JOURNAL OF BACTERIOLOGY, Mar. 1968, p. 925-931
Vol. 95, No. 3

Regulation of Pentitol Metabolism by *Aerobacter aerogenes*

I. Coordinate Control of Ribitol Dehydrogenase and D-Ribulokinase Activities

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Received for publication 28 December 1967

Induction studies on *Aerobacter aerogenes* strain PRL-R3, using ribitol as the inducer-substrate, indicated that two enzymes of ribitol catabolism, ribitol dehydrogenase and D-ribulokinase, are coordinately induced. The utilization of D-arabinose as a substrate resulted in the induction of ribitol dehydrogenase as well as D-ribulokinase. Mutants which were constitutive for ribitol dehydrogenase were also constitutive for D-ribulokinase. In contrast, D-xylulokinase and D-arabitol dehydrogenase did not appear to be coordinately controlled. Induction studies and examination of D-arabitol dehydrogenase constitutive mutants indicated that the three enzymes of the converging pathways for D-arabitol and D-xylose catabolism are under separate control.

With respect to catabolic pathways of carbohydrate metabolism, it appears that coordinate control of the enzymes responsible for the degradation of only one substrate would be a selective advantage for organisms. Alternatively, if two or more substrates are degraded via a converging pathway, it would be advantageous if the enzymes required in common were controlled separately from the earlier enzymes in the pathways. Regulation of catabolic pathways has been studied in diverse systems. Hegeman (8, 9, 10) reported coordinate control of enzymes responsible for the conversion of mandelic acid to benzoate in *Pseudomonas putida*. The benzoic acid oxidase system, responsible for the conversion of benzoate to catechol, is controlled separately in this organism. Furthermore, the enzymes involved in the degradation of catechol to β-ketoadipic acid are also under coordinate control, but such control is separate from the earlier enzymes in the system. Englesberg et al. (6) studied the pathway of L-arabinose degradation in *Escherichia coli*. The four enzymes, permease, isomerase, kinase, and epimerase, required for the conversion of L-arabinose to D-xylulose-5-phosphate were found to be coordinately controlled.

In view of the above considerations, it would seem quite advantageous to study the regulation of a catabolic system which contains highly converging as well as unbranched pathways. The degradative pathways of five-carbon carbohydrates by strains of *Aerobacter aerogenes* provide such a system.

A. aerogenes strain PRL-R3 is able to utilize, as sources of energy, seven of the eight aldopentoses and all four of the pentitols (7, 15). Of these eleven compounds, D-ribose, D-xylose, L-arabinose, D-arabitol, and ribitol are readily available to the organism in nature; on the other hand, D-arabinose, D-lyxose, L-lyxose, L-xylose, xylitol, and L-arabitol are rarely, if ever, found uncomplexed in the natural environment. Metabolism of the pentoses involves (i) the isomerization of an aldopentose to a ketopentose, (ii) phosphorylation to the corresponding pentulose-5-phosphate, and (iii) epimerization of pentulose-5-phosphate to the common intermediate, D-xylulose-5-phosphate. (In the case of D-ribose, phosphorylation to D-ribose-5-phosphate precedes isomerization and subsequent epimerization.) Pentitol utilization initially proceeds via a dehydrogenation to form a ketopentose; the subsequent reactions are identical to those for the pentoses.

The present investigation was undertaken to determine the pattern of regulation in the unbranched pathway of ribitol degradation and in the convergent pathways of D-arabitol and D-xylose utilization in *A. aerogenes*. In this study, the ribitol-degrading system represented the unbranched pathway, whereas the convergent route involved the utilization of D-arabitol and D-xylose. Both of these pathways are presented in

Fig. 1. The first catabolic sequence shown here is normally considered specific for ribitol degradation in *A. aerogenes*. However, it is also employed in the degradation of D-arabinose, provided that the cell possesses an isomerase for the conversion of D-arabinose to D-ribulose. D-Arabinose is an uncommon substrate for which a specific isomerase enzyme does not exist. Camyre and Mortlock (4) have indicated that strains of *A. aerogenes* which are constitutive for L-fucose isomerase are capable of converting D-arabinose to D-ribulose.

