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Metabolism of Some Polyols by *Rhizobium meliloti*[1]

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The utilization of D-mannitol, D-arabitol, and D-sorbitol by *Rhizobium meliloti* was studied in extracts from mannitol-grown cells. Two different polyol dehydrogenases were induced by any of these polyols: (i) a nicotinamide adenine dinucleotide (NAD)-arabitol dehydrogenase and (ii) a NAD-sorbitol dehydrogenase, whereas polyol phosphate dehydrogenases were absent. D-Arabitol dehydrogenase was observed to act on both D-arabitol and D-mannitol, but D-sorbitol dehydrogenase acted specifically on D-sorbitol. D-Arabitol was oxidized to D-xylulose, D-mannitol and D-sorbitol were oxidized to D-fructose. An adenosine triphosphate-linked hexokinase which acts on D-fructose and absence of hexose isomerase were also detected in this organism.

Mannitol and sucrose are the preferred carbon sources for the fast-growing rhizobia (6) and of these two sources, mannitol is the most widely employed in culturing it for experimental or for production purposes. However, little is known about the metabolism of this and other polyols by these bacteria.

In a previous paper (14) Wilson noted adaptation to mannitol, erythritol, glycerol, and acetate by cells of *Rhizobium trifolii* 209 grown on yeast extract medium. Burris et al. (2) found the metabolism of polyols by rhizobia was through some oxidative mechanism and suggested that this oxidation could be effected by inducible enzymes.

In this communication the mechanism of oxidation of some polyols, particularly D-mannitol, D-sorbitol, and D-arabitol, and the existence of several inducible enzymes in *R. meliloti* are reported.

MATERIALS AND METHODS

Reagents. Sodium glucose-6-phosphate, sodium mannose-1-phosphate, sodium fructose-6-phosphate, D-arabitol, L-arabitol, nicotinamide adenine dinucleotide (NAD), nicotinamide adenine dinucleotide phosphate (NADP), reduced nicotinamide adenine dinucleotide (NADH), and adenosine triphosphate (ATP) were obtained from the Sigma Chemical Co., St. Louis, Mo. D-Glucose, D-sorbitol, D-fructose, D-mannitol, D-mannose, and L-sorbose were obtained from E. Merck AG, Darmstadt, Germany. D-Xylitol, dulcitol, and i-inositol were purchased from Nutritional Biochemicals Corp., Cleveland, Ohio. Diethylaminoethyl (DEAE) cellulose was obtained from W. & R. Balston Ltd., London. The authors thank W. A. Wood and J. D. Deupree, Dept. of Biochemistry, Michigan State University, for a sample of D-xylulose and T. Reichstein, Institut für Organische Chemie, Universität Basel, for a sample of 2,3-mono-acetone-D-xylulose. A solution of D-xylulose was obtained from 2,3-mono-acetone-D-xylulose by hydrolyzing 10 mg of this compound with 1 ml of 0.05 N H_2SO_4 until rotation was constant, and then it was neutralized with $BaCO_3$ and centrifuged. The level of contamination of D-arabitol in D-sorbitol or D-mannitol was determined by descending chromatography for 36 hr on Whatman no. 1 paper by using the solvent system *n*-butanol-ethanol-water (4:1:5, v/v; reference 3) and spraying the chromatogram with ammoniacal silver nitrate reagent (3). The level of contamination was found to be lower than 0.2 μmole of D-arabitol for 5-μmole samples of D-mannitol or D-sorbitol. The level of contamination of D-sorbitol in D-mannitol was determined by the same descending chromatographic technique as indicated above and was found to be lower than 0.2 μmole of D-sorbitol in a 5-μmole sample of D-mannitol. The possible contamination of D-mannitol in D-sorbitol or D-arabitol was determined by a specific mannitol dehydrogenase from *L. brevis* (9). The enzyme did not reduce NAD in the presence of D-sorbitol or D-arabitol. This method for determining contamination could not detect as little as 0.02 μmole of D-mannitol in 10-μmole samples of D-arabitol or D-sorbitol. Mannitol-1-phosphate and sorbitol-6-phosphate were prepared by sodium borohydride reduction of mannose-1-phosphate and glucose-6-phosphate, respectively, by the method of Wolff and Kaplan (15).

