

Regulation of the L-Lactate Dehydrogenase from *Lactobacillus casei* by Fructose-1,6-Diphosphate and Metal Ions

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The lactate dehydrogenase of *Lactobacillus casei*, like that of streptococci, requires fructose-1,6-diphosphate (FDP) for activity. The *L. casei* enzyme has a much more acidic pH optimum (pH 5.5) than the streptococcal lactate dehydrogenases. This is apparently due to a marked decrease in the affinity of the enzyme for the activator with increasing pH above 5.5; the concentration of FDP required for half-maximal velocity increases nearly 1,000-fold from 0.002 mM at pH 5.5 to 1.65 mM at pH 6.6. Manganous ions increase the pH range of activity particularly on the alkaline side of the optimum by increasing the affinity for FDP. This pH-dependent metal ion activation is not specific for Mn^{2+} . Other divalent metals, Co^{2+} , Cu^{2+} , Cd^{2+} , Ni^{2+} , Fe^{2+} , and Zn^{2+} but not Mg^{2+} , will effectively substitute for Mn^{2+} , but the pH dependence of the activation differs with the metal ion used. The enzyme is inhibited by a number of commonly used buffering ions, particularly phosphate, citrate, and tris(hydroxymethyl)aminomethane-maleate buffers, even at low buffer concentrations (0.02 M). These buffers inhibit by affecting the binding of FDP.

Lactobacillus casei is one of the few lactobacilli forming only the L(+) isomer of lactic acid (13). However, in a study of the lactate dehydrogenases of this species, Mizushima and Kitahara (12) were unable to detect any nicotinamide adenine dinucleotide (NAD)-dependent L-lactate dehydrogenase (L-lactate:NAD⁺ oxidoreductase, EC 1.1.1.27). They suggested that L-lactic acid formation might be due to the activity of an NAD-independent flavin-containing L-lactate dehydrogenase. De Vries et al. (3) showed, however, that an NAD-linked L-lactate dehydrogenase was present, but that the enzyme required fructose-1,6-diphosphate (FDP) and manganous ions for activity.

FDP is known to be required for activity of L-lactate dehydrogenases of streptococci (6, 14, 15) and of *Bifidobacterium bifidum* (2), but the requirement has not been found in other lactobacilli. Since London et al. (7, 8) have recently demonstrated a close resemblance between both the FDP-aldolase and an inducible malic enzyme of *L. casei* and the corresponding enzymes in *Streptococcus faecalis*, it was of interest to compare the properties of the FDP-activated lactate dehydrogenase of *L. casei* with the streptococcal lactate dehydrogenases.

The present paper reports a partial purification and a study of the effects of pH, FDP, and metal ions on the activity of the L-lactate dehydrogenase of *L. casei* var. *rhamnosus*.

MATERIALS AND METHODS

Organism and growth of cultures. *L. casei* var. *rhamnosus* ATCC 7469 (NCIB 6375) was obtained from the National Collection of Industrial Bacteria, Torrey Research Station, U.K. Cultures were grown in 20 liters of Gasser (3) medium in a 50-liter New Brunswick Fercell fermentor at 30 C. The medium was gassed with nitrogen before inoculation, and a nitrogen atmosphere was maintained over the medium throughout growth. The culture was stirred at an impeller speed of 50 rpm. pH was maintained at 5.9 to 6.0 by addition of 2.5 M NaOH throughout growth. Cells were harvested by centrifugation at the end of the logarithmic phase of growth. Approximately 300 g (wet weight) of cells was obtained from 20 liters of medium.

Chemicals and reagents. Pyruvate and reduced NAD (NADH) were obtained from the Sigma Chemical Co., St. Louis, Mo., and FDP was from Fluka AG Switzerland. Streptomycin sulfate was obtained from Glaxo Ltd., New Zealand. Diethylaminoethyl (DEAE)-cellulose was obtained from Sigma Chemical Co., whereas the DEAE-Proton is an anion-exchange cellulose developed by Tasman Vaccine Laboratories Ltd., Upper Hutt, 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. Bio Gel A-15 M was obtained from Bio-Rad Laboratories, Richmond, Calif.

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

N.Y., and the Aldrich Chemical Co., Milwaukee, Wis., respectively. Cyanogen bromide was obtained from Matheson, Coleman & Bell, Norwood, Ohio.

