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METABOLISM OF MANNITOL BY *COCCIDIOIDES IMMITIS*

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ABSTRACT

LONES, GEORGE W. (National Institute of Allergy and Infectious Diseases, U.S. Public Health Service, Bethesda, Md.), AND CARL PEACOCK. Metabolism of mannitol by *Coccidioides immitis*. J. Bacteriol. **87**:1114–1117. 1964.—Strain M-11 of *Coccidioides immitis* was found to utilize mannitol for growth in the mycelial form but not in the spherule form. Cell-free extracts of both forms, grown on glucose, were capable of reducing nicotinamide adenine dinucleotide with mannitol-1-PO_4 but not with mannitol. The extracts accomplished a rapid oxidation of reduced nicotinamide adenine dinucleotide by fructose-6-PO_4, the expected product of mannitol-1-PO_4 oxidation. Fructose was inactive. Paper electrophoresis and chromatography with several solvent systems demonstrated a substance in extracts of both mycelium and spherules having a migration consistent with that of mannitol.

During a comparative study of the nutrition of the mycelial and spherule forms of the dimorphic fungus *Coccidioides immitus*, strain M-11, it was found that the mycelium grew well on mannitol as a sole source of carbon, but that this polyol did not support the growth of the spherules. The difference in behavior of the two forms prompted an investigation of the metabolism of mannitol by this fungus. The metabolism of the polyols by other organisms was recently reviewed by Touster and Shaw (1962).

MATERIALS AND METHODS

Cultures. C. immitis was maintained in stock culture on modified Sabouraud agar slants at room temperature with transfers at intervals of 2 to 3 months. The production of mycelium and spherules for use was previously described (Lones and Peacock, 1960). The mycelium was grown in a modification of the defined medium of Roessler et al. (1946) having the following composition: K_2HPO_4, 0.015 M; KH_2PO_4, 0.015 M; $MgSO_4$, 0.0016 M; $ZnSO_4$, 3×10^{-5} M; glucose, 0.11 M; ammonium acetate, 0.08 M. The spherule medium was based on that of Converse (1957), and contained K_2HPO_4, 0.003 M; KH_2PO_4, 0.003 M; $MgSO_4$, 0.0016 M; $ZnSO_4$, 3×10^{-5} M; glucose, 0.022 M; ammonium acetate, 0.016 M; and Tamol N (sodium salt of a condensed aryl sulfonic acid), 0.05%. The pH of the media was 6.6 to 6.8.

Substrate utilization studies. The ability to utilize mannitol as a sole source of carbon and energy was compared with the ability to utilize glucose under the same conditions. The media used were those described above for the respective forms of the fungus with the substitution of mannitol for glucose where indicated, and the replacement of ammonium acetate by an equimolar amount of dipotassium ammonium citrate. Unlike acetate, citrate was not utilized by this fungus. Mycelium for inoculation was produced by several serial transfers in shake cultures at 36 C in the glucose-ammonium acetate medium. For the final growth period, the medium was inoculated at a cell density of 1 mg/ml and incubated with shaking for 16 hr. The mycelium was harvested by centrifugation and dispersed by treatment for 15 sec in a Waring Blendor when necessary to facilitate handling with a pipette. The mycelium was washed four times in a centrifuge with sterile medium containing no carbon or nitrogen source. The 5.0-ml test cultures containing glucose or mannitol were prepared in 25-ml flasks. The carbon and nitrogen sources were sterilized separately and added at the time of inoculation. The mycelial inoculum was adjusted to a cell density of approximately 1 mg/ml. Incubation was at 36 C on a rotary shaker with a 1-in. amplitude at 120 cycles per min. At appropriate intervals, flasks were removed and the dry weight of the mycelium was determined by filtration on a weighed Millipore membrane followed by washing and drying to constant weight at 105 C. Spherules for inoculation were from well-established cultures maintained by daily transfers. They were judged to contain less

than 1% mycelium when used. The inoculum was adjusted to provide a final cell density of about 2 mg/ml. This large inoculum was employed because it was found to encourage maintenance of the spherule form (Lones and Peacock, 1960). The mycelial inoculum was about half that of the spherules because of the greater viscosity of the mycelial suspensions.

