

# Erythritol Metabolism in Wild-Type and Mutant Strains of *Schizophyllum commune*

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Received for publication 16 July 1969

Erythritol uptake and metabolism were compared in wild-type mycelium and a *dome* morphological mutant of the wood-rotting mushroom *Schizophyllum commune*. Wild-type mycelium utilized glucose, certain hexitols, and pentitols including ribitol, as well as D-erythrose, erythritol, and glycerol as sole carbon sources for growth. The *dome* mutant utilized all of these compounds except D-erythrose and erythritol. Erythritol- or glycerol-grown wild-type mycelium incorporated erythritol into various cellular constituents, whereas glucose-grown cells lagged considerably before initiation of erythritol uptake. This acquisition was inhibited by cycloheximide. *Dome* mycelium showed behavior similar to wild-type in uptake of erythritol after growth on glucose or glycerol, except that erythritol was not further catabolized. Enzymes of carbohydrate metabolism were compared in cell extracts of glucose-cultured wild-type mycelium and *dome*. Enzymes of hexose monophosphate catabolism, nicotinamide adenine dinucleotide (NAD)-dependent sugar alcohol dehydrogenases, and reduced nicotinamide adenine dinucleotide phosphate (NADPH)-coupled erythrose reductase were demonstrated in both. The occurrence of erythrose reductase was unaffected by the nature of the growth carbon source, showed optimal activity at pH 7, and generated NAD phosphate and erythritol as products of the reaction. Glycerol-, D-erythrose-, or erythritol-grown wild-type mycelium contained an NAD-dependent erythritol dehydrogenase absent in glucose cells. Erythritol dehydrogenase activity was optimal at pH 8.8 and produced erythrulose during NAD reduction. Glycerol-growth of *dome* mycelium induced the erythritol uptake system, but a functional erythritol dehydrogenase could not be demonstrated. Neither wild-type nor *dome* mycelium produced erythritol dehydrogenase during growth on ribitol. Erythritol metabolism in wild-type cells of *S. commune*, therefore, involves an NADPH-dependent reduction of D-erythrose to produce erythritol, followed by induction of an NAD-coupled erythritol dehydrogenase to form erythrulose. A deficiency in erythritol dehydrogenase rather than permeability barriers explains why *dome* cannot employ erythritol as sole carbon source for mycelial growth.

*Schizophyllum commune* is a wood-rotting mushroom capable of utilizing erythritol as sole source of carbon and energy for basidiospore germination (11). Although various enzymes of sugar alcohol (polyol) metabolism occur in glucose-cultured mycelium of *S. commune* (12), a nicotinamide adenine dinucleotide (NAD)-dependent erythritol dehydrogenase is detected only during growth on erythritol or glycerol (7). Erythritol is converted to erythrulose by mycelium of *Fusarium lini* (4), and the oxidation of erythritol occurs during NAD reduction in cell extracts of *Aerobacter aerogenes* (8). In contrast to these pathways, studies of *Propionibacterium pentosaceum* showed that erythritol metabolism

involves phosphorylated intermediates (13, 14). General aspects of the distribution and physiology of erythritol in fungi and green plants are discussed elsewhere (9).

A survey of carbohydrate utilization for mycelial growth of *S. commune* revealed that a *dome* morphological mutant cannot utilize erythritol as sole carbon source. This raises several questions regarding the control of erythritol metabolism, including whether *dome* is deficient in (i) erythritol uptake, (ii) induction of erythritol dehydrogenase, or (iii) ancillary enzymes of carbohydrate metabolism. The present investigation compares the uptake and utilization of this tetritol and related carbo-

hydrates in wild-type and mutant cells of *S. commune*. A preliminary account of this work has appeared elsewhere (M. L. Braun and D. J. Niederpruem, *Bacteriol. Proc.*, p. 37, 1969).

#### MATERIALS AND METHODS

**Cultures, media, and growth conditions.** Homokaryotic mycelium of *S. commune* Fr. was employed throughout this work. Strains 699 *A41B41* and 845 *A51B51* were wild type in that their colonial morphology on agar was normal and they were able to utilize erythrose or erythritol as sole source of carbon and energy. Strain *Sla A1B1* exhibited a *dome* morphology rather than spreading on agar and could not utilize erythrose or erythritol for growth. Mycelium was cultured on a chemically defined liquid medium described elsewhere (7). Incubation was at 25 C ( $\pm 0.5$ ) in the light with aeration (180 oscillations/min) on a shaker-incubator (model G27, New Brunswick Scientific Co., New Brunswick, N.J.).

**Enzyme preparation.** Mycelium was harvested from culture medium by filtration and washed extensively with 0.08 M phosphate buffer, pH 6.8. The mycelial suspension was extracted in a chilled French pressure cell (American Instrument Co., Needham Heights, Mass.) at 10,000 lb/in<sup>2</sup>. The cell extract was centrifuged (1,000  $\times g$ , 10 min) at 4 C in a refrigerated centrifuge (International model B-20) equipped with a model 870 head to remove unbroken hyphae and residual cell walls. The supernatant solution was centrifuged at 10,000  $\times g$  (60 min) to remove subcellular particles containing a reduced nicotinamide adenine dinucleotide (NADH) oxidase which interfered with subsequent assay of pyridine nucleotide reduction by polyol dehydrogenase. The final supernatant solution was used directly as a source of crude enzyme extract or dialyzed against 0.08 M phosphate buffer, pH 6.8, for 18 hr at 4 C. Protein content was always determined on the dialyzed enzyme fraction by the method of Lowry et al. (10), with bovine serum albumin as standard.

