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Production of Phenotypically Epimeraseless Yeast by L-Arabinose[1]

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The previous report from this laboratory that L-arabinose is a gratuitous inducer of the galactose transport system has been found to be an artefact resulting from the combination of galactose contamination of commercial samples of L-arabinose and inhibition by L-arabinose of galactose metabolism by inactivation of uridine-diphosphate-glucose-4-epimerase. As a result of L-arabinose inhibition of the metabolism of the contaminating galactose, galactose itself serves as a gratuitous inducer, producing phenotypically epimeraseless yeast.

In a previous report from this laboratory, L-arabinose was shown to be a gratuitous inducer of the galactose transport system in *Saccharomyces cerevisiae* (6). However, further study of the nature of the induction of the galactose pathway by L-arabinose revealed two anomalies which suggested that the previously reported gratuitous induction might be an artefact. The first of these was the fact that L-arabinose-induced cells can take up and phosphorylate galactose but cannot ferment it. This is unexpected because the structural genes of the three enzymes of the galactose pathway [galactokinase (E.C. 2.7.1.6), uridine-diphosphate-glucose (UDPG): galactose-1-phosphate uridylyltransferase (E.C. 2.7.7.12), and uridine diphosphate glucose-4-epimerase (E.C. 5.1.3.2)] constitute a single operon (7, 8). The inability of L-arabinose-induced cells to ferment galactose was discovered to be the result of inhibition by L-arabinose of the third enzyme of the galactose pathway, UDPG-4-epimerase (1, 2; Azam, Rosen, and Cirillo, Bacteriol Proc., p. 165, 1969).

The second anomaly was the fact that the capacity to take up and phosphorylate galactose induced by L-arabinose is only transient; that is, the induction reaches a maximum level and is followed by deinduction even though L-arabinose is not metabolized and is still present in the medium at its initial concentration. Deinduction should not occur in the continuous presence of a true gratuitious inducer. The inducing activity of L-arabinose was found to be due to the presence of a small amount (ca. 1%) of contaminating D-galactose in some samples of L-arabinose. It is important to note that galactose-free L-arabinose is not an inducer. Ordinarily, the induction by the small amount of D-galactose present as a contaminant of L-arabinose would have gone unnoticed because it would be consumed before the induction reached a high level. However, because L-arabinose is an inhibitor of galactose metabolism, it served to amplify the inducer activity of the small amount of galactose present. The details of the experiments supporting these conclusions are presented below.

MATERIALS AND METHODS

Yeast strains. The following yeast strains were kindly provided by Howard C. Douglas of the Department of Microbiology, University of Washington School of Medicine, Seattle: (i) galactose-positive, inducible: 346-3B; (ii) galactose-positive, constitutive: (122-2C)-1D; and (iii) galactose-negative, epimeraseless: 100-5A.

Growth conditions. The cells were grown in the Douglas and Hawthorne induction medium (7, 8) containing 0.2% D-glucose, 0.2% D-galactose, 2% peptone (Difco), and 1% yeast extract (Difco); galactose was left out for control, uninduced cultures. The cells were grown in 250 ml of liquid medium in 500-ml flasks in a rotary shaker at 30 C for 24 hr or longer as indicated. Each flask was inoculated with the growth from a 24-hr Sabouraud Dextrose Agar (Difco) slant.

Galactose uptake. Yeast suspensions (20 to 50%) in distilled water (wet weight/volume) were mixed with an equal volume of 1 mM D-galactose-*1*-^{14}C (1 mCi/mmole) and incubated at 30 C. The mixture was stirred magnetically; at intervals 0.1-ml samples were run into 5 ml of ice-cold distilled water standing over

[1] A preliminary report was presented at the 69th meeting of the American Society for Microbiology in Miami Beach, Fla. (Azam, Rosen, and Cirillo, Bacteriol. Proc., p. 165, 145, 1969).

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25-mm membrane filters (0.45 μm porosity; Millipore Corp., Bedford, Mass.) on scintered glass filters. The cells were concentrated by suction and washed with two more 5-ml portions of ice-cold distilled water and transferred to scintillation vials containing 10 ml of Bray's solution (3). The radioactivity was measured in a Packard Tri-Carb liquid scintillation spectrometer.

