POLYOL DEHYDROGENASES OF *AZOTOBACTER AGILIS*

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ABSTRACT

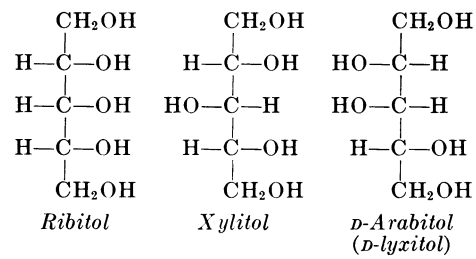
MARCUS, LEON (University of California, Davis), AND ALLEN G. MARR. Polyol dehydrogenases of *Azotobacter agilis*. J. Bacteriol. **82:** 224–232. 1961.—Two soluble diphosphopyridine-linked polyol dehydrogenases are formed by *Azotobacter agilis* (*A. vinelandii*). The first, D-mannitol dehydrogenase is induced by D-mannitol and all of the pentitols except L-arabitol. Ribitol is an excellent inducer of mannitol dehydrogenase although it is not metabolized, nor does the enzyme act upon it. This allows study of the gratuitous induction of mannitol dehydrogenase.

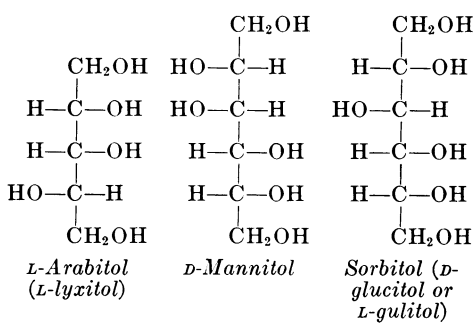
Of the polyols tested, mannitol dehydrogenase oxidizes D-mannitol, D-arabitol, D-rhamnitol, and perseitol, demonstrating its requirement for substrates bearing the D-*manno* configuration. The corresponding 2-ketoses, D-fructose, D-xylulose, and presumably D-rhamnulose, and perseulose are reduced.

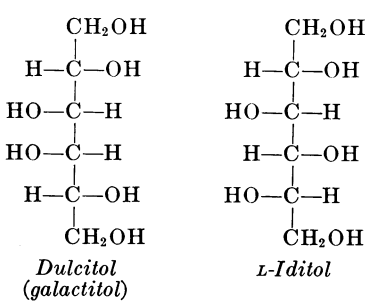
The second enzyme, L-iditol dehydrogenase is induced only by polyols containing the D-*xylo* configuration, i.e., sorbitol and xylitol. L-Iditol dehydrogenase oxidizes D-*xylo* polyols seven times faster than it does D-*ribo* polyols. Substrates oxidized include L-iditol, sorbitol, xylitol, and ribitol. The corresponding 2-ketoses, L-sorbose, D-fructose, D-xylulose, and D-ribulose, are reduced.

The two polyol dehydrogenases have been separated and purified by chromatography on a modified cellulose ion exchanger.

This work was begun with the intent of characterizing a soluble inducible enzyme suitable for the study of the kinetics of induction of enzymes in the azotobacter. Polyol dehydrogenases seemed a reasonable choice for two reasons. First, Burris, Phelps, and Wilson (1943) had established that the metabolism of mannitol was inducible in *Azotobacter agilis* (*A. vinelandii* strain O). Second, a wide variety of polyols which differ in chain length and configuration are available either as natural products or can be obtained by reduction of corresponding sugars; the availability of such an array of compounds may facilitate a more precise definition of the structural specificity of induction and of enzyme activity. The structures of some of the polyols which are most significant in this investigation are as follows:







This paper reports the specificity of induction and activity of two distinct polyol dehydrogenases produced by *A. agilis*, D-mannitol and

L-iditol dehydrogenases. The purification of D-mannitol dehydrogenase and the kinetics of its induction will be discussed elsewhere.