**Enzyme assays.** The reduction of NAD which accompanied polyol oxidation, or the oxidation of reduced nicotinamide adenine dinucleotide phosphate (NADPH) which occurred during aldose reduction, was measured spectrophotometrically at 340 nm in a Zeiss Spectrophotometer (PMQII) at 30 C. Polyol dehydrogenase was assayed as follows: polyol substrate, 100  $\mu$ moles; tris(hydroxymethyl)amino-methane (Tris)-hydrochloride buffer (pH 8.8), 25  $\mu$ moles; NAD, 0.4  $\mu$ mole; enzyme extract and glass-distilled water to 1.2 ml (final volume). Aldose reductase was measured as follows: aldose substrate, 100  $\mu$ moles; phosphate buffer (pH 6.8), 40  $\mu$ moles; NADPH, 0.4  $\mu$ mole; enzyme extract and water to 1.2 ml. Nicotinamide adenine dinucleotide phosphate (NADP) and phosphate buffer (pH 7.4), 40  $\mu$ moles, were used for determining dehydrogenase activity with glucose-6-phosphate and 6-phosphogluconate (5  $\mu$ moles) in 1.2 ml. Phosphoglucoisomerase was evaluated by the enzymatic conversion of fructose-6-phosphate (10  $\mu$ moles) to glucose-6-phosphate and subsequent reduction of NADP as outlined previously.

Cytochrome *c* oxidase was evaluated at 550 nm by the oxidation of reduced cytochrome *c* (0.4 mg) in phosphate buffer, pH 6.8 (60  $\mu$ moles), and the 10,000  $\times g$  sediment, to 1.2 ml. Specific activity represents the change in optical density per minute per milligram of protein.

Analogues of NAD were purchased from P. L. Biochemicals, Inc. (Milwaukee, Wis.) and were employed as 0.4  $\mu$ moles in 1.2 ml. The change in optical density was observed at the absorption maximum peculiar to each analogue (see Circular No. OR-18, P.L. Biochemicals, Inc.).

**Determination of enzyme products.** The product of erythrose reduction was obtained by reacting the following mixture for 4 hr at 30 C in a 50-ml beaker: erythrose reductase, as 2 ml of dialyzed enzyme extract; NADPH, 4  $\mu$ moles; erythrose, 1 millimole; 6-phosphogluconate, 50  $\mu$ moles; endogenous 6-phosphogluconate dehydrogenase to replenish NADPH, phosphate buffer (pH 6.8), 100  $\mu$ moles. In the case of product formation during erythritol oxidation, the following conditions were employed: erythritol dehydrogenase, as 2 ml of dialyzed enzyme extract; NAD, 4  $\mu$ moles; erythritol, 2 millimoles; sodium pyruvate, 2 millimoles; lactate dehydrogenase, 20  $\mu$ g of protein; and Tris-hydrochloride buffer (pH 8.8), 1 millimole. Lactate dehydrogenase served to replenish NAD. Three additional controls were employed and consisted of the above reactions (i) minus substrate, (ii) minus coenzyme, and (iii) minus both coenzyme and substrate. The reactions were terminated by addition of trichloroacetic acid (10%, w/v), and the precipitated proteins were removed by centrifugation. The reaction products were identified by descending paper chromatography, and the spots were revealed with periodate-benzidine (5), silver nitrate (2), or ninhydrin (6) for polyols, sugars, and tetulose, respectively. Two different solvent systems were employed. The first was methyl-ethyl ketone (2-butanone)-acetic acid-boric acid-saturated water (9:1:1); the second was ethyl acetate-pyridine-water (8:2:1). For carbohydrate detection employing borate in the irrigant, the chromatogram was sprayed three times with methanol to diminish the borate complex with carbohydrate.

**Isotope uptake procedures.** To obtain young mycelial pellets for isotope studies *in vivo*, mycelium from a 5-day liquid culture was suspended in sterile water and macerated for 10 sec. For all uptake work, 25 ml of macerate was added to 100 ml of culture medium and incubated for 42 hr. The cells were harvested by centrifugation, and washed twice in sterile 0.08 M phosphate buffer (pH 6.8); the suspension was adjusted to approximately 100 mg (wet weight) per ml.

Beakers (50 ml) containing 12 ml of sterile phosphate buffer and 3 ml of the mycelial suspension were equilibrated to 27 C for 30 min on a Dubnoff Metabolic Shaking Incubator (Precision Scientific Co.) before addition of 20  $\mu$ liters of <sup>14</sup>C-substrate. Samples (1 ml) were withdrawn at intervals and collected by aspiration on a filter (Millipore Corp., Bedford, Mass.; 0.8  $\mu$ m pore size). The cells were washed with sterile phosphate buffer and the filter was removed, dried by

hot air, and placed in scintillation vials containing 10 ml of scintillation fluid [3.0 g of 2,5-diphenyloxazole plus 0.1 g of 1,4-bis-2-(5-phenyloxazole)-benzene per liter of toluene]. Radioactivity was determined by use of a Tri-Carb liquid scintillation spectrometer (model 314 EX, Packard Instrument Co., Inc., Downers Grove, Ill.).

**Extraction of labeled materials in mycelium.** After termination of an isotope incorporation experiment, residual mycelium was extracted in hot water for 20 min, followed by extraction in hot ethyl alcohol (80%, v/v) two times for 20 min each. The supernatant solutions were pooled and concentrated by evaporation. Samples were counted as described above, chromatographed by using methyl-ethyl ketone-acetic acid-boric acid-saturated water as the irrigant, and compared to standard compounds. Strips were cut from the chromatogram and placed in counting vials containing 10 ml of scintillation fluid. The resulting counts were then compared to the migration of reference carbohydrates.

A sample of the mycelium which remained after ethyl alcohol extraction was counted, and the remainder was subjected to 0.5 N acetic acid at 80 C for 30 min. This was repeated twice, and the supernatant solutions were pooled for the evaluation of "glycogen" as outlined by Wessels (15).

The residue was suspended in KOH (5%, w/v) at room temperature and allowed to stand overnight. The alkali-soluble material was removed, and the residue was suspended in KOH for an additional 3 hr. The supernatant solutions were pooled and contained the alkali-soluble (S-glucan) cell-wall material; the final residue consisted primarily of alkali-resistant cell-wall substances (R-glucan plus some chitin) as defined by Wessels (15).

