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Fructose 1,6-Diphosphate-Activated L-Lactate Dehydrogenase from *Streptococcus lactis*: Kinetic Properties and Factors Affecting Activation

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The L-(+)-lactate dehydrogenase (L-lactate:NAD$^+$ oxidoreductase, EC 1.1.1.27) of *Streptococcus lactis* C10, like that of other streptococci, was activated by fructose 1,6-diphosphate (FDP). The enzyme showed some activity in the absence of FDP, with a pH optimum of 8.2; FDP decreased the K_m for both pyruvate and reduced nicotinamide adenine dinucleotide (NADH) and shifted the pH optimum to 6.9. Enzyme activity showed a hyperbolic response to both NADH and pyruvate in all the buffers tried except phosphate buffer, in which the response to increasing NADH was sigmoidal. The FDP concentration required for half-maximal velocity ($FDP_{0.5V}$) was markedly influenced by the nature of the assay buffer used. Thus the $FDP_{0.5V}$ was 0.002 mM in 90 mM triethanolamine buffer, 0.2 mM in 90 mM tris(hydroxymethyl)aminomethane-maleate buffer, and 4.4 mM in 90 mM phosphate buffer. Phosphate inhibition of FDP binding is not a general property of streptococcal lactate dehydrogenase, since the $FDP_{0.5V}$ value for *S. faecalis* 8043 lactate dehydrogenase was not increased by phosphate. The *S. faecalis* and *S. lactis* lactate dehydrogenases also differed in that Mn^{2+} enhanced FDP binding in *S. faecalis* but had no effect on the *S. lactis* dehydrogenase. The FDP concentration (12 to 15 mM) found in *S. lactis* cells during logarithmic growth on a high-carbohydrate (3% lactose) medium would be adequate to give almost complete activation of the lactate dehydrogenase even if the high $FDP_{0.5V}$ value found in 90 mM phosphate were similar to the FDP requirement in vivo.

The L-lactate dehydrogenase of streptococci was shown by Wolin (23) to be activated by fructose 1,6-diphosphate (FDP). The lactate dehydrogenases from three *Streptococcus* species, *S. faecalis* (22), *S. cremoris* (11, 12), and *S. mutans* (2), have subsequently been purified and studied in some detail. The enzymes from these three species appear to differ considerably from each other in respect to a number of kinetic properties. In particular, the reported concentration of FDP required to give half-maximal velocity ($FDP_{0.5V}$) ranges from about 1 to 2 μM for *S. cremoris* lactate dehydrogenase (12) to 5 mM for the *S. mutans* lactate dehydrogenase (2), whereas the *S. faecalis* enzyme (22) has an intermediate $FDP_{0.5V}$ value (50 μM). The effect of FDP on the kinetic parameters V_{max} and K_m was also found to be different for the lactate dehydrogenases from the three streptococcal species. These differences may, in part, be due to the use of different assay conditions, particularly the buffer composition. The kinetic and stability properties of the *S. cremoris* lactate dehydrogenase were affected by phosphate (12), and the $FDP_{0.5V}$ value for the *S. mutans* JC2 lactate dehydrogenase was found to be lower in 0.3 mM phosphate than in 67 mM phosphate (24). The fact that phosphate buffer was used in some of the earlier studies (2, 22) of the streptococcal lactate dehydrogenases may account for some of the differences reported to exist between the different enzymes.

The present paper reports a more detailed comparison of the FDP activation of the *S. lactis* C10 lactate dehydrogenase in phosphate and other buffers and also examines other factors that could influence the activity of the enzyme in vivo.

MATERIALS AND METHODS

Organisms. *S. lactis* C10 (strain 509, National Collection of Dairy Organisms, Reading, England) was obtained from the Dairy Research Institute, Palmerston North, New Zealand. *S. faecalis* ATCC 8043, used in some comparative studies, was obtained from the Department of Microbiology and

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Reagents. Substrates, substrate analogues, and FDP were obtained from Sigma Chemical Co., St. Louis, Mo., with the exception of sodium pyruvate, which was obtained from Fluka AG, Buchs, Switzerland. Aldolase and α-glycerophosphate dehydrogenase plus triosephosphate isomerase were also obtained from Sigma Chemical Co.

The diethylaminoethyl (DEAE)-Protion used in enzyme purification is an anion-exchange cellulose developed by Tasman Vaccine Laboratories Ltd., New Zealand. It differs from the conventional DEAE-cellulose in having a viscose-regenerated cellulose base, which enables a faster flow rate to be obtained.

The reagents 3,3'-diaminodipropylamine and 1-cyclohexyl-3-(2-morpholinoethyl)-carbodiimide metho-*p*-toluene sulfonate, used for preparing the affinity chromatography resin, were supplied by Chemical Procurement Laboratories Inc. and Aldrich Chemical Co. Inc., respectively.

Lactate dehydrogenase assay. Lactate dehydrogenase was estimated by measuring the rate of reduced nicotinamide adenine dinucleotide (NADH) oxidation at 340 nm. The standard assay mixture contained (in a total volume of 3 ml): 90 mM tris(hydroxymethyl)aminomethane (Tris)-maleate-hydrochloride buffer, pH 6.9; 0.167 mM NADH; 10 mM sodium pyruvate; 1 mM FDP; and 0.1 ml of diluted enzyme. The enzyme was diluted in cold 5 mM phosphate buffer, pH 7.0.

