PARALLEL INDUCTION OF D-ARABITOL AND D-SORBITOL DEHYDROGENASES[1]

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ABSTRACT

SCOLNICK, EDWARD M. (Harvard Medical School, Boston, Mass.) AND EDMUND C. C. LIN. Parallel induction of D-arabitol and D-sorbitol dehydrogenases. J. Bacteriol. **84**:631–637. 1962.—Two inducible diphosphopyridine nucleotide-linked dehydrogenases are described in a bacterium isolated from the soil, *Cellvibrio polyoltrophicus* ATCC 14774. The first enzyme catalyzes the dehydrogenation of D-arabitol to D-xylulose and D-mannitol to D-fructose. The data suggest that in vivo this enzyme has the dual function of the utilization of both of these polyhydric alcohols. The second enzyme was found to act only on D-sorbitol, converting it to D-fructose. Evidence for its physiological function as a D-sorbitol dehydrogenase is also given. Both of these enzymes were found to be induced in parallel by any of the three polyhydric alcohols, D-arabitol, D-mannitol, and D-sorbitol. A common stereoconfiguration of the inducers for these eznymes is suggested. The parallel evolution of substrate specificity and inducer specificity is discussed with respect to the functional advantage that such a selective process might offer.

It has been previously reported that a capsulated strain of *Aerobacter aerogenes*, 1033, utilizes D-arabitol as a sole source of carbon, and that D-arabitol induces in this organism a diphosphopyridine nucleotide (DPN)-linked D-arabitol dehydrogenase (Lin, 1961). In vitro, this enzyme was found also to attack D-mannitol, converting it to D-fructose, a metabolizable sugar for this organism. Although the catalytic property of this enzyme permits it to function both as a D-arabitol dehydrogenase and as a D-mannitol dehydrogenase, its physiological function in the latter capacity is precluded by the fact that the metabolism of D-mannitol represses the formation of this enzyme.

In the present communication, we wish to report an inducible dehydrogenase from *Cellvibrio polyoltrophicus*, a newly isolated soil bacterium, manifesting a similar substrate specificity. In this case, the control is such that it enables the organism to utilize *both* D-arabitol and D-mannitol through action of the same enzyme.

During the course of the study of the specificity of the inducers for this enzyme, we encountered yet another DPN-linked polyol dehydrogenase which attacked D-sorbitol. Data on the specificity and the induction of this enzyme are also reported.

MATERIALS AND METHODS

Bacteria. Two strains of bacteria were employed in this study: *A. aerogenes* 1033 and *C. polyoltrophicus*. The former was kindly provided by Boris Magasanik. The latter was isolated from the soil by an enrichment technique with L-arabitol as the sole source of energy and carbon. This organism was able to grow on a number of other polyols, including glycerol, D-arabitol, ribitol, xylitol, D-mannitol, and D-sorbitol. In particular, this organism was able to grow on cellobiose as the sole source of carbon and energy. These cells were found to be strict aerobes and appeared as gram-negative rods. They were unable to grow out as clones on agar plates unless substantial amounts of Tryptone (Difco) were added to the synthetic medium. The general characteristics described seem to fit the genus *Cellvibrio* more than the other groups in *Bergey's Manual of Determinative Bacteriology*. This organism is therefore provisionally designated as *Cellvibrio polyoltrophicus*.

Chemicals. D-Arabitol was purchased from Pfanstiehl Chemical Corp., Waukegan, Ill.; D-mannitol from Eastman Kodak Co., Rochester,

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N.Y.; D-sorbitol from H. M. Chemical Co., Santa Monica, Calif.; and D-arabinose from Nutritional Biochemical Corp., Cleveland, Ohio. The diethylaminoethanol anion-exchange cellulose (Cellex-D) was from California Corporation for Biochemical Research, Los Angeles, Calif. We are grateful to G. Ashwell for a sample of D-xylulose.

