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Mannitol and Mannitol Dehydrogenases in Conidia of *Aspergillus oryzae*

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ABSTRACT

HORIKOSHI, KOKI (The Institute of Physical and Chemical Research, Tokyo, Japan), SHIGEJI IIDA, AND YONOSUKE IKEDA. Mannitol and mannitol dehydrogenases in conidia of *Aspergillus oryzae*. J. Bacteriol. **89**:326–330. 1965.—A sugar alcohol was isolated from the conidia of *Aspergillus oryzae* and identified as D-mannitol. Two types of D-mannitol dehydrogenases, nicotinamide adenine dinucleotide phosphate-linked and nicotinamide adenine dinucleotide-linked, were found in the conidia. Substrate specificities, *p*H optima, Michaelis-Menton constants, and the effects of inhibitors were studied. D-Mannitol was converted to fructose by the dehydrogenases. Synthesis of D-mannitol dehydrogenases was not observed during germination; the content of D-mannitol decreased at an early stage of germination. It was assumed, therefore, that D-mannitol might be used as the source of endogenous respiration and provide energy for the germination.

Germinating fungal conidia are characterized by high levels of endogenous respiration (Yanagita, 1964), but the substrate which sustains this respiration has not been studied much. D-Mannitol was isolated from conidia of *Aspergillus niger* by Sumi (1928), and Takebe (1960) proposed D-mannitol as a possible substrate for endogenous respiration.

The present study was undertaken to investigate the physiological role of D-mannitol in germinating conidia of *A. oryzae*. A large amount of D-mannitol is contained in the conidia and is consumed rapidly in the early stages of germination. D-Mannitol dehydrogenase is also found in the conidia, and it appears that the sugar alcohol is used as the substrate for endogenous respiration after it is converted to fructose by the enzymes. Evidence supporting this view is presented.

MATERIALS AND METHODS

Organism and cultivation. Conidia of *A. oryzae* no. 13 were kindly donated by A. Kuninaka of Yamasa Syoyu Co., Ltd., Chiba, Japan. A 2-g amount of the conidia was inoculated into 1 liter of germination medium of the following composition: glucose, 3%; KH_2PO_4, 0.1%; $MgSO_4 \cdot 7H_2O$, 0.05%; KCl, 0.05%; NH_4NO_3, 0.3%; polypeptone, 0.1%; $FeSO_4 \cdot 7H_2O$, 0.001%. The cultures were incubated at 26 C on a rotary shaker operating at 170 rev/min.

Chromatography. Sugars and sugar alcohols were chromatographed on Toyo Roshi no. 51 paper with the following solvent systems: ethyl acetate-pyridine-water (120:50:40, v/v), isopropanol-water (160:40, v/v), and phenol-ammonia-water (160 g:1 ml:40 ml). Ammoniacal silver nitrate was used as a spraying reagent. The R_G value gives the rate of movement of the compound compared with that of glucose, i.e., R_G for glucose is 1.0.

Assay of D-mannitol dehydrogenase activity. To assay the forward reaction, 1 ml of 0.1 M mannitol solution, 1.0 ml of 0.2 N glycine-NaOH buffer (*p*H 9.8), and 0.1 ml of enzyme solution (about 10 to 200 units) were placed in a cuvette with a 1-cm light path; 0.1 ml of 0.01 M nicotinamide adenine dinucleotide (NAD) or nicotinamide adenine dinucleotide phosphate (NADP) was added and mixed by inversion. At 30-sec intervals, the optical density at 340 mμ was measured at room temperature. For assay of the reverse reaction, 1 ml of 0.1 M fructose solution, 0.1 ml of buffer solution [0.1 M acetate buffer (*p*H 6.0) for NADP-linked enzyme, and 0.1 M tris(hydroxymethyl)aminomethane (Tris) buffer (*p*H 7.0) for NAD-linked enzyme], and 0.05 ml of 0.001 M reduced NADP ($NADPH_2$) or reduced NAD ($NADH_2$) solution were placed in a cuvette; 0.1 ml of enzyme solution (about 300 to 400 units) was added to the mixture, and the reduction of optical density at 340 mμ was measured.

Definition of unit. One unit of the mannitol dehydrogenase is that amount of enzyme which will produce an initial rate of change of optical density of 0.001 per min at 340 mμ under standard conditions. Specific activity is expressed as units per milligram of protein.

