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D-Mannitol Dehydrogenase from *Absidia glauca*. Purification, Metabolic Role, and Subunit Interactions†

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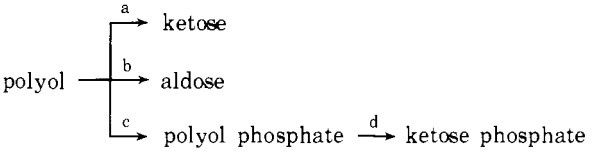
ABSTRACT: When *Absidia glauca* was grown in minimal media with D-mannitol as the only source of carbon, an NAD^+ specific D-mannitol dehydrogenase (EC 1.1.1.67) was induced. The crude extract also gave evidence of mannitol kinase, mannitol-1-phosphate dehydrogenase, phosphofructokinase, and L-iditol dehydrogenase activity. The heat labile purified preparation was judged enzymically homogeneous based on evidence derived from substrate specificity studies and activity staining, following disc gel electrophoresis. The enzymic monomer, with a weight of about 67 000 daltons, slowly polymerizes when stored at −20 °C, giving a multiplicity of protein bands on electrophoresis distributed predominantly across a spectrum from dimer to pentamer, with enzymic activity resident predominantly in even multiples of the monomer. Depolymerization occurred rapidly (hours) when a frozen preparation was brought to and held between 4 and 20 °C. Aggregate fragmentation with sodium dodecyl sulfate showed a time-temperature dependence, terminating in a subunit component of 13 000 daltons. pH optimum for polyol oxidation occurs at 9.6 (NaOH–glycine buffer) while ketose reduction proceeded most rapidly at pH 7.0–7.2 (phosphate buffer). A regulatory role is suggested for this enzyme based on dead-end inhibition by mannitol 1-phosphate, multiple enzyme forms, and its locus at the initiation site for mannitol utilization. The physiological relevance of low-temperature aggregation to regulatory control remains to be established.

While metabolic pathways for polyol utilization and biosynthesis are well established in a variety of plants, animals, and microorganisms (Touster and Shaw, 1962; Touster, 1974) and studies of mannitol utilization have generated key experiments in bacterial genetics and physiology (Berkowitz, 1971), less attention has been directed to the detailed mechanisms of metabolic control which regulate their entrance into the carbohydrate pathway.

As Touster (1974) has indicated, the utilization of polyols by living organisms is initiated by one of the following reactions:



involving (a) oxidation to a ketose; (b) oxidation to an aldose; (c) phosphorylation to the corresponding phosphate. While the direct phosphorylation route c has not been encountered in mammals, the catabolic fate of the phosphate in other tissues is associated frequently with an NAD^+ (or $NADP^+$) linked dehydrogenase (d). However, as Strandberg (1969) has indicated, the presence of two dehydrogenases (a, d) in extracts from mannitol-grown cells of *Aspergillus candidus* has posed difficulties in attempting to assemble a rational picture for mannitol utilization–biosynthesis in this organism.

Boulter and Derbyshire (1957), using conventional and

† From the Department of Chemistry, Seton Hall University, South Orange, New Jersey 07079. *Received August 11, 1975.* This work was supported, in part, by Grant No. GY-2418 from the National Science Foundation.

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low-temperature spectroscopy, demonstrated the presence of reduced cytochromes *a, b,* and *c* in some 20 species of Phycomycetes and 25 species of Basidiomycetes. Although *Absidia glauca* was included in their listing, the mechanism whereby this species can grow on D-mannitol as the sole carbon source appears not to have been previously reported.

In this paper we report the results of a series of studies involving mannitol utilization (as the sole carbon source) by *A. glauca*. The presence of both mannitol and mannitol-1-phosphate dehydrogenase activities is demonstrated and shown to be compatible with the optimum utilization of this polyol. In addition, the self-associative properties of mannitol dehydrogenase are discussed and suggestively assigned a regulatory role in mannitol utilization.

Experimental Procedure

Materials

Polyols. Erythritol, ribitol, D-glucitol (sorbitol), D-arabinitol, D-mannitol, perseitol, xylitol, L-arabinitol, and galactitol were obtained from Pfanstiehl Laboratories. L-Iditol was prepared by the reduction of L-sorbose (Hann and Hudson, 1945). D-Mannose 6-[^{32}P]phosphate, D-fructose 1,6-bis[^{32}P]phosphate, and D-fructose 6-[^{32}P]phosphate were prepared as previously described (Zuleski and McGuinness, 1969). Mannitol 1-phosphate was prepared by the method of Wolfe and Kaplan (1956) from D-mannose 6-phosphate. ^{14}C- and ^{32}P-labeled mannitol 1-phosphate were prepared by the same methods from the correspondingly labeled mannose 6-phosphate.

Nucleotides (ADP, ATP), enzyme cofactors (NAD^+, NADH, $NADP^+$, and NADPH), and various proteins used for calibration and assay runs were obtained from Sigma Chemical Co.