Growth and harvest of cells. The basal medium was made up with the following salt concentrations: 1.26% KH_2PO_4, 0.54% K_2HPO_4, 0.2% $(NH_4)_2SO_4$, 0.02% $MgSO_4 \cdot 7H_2O$, and 0.001% $CaCl_2$. The solution was titrated with NaOH to a final pH of 6.8. Normally, carbon sources were present at a concentration of 2 g per 100 ml. Polyhydric alcohols were generally sterilized in the basal medium at 20 psi of steam pressure for 20 min. Glucose was autoclaved separately and added aseptically to the sterilized medium. D-Fructose and D-arabinose were sterilized by filtration through Millipore filters (0.45-μ pore size).

Cells were grown in liquid culture at 37 C in culture flasks on rotary shakers. Growth was monitored by reading the optical density of the culture in a Klett-Summerson photoelectric colorimeter with a no. 42 filter.

The cells were harvested by centrifugation at 4 C. After the cells had been washed once with distilled water, they were suspended in 0.05 M phosphate buffer (pH 7) and disrupted in a 10-kc Raytheon sonic oscillator. The sonic disruption was carried out for 5 min, during which time the cup was kept at 0 to -2 C. The cell debris was removed by centrifugation at 20,000 $\times$ *g* for 30 min. All of the enzyme activities were found to be present in the supernatant fraction. The enzyme activities were stable for a number of days if kept at -10 C.

Enzyme assays. The reaction was run in a cuvette with a 1-cm light path. In addition to the enzyme extract, the reaction solution contained 0.2 ml of 0.01 M DPN (or other pyridine nucleotide), 0.3 ml of 0.5 M substrate, 2 ml of 0.1 M potassium carbonate buffer (pH 10), and enough distilled water to bring the final volume to 3 ml. Under these conditions, the dehydrogenases were shown to be saturated with respect to both substrate and coenzyme. The activities of the dehydrogenases were measured by following the reduction of DPN at 340 mμ in a Beckman DU spectrophotometer at 25 $\pm$ 1 C. Both enzymes were shown to have a broad pH optimum of 9.0 to 10.5. In this pH range, there was no measurable enzymatic reoxidation or destruction of reduced DPN (DPNH) during the assay. The reaction followed zero-order kinetics for at least 1 min, and the DPNH formation during this first minute was used to calculate the specific activity, which was expressed as μmoles of DPN reduced per min per mg of protein at 25 C. Protein determinations were done by the spectrophotometric method based on the absorption of protein at 260 mμ (Warburg and Christian, 1942).

Fractionation of enzymes by column chromatography. A column of diethylaminoethanol anion-exchange cellulose (30 cm in height and 1 cm in diam) was first washed with 0.05 M KH_2PO_4-K_2HPO_4 (pH 7.0). The crude enzyme extract dissolved in 0.02 M NaCl, was added to the column. Elution was carried out with an increasing gradient of phosphate buffer (pH 7.0); the initial concentration was 0.05 M and the final concentration was 0.5 M.

Keto sugar determination. The products formed enzymatically from D-arabitol, D-mannitol, and D-sorbitol were reacted with resorcinol, and the absorption spectra of the resulting complexes were compared with those of standard compounds (Higashi and Peters, 1950).

RESULTS AND DISCUSSION

Number and specificity of dehydrogenases. This strain of *C. polyoltrophicus* was found to grow on a number of polyhydric alcohols, among them D-arabitol, D-mannitol, and D-sorbitol, as sole sources of energy and carbon. After growth of the cells on any one of the three polyols, the cell-free extracts were found to contain DPN-linked dehydrogenase activities for all three compounds. None of these activities was present in measurable quantities when the cells were grown on glucose or glycerol. Furthermore, the repressive effect of glucose on induced enzyme synthesis was not observed in this organism (Gale, 1943; Monod, 1947; Neidhardt and Magasanik, 1956).

