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Factors Affecting the Activity of the Lactate Dehydrogenase of *Streptococcus cremoris*

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Studies with partially purified extracts of the nicotinamide adenine dinucleotide-linked L(+)-lactate dehydrogenase of *Streptococcus cremoris* US3 showed that fructose-1,6-diphosphate (FDP) was essential for the catalytic reduction of pyruvate in the *p*H range 5.0 to 7.0, outside of which the organism does not grow. In the absence of FDP, enzyme activity was observed only in the region of *p*H 8.0. The optimal *p*H for the oxidation of lactate was approximately 8.0 in the presence and absence of FDP. The FDP-activated enzyme was markedly inhibited by inorganic phosphate. The enzyme lost activity on standing at 5 C in alkaline triethanolamine, was quite stable at *p*H 6.0 to 6.5, and underwent irreversible denaturation below *p*H 5.0. Inorganic phosphate or FDP increased the stability of the enzyme in alkaline buffers. Some distinguishing properties of individual lactate dehydrogenases, activated by FDP, are discussed.

The lactate dehydrogenases of several species of the genus *Streptococcus* have been shown to be markedly activated by the glycolytic intermediate, fructose-1,6-diphosphate (FDP) (1, 7, 8). In a recent survey of the group N streptococci, nicotinamide adenine dinucleotide (NAD)-linked, L(+)-specific lactate dehydrogenase (LDH; EC 1.1.1.27) activity was found in all strains, and in each this activity was markedly increased by FDP (1). The LDH of one strain, *Streptococcus cremoris* US3, was selected for further study. This report describes some factors affecting the activity of this enzyme.

MATERIALS AND METHODS

Bacteria. The methods used for growing cells of *S. cremoris* US3 in quantity and preparing cell-free extracts therefrom have been described elsewhere (5).

The protein concentration of the extracts was measured by the method of Lowry et al. (4) or by measurement of the absorbance at 280 and 260 nm according to the nomograph devised by Adams (California Corporation for Biochemical Research, 3625 Madford St., Los Angeles 63, Calif.).

Estimation of LDH activities. NAD-dependent LDH activity was measured spectrophotometrically as previously described (5).

Partial purification of LDH. The removal of the nucleic acid component in cell-free extracts and fractionation of the extracts with $(NH_4)_2SO_4$ has been described elsewhere (5). The 40 to 60% $(NH_4)_2SO_4$ fraction, previously dialyzed against 0.01 M sodium phosphate, *p*H 7.0, was applied to a diethylaminoethyl-cellulose column (27 by 1.5 cm) which had been previously equilibrated with the same buffer. The enzyme was eluted from the column with a sodium chloride gradient (0–0.5 M) in 0.01 M sodium phosphate buffer, *p*H 7.0, at a flow rate of 0.6 ml/min. Fractions of average volume 7.2 ml were collected and assayed. The enzyme was eluted in fractions 32 through 39 which were pooled and used as the source of LDH in this investigation. A 10-fold increase in specific activity was obtained, but electrophoresis of the pooled fractions on acrylamide gel showed that only partial purification of the enzyme had been achieved.

RESULTS

As shown in Fig. 1, pyruvate reduction by the LDH of strain US3 in triethanolamine-hydrochloride buffer was maximally activated by 0.1 mM FDP at *p*H 6.0, slightly more than 0.1 mM FDP at *p*H 7.0, and 1 mM FDP at *p*H 8.0. In phosphate buffer a much higher concentration of FDP (10 mM) was needed to obtain a reaction velocity equivalent to that obtained with 1 mM FDP in triethanolamine-hydrochloride at the above *p*H values (cf. Fig. 2a and b). The addition of inorganic phosphate to reaction mixtures containing triethanolamine-hydrochloride buffer and 1 mM FDP also resulted in a decreased rate of pyruvate reduction as shown in Fig. 3.