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 by the procedure of Cuatrecasas (1). The terminal amino group was then condensed with oxalate via an amide bond to give the oxamate derivative by the following procedure (V. L. Crow, unpublished information). To each 20 ml of packed resin, 14 ml of 0.74 M oxalic acid was added and the pH was adjusted to 4.7. Four milliliters of a solution containing 0.63 g of 1 cyclohexyl-3-(2-morpho-linoethyl)-carbodiimide metho-*p*-toluene sulfonate was added dropwise while the pH was maintained at 4.7 and the mixture was stirred. The mixture was then stirred gently at room temperature for 24 h and finally washed with 5 liters of distilled water.

Lactate dehydrogenase assay. The routine assay system for lactate dehydrogenase comprised the following: 3 ml of 0.05 M acetate buffer (pH 5.4) containing 0.167 mM NADH, 0.1 mM FDP, and 10 mM pyruvate. At this pH, manganese ions are not required. Assay conditions for particular kinetic determinations are specified in the text.

Protein was determined by the method of Lowry et al. (9).

Enzyme purification: (i) preparation of cell extract. The harvested cells were washed twice in 0.01 M phosphate buffer (pH 7.0) and resuspended in about 400 ml of the same buffer at 4 C. The cells were disrupted by a single passage through an Aminco French pressure cell at 6,500 lb/in². The cell debris was removed by centrifugation at 4 C at 10,000 rpm for 20 min. All subsequent steps were carried out at 4 C.

(ii) Streptomycin sulfate treatment. The crude cell extract was diluted with 0.01 M phosphate buffer (pH 7.0) to a protein concentration of 10 mg/ml. Streptomycin sulfate was added to give a final concentration of 1.5 mg/mg of protein. After 1 h, the precipitate was removed by centrifugation and the supernatant fluid was dialyzed against 0.01 M phosphate (pH 7.0) containing 10% glycerol. This concentration of glycerol was included in the phosphate buffer in all subsequent steps since it gave considerable protection against loss of activity.

(iii) Ammonium sulfate fractionation. To each 100 ml of dialyzed supernatant, 29.1 g of solid ammonium sulfate was slowly added to bring the solution to 50% saturation. After standing for at least 1 h, the precipitate was removed by centrifugation and discarded. The supernatant fluid, which contained virtually all of the lactate dehydrogenase activity, was taken to 70% saturation with additional solid ammonium sulfate. The precipitate was centrifuged and redissolved in a small quantity of 0.01 M phosphate (pH 7.0) plus 10% glycerol. The solution was dialyzed against the same buffer overnight.

(iv) Gradient elution from DEAE-cellulose. The dialyzed 50 to 70% ammonium sulfate fraction was loaded onto a column (6 by 25 cm) of DEAE-cellulose

pre-equilibrated with 0.01 M phosphate buffer (pH 7.0)-10% glycerol and eluted with a linear gradient of 0 to 0.8 M NaCl in the same buffer. Fractions containing greater than 200 U/ml were pooled and dialyzed overnight against 0.01 M phosphate (pH 6.5)-10% glycerol.

(v) Stepwise elution from DEAE-Proton. The dialyzed fractions were loaded (without concentrating) onto a column (6 by 25 cm) of DEAE-Proton pre-equilibrated against 0.01 M phosphate buffer (pH 6.5)-10% glycerol. The column was eluted first with 260 ml of the same buffer, a further 280 ml of the same buffer containing 0.05 M NaCl, and then 260 ml of buffer containing 0.1 M NaCl. This washing procedure removed a considerable amount of inactive protein but no lactate dehydrogenase. The NaCl concentration was then increased to 0.15 M NaCl. Lactate dehydrogenase was eluted almost immediately in a sharp peak.

(vi) Chromatography on an oxamate affinity column. The eluted fractions from the DEAE-Proton column were pooled and dialyzed against 0.01 M phosphate-10% glycerol buffer (pH 6.0). The dialyzed fractions were loaded onto a column (1 by 20 cm) of agarose affinity resin containing an oxamate-binding group. The column was washed with 0.01 M phosphate-10% glycerol buffer (pH 6.0). The enzyme remained bound to the resin during this washing. Lactate dehydrogenase was eluted sharply by increasing the phosphate buffer concentration to 0.05 M.

The active fractions were finally dialyzed against 0.1 M phosphate buffer (pH 7.0) containing 10% glycerol, and concentrated by ultrafiltration. The enzyme could be stored frozen in this buffer for several months without any detectable loss of activity. A summary of the purification procedure is shown in Table 1. Considerable activity loss occurred at each of the column steps so that the final apparent purification achieved was only 25-fold.

