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Sequential Utilization of Mixed Monosaccharides by Yeasts

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Four yeasts (*Saccharomyces cerevisiae, Schizosaccharomyces pombe, Candida utilus*, and *Rhodotorula toruloides*) were tested for their ability to grow and consume D-glucose, D-xylose, D-xylulose, and D-xylitol. Sequential utilization of substrates was observed when D-glucose was mixed with D-xylulose as the carbon source. Catabolite inhibition was tentatively concluded to be responsible for this regulatory mechanism. D-Glucose was also found to inhibit the utilization of D-xylose and D-xylitol in *C. utilus* and *R. toruloides*. D-Xylose, D-xylitol, and D-xylulose were consumed simultaneously by *R. toruloides* and *C. utilus*.

With diminishing energy supply, biomass consisting of cellulose and hemicellulose increases in attraction as an alternative energy source in the form of ethanol. In our laboratory, hemicellulose and cellulose are separately extracted and hydrolyzed into monosaccharides (R. L. Mehlberg, G. T. Tsao, M. R. Ladisch, and K. K. Dyck, 180th Am. Chem. Soc. Meet., Las Vegas, Nev., 1980). Ethanol can be easily produced by yeasts during fermentation of cellulose hydrolysate, which is primarily glucose. Xylose, a major component of hemicellulose hydrolysate, can be fermented into ethanol by bacteria and molds, although by-product formation or slow reaction rate limits their application to ethanol production.

Recently, yeast has been demonstrated to ferment D-xylulose to ethanol (5, 24). Glucose isomerase is known to isomerize D-xylose to D-xylulose (7). This knowledge makes possible the development of several processes which convert D-xylose to ethanol in high yield by coupling enzymatic isomerization and yeast fermentation. *Pachysolen tannophilus, Candida tropicalis*, and a mutant of *Candida* sp. (6, 10, 18) have been reported to convert D-xylose to ethanol, with the formation of xylitol as a by-product. D-Xylulose is better than D-xylose as a fermentation substrate for these three yeasts. D-Xylose, however, is preferentially used by *Mucor* and *Fusarium* spp. when a mixture of D-xylose and D-xylulose is used. We have taken advantage of this property to prepare D-xylulose in large quantity from D-xylose (2).

The presence of glucose in the acid hydrolysate of hemicellulose complicates the pattern of sugar utilization, as indicated by the fact that the rate of ethanol production from hydrolysate by yeast exhibits biphase kinetics (1a). We believe that studies of utilization of mixed sugars in yeasts not only should be the first step in understanding D-xylose metabolism in yeast, but also should help in designing a better process for hemicellulose hydrolysate fermentation.

MATERIALS AND METHODS

Chemicals. D-Xylose (grade II) was provided by Sigma Chemical Co. Immobilized glucose isomerase was purchased from Novo Biochemical Industries Inc. as Sweetzyme type Q. Yeast nitrogen base was supplied by Difco Laboratories. D-Xvlulose was prepared according to Chiang et al. (2). D-Xylose (700 g/liter; 4 liters) was isomerized by immobilized glucose isomerase at 68°C and pH 7.0. The isomerized mixture contained 77% D-xylose and 23% D-xylulose. The resulting D-xylose–D-xylulose mixture was concentrated with a rotary evaporator at 45°C to one-fourth the original volume. Absolute alcohol (3 volumes of the concentrated sugar mixture) was then added to extract D-xylulose at 4°C. The high D-xylulose mixture contained 25% D-xylose and 75% D-xylulose. It was diluted to a 60-g/liter concentration and mixed with proper nutrients. *Fusarium oxysporum* f. sp. *lini* was inoculated, and the sugar composition was constantly checked by low-pressure liquid chromatography. When D-xylose was completely consumed, the remaining D-xylulose solution was concentrated by rotary evaporation at 45°C. The concentrated D-xylulose was extracted into ethanol. After ethanol-insoluble materials were removed, ethanol was evaporated out of the xylulose solution by a rotary evaporator. The purification process was repeated several times.