MATERIALS AND METHODS

Culture. A. agilis (*A. vinelandii* strain O) was grown in Burk's nitrogen-free medium (Wilson and Knight, 1952) modified by reducing the calcium concentration, to contain the following per liter of distilled water: K_2HPO_4, 0.8 g; KH_2PO_4, 0.2 g; $MgSO_4 \cdot 7H_2O$, 0.2 g; $CaSO_4 \cdot 2H_2O$, 0.025 g; $Na_2MoO_4 \cdot 2H_2O$, 0.00025 g; $FeNH_4(SO_4)_2 \cdot 12H_2O$, 0.0086 g; sucrose, 20.0 g.

The phosphate, sulfate, iron, and molybdate salts were dissolved separately. The solutions were combined to give a medium free of turbidity, 100 ml of which were dispensed per 250-ml Erlenmeyer flask. During autoclaving, a slight precipitate forms which dissolves completely on cooling.

Inocula were taken from cultures growing exponentially with a specific growth rate of approximately 0.3 per hour. Cultures were incubated on a rotary shaker at 30 C and harvested during the late logarithmic phase by centrifugation at 5,000 $\times$ *g* for 5 min.

Cell-free extracts. The enzymes were released from the cells by osmotic shock (Robrish and Marr, 1957). An equal volume of 2 M glycerol was added to the centrifuged pellet of cells and mixed thoroughly in the 50-ml plastic centrifuge tube. After allowing at least 1 min for glycerol to enter the cells; five to eight volumes of cold 0.05 M tris(hydroxymethyl)aminomethane acetate (tris acetate) buffer, pH 7.0, were discharged rapidly into the centrifuge tube as the contents were stirred vigorously with a mechanical stirrer. The resulting viscous fluid was centrifuged at 10,000 to 15,000 $\times$ *g* for 10 min to remove residual cells (less than 5% of the initial cells) and the emptied cell envelopes. Centrifugation removes at least 90% of the reduced diphosphopyridine nucleotide (DPNH) oxidase which otherwise would interfere with the dehydrogenase assay. The crude extract was stored at 0 to 4 C.

Dehydrogenase assay. The assay measures the rate of formation of DPNH subsequent to the addition of diphosphopyridine nucleotide (DPN) to a buffered reaction mixture containing polyhydric alcohol and cell extract. The assay was made in the following manner. All solutions except the enzyme preparation were made in the assay buffer and held at 30 C. To a 1-cm silica absorption cell were added in order: 2.3–*x* ml of 0.05 M tris chloride buffer, pH 8.6; 0.5 ml of 0.5 M polyol; and *x* ml of enzyme preparation. Ordinarily, sufficient enzyme was added to give a change in absorbancy at 340 mμ of 0.1 to 0.35 per min. If the activity was low, a maximum of 0.5 ml of enzyme preparation was added, and the rate obtained was reported. Addition of 0.2 ml of 0.02 M DPN started the reaction.

The reduction of ketose was measured by following the oxidation of DPNH. To a 1-cm absorption cell were added in order: 2.4–*x* ml of 0.05 M tris chloride buffer, pH 8.6; 0.5 ml of 0.5 M ketose; and *x* ml enzyme. The reaction was started by adding 0.1 ml of a freshly prepared solution of DPNH containing 2 mg DPNH per ml of buffer.

Formation or disappearance of DPNH was followed at 340 mμ with a spectrophotometer, the cell compartment of which was maintained at 30 C. The spectrophotometer has been modified for recording. The current of the phototube was amplified by a Kiethly electrometer, model 610. The output of the electrometer, which is proportional to per cent transmission, was converted to absorbancy by a diode analogue and recorded by a Varian strip chart recorder, model G-11. Since kinetics for oxidation of polyol are zero order, units of enzymatic activity can be estimated directly from the slope of the recorded change in absorbancy. However, the rate of reduction of ketose is not constant. The rates reported are linear estimates of the rates recorded during the first minute of reaction.

The unit of enzymatic activity is defined as the amount of enzyme which causes a change in absorbancy at 340 mμ of 0.001 per min in the standard assay with polyol. Specific activity is defined as units of enzyme per mg protein as determined with the Folin-Ciocalteau reagent (Lowry et al., 1951) using crystalline serum albumin as the standard.