The *p*H curve for pyruvate reduction and for

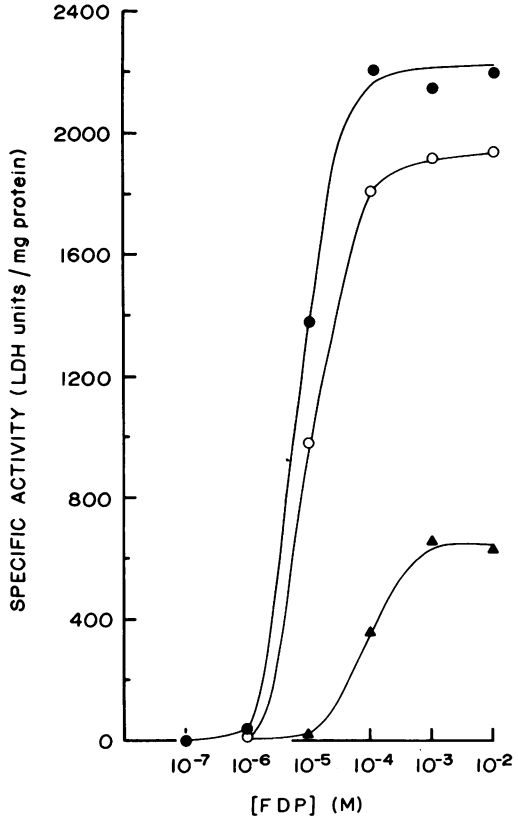

FIG. 1. *Effect of pH on the FDP saturation curve for S. cremoris LDH. The reaction mixture described in Table 2 was used except that FDP concentration was varied as shown. Symbols: pH 6.0, ●; pH 7.0, ○; pH 8.0, ▲.*

lactate oxidation, in the presence and absence of FDP, is shown in Fig. 2. In the absence of FDP a sharp peak of optimal activity was obtained at approximately *p*H 8.0 for both pyruvate reduction and lactate oxidation in both buffer systems. The addition of FDP to the reaction mixture not only resulted in a marked activation of the enzyme but also in a change in the shape of the *p*H curve for pyruvate reduction in both phosphate and triethanolamine-hydrochloride buffers (Fig. 2a and b). The *p*H optimum for the enzyme changed from a relatively sharp peak at *p*H 8.0, in the absence of FDP, to a broad plateau of maximal activity between *p*H 7.0 and 5.0 in the presence of FDP.

Although the *p*H optimum for lactate oxidation was slightly displaced to about *p*H 7.5 on the addition of FDP (Fig. 2c), there was no appreciable change in the shape of the *p*H curve. As with pyruvate reduction, the activation produced by FDP was less in phosphate buffer than in triethanolamine-hydrochloride buffer.

As shown in Fig. 2a the rate of the FDP-activated pyruvate reduction decreased at *p*H values below 5.0 and above 8.0. The loss in activity at the lower *p*H values was found to be due to an effect of *p*H on the stability of the enzyme. Preincubation of extracts of the enzyme for 5 min at 25 C in phosphate-citrate buffer at these low *p*H values, in the presence of 1 mM FDP, followed by assay in the same buffer at *p*H 6.5 resulted in a considerable loss in enzymatic activity (Table 1) accompanied by flocculation of protein.

The decrease in the rate of pyruvate reduction or lactate oxidation above *p*H 8.0 in both triethanolamine-hydrochloride and phosphate buffers, in the presence or absence of FDP, could not be ascribed to the denaturation of the enzyme during the assay period (usually 1 min). Nevertheless, the enzyme was observed to lose activity after longer incubation periods in alkaline triethanolamine buffers. As seen in Table 2 the enzyme retained full activity when incubated for 120 hr in triethanolamine-hydrochloride buffer at *p*H 6.0 and 6.5. However, above *p*H 6.5 the enzyme lost activity, the rate of loss increasing with an increase in *p*H. By contrast, incubation of the enzyme in phosphate buffer resulted in a slower loss of activity which was independent of the *p*H of the buffer.

