Fig. 6).

Figure 7 plots the response of  $\beta$ -Pgal levels when 12-h mannose-grown cells were shifted into broth containing galactose or lactose (0.005 g/ml) and also containing 0.001 g of glucose/ml. Under these conditions, the inducing ability of galactose was almost totally inhibited, whereas the inducing ability of lac-

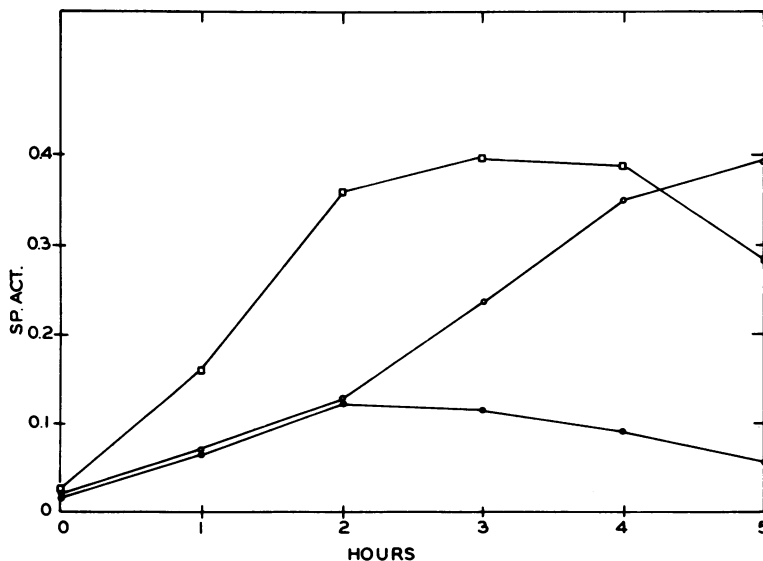


FIG. 4. Effect of shifting 12-h-old mannose-grown cells to 0.002 g of glucose or mannose per ml (●), 0.002 g of galactose per ml (○), or 0.002 g of lactose per ml (□) on the specific activity (units/0.1 mg of cell dry weight) of  $\beta$ -D-phosphogalactoside galactohydrolase in *Streptococcus cremoris* HP.

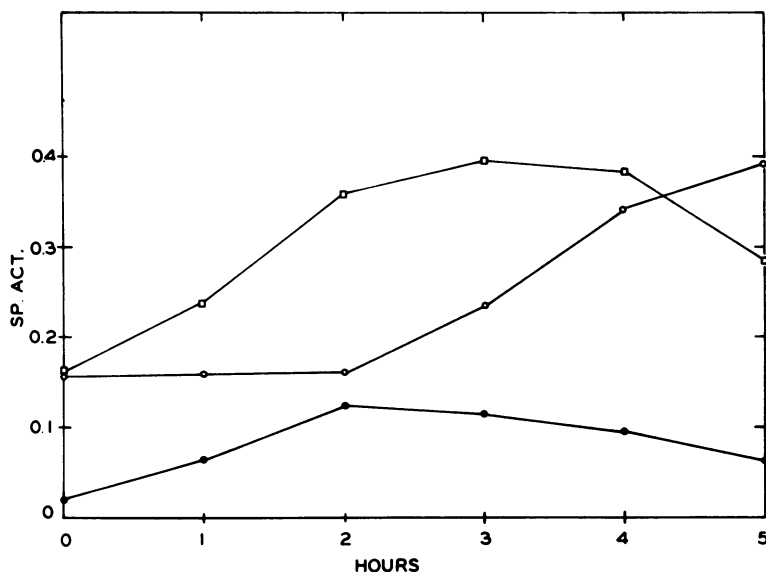


FIG. 5. Effect of shifting 12-h-old galactose-grown cells to 0.002 g of galactose per ml (○) or 0.002 g of lactose per ml (□) on the specific activity (units/0.1 mg of cell dry weight) of  $\beta$ -D-phosphogalactoside galactohydrolase in *Streptococcus cremoris* HP. The control shift curve (mannose to glucose or mannose, ●) also is shown.