Quantitative analysis of D-mannitol in conidia. Conidia (10 mg) were collected by centrifugation, washed three times with water, suspended in 1

ml of water, and boiled at 100 C for 15 min to extract mannitol. This procedure was repeated three times. The consecutive extracts were combined and concentrated in vacuo to 1 ml. Mannitol content in the extract was assayed as follows. To a photometric cuvette were added 2 ml of 0.2 N glycine-NaOH buffer (*p*H 9.8), 0.1 ml of 0.1 M NAD, 0.1 ml of mannitol dehydrogenase solution (about 200 to 300 units), and, finally, 0.2 ml of the extract from the conidia. In parallel, a cuvette containing 0.2 ml of D-mannitol solution (30 to 150 μg) instead of the extract was prepared as the standard solution. The cuvettes were incubated at 30 C for 60 min, and the decrease in optical density at 340 mμ was measured. A linear relationship was found between the change in optical density and the amount of mannitol at least up to 150 μg. Fructose (200 μg), when added to the standard solution (150 μg as mannitol), caused no difference.

Analytical method. Reducing sugar was determined by Somogyi's (1952) method. Hexosamine was determined by the Elson-Morgan method, as described by Tracey (1955).

RESULTS

Isolation of D-mannitol from conidia of A. oryzae. Conidia (2 g) were disintegrated in a Nossal (1953) disintegrator for 2 min. The mixture of ruptured conidia and glass beads was immediately heated in boiling water for 10 min and then subjected to centrifugation at 7,000 × *g* for 10 min. To the clear, brownish supernatant, 2 volumes of ethanol were added, and the mixture was stored overnight in a refrigerator. The precipitate was removed by centrifugation at 5,000 × *g* for 10 min. The supernatant fluid was concentrated in vacuo until white crystals began to appear. The crystals were recrystallized from hot ethanol. About 105 mg of white orthorhombic needles with a melting point of 165 to 169 C were obtained. The crystals were nonreducing and possessed a rotation of +25° (100 mg of crystals plus 128 mg of borax plus 1 ml of water); no hexosamine was detected.

Analysis: $C_6H_{14}O_6$; calculated: C, 39.56; H, 7.74

found: C, 39.1; H, 7.6

The infrared spectra of the crystal and the authentic D-mannitol were identical and exhibited characteristic peaks at 1,420, 1,286, 1,082, 1,020, 883, and 697 cm^{-1}. The R_G values of the compound on paper chromatograms with different solvent systems are shown in Table 1. The compound has typical R_G values for D-mannitol. In addition, a solution of these crystals was oxidized by the D-mannitol dehydrogenase at the same rate as was the authentic D-mannitol.

TABLE 1. *R_G values* of the sugar alcohol isolated from conidia of Aspergillus oryzae*

Solvent system	Sugar alcohol from conidia	Authentic D-mannitol
Ethyl acetate-pyridine-water...	0.92	0.92
Isopropanol-water.....	1.10	1.15
Phenol-water.....	1.15	1.17

* Rate of movement of the compound compared with that of glucose.

These results indicate the identity of the crystalline compound with D-mannitol.

Preparation of D-mannitol dehydrogenases from conidia of A. oryzae. A spore paste (1 g of dried conidia) to which 1 g of alumina (500 mesh) had been added was ruptured by hand grinding for 5 min with a chilled mortar and pestle. The ground paste was suspended in 10 ml of 0.1 M acetate buffer (*p*H 6) and centrifuged at 10,000 × *g* for 10 min. A pale brownish supernatant fluid was obtained. The supernatant fluid was cooled to 0 C and brought to 0.3 saturation with ammonium sulfate. The precipitate was removed by centrifugation, and then the supernatant was brought to 0.7 saturation with ammonium sulfate. The precipitate collected by centrifugation was dissolved in 15 ml of 0.1 M acetate buffer (*p*H 6) and dialyzed against the same buffer. The resulting solution containing 95% of the original activity was stored at −20 C. Unless stated otherwise, the following experiments were performed with this enzyme solution. The preparation thus obtained exhibited no activity of $NADH_2$ or $NADPH_2$ oxidase.

Coenzymes of D-mannitol dehydrogenases. Under the conditions described above, D-mannitol dehydrogenase activities were examined in the presence or absence of NAD or NADP. NAD and NADP were reduced by the enzyme preparation in the presence of D-mannitol (Fig. 1). The specific activity of the NAD-linked D-mannitol dehydrogenase was about 25% of that of the NADP-linked enzyme.