Preparation of cell-free extracts. Mycelium and spherules were harvested after incubation for 16 hr in the glucose-ammonium acetate medium described above for the production of mycelium. Well-established spherule cultures could be grown in this medium for 16 hr without significant reversion to mycelium. Heavy suspensions of washed cells were crushed while frozen in an X-press (Biotech, Biochemical Processes, Inc., New York, N.Y.). The thawed suspension was centrifuged at 1,000 × *g* to remove coarse debris and unbroken cells, and finally at 40,000 × *g* for 1 hr. The supernatant fluid was sterilized by filtration through an HA Millipore membrane and stored at −40 C for up to 1 week. The nitrogen content of the extract was determined by micro-Kjeldahl digestion, distillation, and titration.

Enzyme studies. Mannitol-1-PO_4 dehydrogenase activity was demonstrated by the method of Wolff and Kaplan (1956).

Detection of intracellular mannitol. Heavy, washed suspensions of mycelium or spherules produced as described for the preparation of cell-free extracts were autoclaved at 15 psi for 15 min. The insoluble matter was removed by filtration, and the filtrate was deionized by passage through columns of Amberlite IR 120 (H^+) and IRA 400 (OH^-). The solution was concentrated in a vacuum to a solids content of 3 to 5 mg/ml. Chromatograms were prepared on Whatman no. 1 paper by the descending method with several solvent systems. Reagents for the detection of carbohydrates were the alkaline silver nitrate reagent of Trevelyan, Procter, and Harrison (1950), the *p*-anisidine reagent of Mukherjee and Srivastava (1952), and bromocresol purple in borate buffer (Bradfield and Flood, 1950). Analysis by paper electrophoresis was accomplished with a Spinco model R apparatus with 0.05 M borate buffer at pH 8.3 at 1,000 v for 1 hr. Lead tetraacetate (1%) in benzene was the detection reagent (Gross, 1955).

Materials. Mannitol was commercial material twice crystallized from water. Fructose-6-PO_4 was obtained from Calbiochem. Mannose-6-PO_4, glucose-6-PO_4, nicotinamide adenine dinucleotide (NAD^+), and reduced nicotinamide adenine dinucleotide (NADH) were from Nutritional Biochemicals Corp., Cleveland, Ohio. Tamol N was obtained from Rohm & Haas Co., Philadelphia, Pa.

Mannitol-1-PO_4 was prepared by reduction of mannose-6-PO_4 with an eightfold excess of sodium borohydride at 0 C and pH 7 to 8. Borate was removed from the product by acidification to pH 1 with Amberlite IR 120 (H^+), and evaporation almost to dryness in a vacuum followed by the addition of methanol and evaporation again in a vacuum. The methanol treatment was repeated four times. Tests for borate by the methyl borate flame test were negative. The product was taken up in water, adjusted to neutrality with KOH, and the mannitol-1-PO_4 was determined by oxidation with periodic acid followed by determination of formaldehyde with chromotropic acid (West and Rapoport, 1949). Sorbitol-6-PO_4 was prepared in the same way from glucose-6-PO_4.