In view of the presence of three dehydrogenase activities in extracts of induced cells, it became necessary to determine the number of distinct enzymes which were responsible for these reactions. Since it had been found that the D-arabitol dehydrogenase in *A. aerogenes* also attacked D-mannitol (Lin, 1961), our first attempt was to determine whether D-arabitol and D-mannitol

dehydrogenation was also carried out by the same enzyme in the present organism.

In a test for heat stability, it was found that the activities on D-arabitol and D-mannitol decayed at indistinguishable rates (Table 1). Both activities were inhibited by 25% in the presence of 1×10^{-4} M 1,10-phenanthroline. Under the same conditions, no inhibition of the D-sorbitol dehydrogenase was observed.

Previously, DPN analogues had been employed by Kaplan et al. (1960) for the study of the molecular heterogeneity of enzymes acting with pyridine nucleotides as cofactors. Table 2 shows that the relative activities obtained with DPN and 3-acetyl pyridine DPN, employing D-arabitol as substrate, were experimentally indistinguishable from those obtained by employing D-mannitol as substrate, whereas a different ratio of activities of the coenzyme and the analogue was observed with D-sorbitol. These results indicate that at least two different enzymes were present in the crude extracts: one capable of attacking D-arabitol and D-mannitol, and the other capable of attacking D-sorbitol.

More direct information concerning these three dehydrogenase activities was sought by subjecting cellular extracts to fractionation by diethylaminoethanol anion-exchange column chromatography. The elution patterns of these activities are given in Fig. 1. The activity ratios in different fractions are summarized in Table 3. The D-arabitol and D-mannitol activities appeared concomitantly, and their elution from the column preceded that of the D-sorbitol activity. On the other hand, the D-sorbitol dehydrogenase activity continued to be eluted in later fractions no longer containing activities for the other two polyols.

When a fraction containing only the activities on D-arabitol and D-mannitol was tested in the presence of both substrates at saturating concentrations of enzyme, the activity did not exceed that which was observed with D-arabitol alone.

The D-arabitol dehydrogenase in this preparation was found to exhibit a substrate specificity very similar to that of the D-arabitol dehydrogenase purified from *A. aerogenes* 1033. [Although it will be clear from the data to be presented

TABLE 1. *Comparison of heat stability of D-arabitol and D-mannitol activities of an extract prepared from D-arabitol-grown cells*

Treatment		Activity ratio (D-arabitol/ D-mannitol)	Per cent of original activity
Temp	Heating time		
C	*min*		
0	—	1.9	100
44	60	1.9	40
47	30	1.9	38
50	15	1.9	38

TABLE 2. *Comparison of dehydrogenation activities on D-arabitol, D-mannitol, and D-sorbitol, employing DPN or 3-acetyl pyridine DPN as hydrogen acceptors*

Substrate	Relative activities on		DPN/ analogue ratio
	DPN	Analogue	
D-Arabitol	0.380	0.076	5.0
D-Mannitol	0.198	0.040	4.9
D-Sorbitol	0.380	0.055	6.9