The enzyme lost approximately half of its original activity after incubation for only 4 hr in triethanolamine-hydrochloride, *p*H 8.0 (Table 2). However, the enzyme showed no loss in activity over the same period when 10 mM sodium phosphate was added to the triethanolamine-hydrochloride buffer. The addition of 1 mM FDP to triethanolamine-hydrochloride at pH 8.0 resulted in a 13% decrease in activity after 4 hr, and addition of 2 mM adenosine triphosphate (ATP) resulted in a 94% decrease in activity. At *p*H 7.0 ATP had no observable effect on enzyme activity during the 4-hr incubation period.

It would appear from the double reciprocal plots shown in Fig. 4 that ATP inhibits LDH activity by competition with NADH.

The LDH of *S. cremoris* US3 was completely inactivated at 50 C, but not at 45 C, after incubation for 10 min in 0.01 M sodium phosphate buffer, *p*H 7.0. These conditions of incubation were therefore used to examine the effectiveness of FDP and other compounds on the thermal stability of the enzyme. Increasing levels of phosphate were found to exert a definite protective influence against heat denatur-

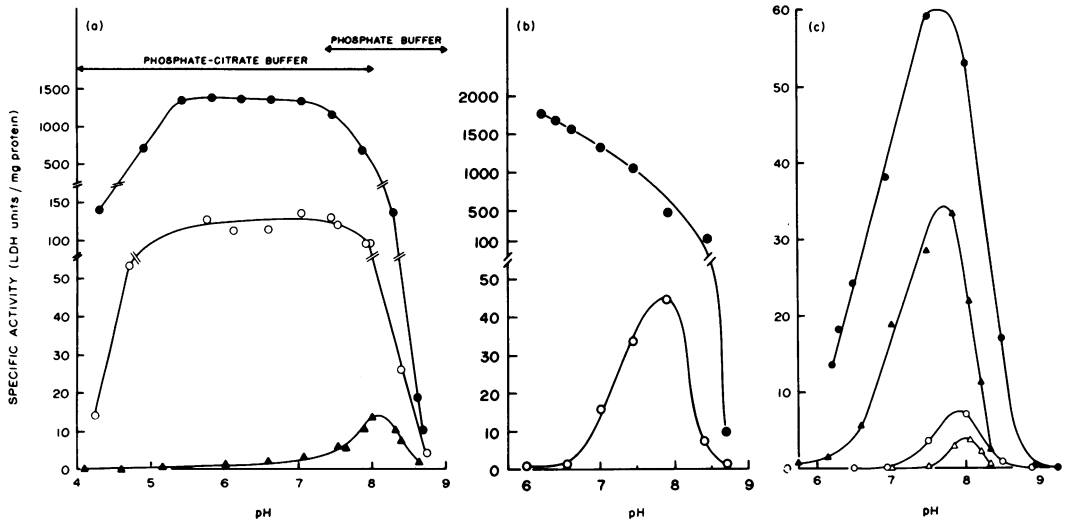

FIG. 2. *Effect of FDP on the pH optima curve for pyruvate reduction (a and b) and lactate oxidation (c) by S. cremoris LDH. The reaction mixture for pyruvate reduction was that described in Table 2 except that in (a) phosphate-citrate buffer was 0.1 M with respect to phosphate. The reaction mixture (total volume, 3 ml) for lactate oxidation contained: 2.1 ml of 0.1 M triethanolamine-hydrochloride or 0.10 M sodium phosphate at the pH values indicated; 0.1 ml of 0.50 M sodium L(+)-lactate; 0.1 ml of water or 0.030 M FDP; and 0.2 ml (0.84 mg of protein) of enzyme solution. (a) In sodium phosphate or sodium phosphate-citrate buffer: no FDP, ▲; 1 mM FDP, ○; 10 mM FDP, ●. (b) In triethanolamine-hydrochloride buffer: no FDP, ○; 1 mM or 10 mM FDP, ●. (c) In triethanolamine-hydrochloride buffer: no FDP, ○; 1 mM FDP, ●. In sodium phosphate buffer: no FDP, △; 1 mM FDP, ▲.*