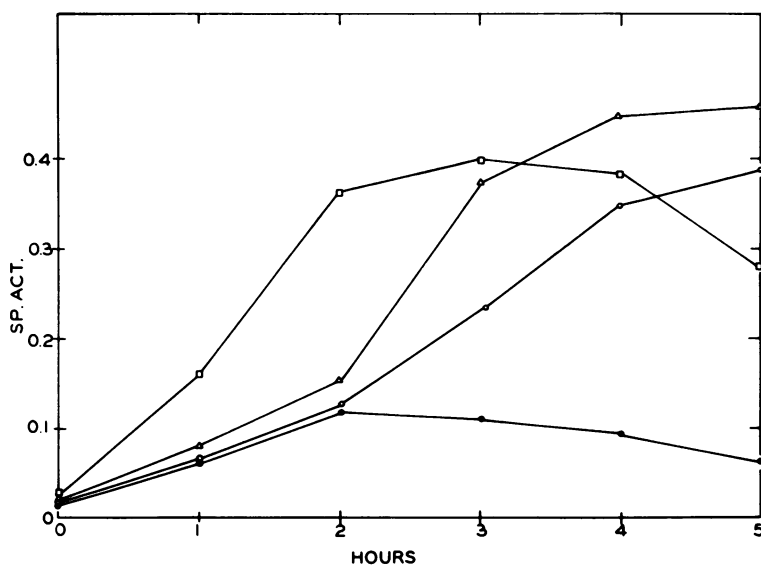


FIG. 6. Effect of shifting 12-h-old mannose-grown cells to mannose or glucose (●), 0.002 g of galactose per ml (○), 0.005 g of galactose per ml (Δ), or 0.002 to 0.005 g of lactose per ml (□) on the specific activity (units/0.1 mg of cell dry weight) of  $\beta$ -D-phosphogalactoside galactohydrolase in *Streptococcus cremoris* HP.

tose was only partially inhibited when compared with the normal induction curves shown in Fig. 4.

#### DISCUSSION

The enzyme  $\beta$ -gal could be demonstrated in only one organism, *S. lactis* 7962 (Table 1); the

other lactic streptococci examined contained various amounts of  $\beta$ -Pgal activity and essentially no  $\beta$ -gal could be detected. These results are consistent with a similar survey undertaken by McKay et al. (16). In the course of the present study, we also found that whole cells of *S. cremoris* HP suspended in phosphate buffer

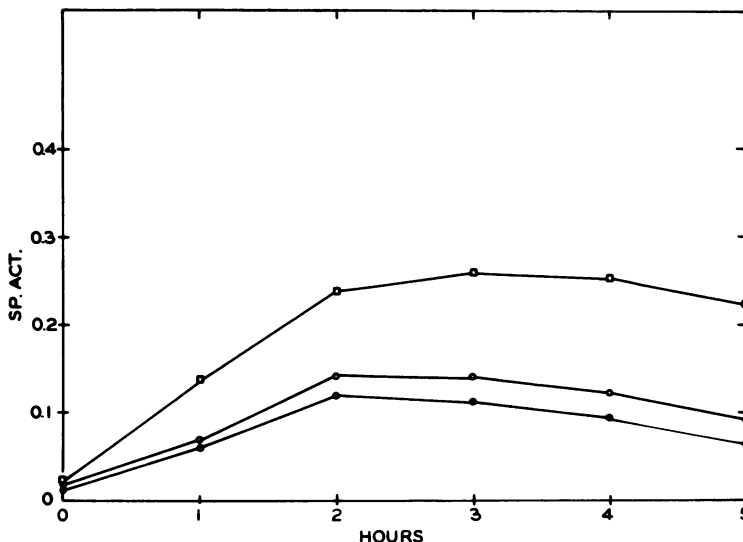


FIG. 7. Effect of shifting 12-h-old mannose-grown cells to mannose or glucose (●), 0.001 g of glucose and 0.005 g of galactose per ml (○), or 0.001 g of glucose and 0.005 g of lactose per ml (□) on the specific activity (units/0.1 mg of cell dry weight) of  $\beta$ -D-phosphogalactoside galactohydrolase in *Streptococcus cremoris* HP.