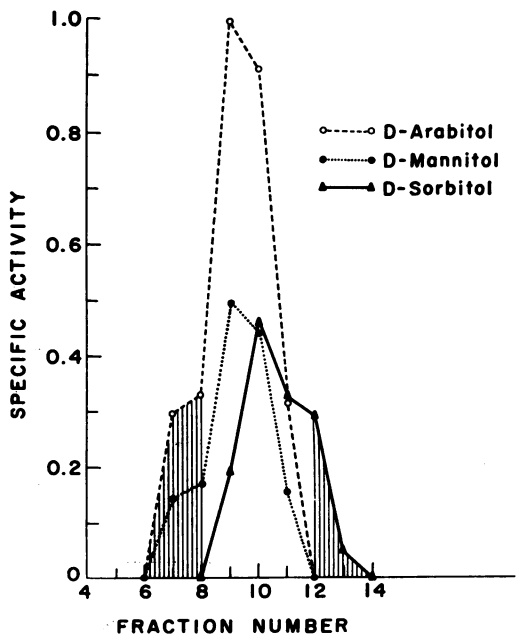

FIG. 1. *Fractionation of D-arabitol dehydrogenase from D-sorbitol dehydrogenase. An extract from D-arabitol-grown cells was fractionated on a diethylaminoethanol column. A total of 40 mg of protein was absorbed on the column, and 25 mg of the material were recovered. Each fraction contained a volume of 4 ml. Fractions 6–8 contained no D-sorbitol dehydrogenase (striped area on the left), and fractions 12–14 contained no D-arabitol dehydrogenase (striped area on the right).*

that the former enzyme serves the dual physiological function of metabolizing both D-arabitol and D-mannitol, we shall refer to the enzyme as D-arabitol dehydrogenase on the basis of its catalytic preference, to keep the nomenclature simple.] Both acted on D-arabitol and D-mannitol but not on glycerol, meso-erythritol, ribitol, xylitol, L-arabitol, D-sorbitol, D-dulcitol, or myo-inositol. The D-sorbitol dehydrogenase-containing fraction acted only on D-sorbitol. Neither D-arabitol dehydrogenase nor D-sorbitol dehydrogenase displayed any appreciable activity when triphosphopyridine nucleotide was substituted for DPN.

Specificity of enzyme induction. Table 4 shows the dehydrogenase activities on D-arabitol, D-mannitol, and D-sorbitol in extracts of cells grown on each of these three compounds. The observation that the ratio of the activities on D-arabitol and D-mannitol was invariant, within experimental error, is again consistent with the belief that these two activities are expressions of the same enzyme. It is noteworthy that D-mannitol and, in particular, the pentitol D-arabitol were better inducers for the D-sorbitol dehydrogenase than was its own substrate, D-sorbitol.

TABLE 3. *Partial resolution of D-arabitol dehydrogenase from D-sorbitol dehydrogenase* (data taken from Fig. 1)

Tube no.	Enzyme activities	
	D-Arabitol/D-mannitol	D-Arabitol/D-sorbitol
7	2.1	100
8	2.0	100
9	2.0	5.6
10	2.0	2.0
11	1.9	1.0
12	—	0
13	—	0

TABLE 4. *Polyhydric alcohol dehydrogenase activities in cells grown on three different carbon sources*

Carbon source	Specific activity on			I/II	I/III
	I D-Arabitol	II D-Mannitol	III D-Sorbitol		
D-Arabitol	0.48	0.25	0.38	1.9	1.3
D-Mannitol	0.30	0.14	0.22	2.1	1.4
D-Sorbitol	0.10	0.050	0.10	2.0	1.0

This would suggest that the cross induction of the D-sorbitol dehydrogenase by the other two polyols was not by prior conversion of these two compounds to D-sorbitol which then acted as the inducer for its enzyme. An alternative hypothesis for the cross induction would be that these three polyols share a molecular configuration which satisfies the specificity requirement for the induction of both enzymes. Indeed, it can be readily seen that these compounds share three carbons with the same configuration (Fig. 2). This hypothesis would be strengthened if another compound possessing this particular configuration, but which was not metabolizable, would also act as an inducer. D-Arabinose was found to satisfy these conditions.