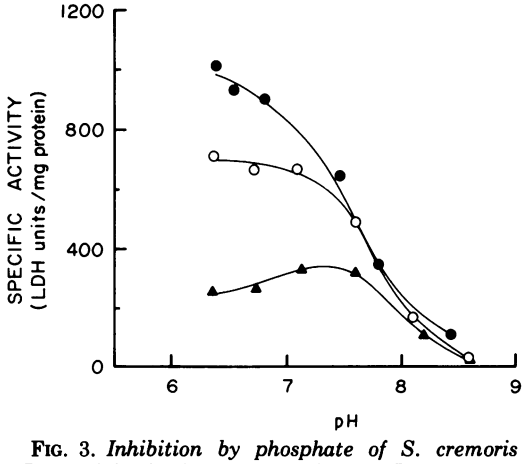

FIG. 3. *Inhibition by phosphate of S. cremoris LDH activity in the presence of 1 mM FDP. The reaction mixture described in Table 2, modified by the addition of 2 ml of 0.10 M triethanolamine-hydrochloride, 0.1 ml of 0.03 M; FDP, and 0.1 ml (3.3 μg of protein) of enzyme, was used. No phosphate, ●; 33 mM sodium phosphate, ○; 67 mM sodium phosphate, ▲.*

TABLE 1. *Acid denaturation of the S. cremoris LDH in the presence of FDP*[a]

pH of incubation mixture	Specific activity (LDH units/mg of protein)
4.0	15
4.5	68
5.0	138
6.0	143
7.0	141
8.0	135

[a] Samples (0.1 ml) of the enzyme solution were added to incubation mixtures containing 2.5 ml of 0.10 M sodium phosphate-citrate at the pH values shown, 0.3 ml of water, and 0.1 ml of 30 mM FDP. After incubation at 25 C for 5 min, 0.1-ml samples were withdrawn and assayed for LDH activity in an assay mixture containing 2.5 ml of 0.10 M sodium phosphate-citrate, (pH 6.5), 0.2 ml of 0.10 M sodium pyruvate, and 0.1 ml of 4 mM NADH.

ation of the enzyme (Table 3). This protective effect could not be ascribed to increasing ionic strength alone, because Na_2SO_4, at equivalent molarities and ionic strengths, did not protect the enzyme to the same extent. FDP, ATP, and glucose-6-phosphate (at a final concentration of 1 mM) gave 80 to 100% protection. The protection provided by glucose-6-phosphate and ATP was not due to hydrolysis of phosphate bonds during incubation. Samples taken before and after incubation at 50 C for 10 min and tested for inorganic phosphate indicated that no hydrolysis had occurred.

The enzyme appeared to be more stable to

TABLE 2. *Stability of the S. cremoris LDH in triethanolamine-hydrochloride and phosphate buffers*[a]

Buffer (0.1 M)	Time of incubation (hr)	Per cent LDH activity remaining after incubation at pH:					
		6.0	6.5	7.0	7.5	8.0	8.5
Triethanolamine-hydrochloride	0	100	100	100	100	100	100
	4	100	100	100	100	44	31
	26	100	97	89	1	4	26
	120	100	100	65	8	5	1
Sodium phosphate	0	100	100	100	100	100	100
	26	100	100	100	100	100	100
	75	76	73	73	57	71	57
	167	63	61	61	57	61	31

[a] The enzyme was diluted to give a protein concentration of 0.45 mg/ml in the above buffers at the pH values shown and stored at 5 C. At intervals, 0.1-ml samples of the enzyme solutions were withdrawn and assayed for LDH activity in a reaction mixture which contained in a total volume of 3.0 ml: 2.6 ml of 0.10 M triethanolamine-hydrochloride, pH 7.0, or 0.10 M sodium phosphate, pH 7.0; 0.2 ml of 0.10 M sodium pyruvate; 0.1 ml of 4 mM NADH; and 0.1 ml of 30 or 300 mM FDP. Results were expressed as per cent of zero-time LDH activity.

heat in phosphate buffer of lower pH. As shown in Table 3 the enzyme was almost completely inactivated at pH 8.0 but showed no loss of activity under the same conditions at pH 6.0. NADH and NAD^+ also protected the enzyme from heat denaturation, the reduced coenzyme providing better protection than the oxidized coenzyme. Sodium pyruvate exerted no protective effect whatsoever. The slight protective effect recorded for sodium L(+)-lactate could be ascribed to ionic strength alone, for the same concentration of NaCl exerted the same degree of protection.