One unit of enzyme activity is defined as a rate of NADH oxidation of 1 μmol per min. Routine assays during enzyme purification were carried out at room temperature, using a Unicam SP 800 spectrophotometer. Kinetic studies were carried out at 25°C, using a Beckman ACTA-3 spectrophotometer.

Protein was determined by the method of Lowry et al. (16).

Preparation of affinity chromatography resin. Bio-Gel A-15m was activated by treatment with cyanogen bromide (250 mg/ml of packed resin) and subsequently reacted with 3,3'-diaminodipropylamine according to the procedure of Cuatrecacas (6). The terminal amino group was then condensed with oxalate via an amide bond to give an oxamate derivative by the following procedure. To each 20 ml of packed resin, 14 ml of 0.7 M oxalic acid was added, and the pH was adjusted to 4.7. A 4-ml amount of a solution containing 0.63 g of 1-cyclohexyl-3-(2-morpholinoethyl)-carbodiimide metho-*p*-toluene sulfonate was added dropwise, the mixture being stirred continuously while the pH was maintained at 4.7. The mixture was then stirred gently at room temperature for 24 h and finally washed with 5 liters of distilled water.

Purification of lactate dehydrogenase from *S. lactis* C10. Cultures were grown at 30°C in 20 liters of the medium described by Jago et al. (11) in a New Brunswick 50-liter Fermacell fermentor. The culture was stirred at an impeller speed of 150 rpm without forced aeration. Thus it would have been anaerobic once the oxygen in the head space was consumed. The pH was maintained between 6.0 and 6.5 by addition of 2.5 M sodium hydroxide during fermentation. Cells were harvested near the end of the logarithmic phase. All subsequent purification steps were carried out at 4°C. A 300-g (wet packed weight) amount of cells was washed three times in 0.005 M phosphate buffer (pH 7.0) and disrupted in the same buffer by two passages through an Aminco French pressure cell at 5,500 lb/in². Cell debris was centrifuged at 13,000 × *g* for 15 min, and the cell-free extract (1,300 ml) was dialyzed against the same phosphate buffer for 15 h. Nucleic acids were precipitated from the dialyzed cell-free extract by dropwise addition of streptomycin sulfate, using 1.75 ml of a 10% (wt/vol) solution for every 100 mg of protein. The resulting suspension was allowed to stand for 4 h, and the precipitate was removed by centrifugation at 13,000 × *g* for 15 min. The supernatant (1,815 ml) was dialyzed against 0.005 M phosphate buffer (pH 7.0) for 15 h. Solid ammonium sulfate was then added to bring the solution to 50% saturation, the precipitate was removed by centrifugation, and the concentration of ammonium sulfate was increased to 65% saturation. After 3 h the precipitate was collected by centrifugation, redissolved in 300 ml of 0.025 M citrate buffer (pH 6.1), and then dialyzed against the same buffer for 24 h.

The dialyzed sample (310 ml) was applied to a DEAE-Protion column (40 by 6 cm) pre-equilibrated with 0.025 M citrate buffer (pH 6.1). The column was eluted with the same buffer until the absorbance of the eluate at 280 nm had dropped to zero. The lactate dehydrogenase was eluted with 0.05 M citrate buffer, and all fractions containing the enzyme at a specific activity greater than 150 U per mg of protein were pooled. Ammonium sulfate was added to bring the concentration to 70% saturation, and the precipitate was centrifuged, redissolved in 60 ml of 0.05 M phosphate buffer (pH 6.7), and dialyzed against the same buffer for 24 h.

Portions (10 ml) of the dialyzed sample were loaded onto a column (20 by 1 cm) of oxamate affinity resin prepared as described above. The use of the oxamate column was based on the work of O'Carra and Barry (18), who used such a column to purify pig heart lactate dehydrogenase. The column was pre-equilibrated with 0.05 M phosphate buffer (pH 6.7). One hour after loading, the column was eluted with 0.1 M phosphate buffer (pH 6.7), which removed a considerable amount of contaminant protein while leaving the lactate dehydrogenase bound to the resin. Activity was removed from the resin by successive elution with 20 ml of 0.15 M and 50 ml of 0.2 M phosphate buffer (pH 6.7). Since binding of the *S. lactis* lactate dehydrogenase did not require the presence of NADH in the loading buffer and removal of the enzyme was achieved simply by increasing the ionic strength of the eluting buffer, the binding was possibly not due to biospecific interaction at the substrate site. However, we have observed that oxamate (but not pyruvate) gives considerable protection against heat inactivation of the enzyme in the absence of NADH. Therefore the enzyme can apparently bind oxamate without NADH being present. This may account for the considerable increase in specific activity achieved by the affinity chromatography procedure used here. High-specific-activity

lactate dehydrogenase fractions were pooled and concentrated by ultrafiltration. The concentrated solution was finally dialyzed against 0.01 M phosphate buffer (pH 7.0) and frozen in small volumes. The frozen enzyme could be stored for several months without appreciable loss of activity or change in properties.