Bacterial strains and growth conditions. The strains used were *R. meliloti* ATCC 9930 and *R. leguminosarum* ATCC 10004; they were obtained from the American Type Culture Collection in lyophilized form. *R. trifolii* WA67 was obtained from the Laboratorio de Control de Inoculantes (M.G.A.), Uruguay.

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The growth medium contained the following: $MgSO_4 \cdot 7H_2O$, 0.28 g; K_2HPO_4, 0.5 g; NaCl, 0.1 g; $CaCl_2$, 0.04 g; yeast extract (Difco), 5.0 g; biotin, 250 μg; thiamine hydrochloride, 500 μg; and carbon source, 10 g; in a total volume of 1,000 ml of distilled water. The medium was sterilized at 121 C for 20 min. Polyols, $CaCl_2$, biotin, and thiamine were sterilized separately in the same conditions. Glucose, fructose, and sucrose were sterilized by filtration through a Seitz EK filter and added aseptically to the medium at the time of inoculation. The final *p*H was 6.8. The organisms were maintained on 2% agar stabs of this composition with sucrose as carbon source and they were transferred every 15 days.

Cultures were grown in liquid medium by incubation at 30 C in a rotary shaker at 140 cycles per min. Culture media were inoculated with 1% of a 48-hr subculture of bacteria and the cells were collected after 72 hr. At this time the optimal enzymatic activities were obtained. The cells were collected by centrifugation in a Sorvall centrifuge at 11,600 × *g* for 10 min at approximately 4 C and washed with 0.05 M sodium phosphate buffer (*p*H 7.2). Cells could be kept frozen or lyophilized for more than 6 months with no loss of the enzymatic activities.

Cell-free extract preparation. Extracts were prepared from bacteria grown on the same day or from frozen cells by grinding for 10 min with three parts of washed alumina (special alumina A-305, Aluminum Co. of America) and extracting the paste with 0.05 M sodium phosphate buffer (*p*H 7.2) containing 2×10^{-4} M mercaptoethanol. The mixtures were centrifuged at 14,000 × *g* for 30 min, and the residue was washed with the same buffer. The supernatant fluids were frozen for 2 hr and centrifuged again at 14,000 × *g* for 30 min. All the operations were carried out between 0 to 4 C. The supernatant fraction could be stored at −20 C for at least 1 month without any significant loss of enzymatic activities. This cell-free extract was employed for the enzyme activity assays and protein concentration. When indicated, a partially purified preparation was used.

Extract purification procedure. Protamine sulfate was added to the clear supernatant fluid to a final concentration of 0.18%. After 20 min at 0 C, the mixture was centrifuged (14,000 × *g*) and the precipitate was discarded. The clear solution was taken to 65% saturation with solid ammonium sulfate, allowed to stand for 10 min at 0 C, and centrifuged, and the supernatant fraction was dissolved in glass-bidistilled water containing 2×10^{-4} M mercaptoethanol. This fraction was dialyzed for 3 hr against 0.1 M KCl, adsorbed on DEAE-cellulose (0.5 g per ml of enzyme preparation), suspended in 0.05 M sodium phosphate buffer (*p*H 7.2), and eluted after 10 min with 0.1 M sodium phosphate buffer (*p*H 7.2). The specific activity of this preparation on D-mannitol, D-sorbitol, and D-arabitol was 10 times that of the original extract, and the activity recovered was about 70%.

Enzyme studies. Polyol dehydrogenase activities were determined by observing the rate of reduction of NAD by the different polyols at 340 nm in a Beckman model DU spectrophotometer. The incubation mixture contained (in a total volume of 1.0 ml): polyol, 50 μmoles; NAD, 0.6 μmole; sodium carbonate-bicarbonate buffer (*p*H 9.7), 50 μmoles; and sufficient diluted enzyme solution to produce an absorbancy change of about 0.030 per min. A unit of enzyme was defined as the quantity required to reduce 1 μmole of NAD per min under the standard assay conditions at 25 C.

Mannitol phosphate dehydrogenase (EC 1.1.1.17) and sorbitol-6-phosphate dehydrogenase were assayed by following the reduction of NAD or NADP by mannitol-1-phosphate or sorbitol-6-phosphate (8). The reaction mixture consisted of (in a total volume of 1.0 ml): sorbitol-6-phosphate or mannitol-1-phosphate, 25 μmoles; tris(hydroxymethyl)aminomethane (Tris) buffer (*p*H 9.0), 70 μmoles; NAD or NADP, 1.5 μmoles; and cell-free extract.