Microorganisms. *Saccharomyces cerevisiae* ATCC 23860, *Schizosaccharomyces pombe* ATCC 24751, *Candida utilus* ATCC 9256, and *Rhodotorula toruloides* ATCC 24196 were used in our study.

Media and cultures. The media used contained only yeast nitrogen base and various concentrations of a monosaccharide(s) (glucose, xylose, xylitol, xylulose). To prevent heat degradation, especially with D-xylulose, all sugar solutions were sterilized by membrane filtration.

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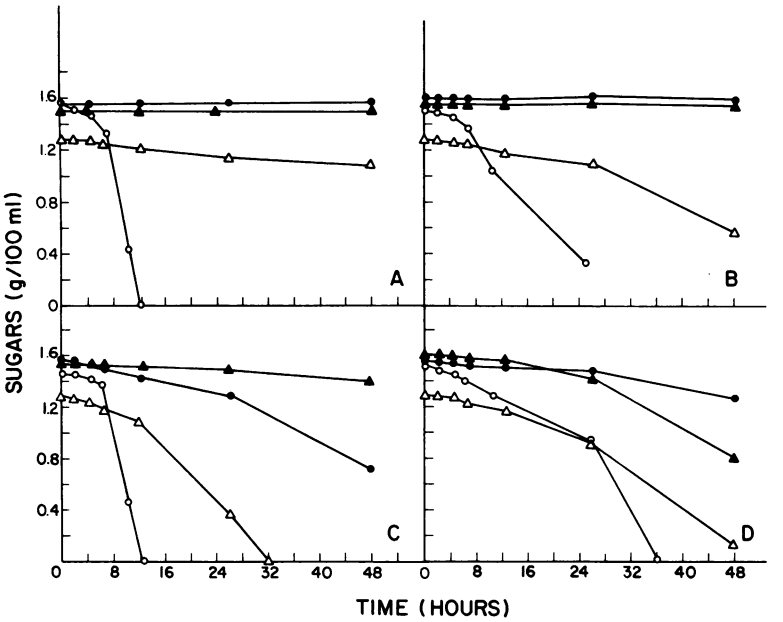

FIG. 1. Utilization of sugar by (A) *S. cerevisiae*, (B) *Schizosaccharomyces pombe*, (C) *C. utilus*, and (D) *R. toruloides*. Experimental details can be found in the text. Symbols: (○) D-glucose; (△), D-xylulose; (●), D-xylose; (▲), D-xylitol.

All slant cultures were grown on potato dextrose agar for 2 days. Inoculation was done by directly transferring a slant culture into a 250-ml shake flask containing 100 ml of sterile medium. Incubation was at 30°C at a shake speed of 250 rpm for 18 h.

To begin the study of sugar utilization by yeasts, 0.5 ml of precultured yeast cells was placed in a 250-ml shake flask containing 125 ml of sterile medium. The incubation temperature was 30°C and the shake speed was 250 rpm. At certain times, portions (2 ml) of yeast solution were centrifuged to remove yeast cells. The sugar concentration in the supernatant was analyzed by low-pressure liquid chromatography, and volatile end products were determined by gas chromatography. Absorbance at 660 nm was used to determine cell concentration.

Analytical methods. Monosaccharides were identified by a low-pressure liquid chromatography technique developed by our laboratory, using a Bio-Rad AG-50 column (20- to 30-μm) (11). The separation of glucose, xylose, xylulose, xylitol, glycerol, arabitol, and ethanol was well defined, and sugar concentrations as low as 0.1 mg/ml could be accurately determined.