Polyacrylamide gel electrophoresis in pH 8.5 tris-(hydroxymethyl)aminomethane-glycine buffer (4) followed by staining of the protein bands with Coomassie brilliant blue revealed two strongly stained sharp protein bands and a weakly stained, faster-running, diffuse band.

RESULTS

Selection of a suitable buffer. In attempting to determine the pH optimum for the enzyme, it was found that many of the buffer systems commonly used to cover the particular pH range over which the enzyme was active strongly inhibited its activity. In particular, phosphate and citrate buffers gave 50% inhibition at 0.005 and 0.006 M, respectively, and complete inhibition at 0.02 M (Fig. 1). Tris(hydroxymethyl)aminomethane-maleate buffer was also inhibitory at somewhat higher concentrations. Imidazole and histidine buffers were less inhibitory, but in these buffers, at a concentration of 0.1 M, a considerably higher concentration of FDP was required to obtain maximal velocity than in

TABLE 1. Purification of lactate dehydrogenase

Purification Step	Total units ($\mu\text{mol}/\text{min}$)	Total protein (mg)	Sp act ($\mu\text{mol}/\text{min}$ per mg of protein)	Purification (fold)	Yield (%)
1. Crude extract	232,000	13,000	17.8	1	100
2. Streptomycin sulfate	243,000	14,000	17.3	1	100
3. $(\text{NH}_4)_2\text{SO}_4$ (50–70% saturation)	210,000	4,800	43.8	2.4	90
4. DEAE-cellulose	106,000	740	144	7.8	45
5. DEAE-Proton	55,000	254	216	12	24
6. Affinity resin	22,400	50	445	25	9.6

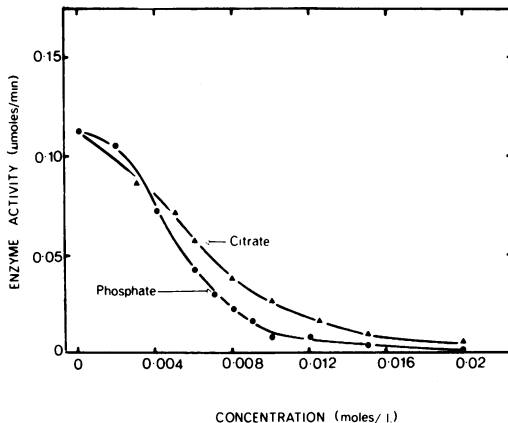


FIG. 1. Inhibition of enzyme activity by phosphate and citrate ions. The reaction was carried out in 0.02 M acetate buffer (pH 5.6) to which pH 5.6 citrate or phosphate buffer was added to give the appropriate final concentration. Concentrations: FDP, 0.1 mM; NADH, 0.167 mM; pyruvate, 10 mM. Symbols: ●, phosphate; ▲, citrate.

0.1 M acetate buffer (Fig. 2). In acetate buffer, activity remained unchanged from 0.01 to 0.2 M concentrations of this buffer even at low FDP concentrations. However, the buffering range of acetate does not cover the full range of enzyme activity. When imidazole-HCl buffer was diluted to 0.05 M, the enzyme showed the same FDP activation curve as in 0.05 M acetate buffer. Consequently 0.05 M acetate and imidazole buffers were selected to cover the pH range of the enzyme.

Effect of pH on activity. The pH profile of the enzyme (Fig. 3) was determined by using 0.05 M acetate and imidazole buffers, 0.167 mM NADH and 10 mM pyruvate (which were shown to be virtually saturating concentrations), and 1 mM FDP. The pH optimum was at pH 5.5, and activity decreased rapidly on either side of the optimum. On the alkaline side, no activity was detectable at pH 7 or above. This pH profile contrasts with that of the FDP-activated streptococcal lactate dehydrogenases. For example, the pH profile of the *S. cremoris* lactate dehydrogenase

showed a broad optimum from 5.5 to 7.5 (6) and was still active at pH 8.5.

When 1 mM MnCl_2 was included in the assay mixture, the pH profile was broadened on either side of the optimum (Fig. 3), particularly on the alkaline side. However, even in the presence of Mn^{2+} , activity fell to zero by pH 7.7.