Substrates. Glycerol, erythritol, ribitol (adonitol), D-arabitol, L-arabitol, xylitol, D-mannitol, sorbitol (D-glucitol), galactitol (dulcitol), and D-rhamnitol were obtained from commercial sources. Perseitol and L-iditol were gifts from C. E. Ballou, Department of Biochemistry, University of California, Berkeley, Calif. D-Xylulose was obtained from G. Ashwell of the National Institutes of Health, Bethesda, Md.

TABLE 1. *Utilization of various compounds as the sole carbon source by Azotobacter agilis*

Growth	No growth
D-Mannitol	Galactitol
Sorbitol	Ribitol
D-Arabitol	L-Arabitol
Xylitol	
Erythritol	
Glycerol	
D-Fructose	D-Mannose
L-Sorbose	D-Ribose
Sucrose	Lactose
D-Glucose	

One hundred milliliters of Burk's nitrogen-free basal medium, containing 0.1 M concentration of the above compounds, was inoculated with 0.5 ml of an exponentially growing culture in Burk's sucrose medium. Cultures were incubated on a rotary shaker at 30 C for 24 hr.

D-Ribulose was prepared by epimerization of D-arabinose in dry pyridine (Glatthaar and Reichstein, 1935). The *o*-nitrophenylhydrazone of D-ribulose was prepared and recrystallized from absolute ethanol (mp 167). The hydrazone was decomposed in an aqueous reaction mixture with excess benzaldehyde. Benzaldehydephenylhydrazone was removed by filtration, the filtrate was extracted with ether, and the aqueous phase was evaporated under reduced pressure yielding D-ribulose as a yellow syrup.

Commercial D-mannitol and sorbitol required purification as explained in the next section. D-Mannitol was recrystallized twice from water. Sorbitol was recrystallized twice from pyridine as the pyridine-sorbitol complex (Strain, 1934). The complex was thermally destroyed and the pyridine was evaporated in a vacuum oven at 50 C giving anhydrous sorbitol which was recrystallized two times from absolute alcohol. Ribitol was recrystallized twice from hot absolute alcohol leaving behind yellow-brown impurities. The remaining polyols were assumed sufficiently pure for the purposes of the present investigation.

DEAE-cellulose was purchased from Bio-Rad Laboratories. After removal of fine particles by flotation it was converted to the free base with NaOH, eliminating a yellow material that absorbs ultraviolet light, and then converted to the acetate with sodium acetate. The exchanger was equilibrated with 0.05 M tris acetate, pH 7.0, before use.

RESULTS

Carbon source for growth. A. agilis grows well in Burk's nitrogen-free basal medium containing a variety of polyols as the sole source of carbon. Table 1 shows the polyols and a few related compounds which support growth.

Induction of polyol dehydrogenases. The initial experiments on the specificity of induction by D-mannitol and sorbitol were misleading because of impurities in both polyols. Although both of these polyols were the best commercial preparations available, with melting points identical with the accepted values, each was sufficiently contaminated, presumably by the other, to confuse the results of induction and assay. *A. agilis* grown on either of the commercial polyols as the sole carbon source gave extracts which oxidized both polyols (Table 2). However, the extracts of cells grown on commercial mannitol oxidized mannitol more rapidly, and cells grown on commercial sorbitol oxidized sorbitol more rapidly. These results suggested that different quantities of two distinct polyol dehydrogenases were produced by growth on either D-mannitol

TABLE 2. *Effect of purification of D-mannitol and sorbitol on the induction of polyol dehydrogenases*

Assay substrate	Growth substrate: Commercial	
	Mannitol	Sorbitol
Recrystallized mannitol	100*	27
Recrystallized sorbitol	20	100*
	Recrystallized	
	Mannitol	Sorbitol
Commercial mannitol	100	10
Recrystallized mannitol	100*	1
Commercial sorbitol	3.8	100
Recrystallized sorbitol	0.7	100*

* The dehydrogenase activities of the extracts are expressed as per cent of the rate with homologous recrystallized polyol as the substrate. Each extract contained approximately 3,000 units per ml; 0.05 to 0.4 ml was used in the assay depending on the activity obtained.

or sorbitol. After growth on purified polyols (*see* Materials and Methods), the ability of extracts to oxidize the heterologous commercial polyol was substantially reduced. The activity of these extracts on purified polyols was almost completely specific; only the homologous polyol was oxidized at a significant rate.