As indicated in Fig. 2, D-arabinose has the minimal configuration postulated to be sufficient for induction. The compound was metabolically inert, in that it could neither be reduced to D-arabitol by extracts containing the D-arabitol dehydrogenase in the presence of DPNH, nor be utilized by this organism for growth even at a concentration of 0.3 M. When cells were grown on glucose in the presence of D-arabinose, both the D-arabitol dehydrogenase and the D-sorbitol dehydrogenase were induced. The specific activities with respect to D-arabitol, D-mannitol, and D-sorbitol in this extract are summarized in Table 5. It should be pointed out that the levels were all considerably lower than those observed when the cells were grown on D-arabitol, D-mannitol, or D-sorbitol. The weak inducing action of D-arabinose is probably due to the fact that at pH 6.5 only 0.13% of a solution of 0.1 M L-arabinose exists as the open-chain aldose (Cantor and Peniston, 1940). Under the same conditions, only 0.012% of D-glucose exists in the open-chain form. It is therefore consistent with our expectation that D-arabinose is a weak inducer and that D-glucose fails to be an inducer at all, even

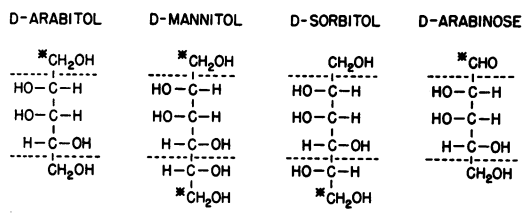

FIG. 2. *Inducers of D-arabitol and D-sorbitol dehydrogenases. The common configuration is included between the broken lines. The conventional designation of carbon 1 is indicated by an asterisk.*

TABLE 5. *Dehydrogenase activities in cells grown with D-arabinose as inducer**

Substrate	Specific activity
D-Arabitol	0.065
D-Mannitol	0.033
D-Sorbitol	0.037
L-Arabitol	0.000
Xylitol	0.000
Ribitol	0.000

* The cells were grown in the presence of 0.15 M D-arabinose with 0.2% glucose as the sole source of energy and carbon. No growth was noticed when D-arabinose was present alone, even at a concentration of 0.3 M.

though this hexose also possesses the minimal stereoconfiguration indicated in Fig. 2. In summary, the simplest way to account for the simultaneous induction of the two dehydrogenases is to postulate that the inducer specificities of these two systems are extremely similar. However, to rule out possible complications of the cross induction of the enzymes as consequences of metabolism of the polyols, it will be necessary to examine the behavior of mutants that lack D-arabitol dehydrogenase or D-sorbitol dehydrogenase.

Physiological roles of the two dehydrogenases. The observations that D-sorbitol induced and was acted on by D-sorbitol dehydrogenase and that both D-arabitol and D-mannitol induced and were acted on by D-arabitol dehydrogenase suggest that these dehydrogenation reactions are relevant to the physiological roles of these two enzymes. If this is true, the immediate products of these reactions should also be utilizable by the cells, provided that there were no serious permeability barriers. For this purpose, the enzyme-catalyzed dehydrogenation products of the three polyhydric alcohols were examined. It was found by the resorcinol test that D-arabitol dehydrogenase converted D-arabitol and D-mannitol to keto sugars indistinguishable from D-xylulose and D-fructose, respectively. Moreover, the enzyme catalyzed the oxidation of DPNH upon the addition of D-xylulose or D-fructose. Similar experiments indicated that D-sorbitol dehydrogenase converted its substrate to D-fructose.

These results suggest that D-fructose could arise as a common intermediate from the action of D-arabitol dehydrogenase and D-sorbitol dehydrogenase during the assimilation of the hexitols. We would therefore expect the organism to grow on D-fructose as the sole source of energy and carbon, and, furthermore, growth on this ketose might induce both the dehydrogenases, in view of the reversibility of DPN-linked reactions. When D-fructose was tested as a growth-supporting sugar, it was indeed found to be utilized readily. When the cell extracts were examined, it was found that both D-arabitol dehydrogenase and D-sorbitol dehydrogenase were induced. Growth studies with D-xylulose were not carried out because of the paucity of the compound.

