vivo action, which is not, however manifest until the tissue is subjected to fractionation procedures. The fact that more breakdown of polyribosomes is observed 11 hours after injection of the drug than at 2½ hours (Table 1) points to a slow and progressive process. This action of actinomycin is reminiscent of the effects of certain hepatotoxins, including carbon tetrachloride (*9, 10*) and ethionine (*11*). It is of interest that carbon tetrachloride leads to breakdown of polyribosomes at dose levels which have no detectable effect on liver RNA synthesis (*10*).

It is clear that the action of high doses of actinomycin D (5 mg/kg) on the protein-synthesizing apparatus is detectable only in liver subjected to fractionation. The effects observed cannot be attributed to the inhibition of RNA synthesis produced by the antibiotic. Thus, inhibition of protein synthesis and a decrease in polyribosomes in liver homogenates of rats given actinomycin in these doses cannot be used as indexes of turnover of messenger RNA.

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Evolution of a Catabolic Pathway in Bacteria

Abstract. *Genetic derepression of ribitol dehydrogenase in a mutant of* Aerobacter aerogenes, *strain 1033, enabled it to grow on xylitol, a substrate of the enzyme, but not its inducer. A derivative strain with an improved rate of growth on xylitol was obtained from the first mutant. The faster growth rate was made possible by the production of an altered ribitol dehydrogenase, as demonstrated by an increase in its activity on xylitol relative to ribitol and by its decreased heat stability.*

Since most compounds in the natural environments of microorganisms can be expected to undergo both acute fluctuations and gradual but profound shifts, it would seem that successful competition by a species should depend not only on its range of phenotypic adaptability but also on the flexibility of its genetic apparatus for the acquisition or deletion of metabolic functions. Such genetic alterations might arise either from mutational events or from recombination with foreign genomes. Although genetic transfers surely must play essential roles in biochemical evolution, this type of process serves mainly to facilitate the eclectic convergence of various genetic traits into more viable systems. Answers to how new functions arise, therefore, should in general be sought in terms of primary mutational events.

The aim of our studies is to discover examples of mutations which would enable an organism to utilize new resources. Assuming that new pathways must be built stepwise and grafted onto the preexisting metabolic network in the manner suggested by Horowitz (*1*), we can then study the problem of developing a new physiologic function for an enzyme (*2*).

Such an innovation might arise as follows: (i) If the structural gene of an inducible enzyme codes a protein which can also act on noninducing substrates, a mutation leading to the constitutive expression of this gene might allow the organism to utilize an additional substrate if its product happens to be an intermediate in the cell's metabolic network. (ii) The same purpose can be achieved if a mutation in a control gene of the above system allows the previously noninducing substrate to induce formation of the enzyme. (iii) If an enzyme is present at significant concentrations most of the time, mutations which alter its specificity, such that a new compound can be attacked, may likewise open up a new pathway.

Several examples are now available in which cells have been enabled to utilize a new substrate by genetic derepression of an enzyme: altrose-galactoside via β-galactosidase (*3*); β-glycerophosphate via alkaline phosphatase (*4*); putrescine via diamine-α-ketoglutarate transaminase (*5*); and D-mannitol via D-arabitol dehydrogenase (*6*).

We have chosen to study short-chain polyols as potential carbon sources not only because they provide interesting stereochemical problems but also because they probably enter bacterial cells freely by diffusion (*7*), thus obviating the necessity for the organism to develop new permeation systems. *Aerobacter aerogenes*, strain 1033, has been used for this study because this organism already has enzyme systems which enable it to grow on a number of polyols. In a preliminary investigation to see if mutations could endow this organism with the ability to use additional polyols, substrains were readily obtained which grew slowly on xylitol. This new growth property was conferred by the genetic derepression of ribitol dehydrogenase, for which xylitol is a substrate but not an inducer