A series of polyhydric alcohols and pertinent related compounds were tested for their ability to induce polyol dehydrogenases. Since some polyols which do not support growth may induce, a complete growth medium containing sucrose was supplemented with the potential inducer. Prior experiments had demonstrated that sucrose did not repress the induction by D-mannitol or sorbitol.

The results of this experiment, shown in Table 3, can be explained most simply by presuming that two distinct enzymatic activities can be induced. D-mannitol, D-arabitol, and ribitol induce an activity for D-mannitol and D-arabitol. Sorbitol induces an activity for sorbitol, D-xylitol, and ribitol. Xylitol induces both enzymatic activities. The first activity mentioned will be designated as D-mannitol dehydrogenase, and the second enzymatic activity, which is identical in substrate specificity to the dehydrogenase isolated from rat liver, has previously been termed L-iditol dehydrogenase (Blakely, 1951; McCorkindale and Edson, 1954).

Although ribitol is an excellent inducer of mannitol dehydrogenase, an enzyme which does not attack ribitol, it fails to induce L-iditol dehydrogenase which does oxidize ribitol. Regardless of whether D-mannitol dehydrogenase is induced by D-mannitol, D-arabitol, or ribitol, the ratio of enzymatic activity against D-mannitol to the activity against D-arabitol is always approximately two. Similarly, the relative rates of oxidation of xylitol, sorbitol, and ribitol by L-iditol dehydrogenase are independent of the inducers.

To demonstrate conclusively that the activities ascribed to D-mannitol dehydrogenase and L-iditol dehydrogenase were in fact the activities of two distinct proteins, we combined an extract of Azotobacter grown on D-mannitol with an extract of cells grown on sorbitol. The combined extracts were chromatographed on DEAE-cellulose. Figure 1 is the chromatogram obtained which demonstrates the resolution of L-iditol dehydrogenase (sorbitol dehydrogenase) from D-mannitol dehydrogenase. L-Iditol dehydrogenase begins eluting at 0.1 M NaCl; the peak is sharpened by the next higher NaCl concentration during stepwise elution. D-Mannitol dehydrogenase elutes with 0.3 M NaCl.

TABLE 3. *Induction of polyol dehydrogenases by various polyols**

Inducer	Specific activity assayed on:				
	D-Mannitol	D-Arabitol	Sorbitol	Xylitol	Ribitol
D-Mannitol	2,200	1,130	<10	<10	<10
D-Arabitol	3,250	1,610	<10	<10	<10
Ribitol	1,670	815	<10	<10	<10
Sorbitol	<10	<10	570	570	85
Xylitol	940	700	330	265	35
None	<10	<10	<10	<10	<10

* *Azotobacter agilis* was grown on Burk's sucrose medium supplemented with 0.1 M polyol or other potential inducer. Extracts of the cells were tested for the reduction of DPN with the substances listed. Erythritol, glycerol, L-arabitol, galactitol, D-fructose, L-sorbose, D-mannose, D-glucose, D-arabinose, D-xylose, and sucrose do not induce dehydrogenases for the polyols listed in the table. Glycerol, erythritol, L-arabitol, and galactitol were not effective substrates in the standard assay of any of the induced systems examined.

The fractions containing L-iditol dehydrogenase were pooled and tested for the ability to oxidize polyols and to reduce ketoses (Table 4). Sorbitol, L-iditol, and xylitol, all of which have the D-*xylo* configuration, were rapidly oxidized with the reduction of DPN. D-Fructose, L-sorbose, and D-xylulose, the corresponding 2-ketoses of these polyols, were all reduced with DPNH. In addition to the D-*xylo* substrates, D-ribulose was reduced but at a lower rate. Thus, the weak activity against ribitol found in crude extracts induced with sorbitol or xylitol is accounted for by the L-iditol dehydrogenase. The relative rates of oxidation of sorbitol and ribitol did not change during purification of the enzyme. Repeated crystallization of ribitol from absolute alcohol did not reduce the activity of L-iditol dehydrogenase for ribitol. Furthermore, the reduction of D-ribulose confirms the additional activity of L-iditol dehydrogenase for D-*ribo* substrates.