RESULTS

Utilization of a single sugar by yeasts. Four yeasts (*S. cerevisiae, Schizosaccharomyces pombe, C. utilus*, and *R. toruloides*) were separately grown on media containing different carbon sources (D-glucose, D-xylose, D-xylitol, or D-xylulose). Consumption of substrate from the media was monitored for the various cultures (Fig. 1). As expected, D-glucose was the best carbon source for growth in all four yeasts. *S. cerevisiae* and *C. utilus* utilized D-glucose faster than did *R. toruloides* and *Schizosaccharomyces pombe*. Among pentoses and pentitol, D-xylulose was consumed faster than D-xylose and D-xylitol in all four yeasts. *S. cerevisiae* utilized D-xylulose poorly, as reported by Wang and Schneider (23). *S. cerevisiae* and *Schizosaccharomyces pombe* were unable to use D-xylose and D-xylitol for growth. D-Xylose was apparently a better carbon source for *C. utilus* than was D-xylitol. *R. toruloides* consumed D-xylitol faster than D-xylose. Optical density at 660 nm (not shown), used to indicate cell concentration, also agreed with the sugar utilization pattern described above. Therefore, results from substrate disappearance studies not only reflected sugar uptake, but also indicated its incorporation into cell mass.

Uptake of mixed sugars. When D-glucose and D-xylulose were both present in the growth medium, an interesting pattern of sugar consumption was observed (Fig. 2). Depletion of D-glucose from the medium was not affected by the presence of D-xylulose. A lag period in the utilization of D-xylulose was observed. The duration of this lag period varied among the four yeasts, with *C. utilus* having the shortest delay and *R. toruloides* having the longest delay in D-xylulose utilization. In *R. toruloides* and *S. cerevisiae*, D-xylulose began to disappear from the media only when D-glucose was totally de-

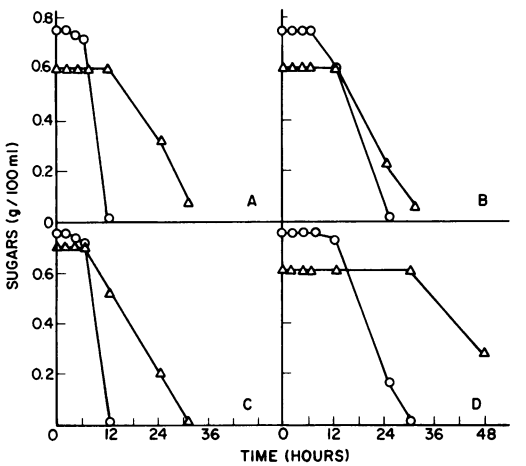

FIG. 2. Utilization of sugars by (A) *S. cerevisiae*, (B) *Schizosaccharomyces pombe*, (C) *C. utilus*, and (D) *R. toruloides*. Symbols: (○), D-glucose; (△) D-xylulose.

pleted. In *Schizosaccharomyces pombe* and *C. utilus*, consumption of D-xylulose was seen before total depletion of D-glucose. The variation described above could be because each yeast strain has a different rate of D-glucose and D-xylulose utilization (Fig. 1). Since the inhibition of utilization of D-xylulose by D-glucose occurred at the early exponential stage, we decided to design more experiments to ensure that the inhibition was not due to the slow rate of D-xylulose uptake when the cell population was low. *C. utilus* was cultured in a medium of 1% glucose for 24 h. Cells were harvested by centrifugation of 4°C and washed with cold water. Half of the cells were mixed with 1% D-xylulose–1% D-glucose. The other half was fed 1% D-xylulose initially and 1% D-glucose 3 h later (Fig. 3 and 4). In Fig. 3, a pattern of sugar utilization similar to that in Fig. 2 was observed. There was an initial inhibition of D-xylulose utilization by D-glucose. As D-glucose was consumed, the inhibitory effect was reversed. The rate of D-xylulose disappearance was unchanged throughout the remainder of the experiment. Induction of the D-xylulose utilization system was another possible explanation for the lag shown in Fig. 3, since *C. utilus* was grown solely on D-glucose. After we examined results from Fig. 4, this possibility was ruled out. In the first 3 h without D-glucose, D-xylulose disappeared from the medium fairly rapidly.