Chromatographic support media were obtained from Pharmacia and LKB Instruments, Inc. (Sephadex gels and Ultrogel AcA34), Reeves Angel (DE-52 ion-exchange cellulose), and Distillation Product Industries (Eastman Chromagram thin-layer sheets). Ammonium sulfate was purchased from Schwarz/Mann. All components of the fungal growth media were obtained from Difco Laboratories.

Methods

Growth Conditions. Absidia glauca (ATCC No. 7852 A) was grown for 3 days on 125 ml of media 5% (w/v) in: D-mannitol; casein amino acid hydrolysate; basal salts ($NaNO_3$, K_2HPO_4, and $MgSO_4 \cdot 7H_2O$) and sequestrene, previously autoclaved (20 min, 120 °C, 18 lb pressure) in 500-ml Erlenmeyer flasks. Growth (at 27 °C) was aerobic (flasks shaken) following inoculation by a spore suspension taken from an agar slant of the organism. The 72-h mycelial mats were Millipore filtered (HA 0.45 μm white grid), washed with three 10-ml portions of triply distilled water, and stored at −20 °C. The yield of wet packed mat was about 15 g/l. of media.

Labeling Studies. The organism was grown as described, but with D-[U-^{14}C]mannitol (20 μCi) or [^{32}P]orthophosphate (15 μCi) added to each flask at inoculation.

Gel Chromatography. The detailed protocol for the use of gel media as applied in these studies has been presented elsewhere (McGuinness and Cullen, 1970).

Thin-Layer Chromatography. Eastman Chromagram sheets (No. 6064) were used as previously described (Zuleski and McGuinness, 1973) for the separation of various sugars, polyols, and corresponding phosphates and for enzyme incubation studies with crude extract.

Polyacrylamide Electrophoresis. Disc gel electrophoresis, performed in 7% polyacrylamide gel and then in 10% gel in the presence of sodium dodecyl sulfate, was carried out by the methods of Davis (1964) and Weber and Osborn (1969), respectively. Activity staining, designed to localize dehydrogenase activity at varying acrylamide gel concentrations, was based on the modification of the Dewey and Conklin method as described by Fine and Costello (1963). The influence of varying polyacrylamide gel concentrations on the size–charge properties of the enzyme was studied following the protocol of Hedrick and Smith (1968), by which a plot of the logarithm of the migration distance relative to that of a marker dye against the gel concentration gives a slope pattern which is uniquely characteristic of the size and/or charge properties of the protein. Comparison of these slopes with that of a slope–molecular weight curve for a series of standard proteins was then used to establish the molecular weight for a migrating species.

Protein Estimation. Protein determinations were routinely performed using the assay methods of Lowry et al. (1951), Warburg and Christian (1941), or the method of Waddell as described by Murphy and Kies (1960). Recrystallized human serum albumin was employed as the standard protein.

Enzyme Assays. Routine dehydrogenase activities were monitored on a Beckman DB-GT spectrophotometer equipped with a Sargent MR recorder at 340 nm by following the rate of reduction of NAD^+ in the presence of the appropriate polyol substrate. Blank and test samples were constituted from co-factor (1.0 ml, 6.0 mM), substrate (0.20 ml, 0.5 M), phosphate buffer (0.06 M, pH 7.6, 1.00 ml), and sufficient deionized-distilled water to bring the final total volume in the 1.0-cm path-length cuvette to 3.00 ml. The reaction was initiated in the test sample by the addition of the appropriate amount (0.01–0.1 ml) of enzyme-active material. Specific activity is reported as units per milligram of protein, where a unit is taken as the amount of enzyme required to convert 1 μmol of NAD^+ to NADH per minute. For these conditions there is a linear correlation between the rate of reaction and enzyme concentration. The reaction mixture for substrate and cofactor specificity studies contained, for polyol oxidation: 120 μmol of the polyol; 6 μmol of NAD^+ or $NADP^+$; 5 μg of enzyme; and 150 μmol of NaOH–glycine buffer, pH 9.6. For ketose reduction the cuvette contained: 120 μmol of the ketose; 0.6 μmol of NADH or NADPH; 5 μg of enzyme; and 60 μmol of phosphate buffer (pH 7.2). The ionic strength was adjusted to 0.1 with NaCl before each run.

Incubation Studies. Evidence for mannitol-1-phosphate dehydrogenase, mannitol kinase, hexokinase, and phosphofructokinase activities in the crude extract was established following the protocol for phosphofructokinase activity we have described elsewhere (Zuleski and McGuinness, 1973).

A typical incubation mixture consisted of: substrate, cofactor(s), phosphate buffer (50 mM, pH 7.4), and crude extract taken from a cut of the effluent fraction at the void volume of a Sephadex G-25 column. R_f values were established for the various metabolites using radiochromatographic scanning, excision followed by liquid scintillation counting, and *p*-anisidine (for fructose) and/or Hanes–Isherwood (1949) spray reagents. Runs were made in the solvent system acetone–trichloroacetic acid (5 g/80 ml)–ammonium hydroxide (6.5:3.0:0.3) and compared to authenti-

