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A Specific Mannitol Dehydrogenase from *Lactobacillus brevis**

G. MARTINEZ,† H. A. BARKER,‡ AND B. L. HORECKER

From the Department of Microbiology, New York University School of Medicine, New York 16, New York

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According to the early studies of Fred, Peterson, and Anderson (1), *Lactobacillus* species fall into two major groups, characterized by their capacity to ferment lactose and to produce mannitol in the fermentation of fructose. The homofermentative species, including *Lactobacillus plantarum*, ferment fructose to lactic acid, with only traces of other products. Heterofermentative species, such as *Lactobacillus brevis*, also produce acetic acid and CO_2 and in addition large quantities of mannitol. In these organisms the fermentation balance is represented approximately by the equation (2, 3):

3 D-Fructose → 2 D-mannitol + lactic acid + acetic acid + CO_2

Edson (4) has suggested that the lactobacilli which produce mannitol possess mannitol dehydrogenase, but such an enzyme has not yet been described in these organisms. Other microorganisms contain mannitol dehydrogenases, which do not, however, possess the required properties. Thus, in some organisms the ketose substrate is D-sorbose rather than D-fructose; in others the enzymes lack specificity and will reduce D-fructose to a mixture of D-mannitol and D-sorbitol. Both *Acetobacter suboxydans* and *Candida utilis* possess DPN-linked polyol dehydrogenases which will form either D-mannitol or D-sorbitol from D-fructose (5, 6). Shaw (7) has described an inducible enzyme in a species of *Pseudomonas* which is capable of oxidizing D-sorbitol to D-fructose but shows no activity with D-mannitol. Although the reaction was not studied in the other direction, it must be assumed that the reduction of D-fructose would yield only D-sorbitol. A specific enzyme capable of catalyzing the formation of D-mannitol as the sole product in the reduction of D-fructose has not previously been reported in any organism.

An alternative pathway for the formation of D-mannitol which would involve the phosphorylated intermediates is suggested by the discovery of mannitol 1-phosphate dehydrogenase in *Escherichia coli* (8). This enzyme catalyzes the reaction:

Mannitol 1-phosphate + $DPN^+ \rightleftharpoons$ fructose 1-phosphate + DPNH + H^+

The function of this enzyme in carbohydrate metabolism in *E. coli* remains obscure. It has not been looked for in heterofermentative species, although it has been found in homofermentative species that do not normally form mannitol from fructose.

We have now isolated a crystalline D-mannitol dehydrogenase from *L. brevis* which catalyzes the reversible reduction of D-fructose to D-mannitol:

D-Fructose + DPNH + $H^+ \rightleftharpoons$ D-mannitol + DPN

The enzyme is specific for the substrates shown and its presence would appear to account for the fermentative production of D-mannitol in this species. The enzyme cannot be detected in *L. plantarum*, which does not produce mannitol. The purification and properties of this enzyme and its use for the identification and quantitative determination of D-mannitol are described.

EXPERIMENTAL PROCEDURE

Lactobacillus brevis strain ATCC 367 was obtained from the American Type Culture Collection. Lactic dehydrogenase was purchased from Böhringer and Söhne. Protamine sulfate was obtained from the Elanco Products Company. Commercial mannitol was purified by recrystallization from alcohol. Other substances were commercial preparations.

Bacterial growth was measured turbidimetrically at 660 mμ in a Zeiss spectrophotometer. Fructose was determined by the method of Roe (9) and glucose with glucose oxidase (10). Mannitol was determined by periodate oxidation (11) or enzymatically as described below. Protein was determined by the method of Bücher (12). Lactic dehydrogenase was assayed by the procedure described by Kornberg and Pricer (13). Other optical density measurements were made with the Beckman model DU spectrophotometer.

Fructose and mannitol were standardized by enzymatic methods with mannitol dehydrogenase under conditions in which the reaction would proceed to completion. For fructose the reaction mixture (1.0 ml) contained 0.1 M sodium acetate buffer (pH 6.0), 0.1 mM DPNH, 0.05 mM D-fructose, and mannitol dehydrogenase (0.02 mg of protein, specific activity = 100 units per mg). The decrease of DPNH absorption at 340 mμ was equivalent to the amount of fructose originally present. For mannitol, the reaction mixture (0.30 ml) contained 0.02 M Tris buffer (pH 8.0), 7.5 mM sodium pyruvate, 2.0 mM DPN, 0.01 mg of lactic dehydrogenase, mannitol dehydrogenase (0.2 mg of protein, specific activity = 100 units per mg), and 1.0 mM mannitol. The mixture was left at room temperature for 180 minutes and fructose was determined colorimetrically (9).