It might be pointed out that, in an analogous case of glycerol utilization by *A. aerogenes* 1033, dihydroxyacetone was postulated as an intermediate which was a product of a DPN-linked glycerol dehydrogenase. When dihydroxyacetone was tested for the support of growth, it was found that it could serve as the sole source of energy and carbon. Cells thus grown were found to be fully induced in the glycerol dehydrogenase as well, presumably by virtue of the ready back-conversion of the triose to glycerol in the presence of trace amounts of glycerol dehydrogenase present in the cells under all conditions (Lin, Levin, and Magasanik, 1960).

Further support for the belief that D-arabitol and D-mannitol were metabolized by the D-arabitol dehydrogenase, and that D-sorbitol was utilized through the D-sorbitol dehydrogenase, was obtained from studies on lag periods (Fig. 3).

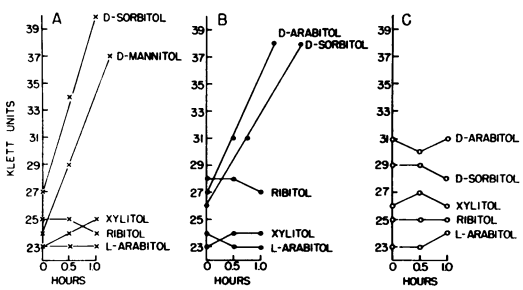

FIG. 3. *Effect of previous induction of enzymes on lag periods. Carbon sources present in a concentration of 5 g per 100 ml.* (A) *Cells were previously grown on D-arabitol;* (B) *on D-mannitol;* (C) *on glucose. The carbon source into which the cells were inoculated is indicated beside each curve. All cultures which showed a lag resumed growth after 1.5 hr.*

Cells previously induced on D-arabitol grew without lag on D-mannitol and D-sorbitol; similarly, cells previously induced on D-mannitol grew without lag on D-arabitol and D-sorbitol. All these cells grew with lag when transferred to xylitol, ribitol, or L-arabitol as a sole source of energy and carbon. Control cells previously grown on glucose showed a lag period when transferred to any of these polyhydric alcohols.

Significance of the induction patterns. Previous studies on the control of enzyme synthesis have revealed that enzymes which were induced or repressed together often belonged to the same operon (Ames and Garry, 1959; Robichon-Szulmajster, 1958; Kalckar, Kurahashi, and Jordan, 1959; Jacob et al., 1960). In the present studies, we have encountered two enzymes which did not catalyze the same series of metabolic reactions, but which nevertheless were simultaneously induced. Although the data so far do not permit us to establish unequivocally whether the simultaneous induction of these two enzymes was due to the fact that they belonged to the same operon, the observation that the relative ratio of D-arabitol dehydrogenase to D-sorbitol dehydrogenase could vary from 1.0 (when D-sorbitol was the inducer) to 1.8 (when D-arabinose was the inducer) strongly suggests that the two enzymes were not synthesized at the same rate and therefore were not part of the same operon. The simultaneous induction was therefore probably the result of the two operons possessing very similar inducer specificity, as suggested in Fig. 2.

In the case of the D-arabitol dehydrogenase, we have an example of one enzyme which utilizes two compounds, giving rise to two different products, and induced by either of the substrates. It seems that the close evolution between the substrate specificity and the inducer specificity has endowed this organism with an economic mechanism by which two sources of carbon and energy could be attacked by a single enzyme.

On the other hand, the induction of the D-arabitol dehydrogenase by D-sorbitol, and the reciprocal induction of the D-sorbitol dehydrogenase by D-arabitol and D-mannitol, offer no apparent functional advantage. Analogous cross inductions of α- and β-galactosidases in various strains of *Escherichia coli* have been reported (Monod, Cohen-Bazire, and Cohn, 1951; Shenin and Crocker, 1961; Lester, 1952; Koppel, Porter, and Crocker, 1953; Porter, Holmes, and Crocker, 1953). It is suspected that many such cases of cross induction of enzymes exist in various organisms and that they remain unnoticed because there is no clear functional connection between the inducers and the enzymes. The evaluation of the biological significance of these cross inductions may have to be postponed until more examples become available for analysis.

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