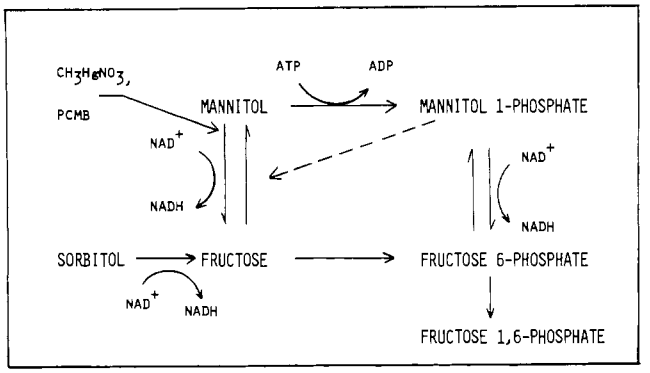

FIGURE 1: Proposed pathway for the initial utilization of polyol as the sole source of carbon by *A. glauca*.

cated samples. Generally between 7 and 12 × 10^3 dpm of ^{32}P activity was delivered to the support media at the origin. Where repetitive spotting was necessary, it was interspersed with spot drying between applications to confine the radioactivity and reduce trailing. Blanks and recovery runs were made simultaneously to correct for thermal degradation of ATP. With scintillation counting as the detection mode, background amounted to 50 ± 12 cpm at counting efficiencies of 57% (^{32}P) and 35% (^{14}C). Recovery of radioactivity amounted to 93%.

Results

Pathway for Polyol Utilization in Absidia glauca. Evidence for the pathway for the initial utilization of sorbitol and D-mannitol by *A. glauca*, as shown in Figure 1, is based on the following types of experiments: (i) thin-layer and column radiochromatographic identification of reactants and products arising from incubation studies with the crude extract; (ii) relative substrate activities examined during the course of purification of D-mannitol dehydrogenase.

Dehydrogenase Activity in the Crude Extract. In addition to evidence for NAD^+-linked enzymic activity with D-mannitol, the crude extract showed the following levels of polyol activity, relative to D-mannitol (100): sorbitol (27); xylitol (39); D-mannitol 1-phosphate (2); and L-iditol (23). As purification proceeds only sorbitol activity persists from this latter group of substrates, falling to a constant, residual level of 4–5% of that for D-mannitol. The low level of mannitol 1-phosphate activity (2%) is due to the high pH (9.6). When the relative activities were compared in phosphate buffer at pH 7.4, the mannitol-1-phosphate dehydrogenase activity amounted to 25% of the mannitol dehydrogenase activity. The crude extract was also active with NADH when either D-fructose or D-fructose 6-phosphate was added as substrate.

Evidence for D-Mannitol 1-Phosphate. The utilization of mannitol by *A. glauca* was accompanied by the appearance of bands of radioactivity at elution volumes of 72 ml (V_o = 1.0) and 112 ml (V_e/V_o = 1.61) when 3.0 ml of the growth medium was chromatographed on Sephadex G-15 (Figure 2). At successive stages of growth there was a decrease in the radioactivity of the D-[1-^{14}C]mannitol peak (V_e = 132 ml, V_e/V_o = 1.86) and a corresponding increase in the 112-ml (D-mannitol 1-phosphate) peak. When ^{32}P as orthophosphate replaced D-[U-^{14}C]- or -[1-^{14}C]mannitol in the culture medium, the single elution band corresponding to labeled orthophosphate (V_e = 118 ml) gradually shifted to an elution volume of 112 ml. Authentic samples of D-[1-^{14}C]mannitol 1-phosphate and D-[1-^{32}P]mannitol 1-phosphate were eluted from Sephadex G-15 at V_e = 112 ml for the conditions described. The fraction cut between 110 and 115 ml was enzymically active when added to a diluted sample of the crude extract containing NAD^+. The band of radioactivity appearing at the void volume was heterogeneous when rechromatographed on Sephadex G-75.