Enzyme Assay—Mannitol dehydrogenase activity was measured by following the rate of oxidation of DPNH by fructose. The incubation mixture (1.0 ml) contained 0.02 M sodium acetate buffer (pH 5.35), 0.1 mM DPNH, 0.1 M D-fructose, and sufficient diluted enzyme solution to produce an absorbancy change of about 0.010 in 30 seconds. The reaction was started by addition of substrate and read every 30 seconds for 3 minutes. Enzyme dilutions were made in 0.05 M sodium acetate buffer (pH 6.0),

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† Fellow of the Organization of American States. Present address, Instituto de Investigacion de Ciencias Biologicas, Avenida Italia 3318, Montevideo, Uruguay.

‡ Guggenheim Fellow and Visiting Professor of Microbiology, New York University School of Medicine. Present address, Department of Biochemistry, University of California, Berkeley.

containing 2×10^{-4} M mercaptoethanol and 1% bovine serum albumin. A unit of enzyme was defined as the quantity required to produce a change in optical density of 1.0 per minute at 340 mμ.

Culture of Organism—Cultures of *Lactobacillus brevis* (ATCC 367) were obtained in lyophilized form. The growth medium (14) contained 0.5% yeast extract, 1.0% nutrient broth, 1.0% sodium acetate·$3H_2O$, 0.001% NaCl, 0.001% $FeSO_4 \cdot 7H_2O$, 0.02% $MgSO_4 \cdot 7H_2O$, 0.001% $MnSO_4 \cdot 4H_2O$, and 1.0% fructose. The organisms were maintained on 2% agar stabs of this composition and transferred every 15 days.

Cultures were grown in liquid medium by incubation at 32° without aeration. The substitution of glucose for fructose in the growth media did not alter the yield of cells but reduced the enzyme activity in the crude extract to about 5% of that found in the fructose medium. For preparation of large amounts of cells for enzyme purification, 10 ml of a 24-hour culture in broth were inoculated into 1 liter of fresh broth which served 24 hours later as inoculum for 16 liters of broth in a 20-liter bottle. After incubation for about 17 hours, at which time nearly all of the fructose had been consumed, the cells were collected by centrifugation in a Sharples supercentrifuge. The harvested cells were washed twice with 150-ml portions of 0.05 M sodium phosphate buffer (pH 6.5) and the packed cell paste stored at −16°. About 2.0 g of cells (wet weight) were obtained per liter of culture.

Purification Procedure—All operations were performed at 0° unless otherwise stated. About one-third of the cells produced in 16 liters of culture were employed for each preparation. Three 3.5-g portions of frozen cell paste were each suspended in 7.0 ml of 0.005 M sodium phosphate buffer (pH 6.5) containing 2×10^{-4} M mercaptoethanol and shaken with 8 g of glass beads in the Nossal tissue disintegrator (15) for four 20-second periods, between which the cartridges were cooled in ice. The suspensions were then centrifuged for 10 minutes at 10,000 × g. The residues were washed twice with a total of 20 ml of the same buffer, and the washings combined with the first supernatant solution (*Extract*, 34 ml, Table I).

The clear extract was treated with 6.0 ml of 2% protamine sulfate solution and centrifuged after 20 minutes at 0°. The precipitate was discarded. To the supernatant solution (*protamine fraction*, 35 ml, Table I), were added 3.0 ml of 0.5 M sodium phosphate buffer (pH 6.5) and 12.85 g of ammonium sulfate. After 10 minutes the precipitate was removed by centrifugation and discarded. The clear solution (42 ml) was treated with 2.86 g of ammonium sulfate; the precipitate was collected after 10 minutes by centrifugation and dissolved in 7.5 ml of 0.05 M sodium phosphate buffer, pH 6.5 (*ammonium sulfate I*, 7.5 ml, Table I). To this solution was added 0.7 ml of 0.5 M sodium phosphate buffer (pH 6.5) containing 2×10^{-4} M mercaptoethanol. The diluted solution was heated in a water bath at 63–65° for 5 minutes, centrifuged for 10 minutes at 10,000 × *g*, and the precipitate discarded. The ammonium sulfate concentration in the supernatant solution (*heated fraction*, 7.3 ml, Table I) was determined with a Barnstead PR2 conductivity meter and adjusted to 70% saturation by addition of solid ammonium sulfate. After 10 minutes, the precipitate was collected by centrifugation at 10,000 × *g* for 5 minutes. This precipitate was extracted with 4.0 ml of 65% saturated ammonium sulfate (pH 7.0), and after 10 minutes the precipitate was again collected by centrifugation and extracted successively as before with 3.0 ml and 2.0 ml of 60% saturated ammonium sulfate. The last two extracts were combined (*ammonium sulfate II*, 5.1 ml, Table I).