Galactose fermentation. Galactose fermentation was measured as CO_2 production by standard Warburg manometry by using 10 mg (wet weight) of yeast per flask in 2% D-galactose in a final volume of 1.5 ml at 30 C in a nitrogen atmosphere. Micromoles of galactose fermented was calculated from the micromoles of CO_2 produced, assuming 2 μmoles of CO_2 are produced per μmole of galactose.

Enzyme preparations. Crude homogenates were prepared by alumina grinding: 0.2 g (wet weight) of yeast was chilled with 0.6 g of alumina powder (A 301, bacteriological grade, Alcoa) in a porcelain mortar and ground by hand for 5 min at room temperature. The ground mixture was taken up to 2 ml of ice-cold 0.16 M KH_2PO_4 buffer (*p*H 7.0) and centrifuged in a refrigerated centrifuge at 13,000 × *g* for 10 min at 3 C. The extracts were diluted appropriately before use. Dialyzed, partially purified epimerase was prepared by the method of Maxwell et al. (12): a 25% yeast suspension was ground with two volumes of glass beads at 4 C for 5 min in a Gifford-Wood Mini-Mill (Hudson, N.Y.). The cell-free supernatant fluid was incubated for 2 hr at 37 C in 0.1 M KH_2PO_4 (*p*H 7.0) to remove nucleic acids by autocatalytic digestion. The precipitate formed between 35% and 50% saturation with $(NH_4)_2SO_4$ was dissolved in distilled water and dialyzed against distilled water containing 0.025% mercaptoethanol for 2.5 hr.

Enzyme assays. Enzyme activities are assayed spectrophotometrically by measuring the change in absorption at 340 nm by using nicotinamide adenine dinucleotide (NAD^+) or reduced NAD (NADH)-linked reactions; kinase was assayed by the method of Heinrich (10), transferase by the method of Kalckar et al. (12), and epimerase by the method of Maxwell et al. (12). Galactokinase was also assayed by the anion-exchange method of Horowitz (9).

Chromatographic separation of products of galactose metabolism. Cells were incubated with radioactive D-galactose and washed as described for measurement of galactose uptake, but the washed cells were transfered to 1 ml of ice-cold absolute ethanol. After 1 hr of extraction in absolute ethanol, 1 ml of distilled water was added and the extraction continued for 1 hr more. The cells were removed by filtration and the extract was concentrated under reduced pressure. The concentrated extracts were applied to Whatman No. 1 filter paper sheets and developed by descending chromatography in the Palladini and Leloir solvent system for the detection of galactose, galactose-1-phosphate, and UDP-galactose (14): 7.5 parts 95% ethanol: 3.0 parts 1 M ammonium acetate (adjusted to *p*H 3.8 with glacial acetic acid). The dried chromatograms were cut into 1-cm strips and counted in 10 ml of Bray's solution.

Sources of reagents. D-Galactose-*1*-^{14}C (specific activity 10 Ci/mole) was purchased from Calbiochem, Los Angeles, Calif. The following reagents used in the enzyme assays were purchased from Sigma Chemical Co., St. Louis, Mo.: D-galactose, L-arabinose, adenosine triphosphate (ATP), UDP-glucose, UDP-galactose, galactose-1-phosphate, β-NAD^+ and β-NADH, phosphoenolpyruvate, lactic dehydrogenase (containing pyruvate kinase), and UDPG-dehydrogenase. All other chemicals were of standard reagent grade. D-Galactose was purified by incubation as a 5% solution with noninduced 346-3B yeast cells (10%, v/v) for 2 hr at 30 C followed by concentration to a syrup, clarification by charcoal, and recrystallization from alcohol. The galactose oxidase reagent was purchased as Galactostat from the Worthington Chemical Co., Freehold, N.J.