Introduction of D-glucose quickly lessened the rate of D-xylulose utilization. When D-glucose was totally consumed, a new rate of D-xylulose utilization was established. The cell population changed insignificantly throughout the experiment, indicated by the fact that little change in

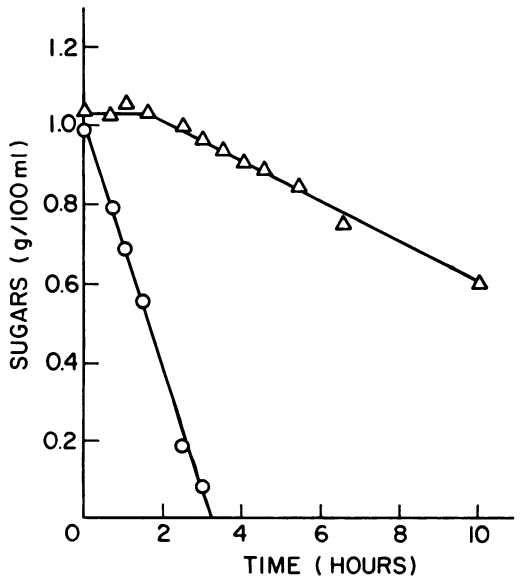

FIG. 3. Utilization of D-glucose and D-xylulose by *C. utilus*. *C. utilus* was grown in 1% D-glucose medium overnight. Cells were harvested, concentrated to half the original volume by centrifugation, and washed with water. The rate of sugar utilization was followed after introduction of a mixture of D-glucose (○) and D-xylulose (△).

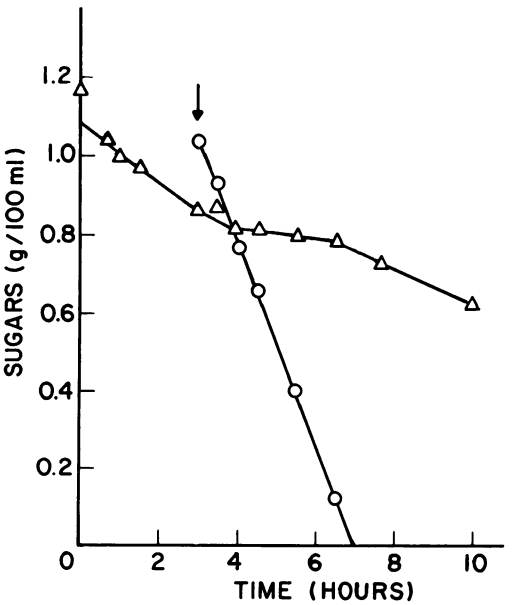

FIG. 4. Utilization of D-glucose and D-xylulose by *C. utilus*. Experimental details were the same as in the legend to Fig. 3, with the exception that D-glucose (●) was introduced into the reaction mixture 3 h later than D-xylulose (△).

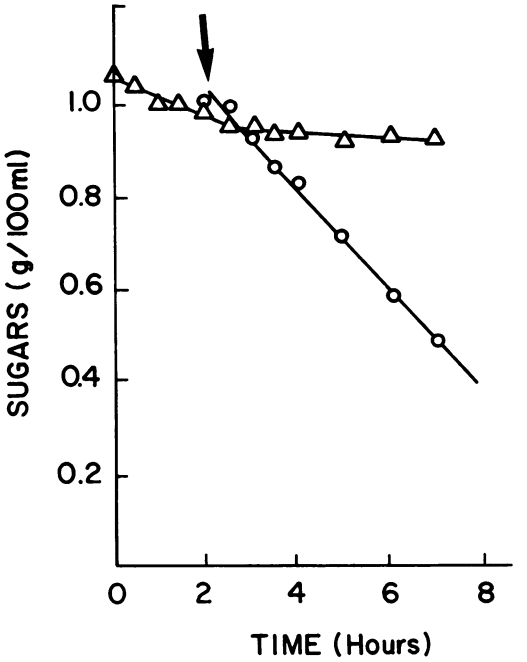

FIG. 5. Utilization of D-glucose and D-xylulose by *C. utilus*. Experimental details and symbols are the same as in the legend to Fig. 3, with the exception that *C. utilus* was precultured in 1% D-xylulose medium.