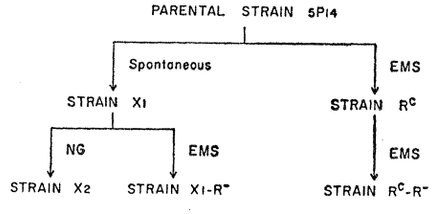

Fig. 1. Genealogy of mutants descended from parental strain 5P14. Strain X1, able to grow on xylitol, was selected by plating about 10^7 cells of strain 5P14 on xylitol agar, from which about one cell per 4×10^5 grew out. To obtain other mutants, ethyl methanesulfonate (EMS) (*19*) or 1-methyl-3-nitro-1-nitrosoguanidine (NG) (*20*) was used as mutagen. The treated cells were allowed a 100-fold growth in simple medium supplemented with arginine and guanine to complete genetic segregation and to eliminate unwanted auxotrophs. The cells were then plated on appropriate media for screening for the desired type of mutant. Strain R^c, selected for its constitutive utilization of ribitol, and strains X1-R^- and R^c-R^-, selected for their inability to ferment ribitol, were isolated from EMS-treated cells (*19, 21*) with appropriate modifications. Strain X2, which grows faster on xylitol, was isolated by recycling NG-treated cells of strain X1 on xylitol as the carbon source.

(*8*, *9*). Similar findings have been reported for *A. aerogenes*, strain PRL-R3 (*10*). We now present data concerning our xylitol-growing mutant and some properties of a new mutant with an improved rate of growth on xylitol.

All mutants in our study were descended from *A. aerogenes*, strain 5P14, an arginine and guanine double auxotroph derived from strain 1033 (*11*). This double nutritional requirement was exploited as a means of distinguishing possible contaminants from the desired mutants, whose genealogy is traced in Fig. 1.

We examined the property of strain X1, the initial mutant able to utilize xylitol, first by determining its growth rate on the new carbon source in liquid medium. The doubling time was 270 minutes. In contrast, no growth of the parental strain could be detected under the same conditions.

Since, in the wild-type organism, several polyols are metabolized by an initial DPN-linked (diphosphopyridine nucleotide) dehydrogenation [D-arabitol (*12*), glycerol (*13*), inositol (*14*), and ribitol (*9*)], a cell-free extract from strain X1 grown on xylitol was examined for a DPN-dependent xylitol dehydrogenating activity. Such an activity was indeed found, though very low (0.066 μmole/min per milligram of protein, measured with 0.05*M* xylitol), so it was suspected that this activity was attributable to an enzyme which acts physiologically on another substrate. In order to see if any of the previously described dehydrogenases might be contributing to this activity on xylitol, the reduction of DPN in the presence of various other polyols was tested. Very high activity was observed with ribitol, suggesting that ribitol dehydrogenase (*9*) was responsible for the in vivo activity on xylitol. This was supported by the observation that a constant ratio of activities on ribitol and xylitol was maintained during heat inactivation. It is most likely then that the mutant, unlike the wild type, is able to produce ribitol dehydrogenase in the absence of its inducer, ribitol, and that this enzyme converts xylitol to a utilizable product.

The high activity of ribitol dehydrogenase in extracts of strain X1 could be the result of either derepression of the enzyme or alteration of its inducer specificity to include xylitol. To distinguish between these possibilities, the enzyme was assayed in extracts of strain X1 grown on casein hydrolyzate alone or with xylitol added to the medium. The results, summarized in Table 1, show that ribitol dehydrogenase is produced constitutively in strain X1, whereas the parental strain produces this enzyme only in the presence of ribitol.

Table 1. Ribitol dehydrogenase activities of strains 5P14 and X1 under different growth conditions. Conditions for growth and the preparation of cell-free extracts have been described (*12*). Arginine and guanine were supplemented at $3 \times 10^{-4}M$ and $5 \times 10^{-4}M$, respectively. Single carbon sources were included at a concentration of 0.2 percent; casein hydrolyzate, at 1 percent. Assays were carried out at *p*H 10.9 by following the formation of DPNH at 340 mμ (*9*). A unit of activity represents 1 μmole per minute. Protein concentrations were measured with the biuret reagent (*18*).

Strain	Polyol added to casein medium	Specific activity: (units/mg of protein)	
		Ribitol	Xylitol
X1	None	5.5	0.051
X1	Xylitol	5.1	0.051
X1	Ribitol	6.1	0.078
5P14	None	0.036	Neg*
5P14	Xylitol	0.024	Neg*
5P14	Ribitol	3.0	0.032

* Negligible.