RESULTS

When cells grown on L-arabinose were tested for their ability to take up and ferment galactose, they were found to take it up but not to ferment it (Table 1). The inability to ferment galactose was found to be due to the absence of epimerase activity (Table 1). When the time-course of the induction of the ability to take up galactose and to phosphorylate it was studied, it was surprising that the induction was only transient. Deinduction in the presence of a supposed gratuitous inducer suggested that L-arabinose was contaminated with a metabolizable inducer, possibly D-galactose itself. This was confirmed by galactose oxidase assay of the L-arabinose (Sigma Chemical Co.) used in these experiments. L-Arabinose contains ca. 1% by weight of D-galactose. Direct demonstration that the previously reported induction by L-arabinose was due to the contaminating D-galactose was provided by the following experiments. L-Arabinose incubated with galactose-induced cells before addition to the induction medium to ferment the contaminating galactose shows no inducer activity. Treatment with uninduced cells has no effect. Finally, the addition of D-galactose to the induction medium containing L-arabinose, which had been purified by fermentation of contaminating galactose, results in approximately the same time-course of induction and deinduction observed with the original, unpurified arabinose.

Since the absence of epimerase activity in cells induced in the presence of L-arabinose could be due either to inhibition by L-arabinose of epimerase induction or to inhibition of epimerase activity after induction, the effect of L-arabinose was tested on nongrowing, fully induced cells and on nongrowing, constitutive cells which synthesize the transport system and the Leloir pathway enzymes without an inducer. Galactose fermentation of both of these types of cells is markedly inhibited by exposure to L-arabinose at room temperature (Table 2). Transport and enzyme assays of the inhibited cells showed that only epimerase activity was affected. Comparison of galactose accumulation products by L-arabinose-inhibited cells and a genetically epimeraseless

TABLE 1. *Galactose fermentation, uptake, and activities of enzymes of the galactose pathway in cells induced by D-galactose and L-arabinose*[a]

Inducer	Activity (nmoles of substrate taken up or used per mg of protein per min)				
	Fermentation	Uptake	Galactokinase	Transferase	Epimerase
None	0 (0)[b]	0.0 (0)	0 (0)	0.0 (0)	0.0 (0)
L-Arabinose	0 (0)	33 (18)	10 (13)	0.3 (19)	0.0 (0)
D-Galactose	50	182	75	1.6	7.2

[a] Fermentation was measured manometrically as CO_2 production at 30 C under a nitrogen atmosphere of a 1% cell suspension in 2% galactose. Uptake was measured as the rate of ^{14}C uptake at 30 C by a 10% cell suspension in 5×10^{-4} M D-galactose-*1*-^{14}C (1 mCi/mmole). Enzyme activities were measured in cell extracts as follows: galactokinase by the anion-exchange method of Horowitz (9) and transferase and epimerase by the spectrophotometric assays of Kalckar et al. (12) and Maxwell et al. (13), respectively. The assays were carried out with cells grown for 15 hr in induction medium containing 0.2% inducer. The L-arabinose used as inducer was the *unpurified* Sigma Chemical Co. product.

[b] Numbers in parentheses represent the per cent of the activity of galactose-induced cells which is taken as 100%.

TABLE 2. *Inhibition of galactose fermentation by L-arabinose*[a]

Incubation with L-arabinose		Rate of fermentation[b]	Inhibition (%)
Concn (%)	Time (hr)		
0	1	7.28	–
0.2	1	6.78	7
2.0	1	1.86	74
0	2	7.00	–
0.2	2	4.09	40
2.0	2	1.41	80

[a] Prior to measuring galactose fermentation, 10% cell suspensions of the constitutive strain (122-2C)-1D were shaken in a 30 C water bath in either 0.2 or 2.0% L-arabinose for 1 or 2 hr as indicated; control cells were suspended in distilled water. After this incubation, the cells were washed by two centrifugations in distilled water at room temperature and resuspended in distilled water. Galactose fermentation was then measured as CO_2 production by a 1% cell suspension in 2% D-galactose in a nitrogen atmosphere by standard Warburg procedures at 30 C.

[b] Micromoles of galactose per gram of yeast per minute.