It seems reasonable to assume, as a working hypothesis, that, since D-arabinose is one of the uncommon pentoses, there would be no selective advantage in evolving a regulatory mechanism for the separate control of the enzymes common to both D-arabinose and ribitol catabolism. On the other hand, when two naturally occurring substrates, such as D-xylose and D-arabitol, share a common pathway, selective pressures may tend to favor the evolution of a separate regulatory system for the individual enzymes concerned.

MATERIALS AND METHODS

Isolation of dehydrogenase-constitutive mutants. Mutants of *A. aerogenes* strain PRL-R3 which are constitutive for ribitol dehydrogenase were selected by growth on xylitol (14). By use of wild-type PRL-R3 or a uracil-requiring auxotroph as the parental strain, 28 independent ribitol dehydrogenase constitutive mutants were obtained. Of these 28 mutants, one mutant was isolated after treatment with ethyl methanesulfonate, four mutants were obtained by treatment with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine, whereas the remaining 23 mutants arose spontaneously after prolonged incubation on 0.5% xylitol-salts-agar, supplemented, when necessary, with 0.005% uracil.

D-Arabitol dehydrogenase constitutive mutants of the organism were isolated by the procedure of Lin, Lerner, and Jorgensen (12).

Cultural conditions. Cells were grown aerobically at 30 C on a minimal medium (2) supplemented with 0.5% carbohydrate, 1% vitamin-free casein hydrolysate, or 2% peptone. The carbohydrates, casein hydrolysate, peptone, and magnesium sulfate were autoclaved separately and were added to the medium after cooling.

To determine dehydrogenase and kinase levels in the ribitol dehydrogenase constitutive mutants, 250-ml Erlenmeyer flasks containing 15 ml of casein hydrolysate- or peptone-medium were inoculated with 5 ml of an overnight culture of cells grown in an identical medium. The cultures were incubated aerobically at 30 C for 3 hr and then were harvested by centrifugation.

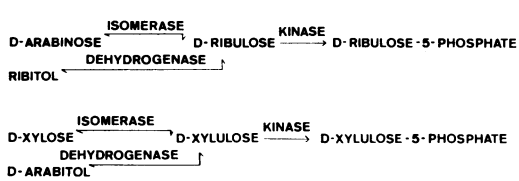

FIG. 1. *Degradative pathways of certain pentoses and pentitols in Aerobacter aerogenes strain PRL-R3.*

In experiments designed to test coordinate repression and derepression of the two enzymes, overnight casein hydrolysate-grown cultures of the constitutive mutants were inoculated into fresh casein hydrolysate medium, and this medium was maintained in the logarithmic phase of growth by periodic subculture into fresh medium. After approximately five generations of growth, cells were transferred to sterile glucose-salts medium and were allowed to go through approximately six generations of exponential growth before being returned to the casein hydrolysate medium.

In all of these experiments, growth was followed by measuring the increase in turbidity by use of a Klett-Sumerson colorimeter equipped with a red filter (660 mμ). The turbidity readings were converted to mg of dry weight per ml using a standard curve. The number of generations was determined on the basis of these dry weight values.

The inoculum used in the induction experiments (Fig. 2 and 5) consisted of PRL-R3 wild-type cells grown on glucose-salts, harvested by centrifugation, and washed once with sterile salts solution. The washed cells were then resuspended in a sterile salts solution to 16% of the original volume and were shaken at 30 C for 2 days. Induction experiments were then begun by inoculating these starved cells into 0.5% of the particular substrates to determine inducer ability.

Preparation of cell-free extracts. Cells were collected by centrifugation at 12,000 × *g* for 10 min at 4 C in an RC-2 refrigerated centrifuge (Ivan Sorvall, Inc., Norwalk, Conn.). The cells were then washed once with an equal volume of cold distilled water. The cell pellets were resuspended in 2 ml of 0.04 M tris(hydroxymethyl)aminomethane (Tris)-chloride buffer, *p*H 7.5, containing 0.0033 M ethylenediamine tetraacetate and 0.0033 M dithiothreitol. The suspended cells were placed in polyethylene tubes, and five such tubes were placed in the cup of a 10-kc magnetostrictive oscillator (Raytheon Co., South Norwalk, Conn.) containing 30 ml of distilled water. The cells were disrupted by exposure to sonic vibration for 15 min at 5 C. The cell debris was removed by centrifugation for 15 min at 27,000 × *g*. The supernatant fractions were collected in chilled tubes, and these fractions constituted the crude extracts.