Effect of pH on the binding of FDP. The effect of varying FDP concentration on enzyme activity was determined at various pH values in 0.05 M acetate and imidazole buffers. The data are shown as a series of Hill plots in Fig. 4. Below the optimum pH value (ca. 5.5) the slope of the Hill plot decreases to a value of one or lower, but at pH values above the optimum it remains constant, giving a value for the interaction coefficient of 1.6 to 1.7. However, the FDP concentration required for half-maximal velocity ($M_{0.5v}$) increases almost 1,000-fold within one pH unit above the optimum from 2.0 μM at pH 5.5 to 1.65 mM at pH 6.6 (Fig. 5).

Effect of manganese ions on the binding of FDP. The response to varying FDP concentration was also determined in the presence of 2.5 mM MnCl_2 at various pH values. $M_{0.5v}$ values were obtained from Hill plots of the data. It can be seen (Table 2) that Mn^{2+} increases the affinity of the enzyme for FDP. The $M_{0.5v}$ value is reduced by 25-fold in the presence of 2.5 mM Mn^{2+} at pH 6.46.

Effect of metal ion concentration. The sigmoidal response to varying Mn^{2+} concentration at three different pH values is shown in Fig. 6. The manganese requirement for half-maximal velocity is largely independent of pH, and very little increase in activity was found at concentrations greater than 2 mM Mn^{2+} at this concentration of FDP (1 mM).

Specificity of metal ion requirement. De Vries et al. (3) reported that Mg^{2+} was unable to replace the Mn^{2+} requirement for the *L. casei* lactate dehydrogenase, but they did not investigate other metal ions. It can be seen (Table 3) that activation by manganese ions at pH values above the optimum is not specific for manganese, which can be replaced by several other

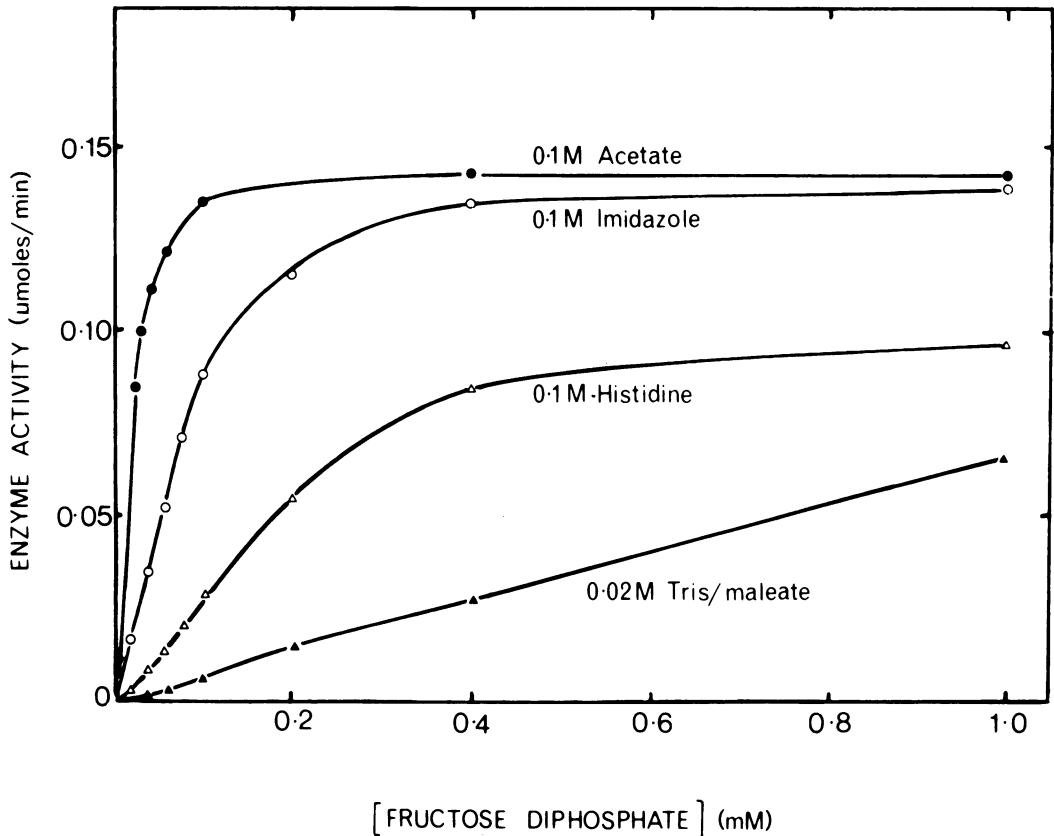


FIG. 2. Effect of FDP concentration on enzyme activity in different buffers. All buffers were at pH 5.8. Substrate concentrations as in standard assay. Symbols: ●, sodium acetate-acetic acid; ○, imidazole-HCl; △, histidine-HCl; ▲, tris(hydroxymethyl)aminomethane-maleic acid.