**Source of chemicals.** Samples of D-erythrose obtained from several commercial sources were impure when evaluated by paper chromatography. Purified

erythrose was obtained after preparative paper chromatography with methyl-ethyl ketone-acetic water-boric acid-saturated water as irrigant. The major substance was eluted and rechromatographed and quantified by the tetrose reaction of Dische and Dische (3).

Erythritol and all other sugar alcohols were purchased from Pfanstiehl Laboratories (Waukegan, Ill.), and phosphorylated sugars and coenzymes were obtained from Sigma Chemical Co. (St. Louis, Mo.). Erythrulose was a kind gift of Dr. Rendig (University of California). Cycloheximide (Acti-Dione) was obtained from the Upjohn Co.

Uniformly labeled  $^{14}\text{C}$ -erythritol was purchased from Nuclear-Chicago with a specific activity of 2.26 mc/mm; uniformly labeled  $^{14}\text{C}$ -glucose and mannitol- $^{14}\text{C}$  were obtained from Tracerlab with specific activity of 11.6 and 13.7 mc/mole, respectively. These were taken up in double-distilled water at concentrations of 50  $\mu\text{c/ml}$  for erythritol and 100  $\mu\text{c/ml}$  for glucose or mannitol.

## RESULTS

**Polyol utilization for mycelial growth.** Individual strains of homokaryotic mycelium of *S. commune* were examined for their ability to use individual polyols as sole carbon and energy sources on solid medium (Fig. 1). Of 12 strains of diverse genetic background examined, only *dome* (strain S1a) was unable to grow on erythritol. None of the strains showed growth on inositol or galactitol. In contrast to considerable strain variation regarding the use of other polyols, *dome* exhibited good mycelial growth on all of these polyols, including glycerol and ribitol, but it was unable to utilize erythritol, regardless of the commercial source of erythritol. Erythritol was

STRAIN NUMBER	POLYHYDRIC ALCOHOLS						
	GLYCEROL	ERYTHRITOL	XYLITOL	RIBITOL	D-ARABITOL	D-MANNITOL	SORBITOL
S 1 a	GOOD	NO GROWTH	GOOD	GOOD	GOOD	GOOD	GOOD
2	GOOD	GOOD	GOOD	GOOD	GOOD	GOOD	GOOD
699	GOOD	GOOD	GOOD	GOOD	GOOD	GOOD	GOOD
38	GOOD	GOOD	GOOD	GOOD	GOOD	GOOD	GOOD
1	GOOD	GOOD	GOOD	GOOD	GOOD	GOOD	GOOD
845	GOOD	GOOD	GOOD	GOOD	GOOD	GOOD	GOOD
3	GOOD	GOOD	GOOD	GOOD	GOOD	GOOD	GOOD
5 h	GOOD	GOOD	FAIR	GOOD	FAIR	GOOD	GOOD
14	GOOD	GOOD	GOOD	GOOD	GOOD	FAIR	GOOD
13	GOOD	GOOD	GOOD	GOOD	GOOD	GOOD	GOOD
19	GOOD	GOOD	GOOD	GOOD	GOOD	GOOD	GOOD
20	GOOD	GOOD	GOOD	GOOD	GOOD	GOOD	GOOD

GOOD   
 POOR   
 FAIR   
 NO GROWTH

FIG. 1. Ability of individual strains of homokaryotic mycelium of *Schizophyllum commune* to use individual polyols as sole carbon and energy sources on solid medium.

not inhibitory when added to glucose cultures of *dome* grown on either solid medium or in liquid shake flasks. Moreover, *dome* could not utilize erythritol for mycelial growth when employing  $(\text{NH}_4)_2\text{SO}_4$  or L-asparagine as sole nitrogen source, even though wild-type mycelium grew under these conditions.

Consistent with the *dome* mutant's inability to grow on erythritol was its failure to utilize erythrose as sole carbon source, although several wild-type strains of *S. commune* utilized this tetrose as well as erythritol for mycelial growth.

**Uptake of glucose and polyols by wild-type and *dome* mycelium.** The failure of *dome* to utilize erythritol as sole carbon source could be explained by a deficiency in a specific uptake process. To explore this possibility, wild-type mycelium was compared with *dome* mycelium as to uptake of labeled substrates after growth on specific carbohydrates.

Glucose-grown cells of wild-type (strain 699) and *dome* mycelium were compared as to uptake of radioactive glucose, mannitol, and erythritol (Fig. 2 and 3). Similar responses were shown by wild-type and *dome* mycelium in that glucose uptake was immediate and rapid, whereas mannitol incorporation lagged from 30 to 60 min and erythritol uptake lagged nearly 120 min. Cells incubated at 4 C or treated by boiling did not show any of these responses. The acquisition of the ability to incorporate radioactive mannitol or erythritol was sensitive to the protein synthesis inhibitor cycloheximide (Acti-Dione). Comparative

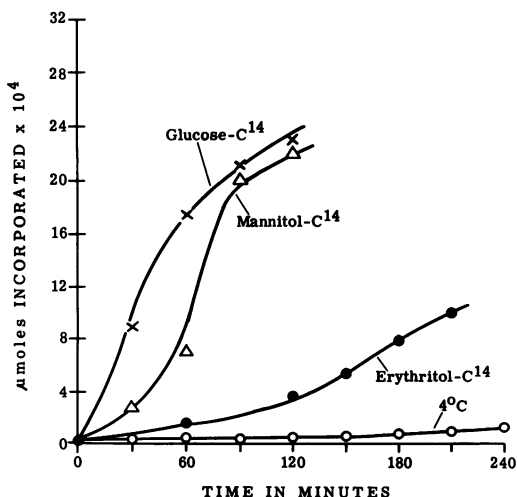


FIG. 2. Uptake of  $^{14}\text{C}$ -labeled compounds at 27 C by glucose-grown wild-type mycelium (strain 699) of *S. commune*. Open circles (4 C) applicable to uptake of glucose, mannitol, or erythritol as controls.