Enzymatic assays. Assays for pentitol dehydrogenase activity were performed spectrophotometrically by observing the rate of reduced nicotinamide adenine dinucleotide ($NADH_2$) oxidation in the presence of ketopentose (18). Kinase assays were based on continuous spectrophotometric measurement of adenosine diphosphate (ADP) formation with the pyruvate kinase-lactic acid dehydrogenase system (3). To measure kinase activity in the presence of large amounts of the $NADH_2$-specific pentitol dehydrogenases, reduced nicotinamide adenine dinucleotide phosphate ($NADPH_2$) was substituted for

the $NADH_2$ (15). A unit of kinase or pentitol dehydrogenase activity was defined as the amount of enzyme necessary to produce an absorbancy change of 1.0 per min at 340 mμ with a light path of 1 cm in a reaction volume of 0.15 ml. The absorbancy change was measured at 25 C in a DU spectrophotometer (Beckman Instruments, Inc., Fullerton, Calif.) and was monitored with a model 2000 recording attachment (Gilford Instrument Laboratories, Inc., Oberlin, Ohio).

Isomerase activity was determined from the rate of pentulose formation by the method of Anderson and Wood (2); at 37 C, one unit of isomerase in 2.0 ml catalyzed the formation of 1 μmole of pentulose per hr. Ketopentose was measured by the cysteine-carbazole test of Dische and Borenfreund (5).

The protein content of the extracts was computed by determining the ratio of absorbancies at 280 mμ and 260 mμ (17).

Chemicals. Vitamin-free casein hydrolysate, ribitol, D-arabinose, xylitol, D-xylose, and D-arabitol were purchased from Nutritional Biochemicals Corp., Cleveland, Ohio. Peptone and Special Agar (Noble) were obtained from Difco. *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine was purchased from the Aldrich Chemical Co., Inc., Milwaukee, Wis., and ethyl methanesulfonate was purchased from Eastman Organic Chemicals, Rochester, N.Y. $NADH_2$ and NADPH were obtained from P-L Laboratories, Milwaukee, Wis., and lactic acid dehydrogenase was obtained from Worthington Biochemical Corp., Freehold, N.J. Dithiothreitol, 2-phosphoenolpyruvate, and D-ribulose ortho-nitrophenyl-hydrazone were purchased from Calbiochem, Los Angeles, Calif. Free D-ribulose was derived from the *o*-nitrophenylhydrazone derivative by the procedure of Müller, Montigel, and Reichstein (16).

RESULTS

Ribitol and D-arabinose pathways. The degradative pathways of ribitol and D-arabinose utilize a common kinase for the phosphorylation of D-ribulose (15). Although wild-type *A. aerogenes* is incapable of isomerizing D-arabinose, the incubation of these cells with D-arabinose as the sole carbon and energy source resulted in the rapid selection of mutants possessing such isomerase activity (4). The inoculation of glucose-grown cells of wild-type PRL-R3 strain into a ribitol-salts medium resulted in the rapid induction of ribitol dehydrogenase and D-ribulokinase (Fig. 2). Similar experiments utilizing D-arabinose as the sole growth substrate resulted in a lag of 10 to 14 hr for the selection of mutants possessing isomerase activity. The induction of both ribitol dehydrogenase and D-ribulokinase was correlated in such experiments (Fig. 2) with the appearance of isomerase activity. The maximal levels of kinase and dehydrogenase induced were comparable in both cases. Since ribitol dehydrogenase was not directly involved in D-arabinose catabolism, these data suggested the possibility of the coordinate induction of ribitol dehydrogenase with D-ribulokinase.