divalent metal ions. Co^{2+} , Cd^{2+} , Cu^{2+} , and Ni^{2+} were just as effective as Mn^{2+} at pH 6.3; Fe^{2+} and Zn^{2+} were less effective; Mg^{2+} was the only divalent metal ion tested that gave no activation. When metal ion activation was determined as a function of pH, a variety of responses was found. Representative types are shown in Fig. 7. Activation by Co^{2+} , Fe^{2+} , Cu^{2+} , and Ni^{2+} showed a pH dependence similar to that of Mn^{2+} , falling rapidly with increasing pH to give only a relatively small activation at pH 6.9. With Cd^{2+} and Zn^{2+} , on the other hand, enzyme activity showed much less change with increasing pH. Cadmium was the best activator at pH 6.9, whereas zinc, which gave no activation at pH 6.2, was a good activator at pH 6.9.

DISCUSSION

From the work of London et al. (7, 8), indicating a close relationship between both the FDP aldolase and the malic enzyme of *L. casei* and the corresponding enzymes of *S. faecalis*, it would be expected that the FDP-activated lac-

tate dehydrogenases of these two species would have similar properties. It is apparent from the present results that the lactate dehydrogenase of *L. casei* differs in a number of respects from that of *S. faecalis* (14). These differences include the low pH optimum of the *L. casei* enzyme, the low concentration of FDP required for maximal activity at the optimum, the metal ion activation, and the high sensitivity to inhibition by phosphate and other buffer ions.

However, many of these differences could be due to relatively minor differences in protein structure in the region of the FDP-binding site. The major difference in the pH profiles of the *L. casei* and streptococcal lactate dehydrogenases appears to be due to the rapid decrease in affinity of the *L. casei* enzyme for the activator, FDP, as the pH is raised above the optimum. The lactate dehydrogenase of *S. faecalis* also shows a decreased affinity for FDP as the pH is raised (14) but to a very much smaller extent. The pK'_3 and pK'_4 values of the two phosphate groups of FDP are in the region of 5.9 and 6.7