The above procedure purified the lactate dehydrogenase by about 100-fold. The results of the purification are shown in Table 1. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (21) of the enzyme purified by this procedure, followed by staining with Coomassie blue, showed that it was not homogeneous. In addition to an intensely staining band (corresponding to a subunit molecular weight of 36,000, in agreement with that obtained [8] for *S. cremoris* lactate dehydrogenase), four weakly staining bands were also present.

Partial purification of lactate dehydrogenase from *S. faecalis* ATCC 8043. Cultures of *S. faecalis* were grown in 1-liter Erlenmeyer flasks at 30°C without forced aeration. The medium of Jago et al. (11) was used except that glucose at a concentration of 15 g/liter replaced lactose. Cells were harvested after 18 h and treated in the same way as described for *S. lactis* C10 to the ammonium sulfate fractionation stage. The enzyme was precipitated in the ammonium sulfate fraction between 50 and 72% saturation. The precipitate was dissolved in 0.01 M phosphate buffer (pH 7.0) and dialyzed against the same buffer for 24 h. This fraction (specific activity = 26 U/mg of protein) was used as the enzyme solution in the studies described here.

Estimation of cellular FDP content. The procedure used was essentially that of Collins and Thomas (4). Samples (25 ml) of *S. lactis* were withdrawn from exponentially growing cultures (using the media and conditions described for enzyme purification). Cells were collected on 0.8-μm membrane filters (type AA; Millipore Corp.) and transferred within 1 min of harvesting onto a stainless-steel block partially immersed in liquid nitrogen. The FDP was extracted with 0.6 M perchloric acid and estimated fluorometrically from the amount of NADH produced in the presence of aldolase, triosephosphate isomerase, glycerol 3-phosphate dehydrogenase, and NAD^+ according to the procedure of Lowry et al. (15). A Turner 430 spectrofluorometer was used set at an excitation wavelength of 350 nm and an emission wavelength of 468 nm. Imidazole (Sigma grade III with low fluorescence) was used as the buffer, and fluorometric blanks were carried out on all components of the reaction mixture. Some NADH was produced in the absence of aldolase, presumably due to the presence of triosephosphates in the extract. The amount of NADH produced in the absence of aldolase was subtracted from the total NADH produced in the presence of aldolase before calculating the FDP content of the extracts.

Intracellular volume was determined from the dry weights of duplicate portions taken at the same time as the samples for FDP estimation. Intracellular volume was determined by J. Thompson of the New Zealand Dairy Research Institute. The determination was made by subtracting the volume, in a given weight of cells, accessible to [^{14}C]sucrose (which penetrates the wall but not the protoplast) from the total water volume in the same weight of cells. Sucrose is not metabolized by *S. lactis* C10. The procedures used for determining the sucrose space and the total water volume have been described previously (1, 3). Determination of the intracellular volume for *S. lactis* C10 has shown that 1 g (dry weight) of cells has an intracellular volume of 1.6 ml. A similar value (1.5 ml/g [dry weight]) has been obtained for *S. lactis* 7962 (13), whereas a slightly lower value (1.3 ml/g) has been obtained for *S. faecalis* (9).

RESULTS

Effect of pH on enzyme activity. The effect of pH on the rate of pyruvate reduction, determined in 90 mM Tris-maleate buffer, is shown in Fig. 1A. In the absence of FDP, optimal activity was observed at pH 8.2 to 8.3. The addition of FDP to a 1- or 10-mM final concentration resulted in marked activation of enzyme and a shift in the pH optimum to 6.9 to 7.0. Virtually identical results were obtained in triethanolamine-hydrochloride, glycylglycine, and imidazole buffers. A similar pH dependence was found in phosphate buffer, except that at 1 mM FDP enzyme activity was only about one-tenth of that at 10 mM (Fig. 1B) and

TABLE 1. *Purification of L-lactate dehydrogenase from S. lactis*[a]

Treatment	Total activity (U)	Total protein (mg)	Sp act (U/mg of protein)	Purification (fold)	Yield (%)
Cell-free extract	4.5×10^5	32,400	13		100
Streptomycin sulfate	4.9×10^5	27,500	18	1.4	116
Ammonium sulfate (50 to 65% fraction)	4.0×10^5	7,300	55	4.3	92
DEAE-Protion (high-specific-activity fractions)	1.95×10^5	650	300	23.2	49
Oxamate affinity column	1.5×10^5	107	1400	109.0	33

[a] 300 g (wet packed weight) of *S. lactis* cells was used for the purification reported. Volumes at each step are given in Materials and Methods.