data obtained for erythritol uptake by glucose-grown wild-type and *dome* mycelium are shown in Fig. 4. When Acti-Dione (10 μg/ml) was added simultaneously with radioactive erythritol, no uptake occurred for 4 hr. However, when Acti-Dione was added 2 hr after the mycelium had been exposed to erythritol, uptake proceeded as in the absence of this poison (see Fig. 4). Glucose uptake was unaffected by the presence of Acti-Dione, whether added initially or later. Consequently, glucose-grown mycelium of either *dome* or wild-type cells showed a temperature-sensitive, protein synthesis-dependent acquisition of the ability to incorporate erythritol. Further support

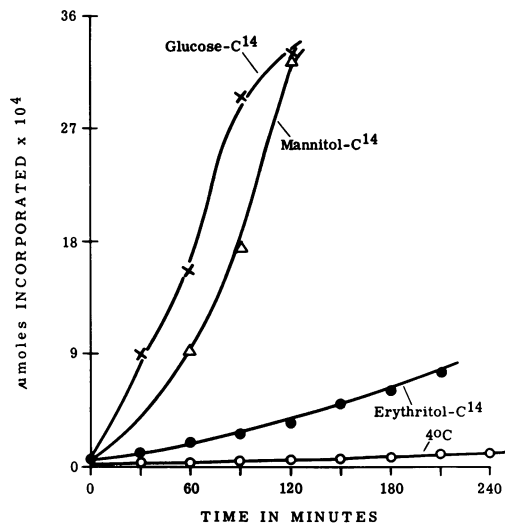


FIG. 3. Uptake of  $^{14}\text{C}$ -labeled compounds by glucose-grown *dome* (strain S1a) mycelium. Open circles as Fig. 1.

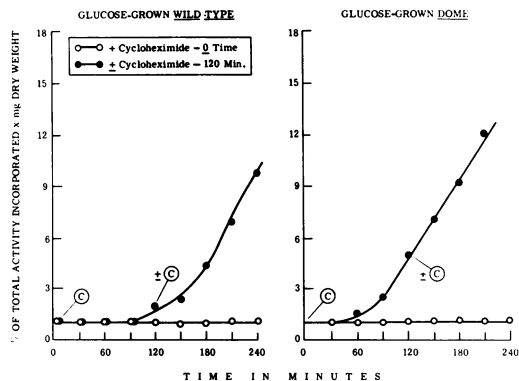


FIG. 4. Effects of cycloheximide (Acti-Dione) at 10 μg/ml, added initially or after 2 hr, on erythritol uptake by glucose-grown wild-type mycelium (strain 699) and *dome*.

for this notion was the finding that erythritol-grown mycelium of wild-type *S. commune* incorporated radioactive erythritol immediately.

Although *dome* could not be cultured on erythritol, glycerol did serve as sole carbon source for growth. Importantly, erythritol or glycerol growth of wild-type mycelium of *S. commune* led to the appearance of an erythritol uptake system and an NAD-coupled erythritol dehydrogenase. Therefore, glycerol induction of the erythritol uptake system was sought in *dome* mycelium. Glycerol-grown mycelium of wild-type and *dome* cells were compared regarding erythritol uptake (Fig. 5). The effects of unlabeled substrates (10  $\mu$ moles/ml) on erythritol uptake were also determined (Fig. 5). Glycerol-cultured *dome* mycelium showed no lag in erythritol uptake. Moreover, erythritol incorporation in both strains of *S. commune* was (i) stimulated by erythrose, (ii) unaffected by either glycerol or ribitol, and (iii) inhibited by glucose or ribose. The non-competitive effects of unlabeled ribitol and glycerol on erythritol uptake suggested separate uptake mechanisms. Moreover, uptake of labeled glycerol or ribitol by either *dome* or wild-type cells was unaffected by unlabeled erythritol (10  $\mu$ moles/ml; data not shown). When viewed as a whole, these data indicate that the failure of *dome* to utilize erythritol as sole carbon source for growth is not explicable in terms of a deficient erythritol uptake system. Alternatively, the subsequent catabolism of erythritol may be impaired in *dome*. To gain further information regarding this possibility, glycerol-grown wild-type and *dome* mycelium were allowed to incorporate labeled erythritol, and the subsequent fate of radioactive material was determined in the alcohol-soluble carbohydrates, the acetic acid-soluble "glycogen" fraction, the alkali-soluble (S-glucan) cell wall fraction, and the alkali-insoluble (R-glucan) cell wall material, as defined by Wessels (15). In this regard, 95% of the total radioactivity incorporated by glycerol-grown mycelium of *dome* was accounted for as erythritol. In contrast, glycerol-grown wild-type mycelium incorporated radioactive material from erythritol into acetic acid-soluble material (1%), S-glucan (20%), R-glucan (13%), and 66% into the ethyl alcohol-soluble fraction primarily as erythritol, with some radioactivity in glucose (2%) and arabitol (4%). Qualitatively similar patterns of isotope distribution were shown by erythritol-grown wild-type mycelium after uptake of labeled erythritol. On the other hand, wild-type cells and *dome* accumulated only labeled erythritol after growth on glucose. Presumably, during the 4-hr uptake study, at least 2 hr was required before induction

of the erythritol uptake system, whereas the remaining time was not sufficient to induce the NAD-dependent erythritol dehydrogenase required for subsequent catabolism in wild-type mycelium. Thus, at least in the case of glycerol-grown cells, radioactive material resulting from the uptake of  $^{14}$ C-erythritol was significantly distributed among the various cellular components of wild-type mycelium but remained essentially as erythritol in the case of *dome* mycelium.

**Control of erythritol metabolism in wild-type mycelium.** Past work established that an NAD-coupled erythritol dehydrogenase occurs only in wild-type mycelium of *S. commune* cultured on erythritol or glycerol as sole carbon source (7). The nature of the metabolic pathway involving C-4 metabolism was therefore first clarified in wild-type mycelium as a necessary preliminary before considering a possible enzyme deficiency in *dome*.