Table 5 summarizes the activities of D-mannitol dehydrogenase. D-arabitol, D-mannitol, D-rham-

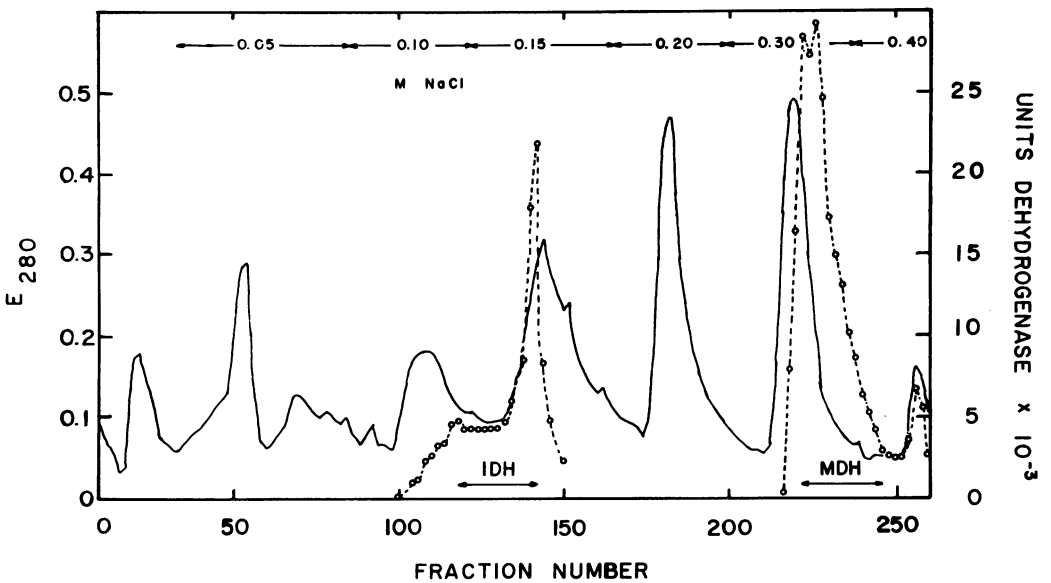


FIG. 1. *Chromatogram on DEAE cellulose acetate of a mixture of extracts of cells grown on* D-*mannitol and of cells grown on sorbitol. The column (31 by 1.5 cm) was buffered with 0.05* M *tris acetate at pH 7.0. Protein was eluted with the same buffer containing sodium chloride at the concentrations indicated. Each fraction was assayed for* L-*iditol dehydrogenase with sorbitol as a substrate and for* D-*mannitol dehydrogenase with* D-*mannitol as the substrate. The solid line is the absorbancy per cm of the effluent at 280 mμ; the dotted line is units of enzyme per 15 ml fraction. Fractions 118 to 142 and 222 to 246 inclusive were pooled separately. The specific activity of each enzyme was increased 20-fold.*

TABLE 4. *Substrate specificity of purified* L-*iditol dehydrogenase**

Polyols oxidized	Activity	Ketose reduced	Activity
Sorbitol	100†	D-Fructose	20
L-Iditol	102	L-Sorbose	11
Xylitol	121	D-Xylulose	92
Ribitol	9	D-Ribulose	39

* The activity of purified L-iditol dehydrogenase was measured in the standard assay (*see* Materials and Methods) except that the one drop of D-xylulose syrup was added in the D-xylulose assay.

† The rate of oxidation of polyols and reduction of ketoses is expressed as per cent of the rate of oxidation of sorbitol. Each assay was made with 150 units of enzyme.