absorbance at 660 nm was observed. Figure 5 shows the sugar depletion pattern when *C. utilus*, precultured in a medium containing 1% D-xylulose for 24 h, was used to study D-xylulose utilization under the influence of D-glucose. This pattern is very similar to that in Fig. 4. The addition of D-glucose to the D-xylulose solution significantly reduced the rate of D-xylulose utilization within a very short time. The results of the above experiments indicate that D-glucose indeed inhibits the utilization of D-xylulose whether or not the yeast is precultured in D-glucose or D-xylulose medium. Similar results were observed when *Schizosaccharomyces pombe* was used (data not shown). The specific rate of D-xylulose utilization (Fig. 4) by glucose-cultured cells was around 0.25 g of D-xylulose absorbed per g of cell mass per h. That for D-xylulose-cultured cells was around 0.28 g of D-xylulose absorbed per g of cell mass per h. In short-term adaptation, the D-xylulose catabolism system of *C. utilus* was not influenced by the carbon source(s). This may not be true for long-term adaptation, since a significant increase in growth rate of *Schizosaccharomyces pombe* in D-xylulose medium was observed (unpublished data).

D-Glucose also strongly inhibited utilization of D-xylose and D-xylitol in *C. utilus* and *R. toruloides* (data not shown). It appears that D-glucose exercises its inhibitory effect on all four yeasts, reducing the utilization rate for D-xylose, D-xylitol, and D-xylulose.

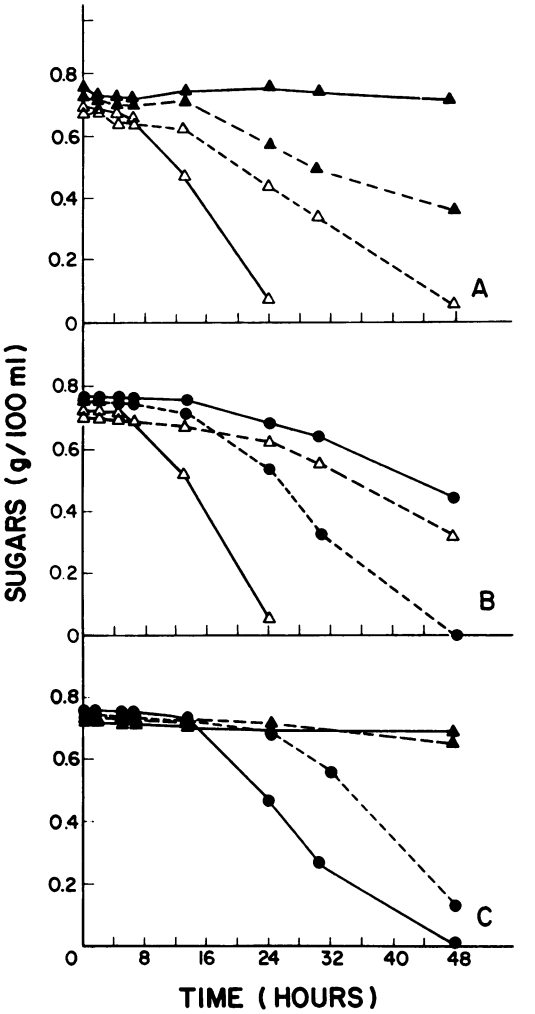

FIG. 6. Utilization of sugars from a mixture of D-xylose and D-xylulose (A), D-xylulose and D-xylitol (B), and D-xylose and D-xylitol (C) by *C. utilus* and *R. toluloides*. Symbols: (△) D-xylulose; (●) D-xylose; (▲) D-xylitol; (——) *C. utilus*; (----) *R. toruloides*.