Cell-free extracts of erythritol-grown wild-type mycelium of *S. commune* contained various NAD-coupled polyol dehydrogenase activities, NADP-dependent enzymatic oxidation of glucose-6-phosphate and 6-phosphogluconate, phosphoglucoisomerase, and aldose reductase, which required NADPH. With regard to C-4 metabolism, an NADPH-coupled erythrose reductase was observed as well as an NAD-dependent erythritol dehydrogenase.

**Properties of erythrose reductase.** The enzymatic oxidation of NADPH by erythrose is shown in Fig. 6. No reaction occurred with NADH nor when the enzyme preparation was heated (60 C, 10 min). Erythrose reductase was not lost upon dialysis and was always higher in activity than NADPH oxidation caused by other

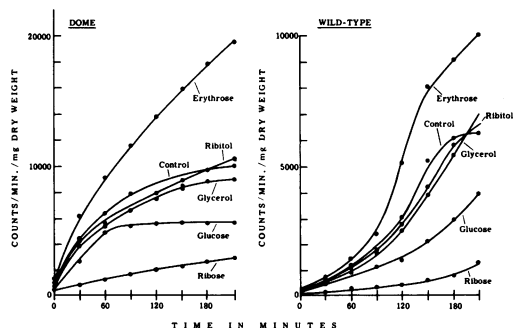


FIG. 5. Effects of unlabeled compounds on uptake of  $^{14}$ C-erythritol by glycerol-grown wild-type mycelium (strain 699) and *dome*. Unlabeled compound (10  $\mu$ moles/ml; final concentration) added simultaneously with  $^{14}$ C-erythritol.

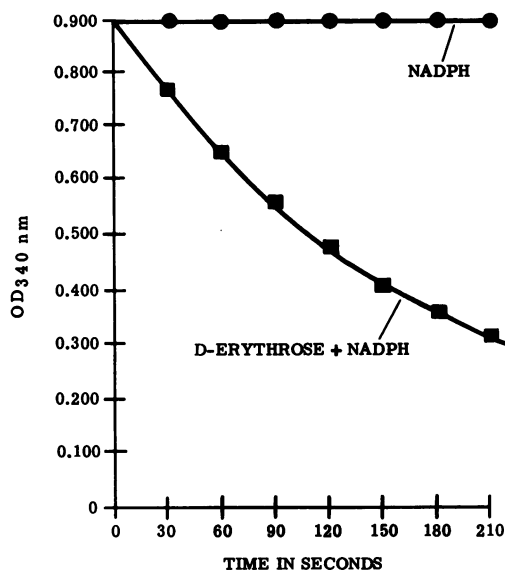


FIG. 6. Erythrose reductase activity in cell extracts of wild-type mycelium (strain 845). Assay conditions as in Materials and Methods.

aldoses, including xylose, ribose, and galactose. The pH optimum of erythrose reductase was compared to erythritol dehydrogenase (Fig. 7). Maximal activity of the reductase at pH 7 and an alkaline pH optimum for erythritol dehydrogenase are common features for these types of enzymes isolated from diverse microbial systems (9). The affinity constant ( $K_m$ ) for erythrose was determined in a dialyzed enzyme preparation from erythritol-grown wild-type mycelium to be 0.005 M.

Erythrose reductase was not recovered in the particulate fraction ( $10,000 \times g$ , 1 hr) which contained all the cytochrome *c* oxidase activity of the enzyme preparation, but rather full activity for the former enzyme remained in the final supernatant fraction. These findings do not exclude the possibility that the microsomal fraction or other particles may contain this particular enzyme.

The enzymatic product of the NADPH-dependent reduction of erythrose was analyzed by descending paper chromatography, with methyl ethyl ketone-acetic acid-boric acid-saturated water as the irrigant. A new spot was discerned which co-chromatographed with authentic erythritol; this substance was absent when the enzyme extract and NADPH were incubated without erythrose.

The occurrence of erythrose reductase in wild-type mycelium of *S. commune* was not dependent upon the particular nature of the sole carbon

source of the culture medium. The specific activity [ $\Delta$  optical density (OD)<sub>340</sub> per minute per milligram of protein] ranged from 0.200 to 0.400, regardless of whether the cells were cultivated on glucose, ribitol, erythrose, erythritol, or glycerol.

Presumptive evidence for the occurrence of separate enzyme species responsible for the NADPH-dependent reduction of aldose substrates was obtained by substrate competition experiments (Table 1). Nearly additive rates of NADPH oxidation were observed when ribose or galactose was employed in combination with erythrose. Other aldose combinations yielded rates of NADPH oxidation considerably less than those expected for two separate enzymes. Thus, erythrose reductase may be a distinct enzyme protein separable from other aldose reductase activities in crude extracts of *S. commune*.

**Properties of erythritol dehydrogenase.** The biochemical characteristics of erythritol dehydrogenase were determined on dialyzed cell-free extracts prepared from erythritol-grown wild-type mycelium. The enzymatic oxidation of erythritol was dependent upon NAD, optimal at pH 8.8 (see Fig. 7), and destroyed by heat (70 C, 10 min). The affinity constant of erythritol dehydrogenase for erythritol was calculated from experimental data obtained at pH 8.8 to be 0.04 M. Low affinity

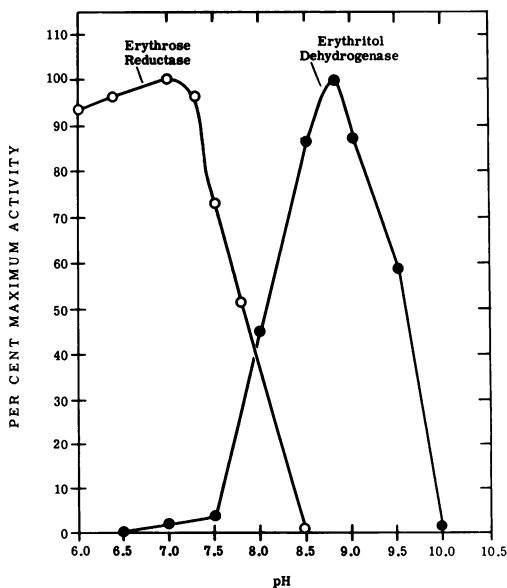


FIG. 7. Effect of pH on erythrose reductase and erythritol dehydrogenase in dialyzed enzyme extract of wild-type mycelium (strain 699) cultured 4 days on erythritol medium. Phosphate buffer (0.08 M), pH 6.0 to pH 8.5; Tris-hydrochloride buffer (0.05 M), pH 8.5 to pH 9.5; glycine buffer (0.05 M), pH 9.5 to pH 10.