Kinetics of D-mannitol dehydrogenases. In the following experiments, an enzyme solution with 50 to 150 units of dehydrogenase activity was used. A linear relationship was observed between the rate of coenzyme reduction and the concentration of enzyme solution under these conditions. The optimal *p*H for NADP-linked enzyme was 9.0 and that for NAD-linked enzyme was 9.8 (Fig. 2). The enzyme solution was dissolved in different buffers (*p*H 7.6 and 5.6) and heated at various temperatures for 3 min. The dehydrogenase activities were determined by the methods

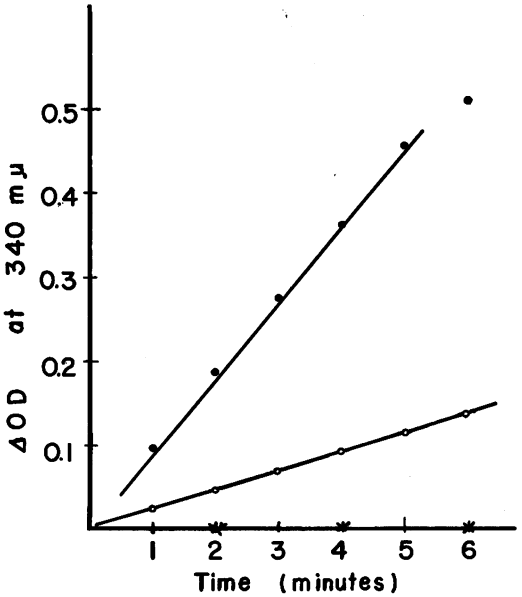

FIG. 1. *Dehydrogenation of D-mannitol by NAD-linked and NADP-linked enzymes. Symbols: ●, NADP-linked; ○, NAD-linked; ×, without coenzyme. Buffer (1 ml), 0.1 ml of enzyme, 0.1 ml of coenzyme, and 1.0 ml of D-mannitol were mixed, and incubated at room temperature. The change in optical density at 340 mμ was measured.*

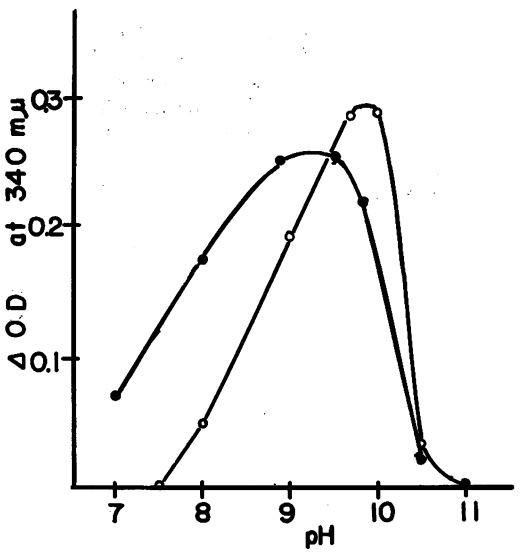

FIG. 2. *Effect of pH on enzyme activities. Symbols: ○, NAD-linked; ●, NADP-linked. Buffer (1 ml), 0.1 ml of enzyme (150 units of NAD-linked enzyme and 130 units of NADP-linked enzyme), and 1.0 ml of D-mannitol were mixed, and incubated at room temperature for 2 min. The change in optical density at 340 mμ was measured.*

TABLE 2. *Heat stability of D-mannitol dehydrogenases**

pH	Temp	Residual activity (%)	
		NAD-linked	NADP-linked
	C		
7.6	60	0	0
7.6	53	0	20
7.6	41	0	100
7.6	37	80	100
5.6	60	0	0
5.6	53	0	0
5.6	41	95	70
5.6	37	110	100

* The enzyme solution was added to an equal volume of buffer solution (pH 7.6 and 5.6), and placed at 60, 53, 41, and 37 C for 3 min. Enzymatic activity was determined under standard conditions.

TABLE 3. *Substrate specificities of D-mannitol dehydrogenases in the presence of NAD or NADP*

Coenzyme	Mannitol	Sorbitol	Inositol	Ducitol	Arabitol
	%	%	%	%	%
NAD.....	100	35	4	10	41
NADP.....	100	7	2	4	4

* Buffer (1.0 ml), 0.1 ml of enzyme, 0.1 ml of coenzyme solution, and 1.0 ml of substrate (0.1 M) were mixed, and were incubated at room temperature for 30 sec. The change of optical density at 340 μ was measured. The activity for mannitol is expressed as 100%.

described above. The NADP-linked enzyme was more heat-stable at pH 7.6 than was the NAD-linked enzyme (Table 2). The Michaelis constants were calculated by the method of Lineweaver and Burk (1934). The values were 6×10^{-2} M in the presence of NADP and 5×10^{-2} M in the presence of NAD. Chromatographic analysis showed the D-mannitol was converted to fructose by the enzyme.