Mutants constitutive for ribitol dehydrogenase were isolated by using xylitol as a selective substrate (14). Of the 28 isolates tested, all were also found to be constitutive for D-ribulokinase. Although the mutants showed differences in enzyme levels, a plot of constitutive dehydrogenase versus kinase activities indicated that they are coordinately controlled (Fig. 3). When incubated with ribitol these mutants could usually be induced to higher levels of dehydrogenase and kinase activity. One hypo-constitutive mutant, i.e., one which synthesized a lower level of dehydrogenase than is normally found in constitutive strains, was isolated by the procedure of Lin et al. (12), after treatment of the wild-type strain with ethyl methanesulfonate. When incubated with ribitol for an extended period of time, this mutant was not induced to higher activity for either ribitol dehydrogenase or D-ribulokinase (Table 1).

Further evidence that ribitol dehydrogenase and D-ribulokinase enzymes are regulated by the same mechanism was obtained from a study of their coordinate derepression. When cells of *A.*

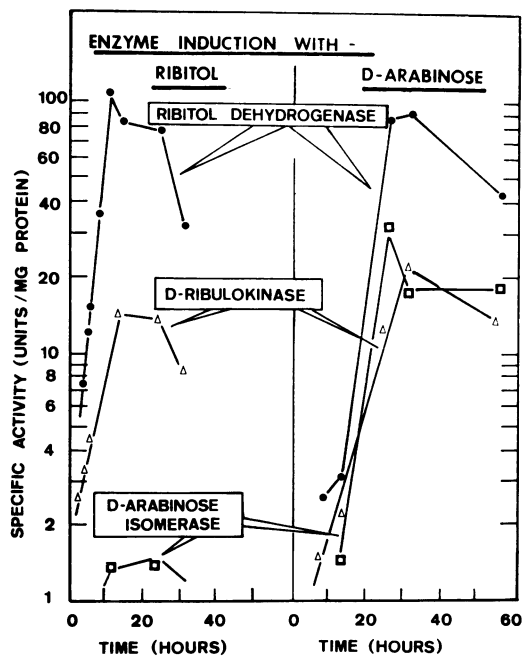

FIG. 2. *Enzymatic activities during growth of wild-type cells on ribitol and on D-arabinose. Cultures were grown at 30 C on a salts medium supplemented with 0.5% ribitol or D-arabinose. Inoculum was grown on 0.5% glucose-salts. Symbols: ribitol dehydrogenase, ●; D-ribulokinase, △; D-arabinose isomerase, □.*

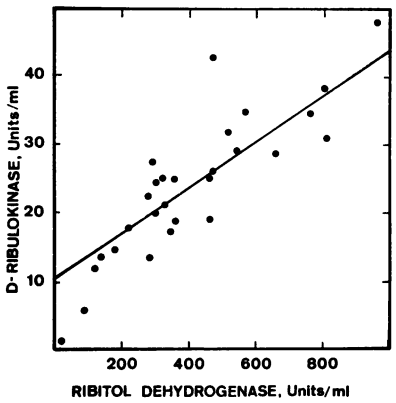

FIG. 3. *Ribitol dehydrogenase versus D-ribulokinase activities in ribitol dehydrogenase constitutive mutants of Aerobacter aerogenes. Extracts of casein hydrolysate-grown cells were disrupted by sonic vibration. The straight line was determined by the method of least squares; data from the wild type were not included in the calculations.*

TABLE 1. *Enzyme activities of a ribitol dehydrogenase, "hypo"-constitutive mutant*

Growth substrate	Strain	Specific activity (units/mg of protein)	
		Ribitol dehydrogenase	D-Ribulokinase
Peptone	Inducible	0	0
	Constitutive	21	1.5
Ribitol	Inducible	117	19.0
	Constitutive	21	1.8

aerogenes strain 126 were maintained on casein hydrolysate for approximately five generations (Fig. 4A) and then were transferred to a glucose-salts medium for six generations, both enzymes were diluted out at a rate closely approximating the theoretical dilution curve (Fig. 4B). The data presented in Fig. 4C indicated a rapid and parallel increase in both enzymes when the cells were released from glucose repression by transfer to casein hydrolysate; maximal levels of activity were obtained after about 2.5 generations of growth.