TABLE I

Purification of mannitol dehydrogenase

Fraction	Total units	Specific activity
		units/mg
Extract	4500	4.7
Protamine fraction	4300	17.0
Ammonium sulfate I	2700	30.5
Heated fraction	1900	53.5
Ammonium sulfate II	1550	104
First crystals	900	160
Second crystals	700	180
Third crystals	500	170

Crystallization—The ammonium sulfate II solution was adjusted to pH 6.0 with 0.1 N acetic acid, and a solution of saturated ammonium sulfate was added until faint turbidity was observed. After standing overnight at 3° the crystals were collected by centrifugation and dissolved in 1.6 ml of 0.1 M sodium acetate buffer (pH 6.0) containing 2×10^{-4} M mercaptoethanol (*first crystals*, 1.6 ml, Table I). This procedure was repeated (*second crystals*, 1.6 ml, Table I) and, after centrifugation to separate denatured proteins, repeated again (*third crystals*, 1.6 ml, Table I). Finally mercaptoethanol (2×10^{-4} M) and ammonium sulfate were added until crystallization was begun, and the crystal suspension was stored at 3°. Because the crystals obtained in this manner were very transparent, for the purpose of visualization another crystallization was carried out in the presence of 10^{-6} M methylene blue (Fig. 1).

Properties of the Enzyme

Stability—The crude extracts show a rapid loss of activity at 2° (Fig. 2). These preparations had been stored for 2 weeks and the specific activity had fallen from an initial value of 5.2 units per mg to 1.9 units per mg. It was brought to 70% of the original value by incubation with 10^{-3} M mercaptoethanol at 37°. The purified enzyme is most stable in solution at pH 6.0 in a 0.02 M sodium acetate buffer containing 10^{-3} M mercaptoethanol. Under these conditions at room temperature we observed a loss of only 5% of activity in 5 hours. Such solutions can be stored for weeks at −16°. The enzyme solutions lost no activity when dialyzed at 3° against oxygen-free water containing 2×10^{-4} M mercaptoethanol for a period of 18 hours. Suspensions of the crystalline enzyme preparations kept in ammonium sulfate at 3° lost about 20% of their activity in 2 months. This loss was largely reversed by the addition of mercaptoethanol to 2×10^{-4} M followed by warming to 37° for 1 hour.

Effect of pH on Reaction Rate—The optimal pH for the conversion of fructose to mannitol, the reaction utilized in the enzyme assay, was 5.35. The optimal pH for the reverse reaction, the conversion of mannitol to fructose, was 8.6 (Fig. 3). These differences in pH curves for the forward and back reactions may be attributed to the participation of H^+ in the reaction.

Specificity—The enzyme was found to be specific for D-fructose and D-mannitol. It did not catalyze oxidation of the following polyols: glycerol, erythritol, xylitol, D-sorbitol, D-arabitol, L-arabitol, dulcitol, inositol, ribitol, milibitol, glycero-D-glucoheptitol. The enzyme did not catalyze reduction of the following sugars: D-sorbose, D-xylulose, D-ribulose, sedoheptulose, fructose