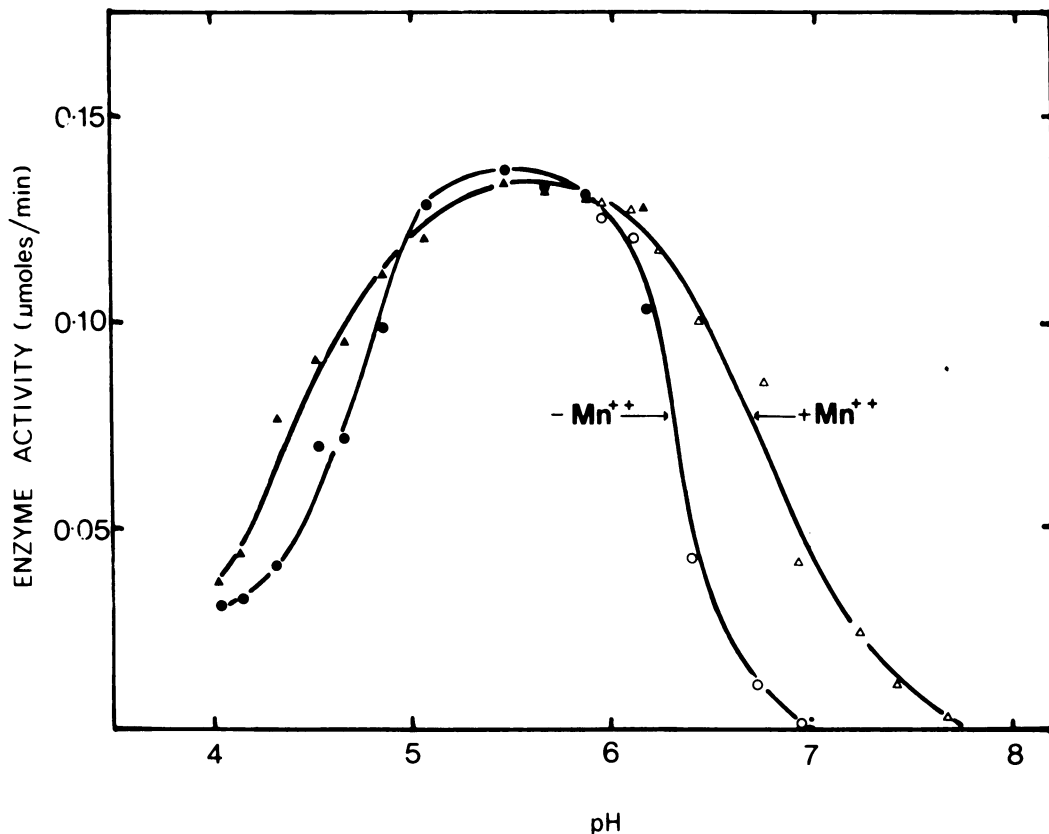


FIG. 3. Effect of pH on enzyme activity in the absence and presence of manganous ions (as $MgCl_2$). Concentrations: FDP, 1 mM; NADH, 0.167 mM; pyruvate, 10 mM. Symbols: ▲, 0.05 M acetate buffer + 1 mM Mn^{2+} ; ●, 0.05 M acetate without Mn^{2+} ; △, 0.05 M imidazole buffer plus 1 mM Mn^{2+} ; ○, 0.05 M imidazole without Mn^{2+} .

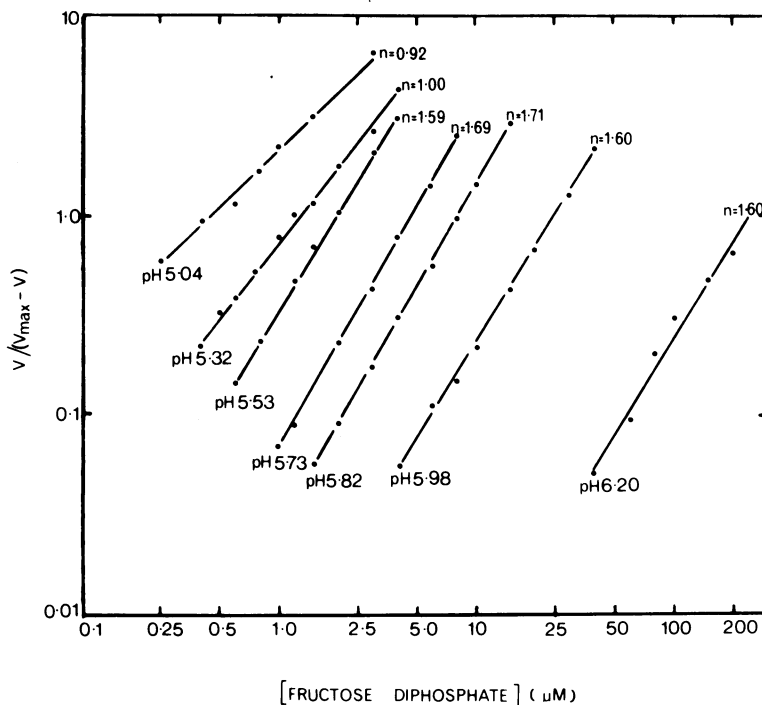


FIG. 4. Hill plots showing the effect of fructose diphosphate concentration on enzyme activity at various pH values in the absence of Mn^{2+} . All determinations except that at pH 6.20 were in 0.05 M acetate buffers; the pH 6.20 determination was in 0.05 M imidazole buffer. Substrate concentrations as in standard assay.

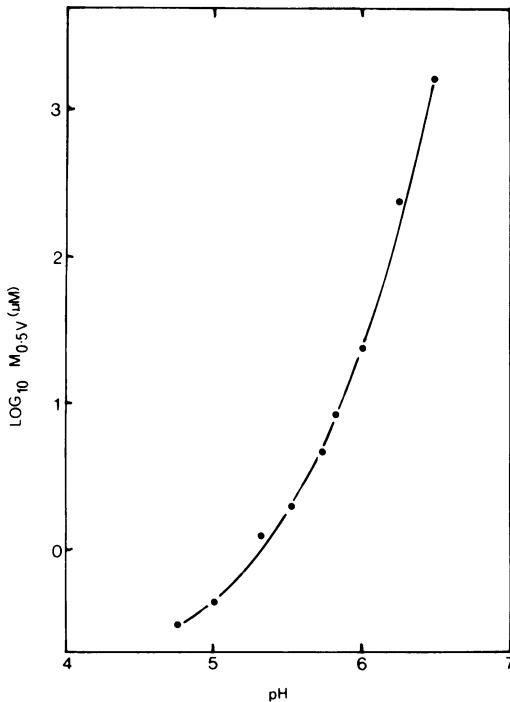


FIG. 5. Relationship between FDP concentration required to give half-maximal velocity ($M_{0.5V}$) and pH. $M_{0.5V}$ values were derived from the data of Fig. 4 plus two additional determinations at pH 4.75 (acetate buffer) and pH 6.47 (imidazole buffer).