D-Arabitol and D-xylose pathways. The induction of D-arabitol dehydrogenase, D-xylose isomerase, and D-xylulokinase upon incubation of *A. aerogenes* strain PRL-R3, with either D-arabitol or D-xylose as the sole carbon and energy sources, is shown in Fig. 5. A comparison of enzyme activities suggested that none of these three enzymes was coordinately induced. Much higher D-xylulokinase activity was obtained when D-xylose was used as the substrate-inducer. D-Xylose (→ D-xylulose) isomerase activity was detected only in the D-xylose culture, and low D-arabitol dehydrogenase activity was also found in extracts of such cells.

To further investigate regulation of the D-arabitol pathway, the procedure of Lin et al. (12) was used to isolate mutants constitutive for D-arabitol dehydrogenase. The constitutive dehydrogenase activities of extracts of such mutants after growth on casein hydrolysate are shown in Table 2. D-Xylulokinase activity could not be detected in such extracts, again indicating the lack of coordinate control for these two enzymes. All of the constitutive strains were capable of normal growth when D-arabitol was used as the substrate, and the enzyme activities of D-arabitol-grown, cell-free extracts are listed in Table 3. D-Xylulokinase activity was induced when D-arabitol was used as the substrate during incubation and growth; thus, the absence of kinase activity in the extracts of casein hydrolysate-grown cells was not due to a defect in the kinase structural gene. The induction of constitutive mutant 203 by D-arabitol is graphically represented in Fig. 6.

The enzyme activities of these four constitutive strains after growth with D-xylose as the substrate are shown in Table 4. It is of interest to note that,

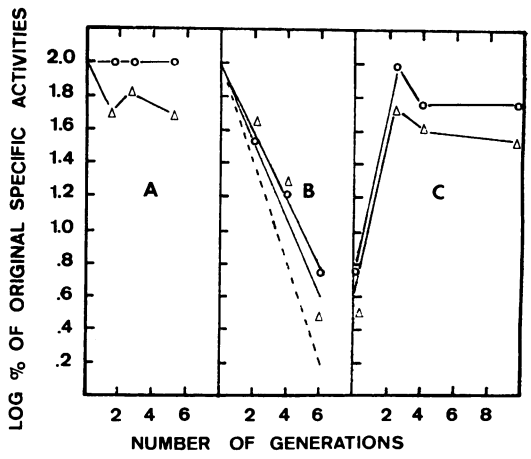

FIG. 4. *Coordinate derepression of ribitol dehydrogenase and D-ribulokinase activities in Aerobacter aerogenes strain 126. (A) Cells maintained in casein hydrolysate; (B) cells from (A) transferred to glucose-salts medium; (C) cells from (B) returned to casein hydrolysate. Symbols: ribitol dehydrogenase, ○; D-ribulokinase, △; theoretical dilution curve, broken line in (B).*

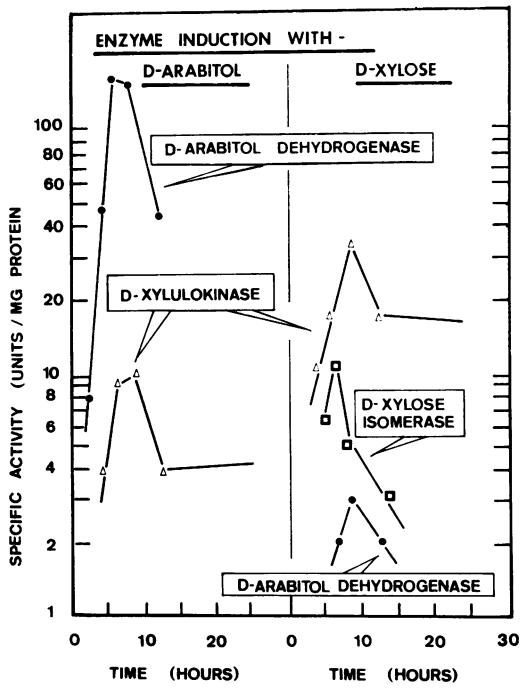

FIG. 5. *Enzymatic activities during growth of wild-type cells on* D-*arabitol and on* D-*xylose. Cultural conditions are similar to those given in Fig. 2. Symbols:* D-*arabitol dehydrogenase,* ●; D-*xylulokinase,* △; D-*xylose isomerase,* □.