Fructokinase activity was measured by following the reduction of NADP by a cell-free extract that had glucose-6-phosphate dehydrogenase (EC 1.1.1.49) and phosphohexose isomerase (EC 5.3.1.9) activities. The reaction mixture contained: fructose, 40 μmoles; ATP, 2 μmoles; Tris buffer (*p*H 8.0), 33 μmoles; $MgCl_2 \cdot 7H_2O$, 5 μmoles; and enzyme preparation. NADP (1 μmole) was added after 15 min of incubation at room temperature, and the increase in optical density at 340 nm was observed.

Glucose-6-phosphate dehydrogenase was determined by following the reduction of NADP. The reaction mixture contained (in a total volume of 1.0 ml): glucose-6-phosphate, 3.0 μmoles; Tris buffer (*p*H 8.0), 60 μmoles; NADP, 0.3 μmole; $MgCl_2 \cdot 7H_2O$, 15 μmoles; and cell-free extract (0.03 mg of protein). Specific activity of glucose-6-phosphate dehydrogenase was expressed as micromoles of NADPH formed per minute per milligram of protein. Phosphohexose isomerase was determined following the transformation of fructose-6-phosphate into glucose-6-phosphate by the method of Wu and Racker (16). Specific activity of phosphohexose isomerase was defined as micromoles of NADPH formed per minute per milligram of protein. Hexose isomerase was determined by following the transformation of glucose into fructose by the method of Yamanaka (17). A unit of this enzyme was defined as the quantity which produced 1 μmole of ketohexose from aldohexose under the assay conditions. A 0.02-μmole amount of fructose formed was the limit of detection in this method. A blank without substrate was run in the determination of all the enzyme activities.

Analytical methods. Fructose was quantitatively determined by the colorimetric method of Roe et al. (11). Mannitol, sorbitol, and arabitol were quantitatively determined by the periodate oxidation method (5). Fructose, sorbose, glucose, and mannose were identified by paper chromatography by using a solvent system of phenol-water (4:1) and spraying the paper chromatograms with aniline reagent (13). Xylulose was determined by the cysteine-carbazole method (4), the resorcinol test (7), and the orcinol method with a 40-min heating time (10). Protein was measured by the method of Bücher (1) which was standardized with bovine serum albumin.

RESULTS

Induction of polyol dehydrogenases. Extracts from *R. meliloti* cells grown on nine different carbon sources were examined for their ability to oxidize some polyols in the presence of either NAD or NADP. NAD-dehydrogenase activities for D-mannitol, D-arabitol, and D-sorbitol were found in cells grown in any of these polyols. These enzymatic activities were 10-fold lower in extracts from dulcitol-grown cells than in the above polyols, and they were not detected in cells grown in L-arabitol, i-inositol, D-glucose, D-fructose, and sucrose (Table 1). Moreover, there was no NADP reduction with D-mannitol, D-sorbitol, or D-arabitol by the nine different extracts prepared. An NAD-inositol dehydrogenase was also specifically induced by i-inositol; however, an NAD or NADP-dulcitol dehydrogenase was not detected in dulcitol-grown cells.

The presence of a second carbon source in the growth media in addition to D-sorbitol and D-mannitol, such as D-glucose and D-fructose in the same final concentration (1%), produced a decrease in the enzymatic activities of about 70%. The induction of the polyol dehydrogenases was of the same magnitude both in cells grown in yeast extract medium or in cells grown in synthetic medium in which the yeast extract was substituted by 0.06% potassium nitrate or by 0.06% ammonium sulfate. Mannitol-1-phosphate dehydrogenase and sorbitol-6-phosphate dehydrogenase were absent in alumina extract and in sonic extract (ten 1-min treatments with a Branson sonifier) from mannitol or sorbitol-grown cells.