(10). The ionization of these two groups could clearly contribute to the decrease in affinity of the enzyme for FDP over the region 5.5 to 7.0 if there was a negatively charged group (or groups) on the enzyme in the vicinity of the FDP-binding site. The metal ion activation is also related to the binding of the activator and could similarly be accounted for by interaction of the negatively charged groups of the enzyme and activator with the metal ions, thus reducing the mutual repulsive forces that lead to loss of affinity. The high sensitivity to inhibition by polyvalent buffer ions such as phosphate and citrate may also be related to the particular nature of the FDP-binding site since these buffer ions appear to inhibit by affecting the affinity for FDP.

Thus, the apparent differences in properties of the *L. casei* and *S. faecalis* lactate dehydrogenases are not necessarily at variance with the idea that there may be a high degree of homology between the enzymes of the two species (8).

The ability of *L. casei* to oxidize pyruvate under aerobic conditions (3, 11) necessitates a control of the alternative pathways of pyruvate utilization. De Vries et al. (3) have found that in

TABLE 2. Effect of Mn^{2+} (2.5 mM) on the fructose-diphosphate concentration required for half-maximal velocity ($M_{0.5V}$)

pH ^a	$M_{0.5V}$ for fructose-diphosphate (μM)	
	No Mn^{2+}	2.5 mM Mn^{2+}
6.11	140	17
6.21	308	23
6.46	1,650	66
6.61	ND ^b	89

^a In 0.05 M imidazole-HCl buffer.

^b Not determined.

TABLE 3. Relative enzyme activity with different metal ions

Metal ion ^a	Relative activity ^b
No metal	1.00
Mg^{2+}	0.97
Zn^{2+}	1.85
Fe^{2+}	2.78
Ni^{2+}	3.34
Mn^{2+}	3.40
Cu^{2+}	3.61
Cd^{2+}	3.75
Co^{2+}	4.17

^a All at 1 mM.

^b Relative to activity in absence of divalent metal ions. Activity determined in 0.05 M imidazole-HCl buffer (pH 6.3) in presence of 1 mM fructose diphosphate.

glucose-limited, continuous cultures of *L. casei* the rate of glucose supply determines the fermentation pattern. At high dilution rates, lactate is the dominant product, whereas, at low dilution rates, little or no lactate is formed and acetate, ethanol, and formate are produced. They suggest that the intracellular FDP level will be regulated by the rate of glucose supply and will determine whether pyruvate is reduced to lactate or oxidized. Both de Vries et al. (3) and Manderson and Doelle (11) noted that lower pH values favor lactate production under aerobic conditions. If the decrease in pH of the medium during growth of a batch culture is reflected in even a small decrease in internal pH, the consequent decrease in the FDP concentration required to activate lactate dehydrogenase would favor lactate production. This would account for the much higher percentage of glucose converted to lactate in batch cultures where the pH was allowed to drop to a low level (3).

The physiological significance of the metal ion activation is less obvious. Although the enzyme does not require metal ions for activity