TABLE 2. *Enzyme activities of* D-*arabitol dehydrogenase constitutive mutants grown on casein hydrolysate*[a]

Mutant strain	D-Arabitol dehydrogenase (units/mg of protein)
200	180.0
201	98.7
202	109.0
203	32.7

[a] No detectable D-xylulokinase or isomerase activity was found in extracts of cells grown on this substrate.

although the data support the concept of separate control for these enzymes, two of the four dehydrogenase constitutive mutants appear to have altered regulation for D-xylulokinase. Mutant strain 202 induced identical levels of D-xylulokinase in response to both D-arabitol and D-xylose as growth substrates, whereas strain 203 produced very low kinase activity during growth on D-xylose. In addition, a fifth dehydrogenase constitutive mutant displayed normal growth when D-arabitol was used as the substrate but was incapable of growth when D-xylose was used as the substrate. This mutant was unable to induce D-xylulokinase in response to the use of D-xylose as an inducer-substrate. D-Xylose isomerase induction, however, was normal.

DISCUSSION

The convergence of the D-arabinose degradative pathway with the ribitol catabolic pathway is the result of an isomerase which converts D-arabinose to D-ribulose. In view of the data indicating that D-arabinose isomerization is catalyzed by an enzyme of the L-fucose pathway, L-fucose isomerase (4), it is not surprising that synthesis of this isomerase is under separate control from the synthesis of ribitol dehydrogenase and D-ribulokinase.

The levels of ribitol dehydrogenase and D-

TABLE 3. *Enzyme activities of* D-*arabitol dehydrogenase constitutive mutants grown on* D-*arabitol*[a]

Mutant strain	Specific activity (units/mg of protein)	
	D-Arabitol dehydrogenase	D-Xylulokinase
200	127.0	3.63
201	117.5	5.67
202	98.0	18.00
203	34.7	7.85

[a] All of the mutants grown on this substrate possessed D-xylose isomerase activities of less than 2.0 units per mg of protein.

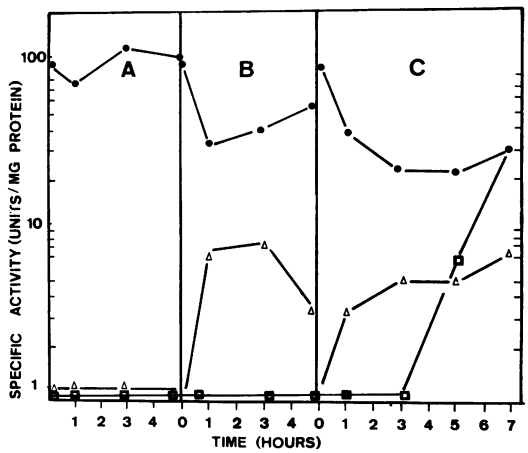

FIG. 6. *Induction of enzyme activities in a* D-*arabitol dehydrogenase constitutive mutant of Aerobacter aerogenes grown in casein hydrolysate (A), in* D-*arabitol (B), and in* D-*xylose (C). Symbols:* D-*arabitol dehydrogenase,* ●; D-*xylulokinase,* △; D-*xylose isomerase,* □.