When a mixture of D-xylose and D-xylulose was used to support the growth of *C. utilus* and *R. toruloides*, the pattern of sugar depletion was quite different from those systems in which D-glucose was also present. D-Xylose and D-xylulose were depleted simultaneously by these two yeasts (Fig. 6B). As expected, the rate of disappearance of D-xylulose from the medium was faster than that of D-xylose in *C. utilus*. This observation is in agreement with the conclusion drawn from Fig. 1C, in which a single type of pentose was used as the carbon source. In *R. toruloides*, D-xylose was consumed faster than

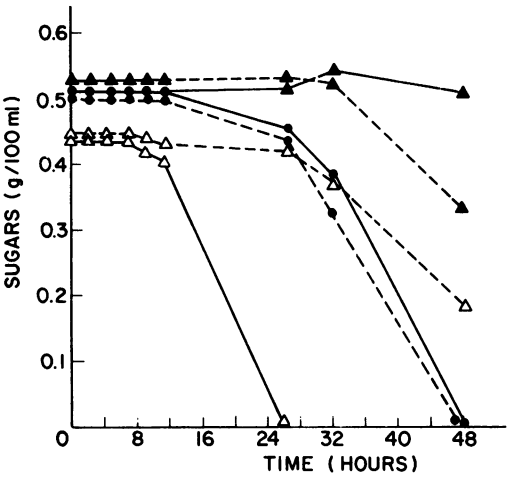

FIG. 7. Utilization of D-xylose, D-xylitol, and D-xylulose by *C. utilus* and *R. toruloides*. Symbols: (○) D-xylose; (▲) D-xylitol; (△) D-xylulose; (——) *C. utilus*; (----) *R. toruloides*.

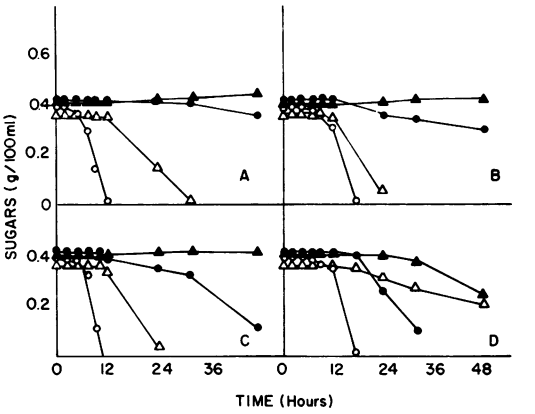

FIG. 8. Utilization of sugars by (A) *S. cerevisiae*, (B) *Schizosaccharomyces pombe*, (C) *C. utilus*, and (D) *R. toruloides*. Symbols: (○) D-glucose; (△) D-xylulose; (●) D-xylose; (▲) D-xylitol.

D-xylulose. This is in contrast to the result shown in Fig. 1D. Figure 6A shows the results when *C. utilus* and *R. toruloides* were cultured in medium containing a mixture of D-xylulose and D-xylitol. D-Xylulose and D-xylitol were consumed simultaneously by *R. toruloides*, although D-xylulose disappeared from the medium faster than did D-xylitol. With *C. utilus*, D-xylulose was depleted rapidly, whereas the D-xylitol concentration slightly increased. This slight increase in D-xylitol concentration perhaps can be explained by the fact that *C. utilus* reduced D-xylulose to D-xylitol and excreted it. In a mixture of D-xylose and D-xylitol, both *C. utilus* and *R. toruloides* utilized D-xylose much faster than D-xylitol (Fig. 6C). A mixture of D-xylose, D-xylitol, and D-xylulose was also used to study sugar utilization (Fig. 7). *C. utilus* utilized sugar in the following order: D-xylulose, D-xylose, and D-xylitol. This is in agreement with the results from earlier studies of single-sugar utilization (Fig. 1C) and two-sugar utilization (Fig. 6). *R. toruloides* consumed sugar in another sequence: D-xylose, D-xylulose, and D-xylitol. Again, this observation agrees with our findings with two-sugar utilization (Fig. 6) and contrasts with the results of the study of single-sugar utilization (Fig. 1D).

A medium containing D-glucose, D-xylose, D-xylulose, and D-xylitol was used to grow the four yeasts (Fig. 8). D-Glucose clearly inhibited utilization of the other three substrates until D-glucose was consumed to a low level. Once D-glucose had been depleted from the medium, D-xylose and D-xylulose were simultaneously consumed by yeasts at various rates. For *R. toruloides*, D-xylitol was also consumed along with D-xylose and D-xylulose.