TABLE 1. *Polyol dehydrogenase activities in extracts of Rhizobium meliloti grown on different carbon sources*

Carbon source in growth media	Specific activity on[a]			
	D-Mannitol	D-Arabitol	D-Sorbitol	*i*-Inositol
L-Arabitol	<0.005	<0.005	<0.005	<0.005
D-Arabitol	0.084	0.380	0.220	<0.005
D-Mannitol	0.100	0.314	0.185	<0.005
D-Sorbitol	0.053	0.250	0.164	<0.005
Dulcitol	<0.005	0.016	0.030	0.010
i-Inositol	<0.005	<0.005	<0.005	0.220
D-Glucose	<0.005	<0.005	<0.005	<0.005
D-Fructose	<0.005	<0.005	<0.005	<0.005
Sucrose	<0.005	<0.005	<0.005	NT[b]
D-Mannitol + D-glucose	0.031	0.112	0.051	NT
D-Sorbitol + D-glucose	0.035	0.134	0.070	NT
D-Mannitol + D-fructose	0.019	0.091	0.050	NT
D-Sorbitol + D-fructose	0.031	0.100	0.052	NT

[a] Specific activity is expressed as micromoles of nicotinamide adenine dinucleotide reduced per minute per milligram of protein. Control without substrate was run with each assay.

[b] Not tested.

Identification of the induced polyol dehydrogenases. The enzymes involved in the dehydrogenase activities for the polyols D-mannitol, D-arabitol, and D-sorbitol in polyol-grown extract were investigated. Two dehydrogenases were detected as shown in the following experiments.

(i) **Stability during incubation at different pH values.** The activity ratio of D-arabitol to D-mannitol did not change on incubating the cell-free extract at various *p*H values for different periods of time, whereas the activity ratio of D-arabitol to D-sorbitol or D-mannitol to D-sorbitol increased as the *p*H of incubation varied from 7.3 to 4.8. Thus, the dehydrogenase activity for D-sorbitol was less stable at low *p*H values than were those for D-arabitol or D-mannitol (Table 2).

The heat stability assay also showed that the activity ratio of D-arabitol to D-mannitol did not change with temperatures ranging from 40 to 50 C. The activity ratio of D-arabitol to D-sorbitol decreased as the temperature increased from 40 to 50 C (Table 3). The tests of *p*H stability and heat stability established differences between

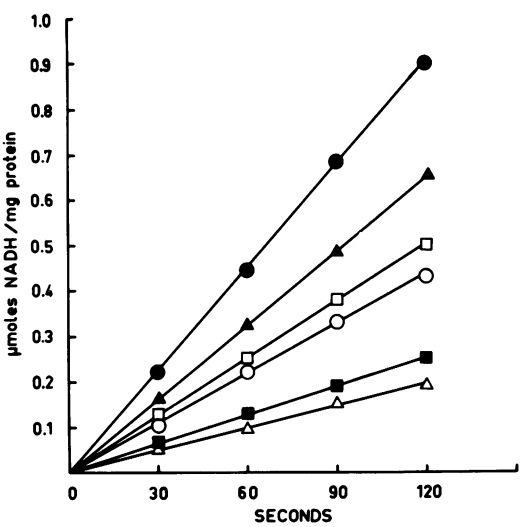

FIG. 1. *NADH formation in the presence of one or two substrates by a cell-free extract from D-mannitol-grown R. meliloti. The incubation mixture contained (1.0 ml): sodium carbonate-bicarbonate buffer (pH 9.7), 50 μmoles; NAD, 1 μmole; cell-free extract (50 μg of protein) and one or two polyols. D-Sorbitol was employed in a final concentration of 50 μmoles/ml. D-Arabitol and D-mannitol were added in a final concentration of 20 μmoles/ml. Symbols: △, D-mannitol; □, D-arabitol; ○, D-sorbitol; ▲, D-mannitol plus D-sorbitol; ■, D-arabitol plus D-mannitol; ●, D-sorbitol plus D-arabitol.*

TABLE 2. *Activity ratio on D-arabitol, D-mannitol, and D-sorbitol at different pH levels in an extract from mannitol-grown Rhizobium meliloti*

pH	Time of incubation	Inactivation on D-arabitol	Activity ratio (D-arabitol/D-mannitol)	Inactivation on D-sorbitol	Activity ratio (D-arabitol/D-sorbitol)
	hr	%		%	
7.3	3	0	4.20	0	1.35
5.0	3	24	4.40	72	3.77
4.9	2	36	4.29	75	3.61
4.8	1	37	4.39	81	5.02
4.8	2	55	4.30	90	5.90

TABLE 3. *Activity ratio on D-sorbitol, D-arabitol, and D-mannitol at different temperatures in an extract from Rhizobium meliloti grown on D-mannitol*