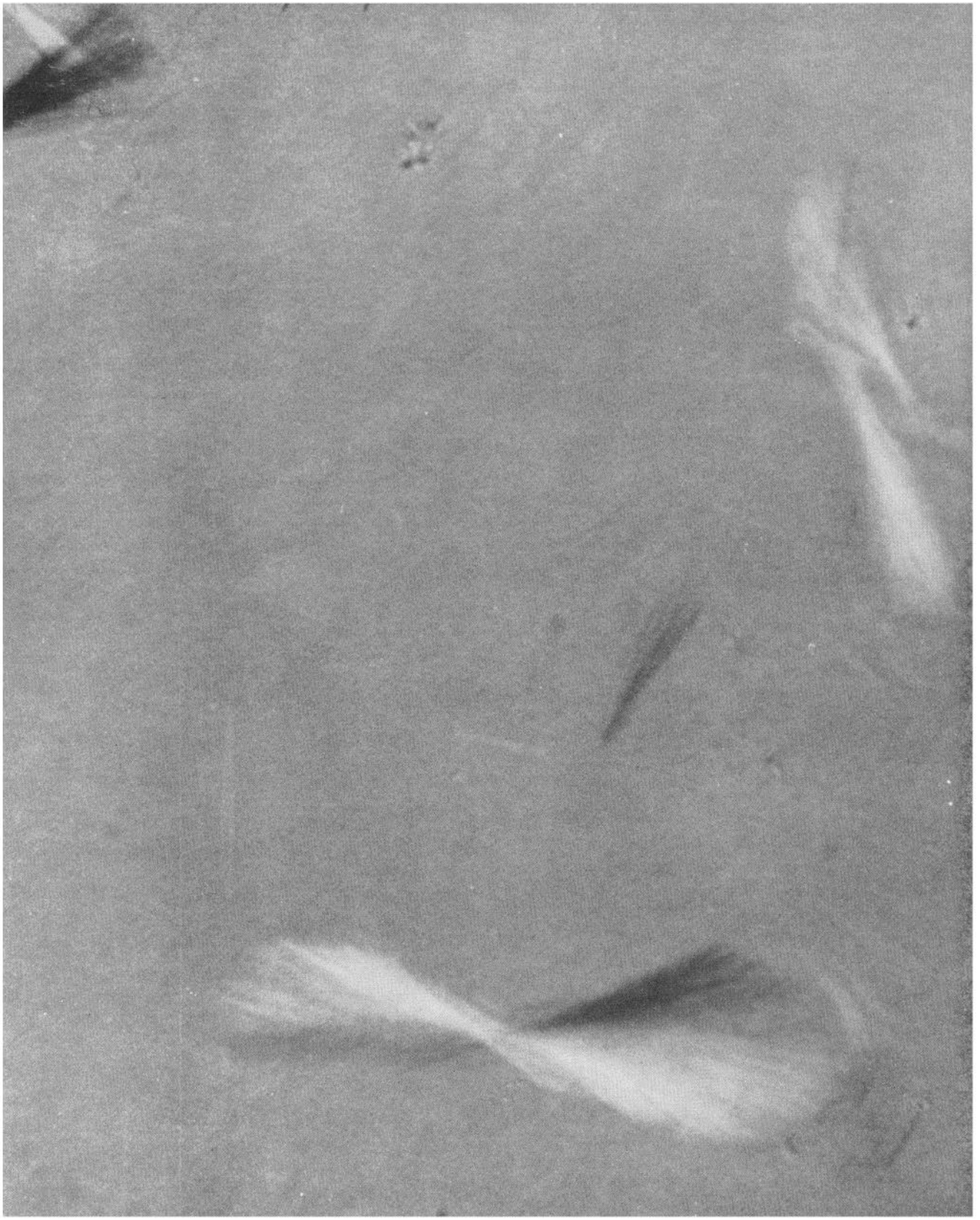

FIG. 1. Crystals of mannitol dehydrogenase purified from *L. brevis*. The crystals (colored with methylene blue) were photographed in polarized light; therefore, those crystals in the plane of polarization appear light, the others appear dark. The longest crystals in this photograph have a length of approximately 8 μ.