TABLE 4. *Enzyme activities of* D-*arabitol dehydrogenase constitutive mutants grown on* D-*xylose*

Mutant strain	Specific activity (units/mg of protein)		
	D-Arabitol dehydrogenase	D-Xylulokinase	D-Xylose isomerase
200	82.0	21.67	12.96
201	73.0	48.12	12.9
202	34.67	18.9	11.24
203	15.4	1.0	7.1

ribulokinase in crude extracts of casein hydrolysate-grown cells were found to vary depending on the particular mutant examined. However, the ratio of the enzyme levels was relatively constant, reflecting their coordinate behavior. Such coordination has been noted in the case of enzymes whose structural genes are closely linked (1, 11). On the other hand, a study of the control mechanisms involved in the L-arabinose gene complex in *E. coli* revealed that coordination of enzyme activities alone is insufficient evidence for linkage of the genes involved (6); in this system, a definite coordination was found between the L-arabinose isomerase and the L-arabinose permease, although the structural genes corresponding to these enzymes are unlinked. Thus, in the absence of a genetic mapping system in *A. aerogenes*, little can be inferred from the coordinate behavior of ribitol dehydrogenase and D-ribulokinase with respect to the relative position of their structural genes on the bacterial genome. One can only state that both structural genes seem to share common regulation.

Coordinate control is not apparent in the case of D-arabitol dehydrogenase and D-xylulokinase (Table 2). The D-arabitol dehydrogenase constitutive mutants possess the genetic capacity to synthesize D-xylulokinase in response to D-arabitol as the inducer-substrate, but this kinase activity is not constitutive. Another mutant had no detectable kinase activity when grown on D-arabitol and was unable to utilize D-xylose as the sole carbon and energy source (D-xylose-negative). However, the growth of this mutant in response to D-arabitol paralleled the growth of the wild-type strain and the other mutant strains on the same substrate.

The results of the induction experiment using strain 203 demonstrated that this constitutive mutant maintained a high D-arabitol dehydrogenase level regardless of whether it was grown on casein hydrolysate, D-arabitol, or D-xylose. There was a slight decrease in dehydrogenase activity in cells grown on the sugar substrates as compared with cells grown on casein hydrolysate. This decrease can be reproduced and may reflect the repression of enzyme synthesis by products of D-arabitol catabolism.

Although the D-xylulokinase from D-xylose-grown cells was partially purified and characterized (13), the enzyme from D-arabitol-grown cells was not carefully studied. It is possible that two species of D-xylulokinase (isozymes) exist, one specifically induced by D-arabitol and the other specifically induced by D-xylose. The alternative explanation of these data would require different levels of induction of a single species of kinase, with higher activity induced by D-xylose and a lower level of activity induced by D-arabitol. Resolution of this problem is dependent upon the purification of D-xylulokinase from cells grown on D-arabitol and a comparison of its properties with the enzyme isolated from D-xylose-grown cells.

The biological advantages of sequential induction can be seen in a situation where two or more catabolic pathways converge. If isozymes are not used to control the common metabolic reactions, then selective pressures may favor segmented control at the branchpoint as the most efficient means of regulating carbohydrate catabolism. If coordinate control were to span the convergence in a metabolic pathway, the result would be the synthesis of unnecessary enzyme protein.

Examples of such expenditures of protein synthesis are found with the xylitol-utilizing and D-arabinose-utilizing strains of *A. aerogenes*. Growth on xylitol results from the selection of mutants constitutive for ribitol dehydrogenase and from the utilization of this nonspecific dehydrogenase to catalyze the oxidation of xylitol to D-xylulose. D-Ribulokinase (coordinately controlled with the dehydrogenase) is also synthesized constitutively by such mutants even though this enzyme has no catalytic function during xylitol catabolism (14). When D-arabinose is used as the substrate, growth is believed to result from the selection of mutants constitutive for L-fucose isomerase and from the utilization of this nonspecific enzyme for the conversion of D-arabinose to D-ribulose. As a consequence of this isomerization, the enzymes of the ribitol pathway are induced. In this case, the induction of ribitol dehydrogenase must be considered superfluous, although D-ribulokinase is essential for the further catabolism of D-arabinose. Presumably, there has been no natural selection for regulatory systems controlling xylitol or D-arabinose degradation.

ACKNOWLEDGMENTS

The authors are indebted to W. A. Wood for his helpful discussions concerning the data presented in

this paper and in whose laboratory this study was initiated. This investigation was supported by Public Health Service research grant AI-06848-02 from the National Institute of Allergy and Infectious Diseases and research grant GB-3864 from the National Science Foundation.

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