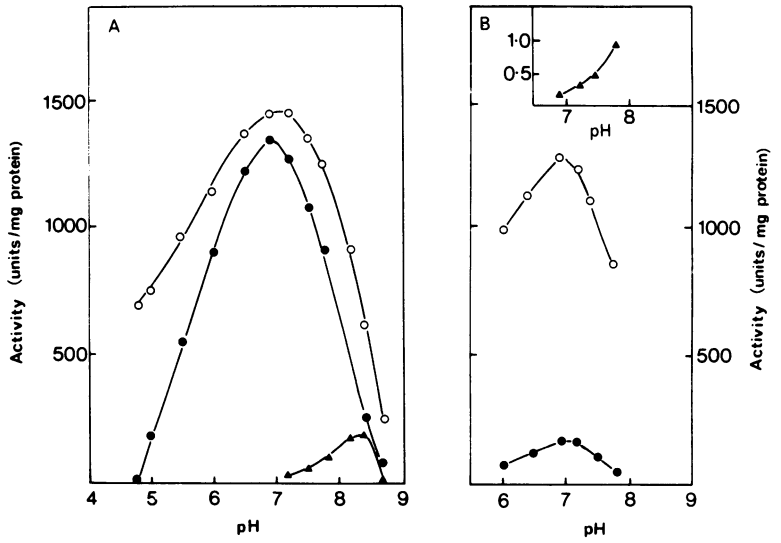

FIG. 1. *Effect of pH on the rate of pyruvate reduction in (A) Tris-maleate buffer and (B) phosphate buffer. The reaction mixture for each assay contained (in a total volume of 3 ml): 90 mM buffer; 10 mM pyruvate; 0.167 mM NADH; 0.1 ml of S. lactis lactate dehydrogenase containing between 0.23 and 2.3 μg of protein. FDP: (▲) zero; (●) 1 mM; (○) 10 mM. For Tris-maleate buffer, the various pH values were obtained by adjusting a stock Tris-maleate solution with HCl to the appropriate pH. The pH values shown are those measured in the complete assay mixture at 25°C.*

activity was only just detectable in the absence of FDP (Fig. 1B, inset).

To ensure that the changes in activity with pH were not a consequence of enzyme inactivation over the assay period, the stability of the enzyme at 25°C was examined at different pH values and in different buffers. Although the enzyme was unstable on prolonged incubation at pH values above 8, no instability over the 3-min assay period was detectable even at the extremes of the pH profile.

Determination of K_m values for pyruvate and NADH. K_m values for pyruvate and NADH were determined in 90 mM Tris-maleate buffer (pH 6.9) containing 0.5 mM FDP. Lineweaver-Burk plots with NADH as the variable substrate are shown in Fig. 2A and with pyruvate as the variable substrate in Fig. 2B. A family of intersecting straight lines was obtained, characteristic of an ordered reaction mechanism. Secondary plots of the data in Fig. 2 gave K_m values for pyruvate of 1.5 mM and for NADH of 0.08 mM.

The double-reciprocal plots for both substrates were quite linear over the range of concentrations used. There was no indication of the nonlinearity with respect to pyruvate as found with the lactate dehydrogenases from certain strains of *S. mutans* (2). However, at concentrations of pyruvate greater than 20 mM, activity became markedly inhibited.

Almost identical K_m values were obtained when the determinations were repeated (at the same FDP concentration) in triethanolamine buffer.

When the determinations were carried out in 90 mM phosphate buffer, the response to increasing NADH was sigmoidal (Fig. 3). A Hill plot of the data gave an interaction coefficient for NADH of 1.7. The $NADH_{0.5V}$ value was 0.14 mM, somewhat higher than the K_m value in the other two buffers. With varying pyruvate, the response was hyperbolic but the K_m value was increased to 5.7 mM. (A higher concentration of FDP [6.7 mM] was used for the determinations in phosphate buffer since 0.5 mM FDP would have been well below saturation in this buffer. Reducing the concentration of FDP further increased the K_m for pyruvate.)

K_m values for L-lactate and for NAD^+ were also determined in triethanolamine buffer (pH 7.9; the pH optimum for the reaction in the direction of lactate oxidation). In the presence of 1 mM FDP, the K_m value was 2.4 mM for NAD^+ and 100 mM for L-lactate. In the absence of FDP, the K_m values were increased to 8.0 mM for NAD^+ and to a very high value (approximately 500 mM) for lactate. These high K_m values, even in the presence of FDP, indicate that the enzyme is unlikely to catalyze lactate oxidation at an appreciable rate under physiological conditions. The enzyme was inactive toward D-lactate.