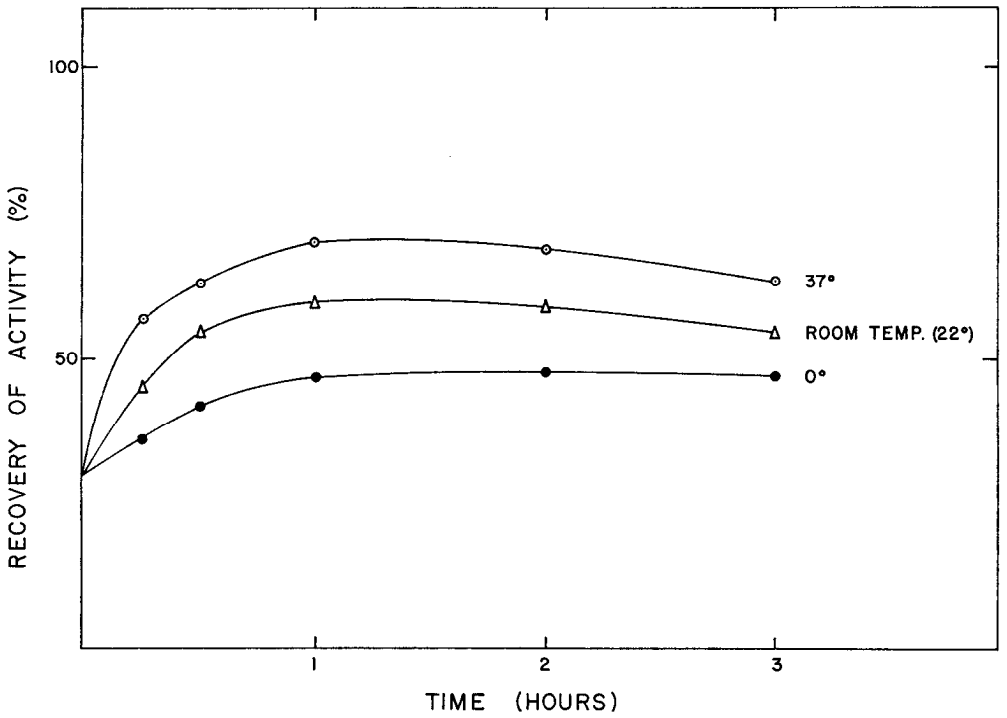

FIG. 2. Reactivation of stored extracts on incubation with mercaptoethanol. The extracts were prepared as described in the tex and contained 18 mg of protein per ml. Mercaptoethanol (10^{-3} M) was added at time zero.

6-phosphate, glucose. TPNH will replace DPNH in the reduction of fructose; with the standard assay conditions the reaction rate is about one-half as fast with this coenzyme. In the reaction between oxidized coenzyme and mannitol at pH 8.6 (see below) the rate with TPN is only 2.5% that with DPN. The kinetic relations suggest that TPN may be inhibitory, but this effect has not been further explored.

Effect of Substrate and Coenzyme Concentration—The Michaelis constants were determined following the method of Florini and Vestling (16). Values for fructose and mannitol were calculated to be 0.07 M and 0.06 M, respectively. For DPN and DPNH the corresponding values were calculated to be 2.3×10^{-4} M and 1.3×10^{-4} M, respectively.

Equilibrium Constants—The equilibrium constant for the reaction:

$$\text{D-Mannitol} + \text{DPN}^+ \rightleftharpoons \text{D-fructose} + \text{DPNH} + \text{H}^+$$

was determined by following changes in the DPNH:DPN ratios (Fig. 4).

The equilibrium constant was calculated to be approximately 5.3×10^{-9} (Table II). At pH 7 and with 50% of DPN present in the reduced form, about 5% of the total substrate is present as ketose. The equilibrium constant is in the same range as that obtained with the polyol dehydrogenase of *Candida utilis* (6).

Enzyme Activity in Glucose-grown Cells—The enzyme appears to be induced by growth on fructose (Table III). Cells harvested following growth on glucose yielded extracts with little enzyme activity, although growth on this carbon source was excellent.

Absence of Activity in L. plantarum—Extracts of *L. plantarum* grown on fructose contained no detectable dehydrogenase. Assay of the crude extracts was difficult owing to the presence