To confirm the requirement of ribitol dehydrogenase for growth on xylitol,

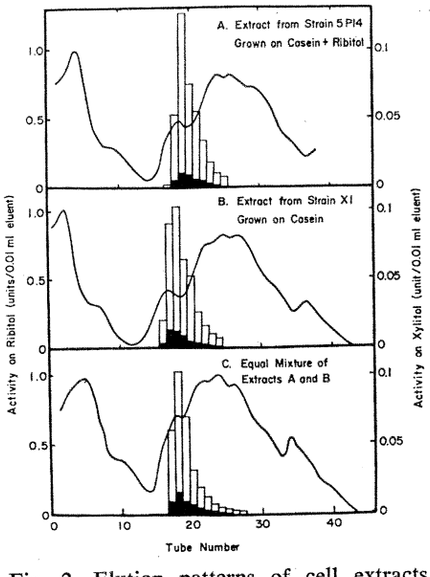

Fig. 2. Elution patterns of cell extracts from DEAE columns. Cell extracts containing about 150 mg protein were placed on a DEAE column (1 × 20-cm). A linear gradient of NaCl (from 0 to 1.0*M* in 500 ml 0.01*M* sodium phosphate, *p*H 7.0) was used. Each tube contained 6 ml of eluent. Fractionation was carried out at 5°C. Curved line, absorbancy at 280 mμ expressed on arbitrary scale; open block, activity on ribitol; solid block, activity on xylitol.

mutants lacking the ability to ferment ribitol were selected from strain X1. One such mutant, strain X1-R$^-$, lacked the dehydrogenase even when grown in the presence of ribitol. When xylitol was tested as a sole source of carbon and energy for this mutant, no growth could be detected. Thus, loss of this enzyme was correlated with failure to grow not only on ribitol but also on xylitol.

To learn whether constitutive production of ribitol dehydrogenase was sufficient to enable the cells to grow on xylitol, a mutant isolated from parental strain 5P14 because of its ability to metabolize ribitol constitutively was studied. This mutant, R^c, not only grew on xylitol but had the same doubling time, 270 minutes, as strain X1. As in the case of strain X1, ribitol dehydrogenase was essential for growth of strain R^c on xylitol, since strain R^c-R$^-$, a ribitol dehydrogenase-negative derivative, failed to grow on both ribitol and xylitol. It therefore seemed that a simple derepression of ribitol dehydrogenase was sufficient to account for the new growth properties of both strains.

If it is true that the acquisition of the ability to grow on xylitol entailed alteration only in the control of enzyme synthesis, then the ribitol dehydrogenase of strain 5P14 and strain X1 should be the same protein. This proposition was supported by the facts that the enzymes from both strains exhibited the same substrate specificity and the same rate of heat inactivation (half-life of 6 minutes at *p*H 7.0 and 60°C), and showed identical patterns of elution from diethylaminoethyl cellulose (DEAE) columns (Fig. 2).

We next investigated the mode of dissimilation of xylitol by identifying the product of its dehydrogenation. A mixture of ribitol dehydrogenase, xylitol, and DPN was incubated until equilibrium was approached. The mixture was then deproteinized, and upon treatment with the resorcinol–ferric chloride reagent, a colored complex was formed with absorption maxima indistinguishable from those of D-xylulose (*12*, *15*). That ribitol dehydrogenase indeed catalyzed the interconversion of xylitol and D-xylulose was shown by the reoxidation of DPNH (reduced DPN) with D-xylulose in the presence of the enzyme.