TABLE 1. Effects of mixed substrates of aldose reductase on NADPH-oxidation\*

Substrate	Specific activity		Actual per cent additivity
	Experimental additivity	Calculated additivity	
Erythrose.....	0.363		
Xylose.....	0.175		
Ribose.....	0.100		
Galactose.....	0.075		
Erythrose + ribose..	0.434	0.463	94
Erythrose + galactose.....	0.417	0.438	95
Erythrose + xylose..	0.450	0.538	84
Xylose + galactose..	0.187	0.250	75
Xylose + ribose....	0.220	0.275	80
Galactose + ribose..	0.112	0.175	65

\* Erythritol-grown mycelium of *S. commune* (strain 699); dialyzed enzyme extract.

for sugar alcohols is a characteristic common to other polyol dehydrogenases in *S. commune* (12) and various fungi. All of the erythritol dehydrogenase activity could be accounted for in the supernatant fraction (10,000 × g, 1 hr) of cell extracts.

The enzymatic product of the NAD-dependent oxidation of erythritol co-chromatographed with authentic erythrose and gave a positive ninhydrin reaction, which is a feature of erythrose (6). Moreover, demonstration of an NADH-dependent reduction of erythrose at pH 6.8 lends further support to the idea that the enzymatic product of erythritol oxidation is erythrose (Fig. 8).

Several indirect lines of evidence suggested that erythritol dehydrogenase is a separate enzyme from other polyol dehydrogenase activities in crude extracts of *S. commune*. For example, ammonium sulfate fractionation of extracts led to a complete loss of ribitol dehydrogenase, although 70% of the original erythritol dehydrogenase activity was still retained. Moreover, ribitol dehydrogenase was always discerned in mycelial extracts prepared from wild-type cells grown on glucose, whereas erythritol dehydrogenase was observed only after growth on erythrose, erythritol, or glycerol.

Experiments utilizing analogues of NAD also suggested several distinct polyol dehydrogenases in crude extracts of mycelium which had been cultured on erythritol (Fig. 9). Oxidation of mannitol or arabitol was most efficient with deamino-NAD, whereas oxidation of xylitol, sorbitol, ribitol, and erythritol was greatest with thionicotinamide-NAD.

Substrate competition experiments, using different polyols with crude extracts and NAD, also provided evidence that separate enzymes exist

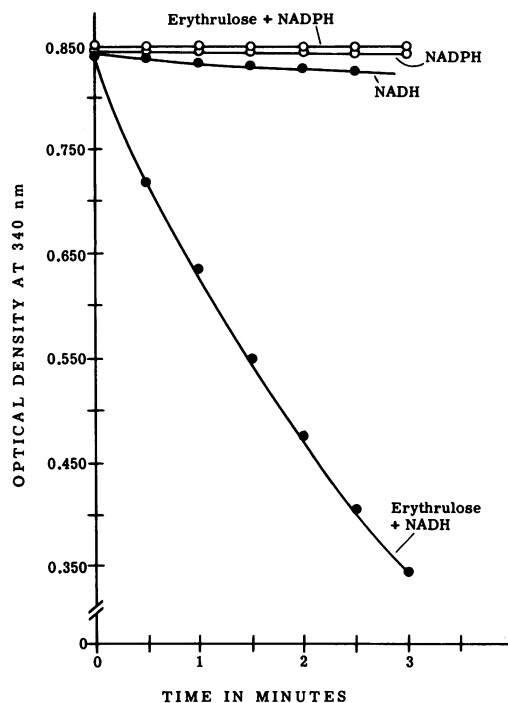


FIG. 8. Enzymatic oxidation of NADH by erythrose in cell extracts of wild-type mycelium (strain 699) cultured 4 days on erythritol medium.

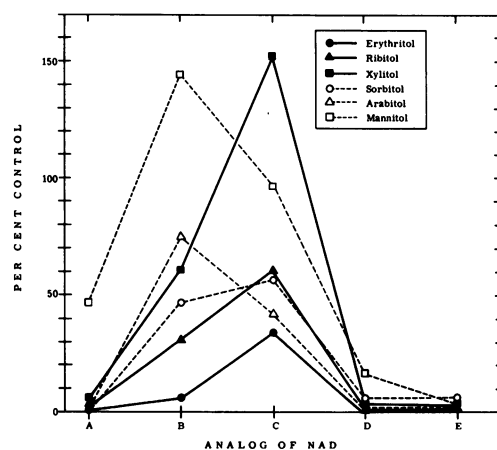


FIG. 9. Utilization of analogues of NAD by polyol dehydrogenase activities in dialyzed enzyme extracts prepared from wild-type mycelium (strain 699) cultured 6 days on erythritol medium. Analogues of NAD: A, 3-pyridinealdehyde-NAD; B, deamino-NAD; C, thionicotinamide-NAD; D, 3-acetylpyridine-deamino-NAD; E, 3-pyridinealdehyde-deamino-NAD.

for the oxidation of erythritol and ribitol, respectively (Fig. 10). Additive rates of NAD reduction were shown with ribitol plus erythritol, or when sorbitol, mannitol, or xylitol was added to erythritol. This was also true when sorbitol or xylitol was added to arabitol. Consequently, several distinct polyol dehydrogenases may occur in erythritol-grown mycelium of *S. commune*.

**Control of erythritol metabolism in dome mycelium.** Glucose-cultured mycelium of *dome* contained all the NAD-coupled polyol dehydrogenase activities observed in wild-type strains of *S. commune* (Table 2); the specific activities of these enzymes were also comparable to wild-type cells. Dehydrogenases capable of oxidizing glucose-6-phosphate and 6-phosphogluconate were similarly present in *dome* extracts and dependent upon NADP. Phosphoglucoisomerase was also discerned here.