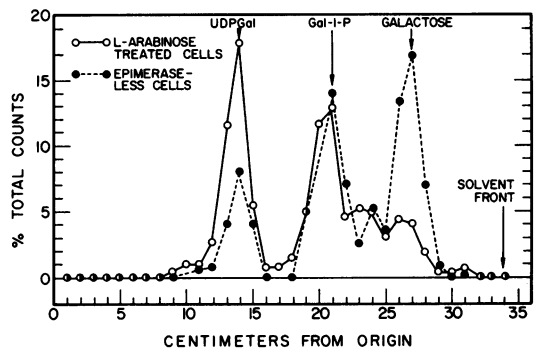

FIG. 1. *Chromotagraphic analysis of galactose accumulation products. Cells of the constitutive [(122-2C)-1D] and epimeraseless (100-5A) strains were grown in induction medium containing 0.2% D-glucose plus 0.2% D-galactose. After harvest and washing in distilled water, one portion of the constitutive cells was exposed to 1% L-arabinose for 2 hr at room temperature and washed in distilled water before incubation with D-galactose. All cells were incubated for 20 min at 30 C with 1 mM D-galactose-1-*^{14}C *(1 Ci/mole), washed with ice-cold distilled water on membrane filters, and extracted successively for 1 hr with absolute ethanol and 50% ethanol. The concentrated alcohol extracts were applied to Whatman No. 1 papers and developed by descending chromatography in the solvent system of Palladini and Leloir at pH 3.8. The dried chromatograms were cut into 1-cm strips and counted in Bray's solution.*

strain (100-5A) confirmed that L-arabinose inactivated epimerase in vivo. Chromatographic analysis of the products formed from D-galactose -*1*-^{14}C in both types of cells show the identical pattern with free galactose, galactose-1-phosphate, and UDP-galactose accounting for more than 90% of the accumulation products. Uninhibited cells contain additional compounds which represent intermediates beyond the epimerase step in galactose fermentation (Fig. 1).

Inactivation of epimerase by L-arabinose can also be demonstrated to occur in vitro by using crude cell homogenates. Exposure of the homogenate to 2% L-arabinose for 15 min at 30 C leads to almost complete inactivation (Table 3). Surprisingly, when dialyzed, 10-fold purified epimerase was used, no sensitivity to L-arabinose was observed (Table 3). However, sensitivity to L-arabinose could be restored by the addition of boiled crude extract to the partially purified dialyzed enzyme.

A cursory survey has been made to determine to what other sugars galactose fermentation is sensitive. For this purpose, cells of the same con-

TABLE 3. *In vitro inactivation of epimerase by* L-*arabinose*

Expt	Enzyme prepn	L-Arabinose preincubation	Epimerase activity[b]
1	Crude	−	770
		+	15
2	Dialyzed	−	6,500
		+	6,500
3	Dialyzed + boiled crude	−	6,400
		+	80

[a] Portions (10 μliter) of crude homogenate (crude) or partially purified and dialyzed extracts (dialyzed) from glucose-grown constitutive cells [(122-2C)-1D] were incubated at room temperature for 15 min in the following solutions: experiment 1: 10 μliters of crude extract + 20 μliters of distilled water *or* 20 μliters of 4% L-arabinose; experiment 2: 10 μliters of dialyzed extract + 20 μliters of distilled water *or* 20 μliters of 4% L-arabinose; experiment 3: 10 μliters of dialyzed extract + 10 μliters of boiled crude extract + 10 μliters of distilled water *or* 10 μliters of 6% L-arabinose. Epimerase activity was measured as the rate of increase in 340-nm absorption at 30 C after the addition of assay mixture containing 1 μmole of uridine diphosphate-galactose, 1 μmole of nicotinamide adenine dinucleotide$^+$ and 400 units of uridine diphosphate-glucose dehydrogenase in a 0.1 M glycine buffer (*p*H 8.75) in a final volume of 1.1 ml.

[b] Nanomoles of uridine diphosphate-glucose epimerized per milligram of protein per minute.