The conversion of xylitol to D-xylulose explains why a simple derepression

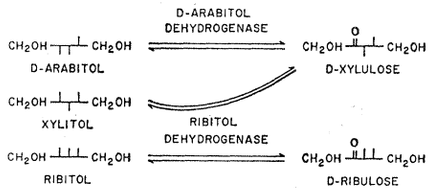

Fig. 3. Steps in metabolism of D-arabitol, xylitol, and ribitol.

of ribitol dehydrogenase is sufficient to allow growth on xylitol, since this ketose is the immediate product of D-arabitol dissimilation by this organism (*12*). In vivo, the presence of D-xylulose during xylitol metabolism was confirmed by the induction of D-arabitol dehydrogenase in cells growing on xylitol. This gratuitous induction of D-arabitol dehydrogenase by xylitol can occur only in cells which possess ribitol dehydrogenase. The D-arabitol dehydrogenase itself, of course, has no activity on xylitol (*12*). The initial steps of D-arabitol, xylitol, and ribitol metabolism are summarized in Fig. 3.

Since D-xylulose is the immediate product of both D-arabitol and xylitol dissimilation, one might expect the growth rates on these two substrates to be similar. In fact this is not so. Cells of strain X1 were able to grow with a doubling time of 40 minutes on D-arabitol, in contrast to a doubling time of 270 minutes on xylitol. In view of the likelihood of free permeation of short-chain polyols, this slow growth on xylitol most likely reflected the slow rate of its dehydrogenation.

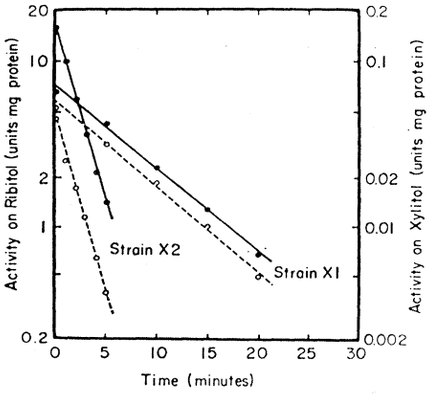

Fig. 4. Heat inactivation of ribitol dehydrogenases from strains X1 and X2 grown on xylitol. Crude extracts containing about 4 mg of protein per milliliter were maintained at 60°C in 0.01*M* sodium phosphate buffer at *p*H 7.0. Solid circles, activity on xylitol; open circles, activity on ribitol.

This postulate was verified by the properties of strain X2, which doubled on xylitol every 110 minutes.

Cell-free extracts from strain X2 showed an increased specific activity of xylitol dehydrogenation—0.164 units per milligram of protein as compared with 0.066 units per milligram of protein in the case of strain X1—while the specific activity on ribitol remained unchanged. This therefore suggested that the protein in strain X2 had been altered, which was also indicated by the fact that the enzyme was more heat labile than that from strain X1 (Fig. 4). However, no change in the Michaelis constant for either pentitol (around $5 \times 10^{-3}M$ for ribitol and $1M$ for xylitol) was noticed, nor was there any change in the fractionation patterns from the DEAE column except that the ratio of activity on xylitol to that on ribitol was increased as expected. Finally, the increase in the specific activity on xylitol by a factor of 2.5 in strain X2 can be correlated, within experimental error, with the improvement of its growth rate on the substrate, thus confirming the belief that ribitol dehydrogenase is the rate-limiting factor in growth on xylitol.

In each case of gain in function so far discovered the initial mutation has involved derepression of an enzyme which already possessed the requisite catalytic property, rather than alteration of its inducer specificity or modification of the structure of an enzyme present in the cells most of the time. Many random mutations can prevent the formation of a functional aporepressor (*16*), but few would be expected to lead to the formation of macromolecules with altered function. However, should the activity of the derepressed enzyme still limit the rate of growth, then a remaining possibility for the improvement of such a pathway would be the production of an altered enzyme with more efficient catalytic function, as illustrated by the present example of "xylitol dehydrogenase." The loss of stability associated with such change of catalytic property is not surprising, since the stability of the wild-type enzyme is most likely the result of numerous cumulative changes in the course of evolution (*17*).

If henceforth xylitol becomes more abundant than ribitol in the habitat of this organism, we can then imagine that the enzyme would become increasingly efficient as a xylitol dehydrogenase at the expense of its activity on ribitol. With time, further amino acid substitutions may occur to restabilize it. In addition, if xylitol is only periodically available as a major source of carbon, the establishment of a repressor system with xylitol as the inducer would confer selective advantage, since it would prevent the wasteful synthesis of the enzyme in the absence of its new physiological substrate.

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