Erythritol dehydrogenase was detected in wild-type mycelium after culture on glycerol, erythritol, or erythrose. The latter is explainable on the basis that NADPH-coupled reduction of erythrose produces erythritol in *S. commune*, which in turn may serve as subsequent inducer for erythritol dehydrogenase. This sort of regulation could not be demonstrated in *dome* mycelium, even though erythrose reductase was shown in

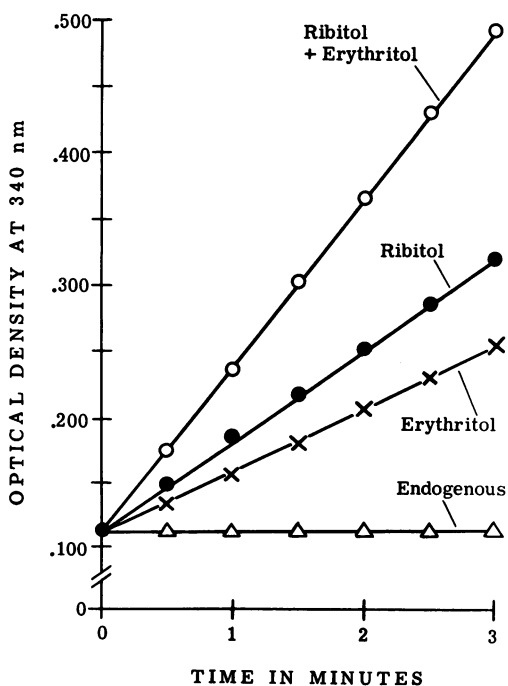


FIG. 10. Effect of mixed substrates on enzymatic reduction of NAD catalyzed by polyol dehydrogenase(s) in mycelial extracts of *S. commune*. Ribitol or erythritol (100  $\mu$ moles each, in 1.2 ml).

TABLE 2. Polyol dehydrogenase activities in dome and wild-type strains of *S. commune* cultivated on glucose<sup>a</sup>

Polyol dehydrogenase substrate	Specific activity <sup>b</sup>		
	<i>Dome</i>	Strain 699	Strain 845
D-Mannitol.....	0.473	0.334	0.208
Sorbitol.....	0.138	0.117	0.116
Xylitol.....	0.062	0.146	0.090
D-Arabitol.....	0.134	0.100	0.183
Ribitol.....	0.066	0.038	0.051
Erythritol.....	0	0	0

<sup>a</sup> Six-day cultures; dialyzed enzyme extracts.

<sup>b</sup> Expressed as change in optical density per minute per milligram of protein.

*dome* extracts of mycelium cultured on glucose, glycerol, or ribitol, because erythrose cannot serve as sole carbon source for mycelium growth of this mutant (Table 3). It is also important to note that, whereas glycerol growth of *dome* did enable this mutant to incorporate erythritol, the NAD-dependent erythritol dehydrogenase could not be demonstrated under these culture conditions. Moreover, ribitol growth of either wild-type mycelium or *dome* failed to elicit the NAD-coupled erythritol dehydrogenase.

The idea that *dome* produces an inhibitor of erythritol oxidation appeared unlikely, because extracts of *dome* mycelium did not inhibit erythritol dehydrogenase of wild-type mycelial extracts. In fact, a dikaryon established from a mating between *dome* and wild-type mycelium grew on erythritol as sole carbon source. The additional finding, that some progeny from this mating were wild type regarding colony morphology yet were unable to utilize erythritol as

TABLE 3. Erythritol metabolism in cell extracts of wild-type mycelium and dome<sup>a</sup>

Growth carbon source	Specific activity			
	Erythritol dehydrogenase		Erythrose reductase	
	Wild type	<i>Dome</i>	Wild type	<i>Dome</i>
Glycerol.....	0.169	0	0.390	0.272
Erythritol.....	0.265	— <sup>b</sup>	0.372	—
D-Erythrose.....	0.172	—	0.435	—
Ribitol.....	0	0	0.180	0.325
D-Glucose.....	0	0	0.180	0.420

<sup>a</sup> Five-day cultures of either wild type (strain 699) or *dome*; dialyzed enzyme extracts.

<sup>b</sup> Indicates *dome* does not grow on either erythritol or erythrose.



sole carbon source, suggests that the *dome* morphological aberrancy and erythritol metabolism are not casually related. However, more extensive genetic data are required to establish whether the erythritol dehydrogenase deficiency involves a single gene change in *S. commune*.

### DISCUSSION

There is currently little information regarding the nature of cellular regulatory devices governing erythritol metabolism in fungi. Barnett (1) showed that yeasts such as *Torulopsis candida*, *Debaryomyces vanriji*, and *Hansenula anomala* can respire erythritol only when cultured on this tetritol. Cell extracts of erythritol-cultured *T. candida* possess an NAD-dependent erythritol dehydrogenase, as in *S. commune*, and in this particular survey no evidence was obtained that phosphorylation was the initial event in polyol catabolism of various yeasts (1). In *Pyrenochaeta terrestris*, Wright and LeTourneau (16) suggested that the lag in growth on erythritol might also be due to induction of specific enzymes.

The present investigation clarifies the nature of control mechanisms governing erythritol metabolism in *S. commune*. Wild-type mycelium can utilize ribitol, erythritol, erythrose, or glycerol for vegetative growth, whereas a *dome* morphological mutant can utilize only ribitol or glycerol.

Wild-type as well as *dome* mycelium contain a NADPH-dependent erythrose reductase, regardless of the carbon source employed for growth. Several lines of indirect evidence suggest that erythrose reductase is a separate enzyme from other aldose reductase activities in crude extracts of *S. commune*. However, enzyme purification is clearly required before more can be said.