TABLE 4. *Inhibition of* D-*galactose fermentation by sugars and sugar derivatives*[a]

Sugar	Inhibition of fermentation (%)
L-Arabinose	90
2-Deoxy-D-glucose	90
2-Deoxy-D-galactose	86
3-Oxymethyl-D-glucose	64
D-Glucosamine	64
L-Glucose	59
D-Mannose	51
L-Mannose	47
α-Methyl-D-glucopyranoside	41
D-Xylose	40
D-Erythrose	38
Sedoheptulose	34
Mannitol	34
N-acetyl-D-glucosamine	34
D-Fucose	33
L-Sorbose	32
Fructose	31
α-Methyl-D-mannopyranoside	30
Trehalose	28
D-Melibiose	28
D-Melezitose	24
D-Mannosamine	24
D-Arabitol	19
L-Arabitol	15
L-Rhamrose	15
β-Methyl-D-xylopyranoside	11
D-Turanose	8
D-Lyxose	8
L-Fucose	0
D-Glucose	0
D-Arabinose	0
Adonitol	−2
D-Ribose	−3
L-Lyxose	−4

[a] Glucose-grown cells of the constitutive (122-2C)-1D strain were incubated as 10% suspensions in 2% solutions of the indicated compounds for 2 hr at 30 C. The cells were washed twice and suspended in distilled water, and galactose fermentation was measured as CO_2 production by standard Warburg procedures by using 1% cell suspensions in 2% D-galactose in a nitrogen atmosphere at 30 C. Cells incubated in distilled water for 2 hr before addition to the Warburg flasks served as controls.

stitutive strain used before were incubated in 2% solutions of the various compounds for 2 hr at room temperature after which they were washed and tested for both glucose and galactose fermentation. The results for those sugars which affected galactose fermentation *but not glucose fermentation* are listed in Table 4. The results show that galactose fermentation is sensitive to a remarkably large number of sugars.

DISCUSSION

These experiments show that the enzyme UDP-glucose-4-epimerase is remarkably sensitive in vivo to glucose and galactose analogues. The basis of the epimerase sensitivity to these sugars and the nature of the cofactors involved have recently been identified by Bertland and Kalckar (1, 2) who showed that highly purified epimerase from *Saccharomyces fragilis* is sensitive to L-arabinose, D-fucose, and other analogues of D-galactose and D-glucose in the presence of pyrimidine nucleotides, notably 5′-uridine monophosphate (UMP). L-arabinose and other sugars in the presence of 5′UMP inactivate the enzyme by reducing the bound NAD^+ molecules on the enzyme. This reduction results in a marked increase in the fluorescence of the enzyme which was the way these investigators originally detected this reaction (1). The sugar oxidation products presumably dissociate from the enzyme leaving it "locked" in the reduced form. It is likely that the factor in the boiled homogenate described here is 5′UMP or a derivative; however, no attempt has been made in this investigation to identify the factor(s) further.

The discovery that L-arabinose and D-fucose

are inhibitors of galactose fermentation coupled with the fact that these sugars are frequently contaminated with small amounts of D-galactose explains the previous erroneous report that these sugars are "gratuitous inducers" (6). The small amount of D-galactose introduced as a contaminant would ordinarily not produce significant levels of induction because it would be consumed within minutes after induction was initiated. However, in the presence of L-arabinose or D-fucose the D-galactose persists to act itself as a "gratuitous inducer" producing phenotypically epimeraseless cells.

Although D-fucose is an effective gratuitous inducer of the galactose operon in *Escherichia coli* (12, 15, 16), Jordan et al. (11) have recently reported that D-fucose may also inhibit the *E. coli* epimerase in vivo but only during late exponential growth. This inactivation required kinase, transferase, and UDPG-pyrophosphorylase activity since mutants lacking any of these enzymes were refractory to D-fucose. No experiments on in vitro inactivation of the bacterial enzyme have yet been reported.

The preliminary screening of sugar and sugar derivatives as inhibitors of galactose fermentation in baker's yeast reported in Table 4 indicated a remarkable sensitivity to a large number of compounds. Although the effect on the activity of the Leloir pathway enzymes has only been studied for L-arabinose and D-fucose, it is likely that many of these inhibitions will prove to be due to inactivation of epimerase. For example 2-deoxy-D-glucose-6-phosphate is a well-known inhibitor of glucose-phosphate isomerase; however, the 2-hr incubation period in 2% deoxyglucose had no affect on glucose fermentation but caused 90% inhibition of galactose fermentation.

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