Effect of FDP on K_m values for NADH and

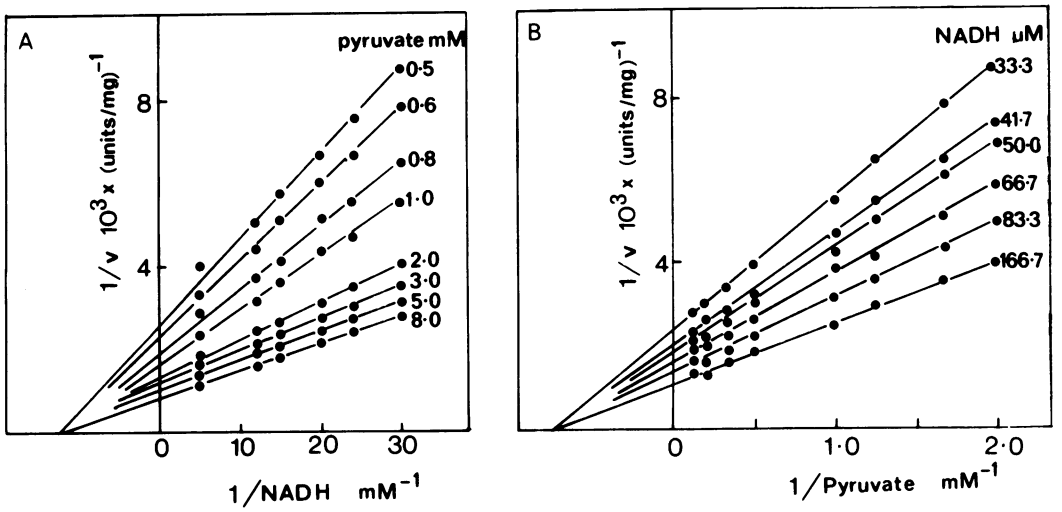

FIG. 2. *Lineweaver-Burk plots of (A) 1/v against 1/NADH at eight different pyruvate concentrations and (B) 1/v against 1/pyruvate at six different NADH concentrations. The reaction mixtures (total volume, 3 ml) contained 90 mM Tris-maleate buffer (pH 6.9), 0.5 mM FDP, and 0.1 ml of diluted S. lactis lactate dehydrogenase containing between 0.3 and 3.0 µg of protein.*

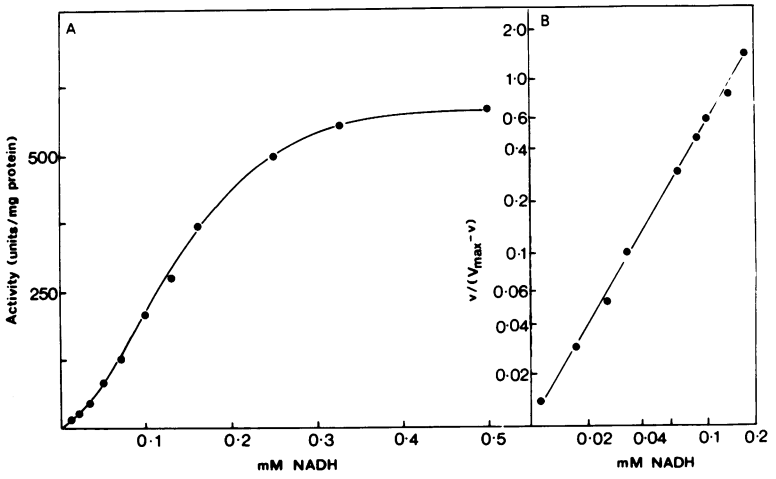

FIG. 3. *Effect of varying NADH concentrations on lactate dehydrogenase activity in phosphate buffer. The reaction mixture contained (total volume, 3 ml): 90 mM phosphate buffer (pH 6.9); 10 mM pyruvate; 6.7 mM FDP; and 0.1 ml of diluted S. lactis lactate dehydrogenase containing between 0.3 and 3.0 µg of protein. (A) Plot of activity against concentration. (B) The same data plotted as a Hill plot.*

pyruvate. The effect of FDP concentration on the K_m for NADH was determined in Tris-maleate buffer (pH 6.9) by varying the NADH concentration at each FDP concentration, with the pyruvate concentration fixed at 10 mM. A comparable set of determinations was carried out at a fixed NADH concentration (0.167 mM), varying the pyruvate concentration at each FDP concentration (Table 2). In neither case was the concentration of the fixed substrate fully saturating, but, since the K_m values for one substrate appear to be largely independent of the concentration of the other (see Fig. 2, where the Lineweaver-Burk plots intersect close to the 1/substrate axis), the apparent K_m determined at a single high concentration of the fixed substrate will be fairly close to the true K_m.

Increasing the FDP concentration lowered the K_m for both pyruvate and NADH. Comparison of the K_m values at two different FDP concentrations in phosphate and triethanolamine

TABLE 2. *Effect of FDP concentration on the K_m values for NADH and pyruvate for the S. lactis lactate dehydrogenase*[a]

FDP concn (mM)	Pyruvate = 10 mM		NADH = 0.167 mM	
	K_m (NADH) (mM)	V_{max}[b] (U/mg)	K_m (pyruvate) (mM)	V_{max}[b] (U/mg)
10.0	0.05	2,220	ND[c]	ND
1.0	0.07	2,020	1.4	1,580
0.5	0.07	1,720	1.6	1,110
0.1	0.10	347	2.7	215
0.05	0.14	202	4.9	95

[a] For each FDP concentration, Lineweaver-Burk plots were constructed from data obtained using a fixed concentration of pyruvate (10 mM) and varying NADH concentrations, or a fixed concentration of NADH (0.167 mM) and varying pyruvate concentrations. 90 mM Tris-maleate buffer (pH 6.9) was used in all assays.

[b] Determined from the intercept of the Lineweaver-Burk plots on the 1/v axis for each FDP concentration at either 10 mM pyruvate or 0.167 mM NADH.

[c] ND, Not determined.

buffers showed a similar lowering of the K_m by increased FDP in these two buffers.

The magnitude of the effect was very similar to that determined (22) for the *S. faecalis* lactate dehydrogenase. With the *S. mutans* lactate dehydrogenase, Brown and Wittenberger (2) found that the K_m for NADH was not altered by the FDP concentration, but this may have been due to their use of a concentration range well above the $FDP_{0.5V}$ value.