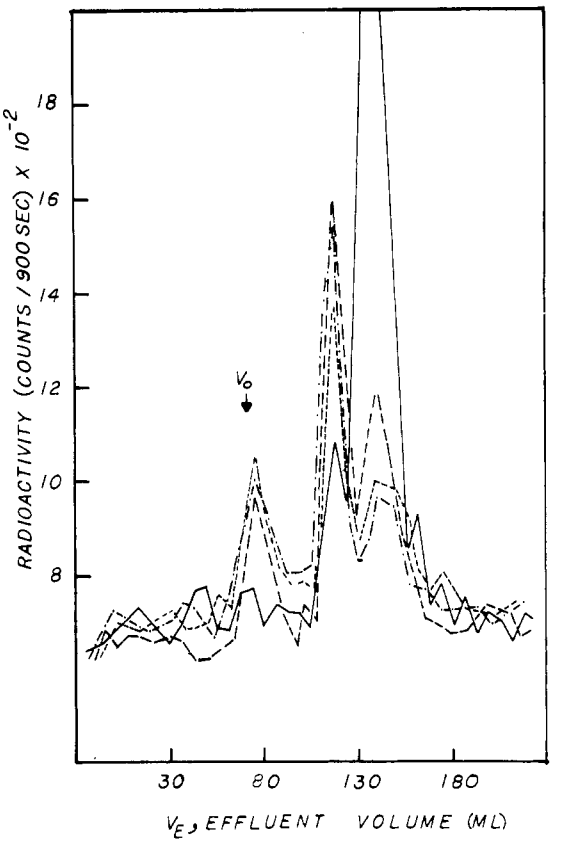

FIGURE 2: Gel chromatographic behavior of the growth media of *A. glauca*. Filtrate (3.0 ml) from the growth media, containing 20 μCi of D-[1-^{14}C]mannitol or 20 μCi of $H_3{}^{32}PO_4$ and 0.5 g of sucrose, was layered on a 2.5 × 41 cm column of Sephadex G-15. The eluting solvent was 0.1 M ammonium acetate, 0.02% in sodium azide. The column effluent was continuously monitored at 4 °C in a 20-ml Packard flow-cell packed with anthracene and mounted in a Nuclear-Chicago (Model 703P) liquid scintillation counter. The flow rate was held constant by adjusting the pressure head using gravity feed. Readings of radioactivity were taken in two channels and collected by automatic print-out at 900-sec intervals. The void volume was established by using blue dextran and myoglobin. Filtrates, taken from the growth media at the 3rd (—), 5th (- - -), 8th (---), and 12th (·--) days, were examined for their elution profile.

Incubation Studies with the Crude Extract. D-Mannitol Kinase. A 50-μl spotting of an incubation mixture consisting of D-mannitol (0.2 ml, 0.2 mM), $MgCl_2$ (0.05 ml, 0.05 mM), [α,β,γ-^{32}P]ATP (0.1 ml, 0.02 mM, 0.1 μCi), buffer (0.9 ml), and crude extract (0.3 ml) gave rise to bands of radioactivity corresponding to mannitol 1-phosphate and adenosine diphosphate. When [^{32}P]ortho- or [^{32}P]polyphosphate replaced the labeled ATP in the incubation mixture these bands disappeared.

D-Mannitol-1-Phosphate Dehydrogenase. Incubation mixtures consisting of D-[^{32}P]fructose 6-phosphate (0.2 ml, 0.2 mM, 0.1 μCi), NADH (0.2 ml, 0.5 mM), buffer (0.85 ml), and crude extract (0.3 ml), when run in the presence and absence of 10^{-4} mM *p*-hydroxymercuribenzoate for various inhibitor exposure times, gave evidence that the inhibition of the enzyme is a time-dependent event. Radioactivity corresponding to mannitol 1-phosphate decreased

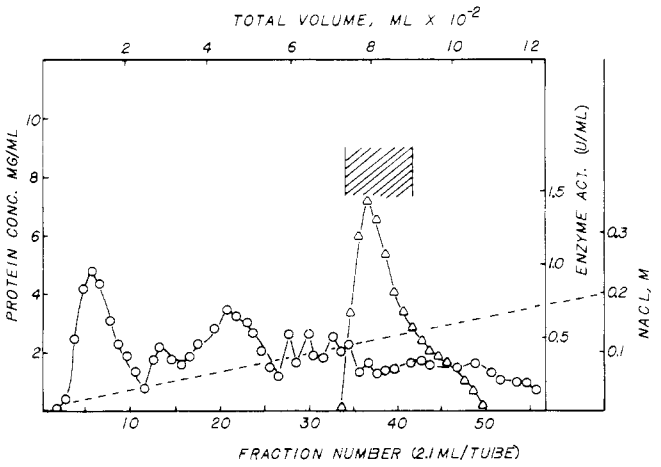

FIGURE 3: Chromatography on DEAE-cellulose. Twenty milliliters of the dialyzed first ammonium sulfate fraction was delivered onto a 2.5 × 30 cm column of Whatman DE-52 ion exchange cellulose, previously equilibrated with 0.02 M phosphate buffer (pH 7.6), 10^{-3} M in dithiothreitol. Elution was carried out with the same buffer and a NaCl gradient from 0 to 0.2 M; flow rate, 54 ml/h; concentration factor, 10.3. Protein (O–O) was assayed by the Lowry et al. (1951) method. Enzyme activity (Δ–Δ) is reported as units/milliliter.

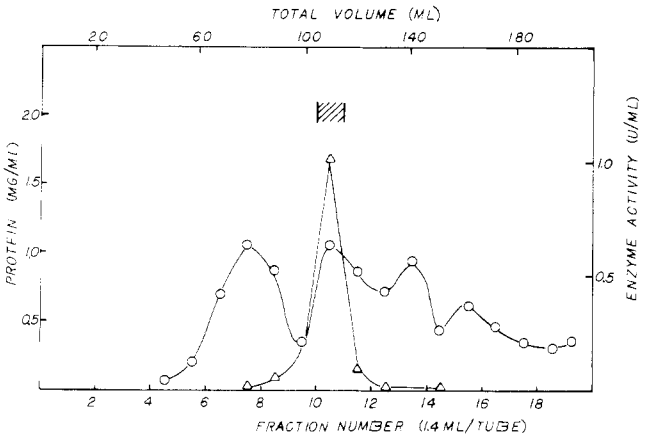

FIGURE 4: Gel filtration pattern on Sephadex G-150. Fractions of 1.4 ml were collected from the eluate concentrator (concentration ratio 7.5/1); flow rate 26.2 ml/h; eluting solvent, 0.02 M phosphate buffer (pH 7.6), 1 mM in dithiothreitol. Protein (O–O) was measured by the method of Lowry et al. (1951); enzyme activity (Δ–Δ) is reported as units/milliliter.

from 399 to 173 to 0 cpm for inhibitor exposure times of 0, 10, and 30 min, respectively.