D-Xylitol was found to be among the end products when D-xylose or D-xylulose or both were the substrates. Arabitol was the end product when D-xylose, D-xylitol, and D-xylulose were the carbon sources for growth of *R. toruloides*. Although D-xylose was unable to support the growth of *S. cerevisiae*, a small portion of D-xylose was consumed, and D-xylitol was detected at the end of the experiment in which D-glucose and D-xylose coexisted in the medium. D-Xylitol was also detected when *S. cerevisiae* was grown on D-xylulose.

DISCUSSION

When microorganisms are exposed to multisubstrates, the phenomena of diauxic growth and sequential substrate utilization are often observed. Epps and Gale (4) first noticed this in bacteria. The glucose effect was named by Monod (15) after he had studied these phenomenona further. Spiegelman and co-workers (19, 20) also reported the existence of the glucose effect in yeasts. Catabolite repression was proposed by Magasanik (12) to be the mechanism specifically regulating the enzymatic response of microorganisms to a multisubstrate environment. Holzer (8) also suggested that catabolite inactivation triggered by D-glucose was a regulatory mechanism in yeast. Sequential substrate utilization was also controlled by catabolite inhibition, a commonly seen regulatory mechanism in bacteria (13, 14, 16, 17, 21, 22).

Several conclusions drawn from our studies can help to determine which control mechanism is responsible for the inhibitory effect of D-xylulose utilization imposed by glucose. Induction of the D-xylulose utilization system was not necessary, since no delay was seen in the disap-

pearance of D-xylulose from the medium in either D-glucose-grown or D-xylulose-grown yeast. More importantly, only a short period of time was needed for the maximal inhibitory effect by glucose on D-xylulose utilization to be reached. The existence of this transition probably excludes the possibility that D-glucose in the medium directly competes with D-xylulose for the same utilization system. As indicated in our experiments, the ability to consume D-xylulose was quickly restored once D-glucose reached a low concentration. This suggests that catabolite inactivation is not responsible for control of sequential substrate utilization. The short transition period and fast recovery of the ability to absorb D-xylulose indicate that catabolite repression is not the regulatory mechanism we are seeking. All of the evidence we gathered supports the idea that inhibition rather than inactivation and repression is the main control. A short transition period also suggests that inhibition is imposed by intracellular glucose or its catabolite. The true identity of the inhibitor, the inhibition site, and the effective exocellular D-glucose concentration for inhibition are currently under study in our laboratory.

The interconversion among D-xylose, D-xylitol, and D-xylulose was well established in *C. utilus* (2, 9). Cirillo (1) reported a high K_m for the binding of D-xylose with a carrier in *S. cerevisiae*, and D-xylose was considered to be a nonmetabolized substrate in this yeast. In our studies, as well as in others (23, 34), D-xylulose could not only support growth but also could be fermented. This suggests that the utilization of D-xylulose and the pentose phosphate shunt works quite well in *S. cerevisiae*. D-Xylitol could be detected whether D-xylose or D-xylulose was the substrate for *S. cerevisiae*, indicating that the pathway used by *C. utilus* to convert D-xylose to D-xylulose probably also exists in *S. cerevisiae*. The reason that *S. cerevisiae* fails to use D-xylose for growth probably is the poor rate of D-xylose uptake, as indicated by Cirillo (1), the low level of enzymes, or a poor supply of cofactors (NADPH, NAD^+) necessary for the conversion of D-xylose to D-xylulose.

In *C. utilus* and *R. toruloides*, D-xylose and D-xylulose were consumed simultaneously. D-Xylitol was also depleted from the medium along with D-xylose and D-xylulose in *R. toruloides*. Contrary to *C. utilus*, *R. toruloides* consumed D-xylose faster than D-xylulose. Judging from structural similarity and simultaneous consumption, it is possible that these three sugars compete for the same utilization system. Of course, the data presented in this communication are not sufficient to exclude other possibilities.

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