Temperature	Time of incubation	Inactivation on D-arabitol[a]	Activity ratio (D-arabitol/D-mannitol)	Inactivation on D-sorbitol[a]	Activity ratio (D-arabitol/D-sorbitol)
C	*min*				
25	60	0.0	4.15	0.0	1.29
40	10	40.0	4.20	18.5	1.05
40	20	52.2	4.16	27.9	0.94
40	40	71.5	4.16	40.5	0.69
45	10	81.0	4.01	45.0	0.48
50	5	91.0	4.24	67.0	0.31

[a] Heat inactivation was carried out in a constant-temperature water bath. The sample (1 ml) was pipetted into a prewarmed tube. After the incubation, the sample was chilled and centrifuged at 14,000 × *g* for 10 min at 4 C.

dehydrogenase activity for D-sorbitol and for D-mannitol or D-arabitol in the crude extract but not in a partially purified preparation. Dehydrogenase activity for D-arabitol and D-mannitol were as unstable at *p*H 4.8 to 4.9 as dehydrogenase activity for D-sorbitol in a partially purified preparation. In the same preparation, dehydrogenase activity for D-sorbitol was as unstable at 50 C as dehydrogenase activity for D-mannitol or D-arabitol.

(ii) Mixtures of two substrates presented to the enzyme preparation. When D-sorbitol and D-mannitol or D-sorbitol and D-arabitol were presented to a cell-free extract, there was an increase in the rate of NADH formation by comparison with the rates found when each substrate was used alone. There was no increase in the rate of NADH formed when both D-arabitol and D-mannitol were added to the same extract (Fig. 1).

(iii) Separation with DEAE cellulose. A partially purified preparation in 0.1 M KCl was passed through a DEAE-cellulose column (1.5 by 22 cm) which had been equilibrated with 0.05 M sodium phosphate buffer (*p*H 7.2). The column was eluted by a linear gradient technique, in which the mixing vessel contained 300 ml of a 0.05 M sodium phosphate buffer (*p*H 7.2) and the reservoir vessel contained 300 ml of a 0.5 M sodium phosphate buffer (*p*H 7.2). Each fraction contained a volume of 3 ml. The elution profile is shown in Fig. 2. Dehydrogenase activity for D-arabitol and D-mannitol appeared together in all the tubes at the same rate of D-arabitol to D-mannitol rate ranging from 4.0 to 4.2. The Michaelis constants were determined by the Lineweaver-Burk method; for this enzyme the K_m was 3.33×10^{-3} M for D-arabitol and 11.4×10^{-3} M for D-mannitol. On the basis of the substrate preference and the specificity data, the enzyme was identified as D-arabitol dehydrogenase. D-Sorbitol dehydrogenase and D-arabitol dehydrogenase did not act on L-arabitol, ribitol, D-xylitol, L-erythritol, dulcitol, and i-inositol in the same experimental conditions as described above for determination of the activities of polyol dehydrogenases. The limit of detection of activity in the assay was 0.2% on D-arabitol.

Induction of D-arabitol and D-sorbitol dehydrogenase in Rhizobium leguminosarum and Rhizobium trifolii. Since most of the *Rhizobium* strains grow on D-mannitol and D-sorbitol as the only carbon source, the presence of the two above-mentioned dehydrogenases and polyol phosphate

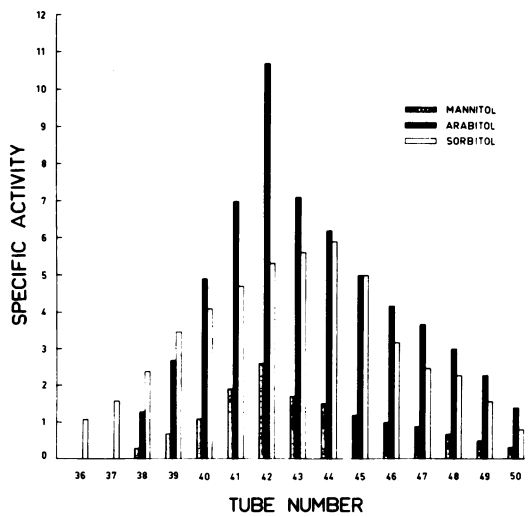

FIG. 2. *Chromatography of dehydrogenases on DEAE-cellulose. The DEAE-cellulose column and the elution are described in the text. A sample from each tube was taken to assay activity on D-mannitol, D-sorbitol, D-arabitol, and protein.*

dehydrogenases was investigated in extracts from strains of *R. leguminosarum* and *R. trifolii*. Table 4 shows that mannitol, sorbitol, and arabitol dehydrogenase activities were found in D-mannitol-grown cells of these species. Mannitol-1-phosphate dehydrogenase and sorbitol-6-phosphate dehydrogenase were absent in both strains.