Phosphofructokinase. When, in the protocol as described (Zuleski and McGuinness, 1973) for phosphofructokinase assay, crude extract replaced the latter enzyme, bands of radioactivity corresponding to adenosine diphosphate and D-fructose 1,6-bisphosphate were evident. An increase in the ratio of radioactivity of the products of this reaction, ADP:fructose 1,6-bisphosphate, upon prolonged incubation times indicated further metabolism of the ketose diphosphate reaction product.

Hexokinase. When, in the protocol for mannitol kinase activity, D-fructose (0.2 ml, 0.2 mM) replaced D-mannitol, the phosphorus-32 label appeared in bands corresponding to ADP, fructose 6-phosphate, and fructose 1,6-bisphosphate. No evidence for either D-mannitol 1-phosphatase or sorbitol-6-phosphate dehydrogenase activity was found in the crude extract.

Purification of D-Mannitol Dehydrogenase. All operations were carried out at 4 °C in the presence of 1 mM dithiothreitol since preliminary experiments revealed the enzyme was heat labile and susceptible to aerobic destruction.

(1) Crude Extract. Small portions of stored mat (3–5 g, wet weight), suspended in 20 ml of phosphate buffer (0.02 M, pH 7.6), were mechanically disrupted using a motor-driven Teflon and glass homogenizer. The resulting homogenate was then centrifuged at 16 000*g* for 20 min and the pellet discarded. Attempts to incorporate protamine or streptomycin sulfate into the purification protocol at this point were unsuccessful, since both destroyed enzyme activity.

(2) Ammonium Sulfate Precipitation. The supernatant from the crude extract was brought to 65% saturation by the addition of solid ammonium sulfate (43 g for 100 ml of crude) as a finely ground powder. The addition was made over a 90-min period in small portions to the continuously stirred solution. The suspension was allowed to stand for 20 min and then centrifuged at 16 000*g* for 30 min.

(3) DEAE-Cellulose Chromatography. The pellet from the ammonium sulfate precipitation was taken up in (20 ml) and dialyzed overnight against 0.02 M phosphate buffer (pH 7.60) and then applied at a rate of 1 ml/min to a column (2.5 × 30 cm) of Whatman DE-52 cellulose previously equilibrated with the phosphate buffer. The column was developed at a flow rate of 54 ml/h using a linear gradient of NaCl (0 to 0.2 M). Column effluent was taken through an Amicon on-line column eluate concentrator fitted with a PM 10 ultrafiltrate membrane. A representative elution profile is shown in Figure 3.

(4) Ammonium Sulfate Precipitation. The pooled fractions from the ion-exchange chromatography step were again brought to 65% saturation with ammonium sulfate and centrifuged, as described above.

(5) Gel Filtration of Sephadex G-150. The pellet from the second ammonium sulfate precipitation was dissolved in 0.02 M phosphate buffer (pH 7.6) and applied to a Sephadex G-150 column (2.5 × 35 cm) previously equilibrated with buffer. Elution with the same buffer was carried out at a flow rate of 26.2 ml/h (Figure 4). The column effluent was again directed through the eluate concentrator described for the ion-exchange step.

(6) Ammonium Sulfate Precipitation. The fractions from the gel chromatography step containing enzyme activity were pooled and the enzyme precipitated by the addition of ammonium sulfate to 65% saturation. The pellet was redissolved in the same buffer, 10^{-3} M in dithiothreitol, and stored at −20 °C.

A representative purification scheme is shown in Table I.

Physical Properties of the Enzyme. Storage Stability. No loss of enzyme activity over a 45-day period was observed when the active preparation was stored as described in step 6. When placed in Tris buffer (pH 8.4) or NaOH–glycine buffer (pH 9.6) under comparable storage conditions, the preparation rapidly deteriorated, as judged by a marked loss of activity after 6–8 days.

Thermal Stability. The preparation was found to retain constant activity for a period of 8–9 h when held at 4 °C. When the enzyme was incubated at a variety of temperatures between 4 and 40 °C for 10 min prior to assay, it showed a rapid and complete loss of activity, starting at 20 °C, with total attenuation occurring by about 40 °C.

pH–Activity Profile. D-Mannitol dehydrogenase catalyzes the reversible oxidation of D-mannitol to D-fructose.