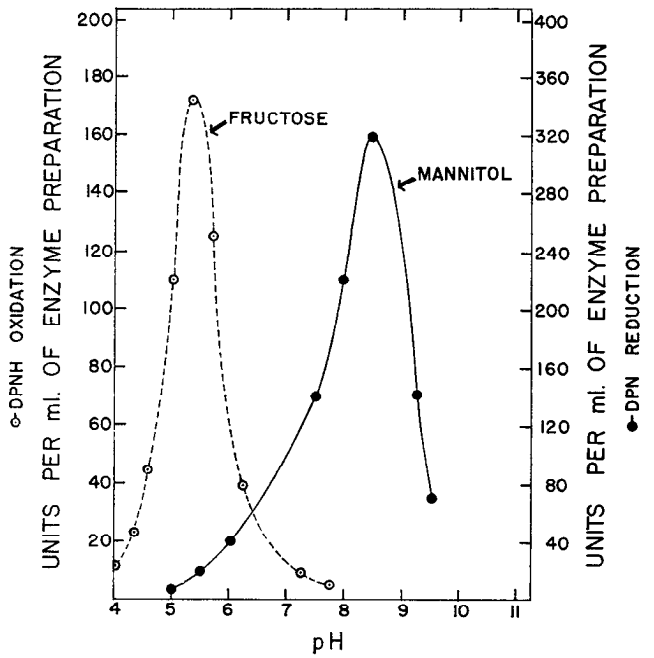

FIG. 3. Effect of pH on the reaction with fructose (⊙-----⊙) or mannitol (●——●) as substrate, In the former case, the incubation mixture (1.0 ml) contained 20 mM buffer, 0.1 M D-fructose, 0.1 mM DPNH, and mannitol dehydrogenase (specific activity = 180 units per mg, 0.2 μg of protein). For the reaction with D-mannitol, the incubation mixture (1.0 ml) contained 40 mM buffer, 0.05 M D-mannitol, 0.8 mM DPN, and mannitol dehydrogenase (specific activity = 110 units per mg, 0.6 μg of protein). For the pH range 4 to 7, the buffer was sodium acetate buffer; for the pH range 6.2. to 80, Tris buffer; for the range 8.0 to 9.5, glycylglycine buffer.

of an active DPNH oxidase. This was destroyed by heating to 60° for 5 minutes or removed by precipitation with protamine sulfate, as in the first step employed with the *L. brevis* extract. Following either of these procedures, no activity with mannitol and DPN or fructose and DPNH was present. The *L. brevis* enzyme activity was not inhibited by these preparations.

Determination of Small Amounts of Mannitol—Since this enzyme is highly specific for mannitol it can be used for the assay of this substance. Although direct spectrophotometric measurement of DPN reduction was not possible, owing to the low affinity of the enzyme for the substrate, mannitol could be assayed by employing a system in which oxidation of this substrate was coupled to the reduction of pyruvate. Both mannitol dehydrogenase and lactic dehydrogenase were added (Fig. 5).

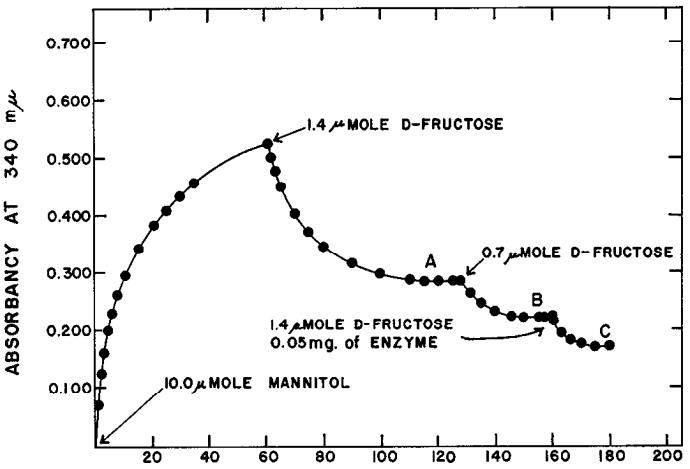

FIG. 4. Determination of the equilibrium constant. The reaction was carried out at 22°. The incubation mixture (1.0 ml) contained 0.16 M Tris buffer (pH 7.2), 10 mM mannitol, 0.11 mM DPN, and 0.05 mg of enzyme (specific activity = 130 units per mg). The pH of the solution was determined for each equilibrium solution. D-Fructose and enzyme were added as indicated. The reaction was followed at 340 mμ.