Product of D-mannitol and D-sorbitol oxidation. The product of oxidation of D-mannitol and D-sorbitol was determined by chromatographic, colorimetric, and enzymatic methods. For this identification, a mixture of NAD, polyol, and polyol dehydrogenase was added to a system able to reoxidize the NADH formed. The addition shifted the equilibrium reaction as far as possible to the complete oxidation of D-mannitol and D-sorbitol. The incubation mixture contained: NAD, 8 μmoles; polyol, (D-mannitol or D-sorbitol), 50 μmoles; sodium carbonate-bicarbonate buffer (*p*H 9.7), 1,200 μmoles; partially purified extract, 1.5 mg of protein; sodium pyruvate, 80 μmoles; lactic dehydrogenase, 0.01 mg; to a final volume of 3.0 ml. A blank without polyol was run in all the experiments. The mixture was incubated at room temperature for 3 hr. The reaction was stopped by addition of cold trichloroacetic acid to a final concentration of 5%, it was then centrifuged, and the supernatant fraction was neutralized with 1.0 N NaOH passed through a column of Amberlite IR-45 (OH^-) and IR-120 [H^+ (2:1)]. The column was washed with distilled water and the eluate was concentrated in vacuo at room temperature. The material was evaporated to dryness and the residue was dissolved in 0.5 ml of glass-distilled water.

The product was identified by descending chromatography on Whatman no. 1 paper in the phenol-water system for 50 hr. D-Glucose, D-fructose, L-sorbose, D-mannose, D-mannitol, D-sorbitol, sodium pyruvate, and sodium lactate were run as controls. The paper was then sprayed with aniline reagent and heated at 100 C for 10 min. D-Mannitol, D-sorbitol, pyruvate, and lactate gave no spot with the aniline reagent. The spot for the D-glucose standard was at 16 to 17 cm; for the L-sorbose standard, 19.5 to 20.2 cm; for the D-mannose standard, 18 to 19 cm; and for the D-fructose standard, 25.5 to 26.5 cm. A single spot was observed with the mannitol and sorbitol incubation mixtures at 24.7 to 26.2 cm. This spot was coincident with D-fructose. This product was also identified enzymatically by using mannitol dehydrogenase from *L. brevis* (9) as D-fructose. Furthermore, NADH oxidation was demonstrated with D-fructose as the substrate by an extract from mannitol or sorbitol-grown cells. There was no NADH oxidation by the same extracts without addition of D-fructose. The amount of fructose formed in both mannitol or sorbitol incubation mixtures was determined by the method of Roe et al. (11). Pyruvate and lactate did not give a positive Roe test. The percentage of fructose formed from D-mannitol was 97%; from D-sorbitol, 88%.

TABLE 4. *Induction of polyol dehydrogenases in extracts from mannitol-grown Rhizobium leguminosarum and R. trifolii*

Strain	Specific activity on[a]		
	D-Mannitol	D-Arabitol	D-Sorbitol
R. leguminosarum	0.072	0.140	0.106
R. trifolii	0.083	0.183	0.132

[a] Expressed as micromoles of nicotinamide adenine dinucleotide reduced per minute per milligram of protein.

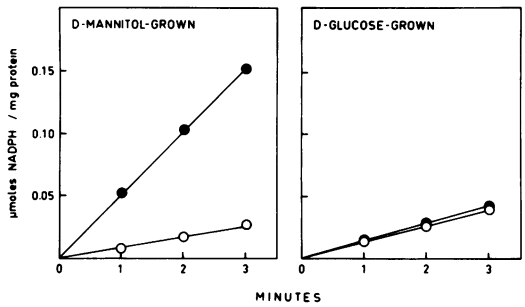

FIG. 3. *Fructokinase activity in extracts from D-mannitol and D-glucose-grown Rhizobium meliloti. The fructokinase activity is expressed as micromoles of NADPH formed per milligram of protein and is measured as described in the text. Symbols: ●, D-fructose; ○, D-fructose (minus ATP).*