TABLE 5. *Substrate specificity of purified* D-*mannitol dehydrogenase**

Polyols oxidized	Activity	Ketoses reduced	Activity
D-Mannitol	100†	D-Fructose	98
D-Arabitol	64	D-xylulose	232
		D-Ribulose	0
D-Rhamnitol	112		
Perseitol	130		

* The activity of purified D-mannitol dehydrogenase was measured in the standard assay (*see* Materials and Methods) except that one drop of D-xylulose syrup was added in the D-xylulose assay.

† The rates of activity of the enzyme on the various substrates are expressed as per cent of the rate of oxidation of D-mannitol; each assay was made with 105 units of D-mannitol dehydrogenase.

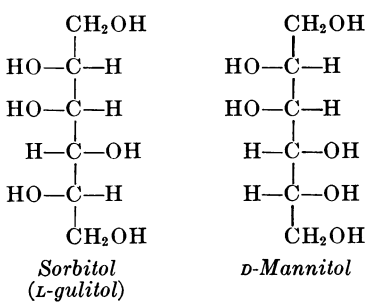
nitol, and perseitol, all of which have the D-*manno* configuration, were oxidized with the reduction of DPN. D-Fructose, the 2-ketose corresponding to D-mannitol, was reduced. D-Xylulose and D-ribulose are alternative 2-ketoses of D-arabitol; only D-xylulose was reduced by D-mannitol dehydrogenase. Although sorbitol, viewed as L-gulitol, differs from D-mannitol only in the configuration of carbon 5, the purified L-mannitol dehydrogenase oxidizes sorbitol at less than 1% the rate of D-mannitol and does not reduce the corresponding 2-ketose, L-sorbose.

DISCUSSION

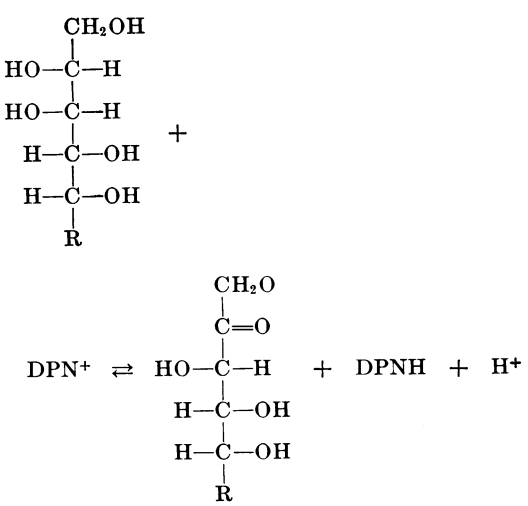
Structural specificity for D-*mannitol dehydrogenase induction.* A chain length of at least five

carbons is presumably a minimal requirement for the induction of D-mannitol dehydrogenase since glycerol and erythritol fail to induce; however, as shown in Table 3, all of the pentitols except L-arabitol induce the enzyme. Ribitol is a rare example of a compound which induces an enzyme for which it is not itself a substrate, yet fails to induce a related enzyme for which it is a substrate. Since ribitol is not metabolized, it lends itself to the study of gratuitous induction; that is, the kinetics of induction are not complicated by utilization of the inducer.

Of the three hexitols tested, only D-mannitol induced D-mannitol dehydrogenase; sorbitol and galactitol were ineffective. Thus, structural requirements for induction by hexitols are far more stringent than for pentitols. The sole dissimilarity between sorbitol, viewed as L-gulitol, and D-mannitol is in the configuration of C-5, yet mannitol is an inducer and sorbitol is not.

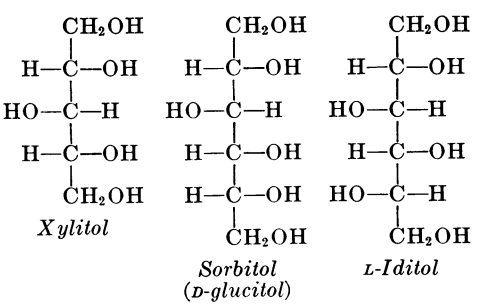


Substrate specificity of D-mannitol dehydrogenase. The D-*manno* configuration is an obligatory requirement for a substrate of D-mannitol dehydrogenase which catalyzes the reaction:

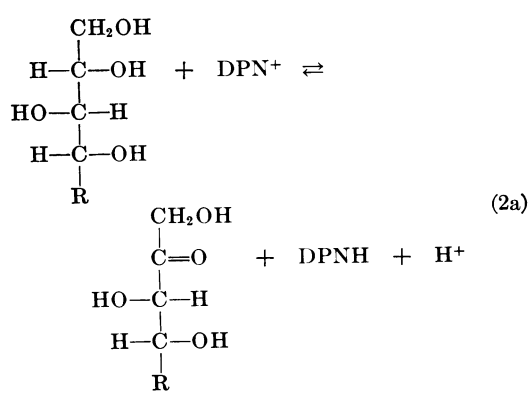


D-arabitol (R = H), D-mannitol (R = CH_2OH), D-rhamnitol (R = CH_3), and perseitol (R = $CHOH–CH_2OH$) were the only polyols oxidized by D-mannitol dehydrogenase. The ketoses reduced by the enzyme are consistent with this rule of specificity. D-Fructose is reduced, presumably to D-mannitol; and D-xylulose, but not D-ribulose, is reduced, presumably to D-arabitol. Thus, the D-mannitol dehydrogenase of *A. agilis*, which requires a specified configuration of 5 asymmetric carbons, has the most stringent structural requirements for substrates of any of the previously described polyol dehydrogenases (Edson, 1953; Shaw, 1956).

Structural requirements for induction of L-iditol dehydrogenase. Of the polyols tested only xylitol and sorbitol induce this enzymatic activity. D-Mannitol, ribitol, D- and L-arabitol, as well as erythritol, glycerol, and galactitol, are ineffectual. The configuration common to the inducers is the D-*xylo* configuration. L-Iditol was not available in quantities sufficient for induction trials but it presumably would act as an inducer.



Substrates for L-iditol dehydrogenase. Both the D-*xylo* and D-*ribo* configuration are oxidized at carbon 2, although the D-*xylo* configuration apparently is preferred. The reactions catalyzed by L-iditol dehydrogenase are:



(2a)

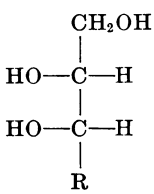
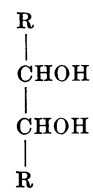
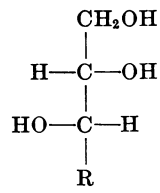
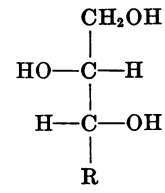
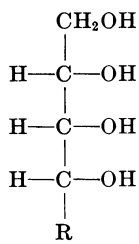
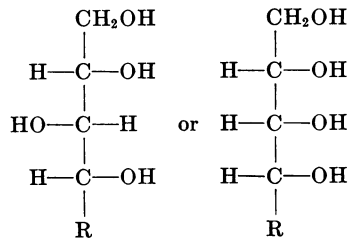
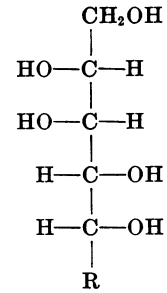
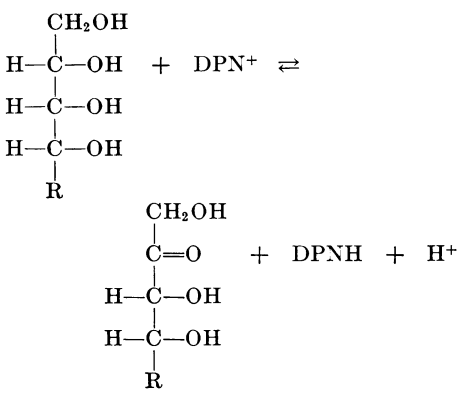
Polyol oxidase (Bertrand-Hudson enzyme)	Glycerol and glycol dehydrogenases	Galactitol dehydrogenases	D-Iditol dehydrogenase
			
Ribitol dehydrogenase	**L-Iditol dehydrogenase**		**D-Mannitol dehydrogenase**
			

FIG. 2. *Substrate specificities for various bacterial polyol dehydrogenases*



(2b)

Thus, L-iditol dehydrogenase oxidizes xylitol (R = CH_2OH), sorbitol (R = H—C—OH, CH_2OH) and L-iditol (R = HO—C—H, CH_2OH) and reduces the corresponding 2-ketoses, D-xylulose, D-fructose, and L-sorbose, by reaction 2a. The oxidation of ribitol (R = CH_2OH) and reduction of D-ribulose suggest a second general reaction, 2b. L-Iditol dehydrogenase from *A. agilis* is thus identical in substrate specificity with the L-iditol dehydrogenase isolated from rat liver, studied by Blakely (1951) and McCorkindale and Edson, (1954).