RESULTS AND DISCUSSION

A comparison of the growth of mycelium and spherules on glucose and mannitol as sole carbon sources appears in Table 1. Both glucose and mannitol supported growth of the mycelium, whereas only glucose was utilized by the spherules. Mycelium which was obtained by reversion of spherules, brought about by several transfers in the more concentrated glucose medium described above, regained the ability to grow on mannitol. The difference in growth behavior of the two forms of the fungus in mannitol medium was not due to the difference in concentration of

TABLE 1. *Effect of carbon source on growth of Coccidioides immitis**

Inoculum	Glucose				Mannitol			
	0 hr	16 hr	40 hr	96 hr	0 hr	16 hr	40 hr	96 hr
Mycelium	4.3	10.0	23.9		4.3	13.3	24.4	
Spherules	9.0	18.3	17.7	15.2	9.0	8.5	8.5	7.7
Mycelium from spherules	4.2	10.2	31.4		4.2	19.9	41.4	

* Results are expressed as dry weight of cells in milligrams.

the substrate, since the spherules did not grow when inoculated into the more concentrated mannitol medium. Moreover, growth of a mycelial inoculum was obtained at the lower concentration of mannitol found in the spherule medium. Similar studies with sorbitol resulted in no growth of either form of the fungus.

In view of the difference in behavior of the two forms of the fungus with respect to mannitol, it was of interest to determine the enzymatic basis for the difference. Cell-free extracts of glucose-grown mycelium were capable of bringing about reduction of NAD^+ by mannitol-1-PO_4 (Fig. 1). The expected reverse reaction, the oxidation of NADH by fructose-6-PO_4, was readily demonstrated. Mannitol and fructose were inactive when tested in the corresponding systems, and sorbitol-6-PO_4 had about 10% of the activity of mannitol-1-PO_4. Growth of the mycelium on mannitol did not enhance the mannitol-1-PO_4 dehydrogenase activity of the extract. Cell-free extracts of spherules also possessed mannitol-1-PO_4 dehydrogenase activity (Fig. 2). The oxidation of mannitol-1-PO_4 appears to occur in the same manner in this fungus as was reported

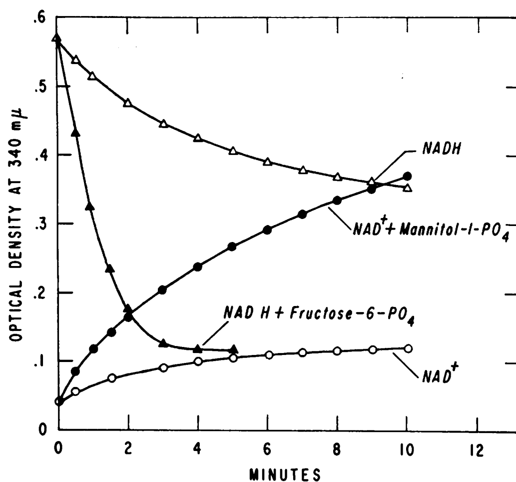

FIG. 1. *Mannitol-1-PO_4 dehydrogenase activity of cell-free extract of Coccidioides immitis mycelium. Symbols:* ○ = *sodium carbonate buffer (pH 9.0, 0.05 M), cell-free extract (0.20 mg of nitrogen), NAD^+ (0.31 mM);* ● = *with mannitol-1-PO_4 (0.017 M);* △ = *potassium phosphate buffer (pH 6.0, 0.05 M), cell-free extract (0.20 mg of nitrogen), NADH (0.31 mM);* ▲ = *with fructose-6-PO_4 (0.017 M). Final volume, 3.0 ml. Temperature, 33 C.*