The Michaelis constant (K_m) for pyruvate and NADH was determined according to the method described by Dixon and Webb (2) in a reaction mixture containing triethanolamine-hydrochloride buffer at pH 8.0 in the absence of FDP (Fig. 5) and at pH 6.0 in the presence of 1 mM FDP (Fig. 6). The specific activities of the enzyme were measured at four different pyruvate concentrations at four different concentrations of NADH. By using the method of least squares to ensure accurate plotting of straight line data, four straight lines were obtained by plotting reciprocal specific activities ($1/V$) versus reciprocal pyruvate concentrations (1/pyruvate) at four fixed levels of NADH (Fig. 5a). The four intercepts on the $1/V$ axis ($1/V_{max}$ apparent) were equal to $1/V_{max}$ [1 + ($K_{m\,NADH}$/NADH)]. A secondary plot of the four intercepts versus 1/NADH yielded a straight line with an intercept on the ($1/V_{max}$ apparent) axis equal to $1/V_{max}$, and an intercept on the 1/NADH axis equal to $-(1/K_{m\text{-}NADH})$ (Fig. 5b). An analogous procedure was employed for the determination of $K_{m\ pyruvate}$ (Fig. 5c and 5d). It would appear from the K_m and V_{max} values obtained by the above procedures (cf. Fig. 5 and 6) that FDP caused a change in all three kinetic parameters.

DISCUSSION

Although the lactate dehydrogenases specific for the L(+) isomer of lactic acid from several species of streptococci have been shown to be activated by FDP (1, 7, 8), little is known about the distinguishing properties of the individual enzymes.

As yet, the inhibition of FDP activation by inorganic phosphate, observed in this investigation, appears to be a unique property of the LDH of group N streptococci. Phosphate inhibition of FDP activation might possibly be explained by competition for the binding site of FDP. However, although phosphate does not

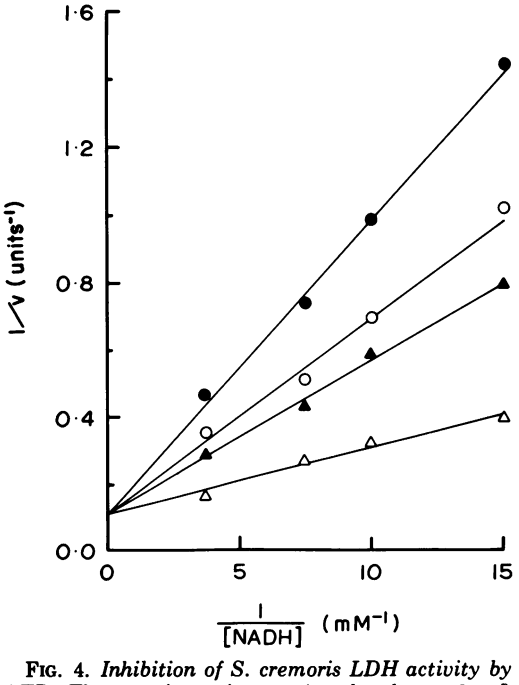

FIG. 4. *Inhibition of S. cremoris LDH activity by ATP. The reaction mixtures (total volume, 3 ml) contained: 2.0 ml of 0.10 M triethanolamine-hydrochloride, pH 8.0; 0.2 ml of 0.10 M sodium pyruvate; 0.1 ml (0.53 mg of protein) of enzyme; NADH at the concentrations shown and ATP in the following concentrations: 3.33 mM, ●; 2.50 mM, ○; 1.67 mM, ▲; 0.83 mM, △.*