TABLE II

Equilibrium constants

Equilibrium mixture	K_{eq}*
First (A)	5.0×10^{-9}
Second (B)	6.6×10^{-9}
Third (C)	4.3×10^{-9}
Mean	5.3×10^{-9}

$$* K_{eq} = \frac{(\text{fructose})\ (\text{DPNH})\ (H^+)}{(\text{mannitol})\ (\text{DPN})}$$

TABLE III

Enzyme activity in cells grown on fructose or glucose

Growth on*	Enzyme activity	Protein in extract
	units/g cells (wet weight)	*mg/g cells (wet weight)*
Glucose	23	62.0
Fructose	420	80.0

* Cells were grown at 32° on 1% glucose or 1% fructose as described in the text. One liter of growth medium yielded 2.5 g of washed cell mass (wet weight) in growth on fructose for 18 hours and 2.0 g of cell mass (wet weight) in growth on glucose for 24 hours. Extracts were prepared as described in the text.

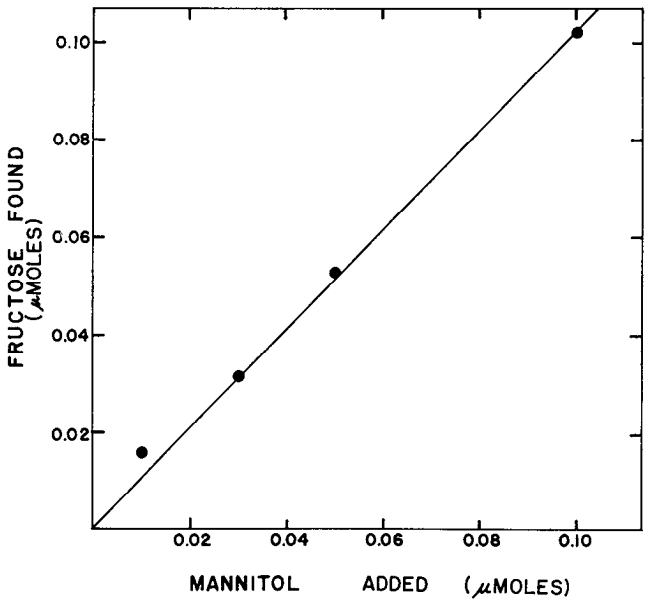

FIG. 5. Determination of mannitol. The incubation mixtures (0.3 ml) contained 7.5 mM sodium pyruvate, 2 mM DPN, 20 mM Tris buffer (pH 8.0), 0.01 mg of lactic dehydrogenase, mannitol dehydrogenase (specific activity = 140 units per mg, 0.06 mg of protein), and mannitol in the quantities indicated. The mixture was incubated at 23° for 3 hours and the fructose formed was determined colorimetrically.

$$\text{Mannitol} + \text{pyruvate} \xrightarrow{\text{DPN}} \text{fructose} + \text{lactate}$$

In the presence of excess pyruvate and catalytic quantities of DPN, the quantity of fructose formed was equal to the quantity of mannitol added. Fructose was determined with the Roe reaction (9). With this procedure, quantities of mannitol as small as 0.02 to 0.03 μmole can be determined with good precision, even in the presence of large quantities of other polyols.

DISCUSSION

The production of D-mannitol in the fermentation of D-fructose by *Lactobacillus brevis* has now been shown to be catalyzed by an inducible mannitol dehydrogenase. The enzyme is present in extracts of fructose-grown cells in quantities equivalent to about 3% of the soluble protein. It is absent from the non-mannitol-producing strain *L. plantarum*. The formation of mannitol from fructose, therefore, appears to depend upon the presence of this enzyme.

The crystalline enzyme is highly specific for mannitol and for fructose, unlike similar dehydrogenases from other species, which attack a variety of polyols and ketoses of variable chain length. This property makes the enzyme useful for the determination of mannitol, and a procedure for this determination is described.

SUMMARY

A specific and very active mannitol dehydrogenase has been isolated from extracts of *Lactobacillus brevis*. The enzyme has

been purified about 35-fold and obtained in crystalline form. It catalyzes the reaction

$$\text{Fructose} + \text{DPNH} + \text{H}^+ \rightleftharpoons \text{mannitol} + \text{DPN}$$

The equilibrium constant for the reaction with DPN has been determined and agrees with values obtained previously with similar enzymes from other sources.

The Michaelis constants for fructose and mannitol are of the order of 0.06 M. A method for the determination of mannitol has been developed which depends upon coupling the oxidation of mannitol to the reduction of pyruvate in the presence of mannitol dehydrogenase and lactic dehydrogenase. With this method quantities of mannitol as small as 0.02 μmole can be determined.

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G. Martinez, H. A. Barker and B. L. Horecker

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