could not hydrolyze ONPG-6-P, but could slowly hydrolyze ONPG if incubated for 1 h or more. Whole cells treated with toluene-acetone were able to hydrolyze ONPG-6-P (10-min assay) but could no longer hydrolyze ONPG, even when the assay time was considerably extended. *S. lactis* UN exhibited a similar response (16). These results indicate that an untreated cell still has a phosphotransferase system capable of slowly phosphorylating ONPG, thus making it available for hydrolysis by  $\beta$ -Pgal. Intact whole-cell membranes, however, are impermeable to ONPG-6-P. Treatment with toluene-acetone apparently results in a loss of a functional phosphotransferase system and thus loss of the ability to hydrolyze ONPG, but at the same time yields a membrane that is no longer impermeable to ONPG-6-P and hydrolysis of this compound is possible.

A rapid rate of lactic acid production from lactic streptococci is important in many industrial fermentations. It has been shown that slow acid production can be the result of a loss of proteolytic ability of the cells (4). Since the peptide and amino acid content of fresh milk is low (2), an organism with impaired proteolytic ability would be at a disadvantage. The addition of certain protein hydrolysates to milk greatly increases the ability of slow lactic acid producers to produce acid despite their poor proteolytic ability (12). We were interested in determining whether slow lactic acid-producing cells with reduced proteolytic ability also

had decreased levels of  $\beta$ -Pgal and whether or not the addition of protein hydrolysates would affect enzyme level.

Our results indicate that the rate of lactic acid production is independent of the level of  $\beta$ -Pgal present in the cell (Table 2). Although *S. cremoris* 175 when grown in plain 11% nonfat milk produced very little lactic acid over a 13-h period, addition of protein hydrolysates greatly increased the amount of acid produced. This increase was accomplished without significant change in the level of  $\beta$ -Pgal. It appears, therefore, that impaired protein synthesis which may result from an inability of the cell to hydrolyze the milk protein efficiently affects  $\beta$ -Pgal synthesis.

Lactose was the best inducer of  $\beta$ -Pgal over the range of concentrations tested. Galactose-grown cells, although eventually reaching the same maximal level of  $\beta$ -Pgal as lactose-grown cells, exhibited a slower rate of induction, especially during the initial hours after shifting.  $\beta$ -Pgal activity of cells grown in sugars normally considered noninducers showed some degree of fluctuation. The characteristic low specific activity of 12-h-old cells grown in mannose or glucose showed an increase during the first few hours after shifting into fresh medium containing the same sugar. Although cells carried on lactose or galactose showed considerable fluctuation in enzyme levels also, the level of  $\beta$ -Pgal in these cells was always found to be higher than the level in glucose- or mannose-grown cells. Cells shifted at 12 h from

mannose broth to broth containing both galactose (0.005 g/ml) and glucose (0.001 g/ml) gave only a basal level of enzyme typical of glucose- or mannose-grown cells (Fig. 7). The inducing action of galactose was prevented by trace amounts of glucose. Lactose induction was only partially inhibited by the presence of glucose under similar conditions. This would indicate that lactose is an ideal combination of a rapidly metabolizable sugar (glucose) and also a form of the actual inducer (galactose), since a mixture of the free sugars does not show any inducing ability. When galactose served as the sole carbohydrate, *S. cremoris* HP grew very slowly, indicating that the rate of  $\beta$ -Pgal induction, which is also slow in broth containing only galactose, may be a direct reflection of the organism's ability to metabolize the inducer.

The basal level of enzyme in the presence of noninducing sugars and the nature of the fluctuations found may indicate a kind of repression-derepression of basal  $\beta$ -Pgal always present in the cell and not an actual synthesis of new enzyme. This is indicated by the fact that this basal increase occurs even when 12-h mannose-grown cells are shifted to starvation conditions and actual cell growth does not occur. Also, trace amounts of glucose, in the presence of galactose, stop production of  $\beta$ -Pgal above the characteristic basal level.

Current work is being directed into the nature of the control of synthesis of  $\beta$ -Pgal. The reason for the slight increase in  $\beta$ -Pgal levels during growth in mannose or glucose, whether actual synthesis or activation of already existing enzyme, is not known. If the enzyme exists in both an active and inactive form, it should be possible to examine the mechanisms involved in this process.

#### ACKNOWLEDGMENTS

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