TABLE 3. *Effect of phosphate concentration and pH on the rate of heat denaturation of the S. cremoris LDH*[a]

Phosphate buffer		Per cent LDH activity remaining after incubation at 50 C for:	
pH	Molarity (M)	5 min	10 min
6.0	0.02	99	98
7.0	0.01	20	0
	0.02	49	34
	0.05	99	97
8.0	0.02	3	2
	0.05	11	3

[a] The enzyme, contained in sodium phosphate buffers at the concentrations and pH values shown, was heated at 50 C for 5 or 10 min. Samples (0.1 ml) were withdrawn and tested for LDH activity as described in Table 2. Results were expressed as per cent LDH activity of unheated extract.

activate the enzyme, like FDP it affords protection against heat denaturation.

The protection given by FDP, at concentrations as low as 10^{-5} M, against thermal inactivation of the LDH of *S. cremoris* US3, was in direct contrast to that found for the FDP-activated LDH of *Streptococcus faecalis* which became more labile to heat in the presence of FDP (7). The observation that NADH and NAD^+ [but not pyruvate or L(+)-lactate] were able to protect both of these enzymes from heat denaturation, presumably by stabilizing the active site, is consistent with a compulsory binding order of substrate and coenzyme in which the coenzyme is bound first (5). Since ATP was shown to be a competitive inhibitor of NADH, it was not surprising to find that this compound also increased the heat stability of the *S. cremoris* enzyme.

It has been reported that FDP-activated LDH species require the presence of phosphate

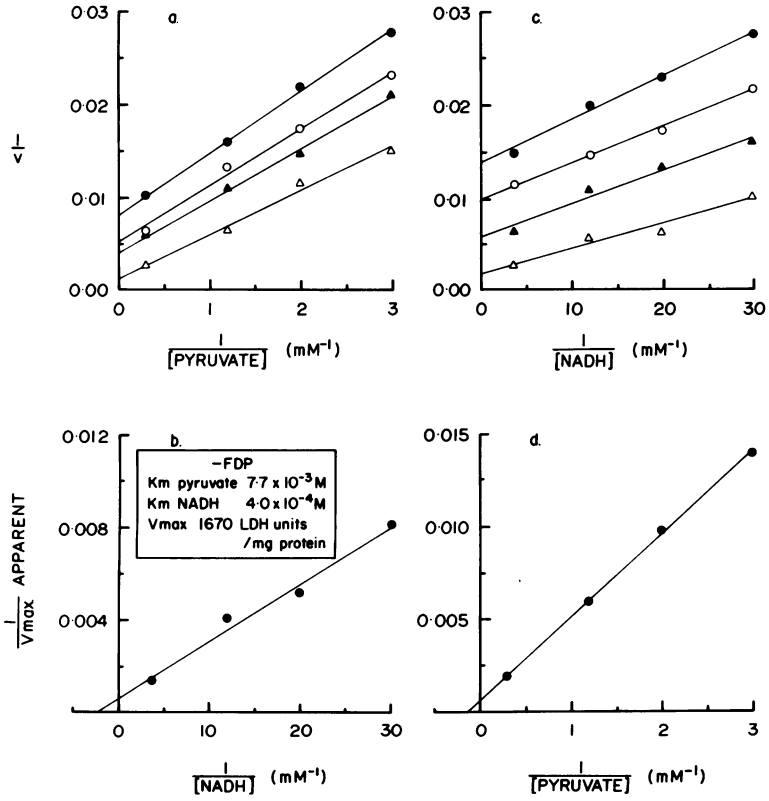

FIG. 5. *K_m values for NADH and pyruvate for S. cremoris LDH in the absence of FDP. The reaction mixtures contained 1.8 ml of 0.10 M triethanolamine-hydrochloride (pH 8.0), 0.1 ml (0.13 mg of protein) of enzyme, and NADH and pyruvate in the following concentrations. (a) NADH—0.033 mM, ●; 0.050 mM, ○; 0.083 mM, ▲; 0.267 mM, △. (c) Pyruvate—0.33 mM, ●; 0.500 mM, ○; 0.833 mM, ▲; 3.33 mM, △. Data for plots b and d were obtained from plots a and c, respectively.*