Table I: Purification Scheme for D-Mannitol Dehydrogenase from *A. glauca*.[a]

Fraction	Vol (ml)	Protein (mg)	Activity: Total Units	Activity: Specific Units/mg
1. Crude extract	100	395	37.4	0.0947
2. $(NH_4)_2SO_4$ (35–65%)	21	244	29.4	0.119
3. DE-52 cellulose	12.8	13.5	17.2	1.27
4. 2nd $(NH_4)_2SO_4$	2.4	5.3	16.4	3.09
5. Sephadex G-150	1.2	1.26	12.3	9.77
6. 3rd $(NH_4)_2SO_4$	0.5	1.19	12.2	10.24

[a] The enzyme activity assay system used during purification contained 100 μmol of D-mannitol, 6 μmol of NAD^+, and up to 0.1 ml of the preparation in a final volume adjusted to 3.00 ml with sodium phosphate buffer (pH 7.6). The reaction was initiated by the addition of the enzyme and the absorbance change at 340 nm was followed at 25 °C. A unit of activity is defined as that amount of enzyme capable of producing 1 μmol of NADH per min. Protein was routinely estimated by the method of Lowry et al. (1951).

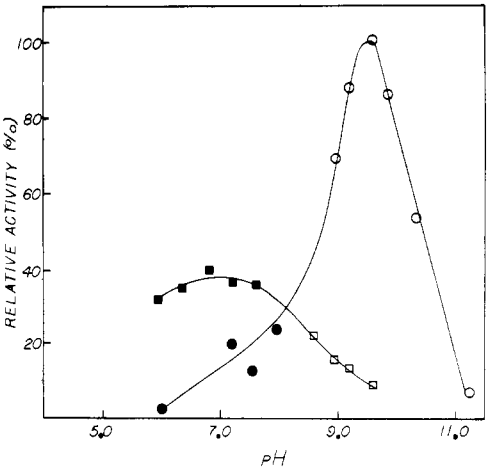

FIGURE 5: Influence of pH on the activity of D-mannitol dehydrogenase. For mannitol oxidation (●–○) each cuvette contained NAD^+ (5 μmol), mannitol (100 μmol), buffer (phosphate (60 μmol) or NaOH–glycine (150 μmol)) and enzyme (5 μg) in a total final volume of 3.0 ml. Fructose reduction (■–□) was followed in a system consisting of NADH (0.5 μmol), fructose (100 μmol), buffer (same as forward reaction), and enzyme (0.1 ml) brought to 3.0 ml. Ionic strength was held at 0.1 in all runs with NaCl. Reaction was initiated by the addition of enzyme. Temperature was held at 25 °C: (■,●) phosphate buffer; (□,○) NaOH–glycine buffer.

The influence of pH on the activity of the enzyme was studied over the pH range 6.0–11.0 in phosphate and NaOH–glycine buffers (Figure 5). The apparent pH optimum for mannitol oxidation occurred at 9.6, while that for D-fructose reduction occurred at about 7.0–7.2.

Thiol Group Activity. The enzyme was completely inhibited by methyl mercurinitrate and *p*-hydroxymercuribenzoate. The inhibition by the alkyl mercurial was complete in the time required to add the reagent and position the cuvette in the spectrophotometer (10 s). The aryl mercurial produced complete inhibition in 3 to 4 min.

Substrate and Cofactor Specificity. Substrate specificities, as judged by the relative rates of polyl oxidation, referred to NAD^+ and D-mannitol (100), were: erythritol (0), ribitol (2), sorbitol (6), D-arabinitol (29), perseitol (13), xylitol (0), L-iditol (0), L-arabinitol (0), and galactitol (0). Reactivity with $NADP^+$ relative to NAD^+ (100) was: D-mannitol (4.3); all other polyols (0). For the reverse reaction (pH 7.2) the relative rates of ketose reduction were: D-fructose (100), L-sorbose (5.1), and D-tagatose (6.3), with NADH as cofactor. Reactivity with NADPH, relative to NADH (100) for the three ketoses, was: D-fructose (7.2); L-sorbose (5.1); and D-tagatose (6.5). Based on this evidence we judge the preparation cited in Table I to be enzymically homogeneous and specific for NAD^+ and D-mannitol. Since the hydroxyl of the secondary alcohol involved in the oxidation and the cis-vicinal secondary hydroxyl must have a D configuration with respect to the primary alcohol adjacent to the site of oxidation, the enzyme is properly classed as a D-mannitol dehydrogenase (McCorkindale and Edson, 1954).

In contrast to the clear criteria and evidence for evaluating enzymic homogeneity, the comparable picture for protein homogeneity is less definitive. Thus, a fresh preparation, when subjected to protein staining following electrophoresis, gave one major band (amounting to some 80%, at 67 000) and three minor bands (totaling about 20%), all of lesser mobility (larger size). None of these minor bands showed any evidence of enzymic activity. Therefore, either these minor bands are contaminating protein and our preparation is judged 80% pure on the basis of protein, or some aggregation into nonenzymic multimers has been engendered during the electrophoretic run.

Studies of several factors that influence the extensive self-association exhibited by D-mannitol dehydrogenase were carried out using gel permeation chromatography and polyacrylamide disc gel electrophoresis.

Gel Chromatography. The elution profile of a 12-day frozen preparation run on LKB Ultrogel AcA-34 at 25 °C gave bands corresponding to molecular weights of 67 000 and 133 000. When this preparation was carried through pretreatment and gel chromatography with sodium dodecyl sulfate according to the protocol of Fish (1971), a single constituent polypeptide chain of molecular weight 13 000 was obtained.