The "apparent V_{max}" values (determined from the intercepts of the Lineweaver-Burk plots at the fixed concentrations of pyruvate and NADH) were also markedly increased by increasing the FDP concentration. In a similar study of the *S. faecalis* lactate dehydrogenase (22), it was found that the intercept values were unchanged when the FDP concentration was varied.

Factors affecting the binding of FDP. The response of the *S. lactis* lactate dehydrogenase to increasing FDP concentrations was studied at different substrate concentrations and in different buffers to allow comparisons with the results obtained by other workers.

Interaction coefficients between the binding sites (n_H values) and $FDP_{0.5V}$ values were obtained from Hill plots of the data at three different concentrations of both pyruvate and NADH in 90 mM Tris-maleate buffer (pH 6.9) (Table 3). Changing the concentration of either substrate had only a small effect on both parameters. The n_H value of 1.7 to 2.1 was similar to that obtained by other workers (2, 12, 22) and indicated the presence of at least two interacting FDP binding sites. The $FDP_{0.5V}$ value (0.16 to 0.22 mM) was somewhat higher than the value found for *S. faecalis* (0.05 mM), but contrasted markedly with the high value of 5 mM found for *S. mutans* 10449.

The $FDP_{0.5V}$ values in three different systems are compared in Table 4. Although the n_H value was similar in all three buffers, the $FDP_{0.5V}$ value was clearly very sensitive to the particular buffering ions present in solution. In triethanolamine buffer, the $FDP_{0.5V}$ was only 2 μM, very much lower than the value found in Tris-maleate buffer but in good agreement with previous values obtained for the *S. cremoris* lactate dehydrogenase (12, 19), which were also determined in triethanolamine buffer. On the other hand, phosphate buffer, which was used at a similar concentration in studying the properties of both the *S. mutans* lactate dehydrogenase (2) and the *S. faecalis* lactate dehydro-

TABLE 3. *Effect of NADH and pyruvate on FDP activation of the S. lactis lactate dehydrogenase*[a]

Substrate concn	n_H[b]	$FDP_{0.5V}$[b]	V_{max}[c] (U/mg)
Pyruvate = 10 mM			
NADH = 0.05 mM	1.8	0.22	950
0.1 mM	1.7	0.17	1,275
0.167 mM	1.7	0.16	1,450
NADH = 0.167 mM			
Pyruvate = 0.5 mM	2.1	0.20	360
2.0 mM	1.85	0.19	915
10 mM	1.7	0.16	1,450

[a] The activity at different FDP concentrations, for each different NADH and pyruvate concentration, was determined in 90 mM Tris-maleate buffer (pH 6.9).

[b] Obtained from Hill plots.

[c] Determined from the intercepts of the Lineweaver-Burk plots on the 1/v axis for each NADH-pyruvate combination.

TABLE 4. *Effect of buffer composition on FDP activation of S. lactis lactate dehydrogenase*[a]

Buffer	$FDP_{0.5V}$ (mM)	n_H
Tris-maleate	0.2	2.1
Triethanolamine-hydrochloride	0.0022	1.7
Phosphate (KH_2PO_4 + NaOH)	4.4	2.1

[a] For each buffer, the activity was measured at 12 different FDP concentrations. $FDP_{0.5V}$ values and interaction coefficients (n_H) were obtained from Hill plots of the data. Standard assay conditions were: 90 mM buffer (pH 6.9); 0.167 mM NADH; 10 mM pyruvate; and 0.1 ml of enzyme solution containing between 0.3 and 3.0 μg of protein.

genase (22), increased the $FDP_{0.5V}$ of the *S. lactis* dehydrogenase to 4.4 mM. This is very similar to the value (5 mM) found for the *S. mutans* lactate dehydrogenase (2). However, the *S. faecalis* lactate dehydrogenase had a relatively low $FDP_{0.5V}$ (0.05 mM) in 0.1 M phosphate buffer (22). To ascertain whether the *S. faecalis* lactate dehydrogenase was affected by phosphate, the FDP activation of a partially purified preparation was compared in phosphate and Tris-maleate buffers (Table 5). The affinity for FDP was considerably higher than that of the *S. lactis* lactate dehydrogenase in both buffers. However, the most interesting result was the absence of phosphate inhibition of FDP binding. In fact, activity was slightly higher in phosphate buffer than in Tris-maleate buffer at all concentrations of FDP.

Effect of varying phosphate concentrations on activity. The effect of varying phosphate concentrations at a fixed concentration of FDP (1 mM) was investigated. A plot of activity against phosphate concentration (Fig. 4) shows the sigmoidal nature of the inhibition at the concentration of FDP used. It is clear from the pH profiles in Fig. 1B that, at a higher FDP concentration (10 mM), phosphate inhibition was much less significant. A Hill plot of the data (Fig. 4, inset) indicates that phosphate inhibition is highly cooperative ($n_H = -2.4$). Replacement of phosphate by sodium sulfate (to a final concentration of 175 mM) or by potassium chloride (to 200 mM) did not inhibit enzyme activity.