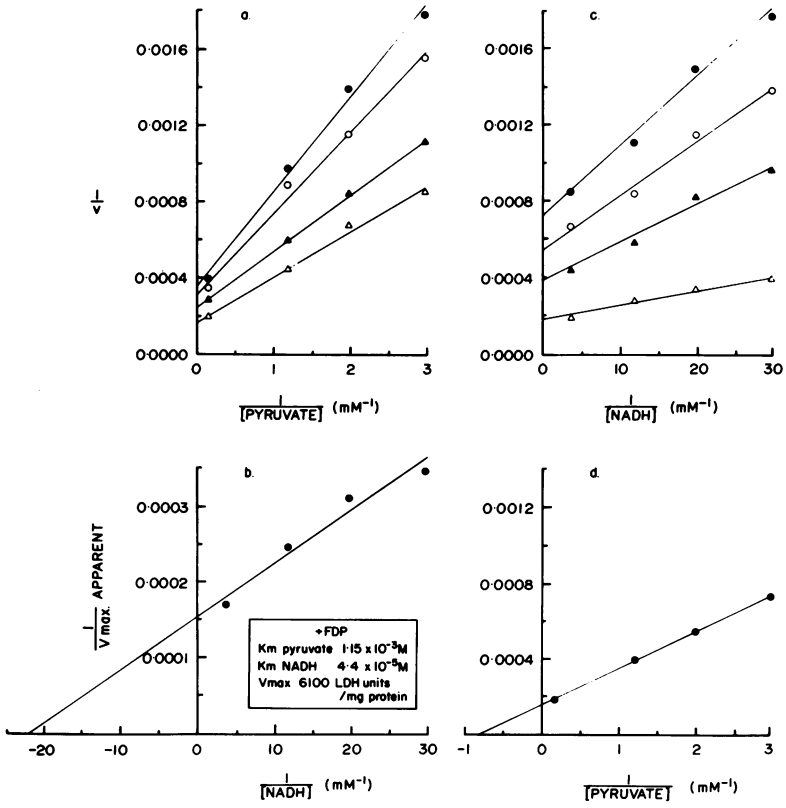

FIG. 6. *K_m values for NADH and pyruvate for S. cremoris LDH in the presence of FDP. The reaction mixtures contained 1.8 ml of 0.10 M triethanolamine-hydrochloride (pH 6.0), 0.1 ml of 0.03 M FDP, 0.1 ml (1.7 μg of protein) of enzyme, and NADH and pyruvate in the following concentrations. (a) NADH—0.033 mM, ●; 0.050 mM, ○; 0.083 mM, ▲; 0.267 mM, △. (c) Pyruvate—0.333 mM, ●; 0.500 mM, ○; 0.833 mM, ▲; 6.67 mM, △. Data for plots b and d were obtained from plots a and b, respectively.*

or FDP for stability on storage (8). In this investigation loss of enzymatic activity occurred on storage in triethanolamine-hydrochloride buffer only above *p*H 6.0 to 6.5. Recent physicochemical studies (3) have shown that a structural unfolding and dissociation of the enzyme occurs on storage in alkaline triethanolamine. This structural change in the enzyme was not observed in alkaline phosphate buffers under the same conditions of *p*H, time, and temperature. This is in keeping with the greater stability of the enzyme in these buffers above *p*H 7.0, as shown in Table 3.

Another characteristic of the LDH of group N streptococci not previously reported for other FDP-activated LDH species is the small peak of activity observed in the region of *p*H 8.0 in the absence of FDP (Fig. 2). One explanation for the appearance of this activity in the region of *p*H 8.0 could be that the conformational changes, which lead to unfolding and dissociation of the enzyme on standing in alkaline triethanolamine, allow binding of the substrates in the absence of FDP. Evidence in support of this hypothesis and of its possible relationship to the action of FDP is given elsewhere (3).

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