Utilization of D-fructose. Since D-fructose was the oxidation product of D-mannitol and D-sorbitol by arabitol dehydrogenase and sorbitol dehydrogenase, respectively, the mannitol-grown cells must have a mechanism of utilizing the fructose. Hexoisomerase, which transforms fructose into glucose, was absent in extracts from mannitol-grown cells. Figure 3 gives evidence of an enzyme able to phosphorylate D-fructose. This enzyme was shown by reduction of NADP by glucose-6-phosphate produced from newly formed fructose-6-phosphate. Phosphohexose isomerase, which transforms fructose-6-phosphate into glucose-6-phosphate, and glucose-6-phosphate dehydrogenase, which reduces NADP in the presence of glucose-6-phosphate, were detected in the enzyme preparation from *R. meliloti*. Their specific activities were 0.120 and

0.156, respectively. The fructokinase rates were minimal values, since the two coupling enzymes were not in excess in the extract. This fructokinase was induced in D-mannitol, D-sorbitol, or D-fructose-grown cells but it was not detected in D-glucose-grown cells (Fig. 3).

Product of D-arabitol oxidation. The product of oxidation of D-arabitol was studied in an incubation mixture containing: NAD, 8 μmoles; D-arabitol, 50 μmoles; sodium carbonate-bicarbonate buffer, 1,200 μmoles (*p*H 9.7); sodium pyruvate, 80 μmoles; lactic dehydrogenase, 0.01 mg; and partially purified enzyme preparation, 1.5 mg of protein. The total volume was 3 ml and the mixture was incubated at room temperature. A blank without D-arabitol was run. The time of incubation and the passage through the Amberlite column was similar to the determinations indicated above for identification of the D-mannitol oxidation product. The eluate from the column was concentrated in vacuo, and the residue was dissolved in 0.005 M sodium tetraborate, passed through a Dowex 1-X4 tetraborate column, and then eluted with 0.02 M sodium tetraborate, concentrated in vacuo, and dissolved in 0.5 ml of glass-bidistilled water. The product of D-arabitol oxidation was determined in the solution by the (i) orcinol test (10), (ii) cysteine carbazole method (4); and (iii) resorcinol test (7). The absorbancy ratio at 540 and 670 nm in the orcinol test was of 0.50 (the absorbancy ratio for xylulose standard was 0.52 and for ribulose standard was 0.90). A maximal color development in the cysteine-carbazole method was obtained in 100 min (xylulose standard, 100 min; ribulose standard, 15 min). The resorcinol test revealed a peak at 640 nm which was coincident with the xylulose standard. Consequently, the product of D-arabitol oxidation was identified as xylulose. The amount of xylulose formed was measured by the resorcinol test (640 nm). The percentage of xylulose formed in the arabitol incubation mixture was 90%.

DISCUSSION

According to the data presented here, the polyols, D-mannitol, D-arabitol, and D-sorbitol were oxidized via NAD-polyol dehydrogenase by *R. meliloti*, *R. leguminosarum*, and *R. trifolii*, organisms which do not possess polyol phosphate dehydrogenases to metabolize these polyols.

The presence of an induced NAD-sorbitol dehydrogenase which oxidizes D-sorbitol, and an NAD-arabitol dehydrogenase which oxidizes D-mannitol and D-arabitol was also established.

Several lines of evidence support the conclusion that D-arabitol and D-mannitol are oxidized by a single enzyme. (i) The relative activities with both substrates remained unchanged during the thermal and *p*H inactivations, and (ii) the substrates were shown to be competitive in experiments with pairs of substrates.

The induction of the D-arabitol dehydrogenase by its substrates and by D-sorbitol, and the reciprocal induction of D-sorbitol dehydrogenase by its substrate and by D-arabitol and D-mannitol were found in our material. An analogous cross induction was described by Scolnick and Lin (12) in *Cellvibrio polyoltrophicus*. These authors assumed that this induction was probably due to the fact that the three polyols shared three carbons with the same configuration. Moreover, they observed no inhibitory effect of glucose on enzyme induction. On the contrary, we found that glucose and fructose produced inhibition of the synthesis of the two enzymes.

Scolnick and Lin also demonstrated the induction of D-arabitol and D-sorbitol dehydrogenases by D-fructose in *C. polyoltrophicus*. In contrast, D-fructose did not induce arabitol and sorbitol dehydrogenases in *R. meliloti*. On the other hand, we found that D-fructose induced the formation of an ATP-dependent fructokinase. Besides, since we did not detect hexose isomerase in *R. meliloti*, we think that the fructose could probably be phosphorylated to fructose-phosphate.

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