Effect of Mn^{2+} on activity. DeVries et al. (7) found that the FDP-requiring lactate dehydrogenase of *Lactobacillus casei* was activated by manganese, and subsequent work (10) has shown that Mn^{2+} increases the affinity of this enzyme for FDP at pH values above 5.5. The effect of $MnCl_2$ on the *S. lactis* lactate dehydrogenase was examined over a range of $MnCl_2$ concentrations in Tris-maleate and triethanolamine buffers at different pH values and FDP concentrations. In no case was Mn^{2+} found to

TABLE 5. *FDP activation of S. faecalis lactate dehydrogenase*[a]

Buffer	V_{max} (U/mg of protein)	$FDP_{0.5V}$ (mM)	n_H
Phosphate	37	0.008	1.5
Tris-maleate	25	0.01	1.7

[a] Twelve different FDP concentrations were used. $FDP_{0.5V}$ and n_H values were obtained from Hill plots. Standard assay conditions were: 90 mM buffer (pH 6.9); 0.167 mM NADH; 10 mM pyruvate; and 0.1 ml of enzyme solution containing 4.0 to 10.0 μg of protein.

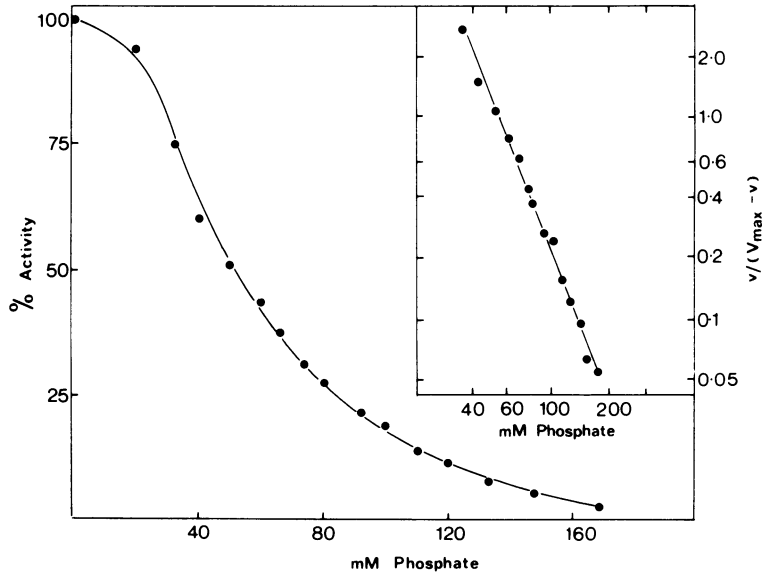

FIG. 4. *Inhibition of the rate of pyruvate reduction by phosphate. Phosphate (as KH_2PO_4) was added to the reaction mixtures at the concentrations indicated, and the pH was standardized to 6.9. The reaction mixtures contained, in addition to phosphate: 90 mM Tris-maleate buffer (pH 6.9); 10 mM pyruvate; 0.167 mM NADH; 1 mM FDP; and 0.1 ml of diluted S. lactis lactate dehydrogenase containing between 0.23 and 1.9 μg of protein. The inset shows the same data plotted as a Hill plot.*

affect the activity of *S. lactis* lactate dehydrogenase in any way.

However, addition of $MnCl_2$ to the *S. faecalis* lactate dehydrogenase did result in significant activation (Fig. 5), increasing the affinity for FDP as found for the *L. casei* lactate dehydrogenase. It is also interesting to note that with $MnCl_2$ present, the *S. faecalis* lactate dehydrogenase had considerable activity in the absence of added FDP in the assay system. Since the enzyme was not highly purified, this may have been due to a low residual FDP concentration, although the preparation had been thoroughly dialyzed before assay.

Determination of the FDP concentration in vivo. In view of the widely differing values for the FDP requirements of the *S. lactis* lactate dehydrogenase in vitro, depending on the nature of the assay buffer used, it is important to ascertain the in vivo FDP concentration in growing cultures of this organism.

Samples were taken from batch cultures of *S. lactis* C10 growing on the same medium as that used for obtaining cells for enzyme purification. This medium has a high lactose content (3%, wt/vol). Samples were taken from mid-logarithmic phase to stationary phase, and the FDP content was determined (Table 6). The in vivo FDP concentration remained at a fairly steady concentration of 12 to 14 mM, and only in the stationary phase of growth did it drop appreciably. The concentration present in logarithmic-phase cells is in good agreement with the values determined for *S. lactis* ML3 (18 mM; 4), for *S. mutans* JC2 (approximately 16 mM, assuming the same intracellular volume/dry-weight factor used in the present study; 24), and for *Lactobacillus plantarum* (17 mM; 17).