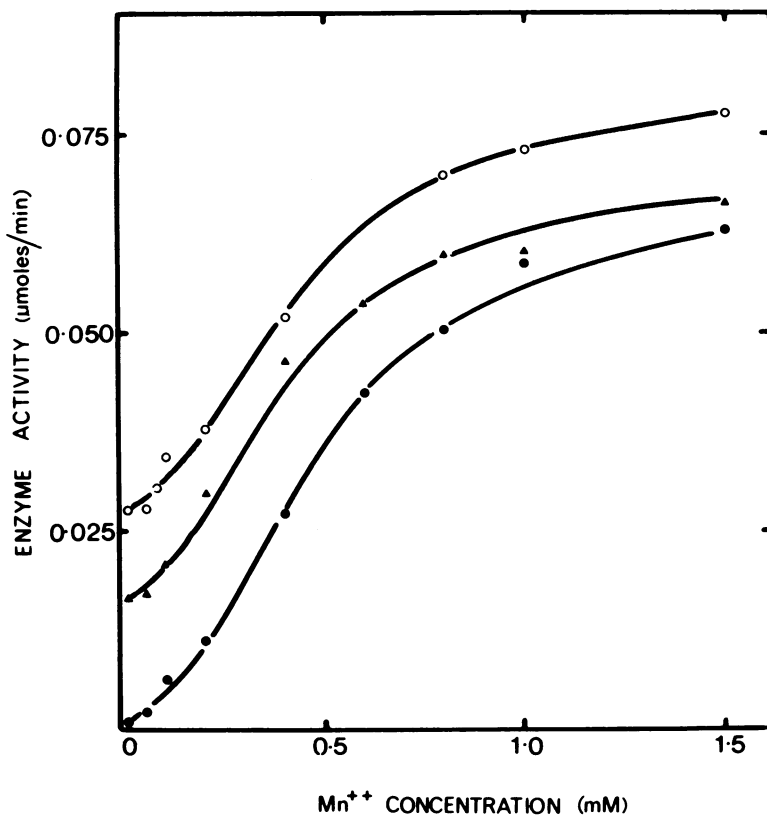


FIG. 6. Effect of varying Mn^{2+} concentration on enzyme activity at three different pH values. Concentrations: FDP, 1 mM; NADH, 0.167 mM; and pyruvate, 10 mM. Symbols: O, pH 6.26; ▲, pH 6.46; and ●, pH 6.75 in 0.05 M imidazole buffers.

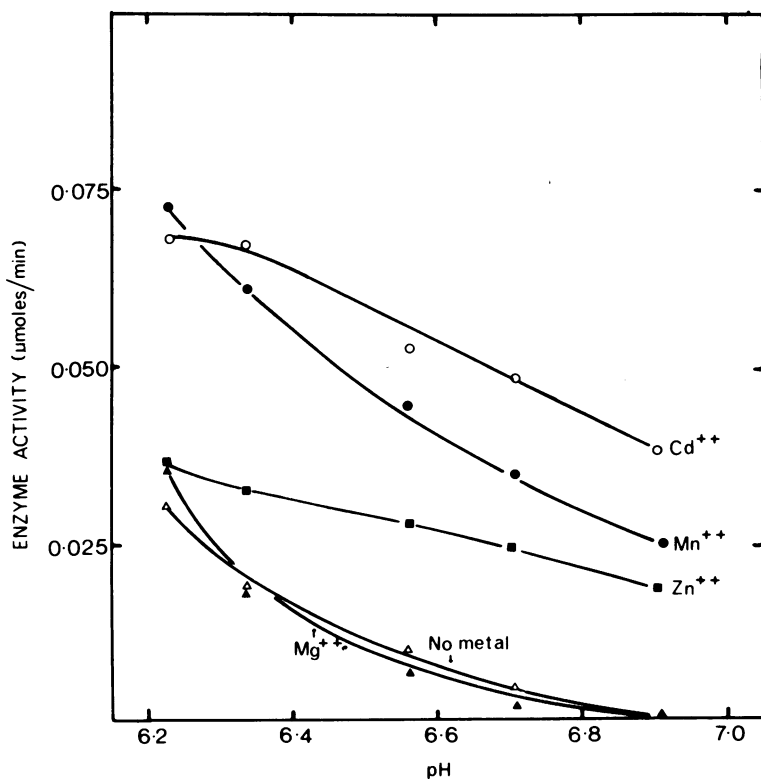


FIG. 7. Effect of pH on enzyme activity in 0.05 M imidazole buffers in the presence of different divalent metal ions. Concentrations: FDP, 1 mM; metal ion, 1 mM; NADH, 0.167 mM; and pyruvate, 10 mM. Symbols: O, $CdCl_2 \cdot 2\frac{1}{2}H_2O$; ●, $MnCl_2 \cdot 4H_2O$; ■, $ZnSO_4 \cdot 7H_2O$; ▲, $MgCl_2 \cdot 6H_2O$; △, no metal.

at low pH values, it is highly probable that the in vivo pH will be in the region where metal ion activation is important. Manderson and Doelle (11) consider the possibility that oxidation of manganous ions to a higher valency state could be important in directing pyruvate metabolism away from lactate under aerobic conditions. The relatively nonspecific nature of the metal ion requirement for enzyme activity in vitro is difficult to reconcile with the idea of a specific regulatory role of any one metal ion. However, the concentration required for activation appears to be quite high so the number of different divalent ions that would be present at a suitable concentration in vivo may be very limited. Studies on the internal concentration of these ions under different nutritional and environmental conditions and its relation to the nature of the fermentation products could be of interest.

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