When wild-type mycelium is cultured on erythrose or erythritol, an NAD-dependent erythritol dehydrogenase capable of producing erythrulose is observed. In addition, glycerol growth of wild-type mycelium also serves to induce erythritol dehydrogenase. Induction of erythritol dehydrogenase by erythrose is most easily understood by the finding that the constitutive erythrose reductase serves to generate erythritol, which may then serve as subsequent inducer. The situation involving glycerol induction of erythritol dehydrogenase is more difficult to explain, although structural considerations of the nature of glycerol and erythritol make this tenable. An additional restriction concerning the nature of the inducer involves the chain length of the particular acyclic compound because ribitol is inactive in this regard. The problem is further complicated by the occurrence of an inducible erythritol uptake system. Interestingly, prior mycelial growth on either glycerol or

erythritol also supports the formation of the uptake process. The nature of control mechanisms regulating erythritol metabolism of yeast differs in this regard, because prior growth on erythritol but not glycerol sustains the erythritol oxidation capacity of *T. candida* and *H. anomala* (1).

Although glycerol is utilized as sole carbon source for mycelial growth of the *dome* mutant of *S. commune* and yields cells capable of erythritol uptake, this mutant does not produce a functional erythritol dehydrogenase under these conditions, nor can growth occur on erythrose or erythritol. In addition, erythrose reductase is also a constitutive enzyme in *dome*. A further similarity regarding erythritol uptake by glycerol-grown wild-type mycelium or *dome* involves the effects of unlabeled sugars and polyols. Both systems were stimulated by D-erythrose, unaffected by ribitol or glycerol, and inhibited by glucose or ribose. The mechanisms underlying most of these effects remain to be established; however, glucose may exert an inhibition through catabolite repression because wild-type growth of *S. commune* on glucose plus erythritol leads to a depression of erythritol dehydrogenase (7). Consequently, the failure of *dome* mycelium to utilize substrates containing the *erythro*-configuration cannot be explained by either a permeability barrier or an initial enzyme deficiency regarding erythrose catabolism, but rather is explicable in terms of a specific loss in the ability to produce erythritol dehydrogenase.

The subsequent fate of erythrulose produced by erythritol oxidation of wild-type mycelium of *S. commune* is not known. In *P. pentosaceum*, erythritol is phosphorylated to L-erythritol 1-phosphate, which is then oxidized in the presence of NAD to form L-erythrulose 1-phosphate, and subsequent cleavage produces formaldehyde and dihydroxyacetone phosphate (13). An enzymatic cleavage of erythrulose may therefore be anticipated in *S. commune*, although evidence is still lacking on this point. It also remains to be established whether the erythritol phosphorylation pathway of *P. pentosaceum* is peculiar to this bacterium or may have a more general distribution among other bacteria or fungi.

Because *dome* still possesses an inducible erythritol uptake system yet is deficient in subsequent catabolism of erythritol, the employment of this particular mutant of *S. commune* may prove particularly valuable for future studies regarding mechanisms of sugar alcohol permeation in fungi.

### ACKNOWLEDGMENTS

This investigation was supported by a research training grant fellowship from the Public Health Service to M.L.B. and a re-

search grant from the National Science Foundation (GB 8327) to D.J.N.

We gratefully acknowledge the technical assistance of Karen Aronoff.

#### LITERATURE CITED

1. Barnett, J. A. 1968. The catabolism of acyclic polyols by yeasts. *J. Gen. Microbiol.* **52**:131-159.
2. Dawson, R. M. C., D. C. Elliott, W. H. Elliott and K. M. Jones. 1959. *Data for biochemical research*. Oxford University Press, Inc., New York.
3. Dische, L., and M. R. Dische. 1958. Methods for the qualitative and quantitative determinations of tetroses by two new specific color reactions. *Biochim. Biophys. Acta* **27**:184-188.
4. Goepfert, G. J., and F. F. Nord. 1942. On the mechanism of enzyme action. 20. Chemistry and dehydrogenations with and without training of *Fusarium lini* Bolley (FIB). *Arch. Biochem.* **1**:289-301.
5. Gordon, H. T., W. Thornburg, and L. N. Werum. 1956. Rapid paper chromatography of carbohydrates and related compounds. *Anal. Chem.* **28**:849-855.
6. Hu, C. L., V. V. Rendig, and E. A. McComb. 1965. Evidence for the ninhydrin-positive reaction of some ketoses. *J. Chromatog.* **19**: 622-623.
7. Isenberg, P., and D. J. Niederpruem. 1967. Control of erythritol dehydrogenase in *Schizophyllum commune*. *Arch. Mikrobiol.* **56**:22-30.
8. Jakoby, W. B., and J. Fredericks. 1961. Erythritol dehydrogenase from *Aerobacter aerogenes*. *Biochim. Biophys. Acta* **48**:26-32.
9. Lewis, D. H., and D. C. Smith. 1967. Sugar alcohols (polyols) in fungi and green plants. I. Distribution, physiology and metabolism. *New Phytol.* **66**:143-184.
10. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193**:265-275.
11. Niederpruem, D. J., and D. W. Dennen. 1966. Kinetics, nutrition and inhibitor properties of basidiospore germination in *Schizophyllum commune*. *Arch. Mikrobiol.* **54**:91-105.
12. Niederpruem, D. J., A. Hafiz, and L. Henry. 1965. Polyol metabolism in the basidiomycete *Schizophyllum commune*. *J. Bacteriol.* **89**:954-959.
13. Wawszkiewicz, E. J. 1968. Erythritol metabolism by *Propionibacterium pentosaceum*. The role of L-erythrulose 1-phosphate. *Biochemistry* **7**:683-687.
14. Wawszkiewicz, E. J., and H. A. Barker. 1968. Erythritol metabolism by *Propionibacterium pentosaceum*. The over-all reaction sequence. *J. Biol. Chem.* **243**:1948-1956.
15. Wessels, J. G. H. 1965. Morphogenesis and biochemical processes in *Schizophyllum commune* Fr. *Wentia* **13**:1-113.
16. Wright, J. R., and D. LeTourneau. 1965. Utilization and production of carbohydrates by *Pyrenochaeta terrestris*. *Physiol. Plant.* **18**:1044-1053.