Substrate specificities of the enzymes. Specificities of the enzymes were examined by substituting various sugar alcohols for D-mannitol. No reduction of NADP occurred with sorbitol, inositol, arabitol, and dulcitol (Table 3). On the other hand, arabitol and sorbitol were oxidized to some extent in the presence of NAD.

Inhibition of enzyme activities by chemical agents. The effect of several possible inhibitors was studied by comparing the velocity of the uninhibited reduction of NAD and NADP in the presence of D-mannitol with the velocity of the same reaction in the presence of inhibitors. Table

4 shows that both the NADP-linked and NAD-linked enzymes were strongly inhibited by *p*-chloromercuribenzonate (5×10^{-5} M), but not by iodoacetate (10^{-3} M). Ethylenediaminetetraacetate (10^{-3} M) had no effect; however, 8-hydroxyquinoline (2×10^{-3} M) inhibited NAD-linked enzyme.

TABLE 4. *Effect of inhibitors on enzyme activity*

Inhibitor	Per cent inhibition	
	NAD-linked enzyme	NADP-linked enzyme
None	0	0
8-Hydroxy-quinoline (2×10^{-3} M)	93	20
Monoiodo acetic acid (10^{-3} M)	7	0
Ethylenediaminetetraacetate (10^{-3} M)	7	0
p-Chloromercuribenzoate (5×10^{-5} M)	100	75

* Buffer (1 ml, with or without inhibitor), 0.1 ml of coenzyme, and 1.0 ml of mannitol were mixed, and were incubated at room temperature for 30 sec. The change in optical density at 340 mμ was measured.

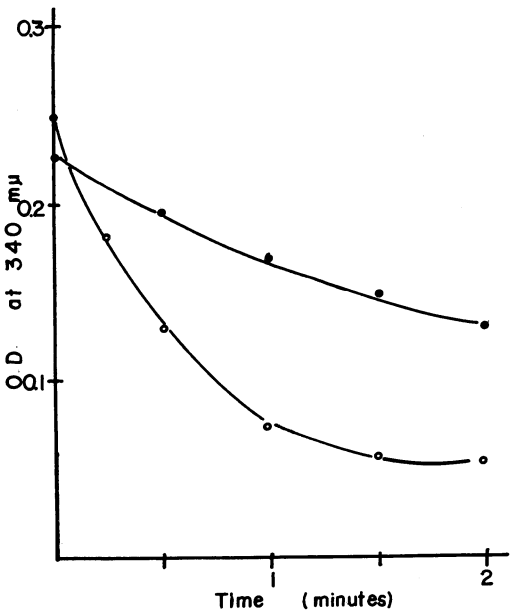

FIG. 3. *Reduction of fructose by the enzymes. Symbols:* ●, *NAD-linked;* ○, *NADP-linked. Fructose (1 ml), 1.0 ml of buffer, 0.05 ml of coenzyme* [b]*(10^{-3} M), and 0.1 ml of enzyme (340 units as NADP-linked enzyme) were mixed, and incubated at room temperature. The change in optical density at 340 mμ was measured.*

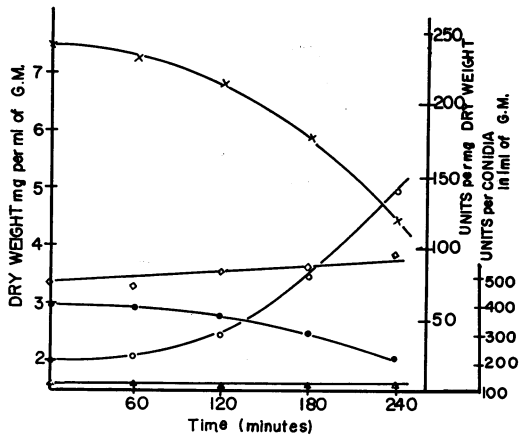

FIG. 4. *Change of* D*-mannitol dehydrogenase activity during germination. Symbols:* ○, *dry weight of conidia during germination;* □, *activity of NADP-linked enzyme per conidium in 1 ml of germination medium;* △, *activity of NAD-linked enzyme per conidium in 1 ml of germination medium;* ×, *activity of NADP-linked enzyme expressed as units per milligram (dry weight) of conidia;* ●, *activity of NAD-linked enzyme expressed as units per milligram (dry weight) of conidia. G.M. = germination medium.*