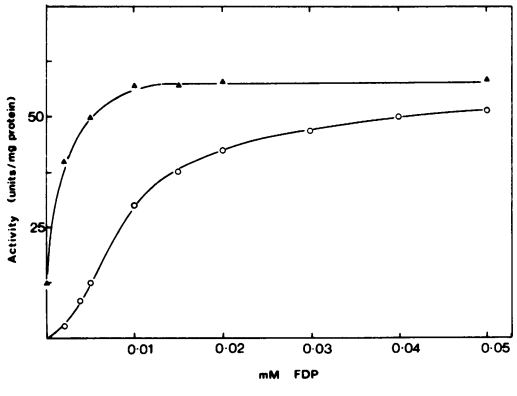

FIG. 5. *Effect of Mn^{2+} on FDP activation of S. faecalis lactate dehydrogenase. The reaction mixture contained (in a total volume of 3 ml): 90 mM Tris-maleate buffer (pH 6.9); 10 mM pyruvate; 0.167 mM NADH; and 0.1 ml of diluted enzyme solution containing between 4.0 and 10.0 μg of protein. Symbols: (○) Without $MnCl_2$; (▲) in the presence of 1 mM $MnCl_2$.*

TABLE 6. *Concentration of FDP and triosephosphates in S. lactis cells*

Wet wt of cells[a] (g/25 ml of culture)	FDP concn[b] (mM)	Triosephosphate concn[b] (mM)
0.055	14.9	4.5
0.109	14.9	4.0
0.118	13.2	3.2
0.155	13.2	2.2
0.189	12.7	2.3
0.364	3.9	3.0

[a] Samples were taken from a batch culture of *S. lactis* C10 on a lactose (3%)-tryptone-yeast extract medium at various times from mid-logarithmic to stationary phase. Wet weights were determined from the weight of cells collected on a membrane filter minus the weight of the filter prerinsed with washing liquid.

[b] Determined as described in Materials and Methods. The FDP concentrations shown have been corrected for interference arising from the triosephosphates present.

DISCUSSION

The main purpose of the present study was to investigate possible reasons for the widely differing values that have been reported for the FDP requirement for activation of the streptococcal lactate dehydrogenases. Yamada and Carlsson (24) have shown that a change from glucose excess to glucose-limited growth conditions for *S. mutans* and *S. bovis* is accompanied by a large decrease in the intracellular level of FDP and a change in product formation from a virtually homolactic fermentation to one in which formate, acetate, and ethanol are the dominant fermentation products. This clearly demonstrates the importance of the FDP activation in determining the fate of pyruvate during fermentation under conditions of different carbohydrate availability. However, the reported values for the $FDP_{0.5V}$ value (which should be an indicator of the physiologically significant range over which FDP will control activity) vary enormously. Thus the reported $FDP_{0.5V}$ value for the lactate dehydrogenase of *S. cremoris* is of the order of 0.002 mM (11) and that for *S. faecalis* is 0.05 mM (22), whereas that for several *S. mutans* strains is 5 mM (2). It would seem unlikely that the physiologically significant range of FDP concentrations will differ by a factor of 2,000 in the different species.

The differences between the reported $FDP_{0.5V}$ values could possibly be due to differences in

the purification procedures used or the degree of purification achieved. However, we have found that the $FDP_{0.5V}$ value for the *S. lactis* lactate dehydrogenase determined either in the dialyzed crude cell extract or after ammonium sulfate fractionation is virtually identical with that for the purified enzyme. It is clear from the results of the present study that the main reason for the widely differing $FDP_{0.5V}$ values reported is the sensitivity of FDP binding to the presence of other ions in solution. Thus the high $FDP_{0.5V}$ found for the lactate dehydrogenase of *S. mutans* 10449 (2) may well have been due to the fact that phosphate buffer was used in studying this enzyme. A similar value was obtained in the present study for the *S. lactis* lactate dehydrogenase when determined in 90 mM phosphate buffer, and Yamada and Carlsson (24) noted a substantial decrease in the $FDP_{0.5V}$ for *S. mutans* JC2 lactate dehydrogenase when the ionic strength of the phosphate buffer was decreased.

The effect of phosphate on the FDP requirement for the activation of the lactate dehydrogenase in vitro raises the question of the physiological significance of this effect in vivo. The in vivo concentration of inorganic phosphate in *S. lactis* has not been determined, but in a study of phosphate uptake by *S. faecalis* (9), a cellular concentration of inorganic phosphate of 75 mM was found after overnight growth in a medium containing 10 mM phosphate. The in vivo FDP concentration in log-phase cells of *S. lactis* growing on a high-carbohydrate medium is sufficient to activate the lactate dehydrogenase fully, even if the high FDP requirement found in 90 mM phosphate approximates the in vivo situation. However, the much greater sensitivity of the FDP-activated pyruvate kinase of *S. lactis* to inhibition by phosphate (50% inhibition at 1 mM phosphate; 5) suggests either that the inorganic phosphate concentration in *S. lactis* is kept at a low level or that other factors must overcome or compensate for the phosphate inhibition in vivo. The pyruvate kinase of lactic streptococci is known to have a much wider activator specificity than the lactate dehydrogenase (19, 20), so it is more difficult to predict the in vivo significance of phosphate inhibition of this enzyme.

It is also clear the phosphate does not affect FDP activation similarly in all streptococcal lactate dehydrogenases. Thus the *S. faecalis* lactate dehydrogenase is not sensitive to phosphate inhibition, which accounts for the relatively low $FDP_{0.5V}$ value reported for this species (22) even though it was studied in phosphate buffer. The *S. faecalis* lactate dehydrogenase also differs from the *S. lactis* lactate dehydrogenase in that the binding of FDP is enhanced by Mn^{2+} in the former enzyme but not in *S. lactis*. In this respect, the *S. faecalis* lactate dehydrogenase resembles that from *L. casei*, a similarity that has already been thoroughly documented for other enzymes in these two species (14).

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