Disc Gel Electrophoresis. A freshly prepared sample, not subject to storage freezing, showed a single band with a molecular weight corresponding to 67 000 when run on 5, 7 (Figure 6A), 7.5, and 10% gels and subjected to activity staining with D-mannitol, NAD^+, phenazine methosulfate, and nitro blue tetrazolium. This evidence of enzymic homogeneity for the preparation agrees with that derived from substrate specificity studies cited above. When a 45-day frozen dialyzed preparation was examined using the activity staining technique, multimers of 136 000, 198 000, and 262 000 daltons showed D-mannitol dehydrogenase activity (Figure 6D).

When samples, subjected to prolonged storage (40–45 day frozen preparations) and prolonged storage followed by overnight dialysis against phosphate buffer (0.02 M, pH 7.6), 0.1 M in NaCl and 10^{-3} M in dithiothreitol, were stained with amido black following electrophoresis on 5, 7 (Figure 6B,C), 7.5, and 10% gels, multiple banding was evident. Prolonged storage gave rise to five major bands of molecular weights 201 000, 214 000, 232 000, 265 000, and 292 000 (Figure 6B). Prolonged storage, followed by dialysis (Figure 6C), gave rise to three major bands of molecular weights 137 000, 205 000, and 269 000 (compare to Figure

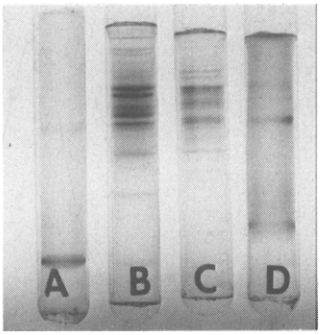

FIGURE 6: Disc gel electrophoresis of the mannitol dehydrogenase preparation. Samples of protein (80 μg) were run on 5, 7, 7.5, and 10% gels using the protocol of Davis (1964). Only 7% gels are shown. Samples A and D depict a freshly prepared sample and a 45-day frozen dialyzed sample, respectively, visualized by activity staining. Samples B and C depict 40-day frozen and 45-day frozen dialyzed preparations respectively, stained for protein with amido black. Direction of migration is from top to bottom. See text for additional details.

6D), and, in addition, evidence of some fragmentation of the preparation, as judged by the appearance of minor bands of 13 000, 25 000, and 50 000 daltons.

When polymerized samples (24-days frozen) were subjected to polyacrylamide gel electrophoresis in sodium dodecyl sulfate, the extent of depolymerization was dependent on the time–temperature cycle of the pretreatment with 0.1% of this surfactant. Thus, treatment with 0.1% sodium dodecyl sulfate at 37 °C for 3 h was sufficient to cause complete dissociation of the protein to the constituent polypeptide chain (13 000 daltons), whereas treatment with 0.1% sodium dodecyl sulfate for 1 h at 25 °C gave rise to a partially depolymerized material with major bands of molecular weights 80 000, 67 000, and 54 000.

The molecular weights of the associated species were established by determining the relative mobilities of the preparation as a function of the polyacrylamide gel concentration. The common intercept pattern obtained in the region of 0% gel concentration when the plots of the logarithm of the relative mobilities against the several gel concentrations were extrapolated to the ordinate is taken as evidence for a size-isomer family of proteins (Hedrick and Smith, 1968). (The authors will be pleased to furnish the interested reader with a tabular correlation of aggregate molecular weights and the factors that enhance this self-association.)

An investigation of the mechanism of mannitol oxidation by this enzyme, based on initial velocity, and product and dead-end inhibition studies (to be reported elsewhere), reveals that the reaction follows a sequential, ordered pathway in which mannitol 1-phosphate acts as a dead-end inhibitor.

Discussion

On the basis of the cumulative evidence presented in this paper it is reasonable to suggest that the initial utilization of mannitol by this organism proceeds by either of two pathways: (1) a quantitatively dominant oxidation to D-fructose by mannitol dehydrogenase, thence by conventional catabolic pathways to the cytochrome terminus for the fabrication of ATP and reducing equivalents; (2) in the presence of adequate supplies of ATP and NADH, mannitol 1-phosphate, arising from mannitol kinase or mannitol-1-phosphate dehydrogenase directed catalysis, acts as a dead-end inhibitor to mannitol dehydrogenase and, in turn, serves as a precursor of fungal wall material.

The crude extract contains an induced L-iditol (sorbitol) dehydrogenase, probably arising from the small amount of sorbitol present in the growth media. The total activity manifested by sorbitol as substrate (27%) is attributed to its reactivity to its own enzyme (23%) and to the mannitol dehydrogenase (4–5%). As purification proceeds only the latter activity remains. In a similar way the xylitol activity in the crude extract may arise from the presence of an induced xylitol dehydrogenase, reaction with L-iditol dehydrogenase, or both. However, this point was not further investigated.