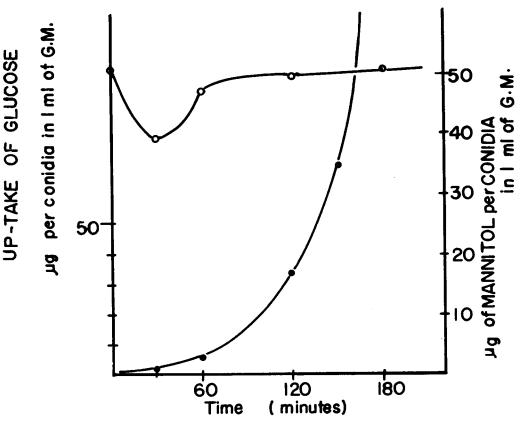

FIG. 5. *Glucose uptake and mannitol content in conidia during germination. Symbols:* ●, *glucose uptake;* ○, *mannitol in conidia. Glucose uptake was measured by incorporation of radioactive glucose. Mannitol in conidia was determined by* D*-mannitol dehydrogenase as described in Materials and Methods. G.M. = germination medium.*

Oxidation of $NADH_2$ and $NADPH_2$ by the enzyme solution. In the presence of fructose, $NADH_2$ and $NADPH_2$ were oxidized under the standard conditions described above (Fig. 3). The optimal *p*H for NAD-linked enzyme was 7, and that for $NADPH_2$-linked enzyme was 6.

D-Mannitol dehydrogenase activities during germination. The germination medium (1,000 ml) which had been inoculated with 2 g of conidia was placed in a 5-liter Erlenmeyer flask and incubated at 26 C on a rotary shaker; 250 ml of the broth were withdrawn from the culture after 0, 60, 120, and 240 min. Microscopy revealed that more than 90% of the conidia sprouted germ tubes after 240 min of incubation. Dry matter in 20 ml of each culture was weighed, and the remaining culture fluid (200 ml) was centrifuged at 3,000 × *g* for 10 min to collect the conidia. From these conidia, 30 ml of the crude enzyme solutions were obtained. The ammonium sulfate precipitation was omitted in this experiment. As clearly demonstrated in Fig. 4, the mannitol dehydrogenase activities, expressed as units per milligram (dry weight) of conidia, gradually decreased as germination proceeded, and the activities per conidia in 1 ml of germination medium remained unchanged.

Uptake of glucose. Conidia (3 mg) were inoculated into 3 ml of the germination medium supplied with glucose-*U*-C^{14} (1.2×10^6 count/min) and incubated aerobically at 26 C; 0.5-ml samples were withdrawn after 0, 15, 30, 60, and 120 min. Conidia were collected on filter paper by filtration and washed with 4 ml of water; radioactivity of conidia on the filter paper was measured. Uptake of radioactive glucose by germinating conidia proceeded actively after a lag of about 60 min (Fig. 5).

D-Mannitol in germinating conidia. Samples (10 ml) were withdrawn from 60 ml of culture after 0, 30, 60, 120, and 180 min, and mannitol content in the conidia was determined by the method described above. The content of mannitol decreased at an early stage of germination and returned to the original level after 60 min (Fig. 5). This showed that the decrease of mannitol content stopped when uptake of exogenous glucose started.

DISCUSSION

In conidia of *A. oryzae*, there are two kinds of D-mannitol dehydrogenases: NAD-linked and NADP-linked. They differ in their *p*H optima, heat stability, and substrate specificity. The pattern of inhibition by chemical agents is also different. Both enzymes are active at an alkaline *p*H. Similar enzymes, L-alanine dehydrogenases, in *A. niger* and in *Bacillus cereus* have been reported (O'Connor and Halvorson, 1960; Hoshino, Nishi, and Yanagita, 1962), and the optimal *p*H of both enzymes is about 11. Recently, Edmundowicz and Wriston (1963) reported NAD-linked and NADP-linked D-mannitol dehydrogenases in *Agaricus campestris*. The activity of NAD-linked enzyme was very low (5 to 10% of NADP-linked enzyme). We found that the specific activity of NAD-linked mannitol dehydrogenase was about 25% of that of NADP-linked enzyme. D-Mannitol dehydrogenase activities, expressed as units per milligram (dry weight) decreased as the germination process proceeded. Synthesis of D-mannitol dehydrogenases has not been observed during germination. These observations suggest that the enzymes play an important role at the early stage of germination. The consumption of D-mannitol in conidia began after 15 min of incubation, and the uptake of exogenous glucose was observed after 60 min. It is presumed, therefore, that the first steps of germination are sustained by D-mannitol, and the later steps, by glucose; the change may occur at 60 min. D-Mannitol may be used as the source of endogenous respiration and provide energy for germination after it is converted to fructose.

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