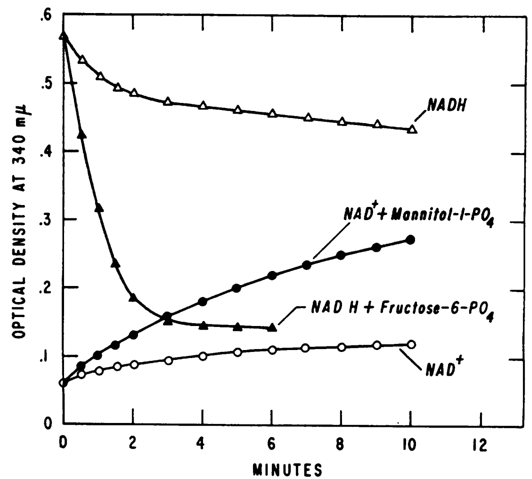

FIG. 2. *Mannitol-1-PO_4 dehydrogenase activity of cell-free extract of Coccidioides immitis spherules. Symbols:* ○ = *sodium carbonate buffer (pH 9.0, 0.05 M), cell-free extract (0.20 mg of nitrogen), NAD^+ (0.31 mM);* ● = *with mannitol-1-PO_4 (0.017 M);* △ = *potassium phosphate buffer (pH 6.0, 0.05 M), cell-free extract (0.20 mg of nitrogen), NADH (0.31 mM);* ▲ = *with fructose-6-PO_4 (0.017 M). Final volume, 3.0 ml. Temperature, 33 C.*

for a variety of other microorganisms (Wolff and Kaplan, 1956; Touster and Shaw, 1962).

The occurrence of mannitol-1-PO_4 dehydrogenase in glucose-grown cells of this fungus suggested that mannitol is a normal metabolite. Supporting evidence was found in chromatograms of deionized extracts of both mycelium and spherules. Both extracts contained a substance having a mobility in three solvent systems consistent with that of mannitol (Table 2). Moreover, the substance in question did not give the reaction with *p*-anisidine characteristic of aldoses and ketoses. A positive test characteristic of the polyols was obtained with bromocresol purple in borate buffer, and oxidation by alkaline silver solution was strongly inhibited by borate, a property of the sugar alcohols. Additional evidence for the presence of mannitol was obtained by electrophoresis in borate buffer (Table 3). It is of some interest that the chromatograms revealed a substance in both mycelium and spherules with R_F values identical with those of trehalose. This is not surprising in view of the numerous reports of the occurrence of this carbohydrate in fungi. Two of the solvent systems resolved a third

TABLE 2. *Partition chromatography of extracts of Coccidioides immitis*

Substance	R_F value in solvent*		
	1	2	3
Mannitol	0.48	0.33	0.25
Sorbitol	0.52	0.33	0.22
Fructose	0.55	0.36	0.27
Glucose	0.39	0.31	0.23
Sucrose	—	0.22	0.19
Trehalose	0.29	0.17	0.15
Mycelium extract	0.48, 0.42, 0.29	0.33, 0.17, 0.13	0.25, 0.15
Spherule extract	0.48, 0.29	0.34, 0.18	0.25, 0.15

* Solvent 1, phenol–water (4:1); solvent 2, *n*-propanol–ethyl acetate–water (6:1:2); solvent 3, *n*-butanol–ethanol–water (4:1.1:1.9).

TABLE 3. *Paper electrophoresis of extracts of Coccidioides immitis*

Substance	R_M*
Mannitol	100
Sorbitol	109
Dulcitol	121
Arabitol	89
Ribitol	56
Erythritol	9
Mycelium extract	101
Spherule extract	100

* Distance of migration as per cent of that of mannitol.

substance, as yet unidentified, as a faint spot seen after treatment with the silver reagent and obtained only with the mycelial extract.

The properties of strain M-11 which have been described are not shared by all strains of *C. immitis*. A second strain, 6207, which we examined, did not grow on mannitol in either the mycelial or spherule forms. However, this strain did contain mannitol-1-PO_4 dehydrogenase. A third strain, 6228, appeared to resemble M-11, but growth studies with the spherule form of this strain were less definitive because of a tendency of the spherules to revert to mycelium in mannitol medium.

Although a positive identification of mannitol would require isolation and chemical characterization of the substance, the evidence is sufficient to justify a tentative identification of mannitol as a component of the mycelium and spherules of *C. immitis*. It is possible that mannitol serves as an intracellular carbon and energy reserve.

The inability of the spherules to utilize extracellular mannitol may be due to impermeability or lack of an active transport system. Alternatively, the spherules may be deficient in mannitol kinase. Attempts to detect mannitol kinase in extracts of mycelium and spherules have not been successful.

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