The fresh (i.e., unfrozen) purified preparation of mannitol dehydrogenase satisfies two distinct criteria of enzymic homogeneity: (i) a unique substrate specificity to D-mannitol; (ii) a single band on variable (percent) gel electrophoresis when a freshly prepared sample is subjected to activity staining. The absence of multiple banding under these conditions also precludes the possibility of dissociation or association of the (67 000 mol wt isomer) preparation into active subunits or multimers during the course of the electrophoretic run.

We became suspicious that the enzyme readily aggregated when a 67 000 mol wt isomer preparation (subsequently frozen), examined by sedimentation velocity ultracentrifugation (following overnight dialysis at 4 °C against phosphate buffer, 0.1 M in NaCl), was shown to be inhomogeneous, with a sedimentation pattern characteristic of proteins of mol wt about 250 000.

The results of the various electrophoretic mobility runs clearly demonstrate that the preparation we have described exhibits an extensive capacity to polymerize and dissociate, and is markedly responsive to changes in the physical conditions of its environment. Thus, upon storage (−20 °C), the enzyme slowly polymerizes and the single band at 67 000 gradually decreases in intensity, disappearing entirely when the sample is exposed to overnight dialysis against 0.1 M NaCl. Although prolonged storage and dialysis against salt enhance polymerization, they also give rise to some fragmentation as evidenced by the appearance of minor bands of 13 000, 25 000, and 50 000 sized isomers. These three sized isomers are not enzymically active since the activity staining detection method is sufficiently sensitive to have revealed them. If we view the 25 000 and 50 000 size isomers as dimers and tetramers of the sodium dodecyl sulfate generated subunit polypeptide chain, then we conclude that the latter units are also devoid of enzyme activity. It is tempting to suggest that sodium dodecyl sulfate degradation of the oligomers occurs by the sequential removal of a basic polypeptide chain, since partially degraded fragments differ by the weight of the basic unit.

No evidence from activity staining under a variety of conditions has been adduced to suggest that enzymic activity is resident in any size isomer smaller than 67 000 daltons. Consequently, if we assign to this entity the role of enzymic monomer, mannitol dehydrogenase activity is resident in the monomer, dimer (136 000), and tetramer (262 000), but only to a significantly lesser extent in the trimer (minor activity, major protein band at 198 000), and not at all in the pentamer (336 000).

The extensive capacity of the enzyme involved in mannitol utilization to self-assemble and its susceptibility to control by mannitol 1-phosphate as a dead-end inhibitor point to this initial catalysis site as the locus of regulatory control for mannitol utilization by *A. glauca*. Since mannitol, as the sole carbon source, is sufficient for growth, we regard

this site as the first step in a branched pathway that leads ultimately to the biosynthesis of a large number of compounds.

While the physiological relevance of these aggregration phenomena to regulatory control remains to be established, the evolutionary significance of isozymic regulatory control for several dehydrogenases is now well recognized (Markert et al., 1975).

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Glutamine Synthetase of *Bacillus stearothermophilus.* Regulation, Site Interactions, and Functional Information†

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ABSTRACT: The action of various feedback modifiers on *Bacillus stearothermophilus* glutamine synthetase has been investigated by initial velocity kinetics, using the Mn^{2+}-stimulated biosynthetic assay at 55 °C. The most potent inhibitors, used singly, are AMP, L-glutamine, and L-alanine. Other modifiers of significance include glycine, CTP, L-histidine, glucosamine 6-phosphate, and GDP. Marked synergism of action is observed for AMP in the presence of L-glutamine, L-histidine, ADP, or glucosamine 6-phosphate (glucosamine-6-P), and for CTP with ADP or GDP. Inhibition by saturating levels of many modifiers is either <100%, or is not overcome by elevated substrate levels, or both. This argues for modifier binding sites separate from substrate sites, notably in the cases of AMP, L-glutamine, glycine, L-alanine, glucosamine-6-P, and CTP. Glycine and L-alanine are V_{max} inhibitors, whereas L-glutamine, glucosamine-6-P, GDP, and CTP alter the binding of L-glutamate. ADP and L-histidine apparently can compete directly with MnATP, but AMP alters Mn-ATP binding from a separate site. The action of several modifiers requires or is enhanced by bound substrates. Considerable antagonistic interaction is observed in experiments with modifier pairs, but the most potent inhibitors show synergistic or cumulative (independent) interactions. One may interpret antagonistic effects as due to (a) overlapping modifier domains, or (b) separate but antagonistically interacting sites. Either interpretation leads to a scheme for modifier–substrate and modifier–modifier site interactions in which the thermophilic enzyme must maintain and stabilize a great deal of complex functional information under extreme environmental conditions.

The regulation of glutamine synthetase activity in microorganisms can occur primarily by end-product metabolite feedback inhibition, and in some cases by direct product inhibition (Shapiro and Stadtman, 1970). Specific mechanisms of regulation from one source to another are quite variable, however. Among mesophiles, for enzymes from gram-negative organisms, such as *Escherichia coli,* sensitivity to inhibitors is modulated by covalent adenylylation–deadenylylation, whereas for enzymes from gram-positive organisms, such as *Bacillus sp.,* inhibitors act synergistically and product inhibition by glutamine is a key feature (Hubbard and Stadtman, 1967a,b; Deuel and Prusiner, 1974).

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