It is of some interest to compare the substrate specificities of the polyol dehydrogenases produced by bacteria. Figure 2 shows the structural specificities written for oxidation at carbon 2 of several bacterial enzymes. For some of these enzymes the specificity has not been definitely established but is postulated on the basis of polyols oxidized by crude extracts. The familiar Bertrand-Hudson enzyme(s) of Acetobacter is an insoluble, cytochrome-linked oxidase with an acid pH optimum (Arcus and Edson, 1956) located in the cell envelope (Stouthamer, 1959). Configurational requirements for the substrate are simple; the enzyme requires the L-*erythro* configuration and a primary alcohol at carbon 1. In many cases, however, a methyl group may be substituted for the primary alcohol (Richtmeyer, Stewart, and Hudson, 1950). The remaining polyol dehydrogenases are soluble, DPN-requiring enzymes.

A specific DPN-linked glycerol dehydrogenase from *Escherichia coli* oxidizes glycerol to dihydroxyacetone but does not oxidize sorbitol, D-mannitol, erythritol, or L-2,3-butanediol (Asnis

and Brodie, 1953). A glycerol dehydrogenase similar in substrate specificity and heat resistance has been isolated from the halophile *Pseudomonas salinaria* (Baxter and Gibbons, 1954). A second class of enzymes oxidize a variety of glycols such as 1,2-propane diol and L-2,3-butanediol in addition to glycerol; glycol dehydrogenases have been described in *Aerobacter aerogenes* (Burton and Kaplan, 1953; Lamborg and Kaplan, 1960), *E. coli* (Strecker and Harary, 1954), *Vibrio costicolus* (Baxter and Gibbons, 1954), and *Acetobacter suboxydans* (Goldschmidt and Krampitz, 1954).

The polyols oxidized by extracts of Pseudomonas suggest at least two novel polyol dehydrogenases which are distinguished by a difference in stability. One, galactitol dehydrogenase, requires the L-*threo* configuration and, thus, is responsible for the oxidation of galactitol, L-iditol, L-arabitol, and xylitol. Another, D-iditol dehydrogenase, which requires the D-*threo* configuration, is active against D-iditol, D-gulitol, D-talitol, and xylitol (Shaw, 1956).

Ribitol dehydrogenase (Wood and Tai, 1958; Fromm, 1958) produced by *A. aerogenes* differs from L-iditol dehydrogenase in that ribitol dehydrogenase does not oxidize D-*xylo* substrates and differs from glycol dehydrogenase in that it does not oxidize glycerol or erythritol. The precise substrate specificity of ribitol dehydrogenase has not been reported.

L-Iditol dehydrogenase, which we have found in *A. agilis*, was first isolated from rat liver (Blakely, 1951) and has previously been reported to be formed by Acetobacter (Arcus and Edson, 1954). Both the D-*xylo* and D-*ribo* polyols are substrates.

D-Mannitol dehydrogenase has been definitely established only in *A. agilis*. Other genera including *Acetobacter* (McCorkindale and Edson, 1954) and *Pseudomonas* (Shaw, 1956) yield extracts that oxidize D-mannitol with the production of DPNH; however, the responsible enzyme may not be identical in substrate specificity with the D-mannitol dehydrogenase of *A. agilis*.

A possible alternative route for the metabolism of D-mannitol is by phosphorylation followed by oxidation of D-mannitol phosphate to D-fructose 6-phosphate. Mannitol 1-phosphate dehydrogenase has been found in *E. coli* (Wolff and Kaplan, 1956) but has not been detected by us in *A. agilis*.

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