Relationship between UDP-Galactose 4′-Epimerase Activity and Galactose Sensitivity in Yeast*§

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UDP-galactose 4′-epimerase (GALE) catalyzes the final step of the highly conserved Leloir pathway of galactose metabolism. Loss of GALT in humans results in a variant form of the metabolic disorder, galactosemia. Loss of GALT in yeast results in galactose-dependent growth arrest. Although the role of GALT in galactose metabolism has been recognized for decades, the precise relationship between GALE activity and galactose sensitivity has remained unclear. Here we have explored this relationship by asking the following. 1) Is GALE rate-limiting for galactose metabolism in yeast? 2) What is the relationship between GALE activity and galactose-dependent growth arrest in yeast? 3) What is the relationship between GALE activity and the abnormal accumulation of galactose metabolites in yeast? To answer these questions we engineered a strain of yeast in which GALE was doxycycline-repressible and studied these cells under conditions of intermediate GALE expression. Our results demonstrated a smooth linear relationship between galactose metabolism and GALE activity over a range from 0 to ~5% but a steep threshold relationship between growth rate in galactose and GALE activity over the same range. The relationship between abnormal accumulation of metabolites and GALE activity was also linear over the range from 0 to ~5%, suggesting that if the abnormal accumulation of metabolites underlies galactose-dependent growth-arrest in GALE-impaired yeast, either the impact of individual metabolites must be synergistic and/or the threshold of sensitivity must be very steep. Together these data reveal important points of similarity and contrast between the roles of GALE and galactose-1-phosphate uridylyltransferase in galactose metabolism in yeast and provide a framework for future studies in mammalian systems.

Galactose is metabolized in species ranging from Escherichia coli to mammals via a series of reactions collectively known as the Leloir pathway. The three enzymes that catalyze these sequential reactions are galactokinase (EC 2.7.1.6), galactose-1-phosphate uridylyltransferase (GALT, EC 2.7.7.12), and UDP-galactose 4′-epimerase (GALE, EC 5.1.3.2) (see Fig. 1 (1)). Deficiency of any one of these enzymes in humans results in a form of the inherited metabolic disorder, galactosemia (OMIM entry 230400), which affects about 1 in 30,000–60,000 live births and results from profound impairment of the GALT enzyme. Although typically asymptomatic at birth, patients with classic galactosemia develop escalating symptoms after exposure to a milk-based diet. In the absence of intervention these symptoms, which include vomiting, diarrhea, cataracts, hepatomegaly, and E. coli sepsis, can be lethal within the first few days to weeks of life. Although a dietary restriction of galactose, the current standard of care, relieves or prevents the acute and potentially lethal symptoms, many patients with classic galactosemia go on to develop long term complications. The most common complications include speech and/or learning disabilities in 30–50% of all patients and primary or premature ovarian failure in almost 85% of females (3).

Perhaps the least well understood form of galactosemia is epimerase (GALE) deficiency galactosemia (OMIM entry 230350). As illustrated in Fig. 1, human GALE catalyzes not only the interconversion of UDP-galactose (UDP-gal) and UDP-glucose (UDP-glc) but also the interconversion of UDP-N-acetylgalactosamine and UDP-N-acetylglucosamine (e.g. Refs 4 and 5). Originally described as a “peripheral” clinically benign condition in which GALE deficiency is restricted to the circulating red and white blood cells (6–8), GALE deficiency was later demonstrated to exist also in an extremely rare but clinically severe “generalized” form characterized by enzyme impairment in multiple tissues and symptoms reminiscent of classic galactosemia (9–11). Most recently, GALE deficiency has been described as a continuous disorder, with a spectrum of enzyme impairment and corresponding metabolic compromise impacting a variety of tissues in affected individuals (12–17).

Despite decades of study, the underlying bases of pathophysiology in both classic and epimerase-deficiency galactosemia remain unknown (2). For example, although it is clear that patients with both disorders accumulate high levels of galactose-1-phosphate (gal-1P) on galactose-containing diets, whether gal-1P causes pathophysiology or simply correlates with pathophysiology remains unclear. In the absence of an animal model of galactosemia that recapitulates the human phenotype (18–21), we and others have turned to the single celled yeast, Saccharomyces cerevisiae, as a biochemically and genetically amenable model to explore the metabolic and cellular consequences of impaired GALT and GALE function in eukaryotes.

Studies dating back more than 40 years (22) demonstrate that both GALT and GALE-null S. cerevisiae arrest their growth in response to even trace quantities (0.05%) of environmental galactose despite the presence of an alternate, metabolize-able carbon source (e.g. glycerol/ethanol). More recent studies by our group and others (23–28) have both confirmed and extended that result. For example, we found that GALE-null yeast arrest growth at galactose exposures 10-fold lower than do GALT-null yeast, although the GALT-null yeast accumulate levels of gal-1P greater than or equal to their GALE-null counterparts (28). We also found that although GALT-null yeast remain at least marginally competent to deplete their medium of galactose, GALE-null...
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![The Leloir pathway of galactose metabolism.](image)

yeast cannot do so, even after prolonged incubation (28). Of note, this same observation was corroborated recently in mammalian and patient cells (17, 29). These data raise the possibility that although the acute phenotypes of profound GALT or GALE impairment may be similar in patients and in yeast, the mechanism(s) of pathophysiology of galactose sensitivity for GALT- versus GALE-impaired cells may be distinct.

Previously we addressed the quantitative relationship between GALT impairment and galactose sensitivity by expressing each of 16 differentially impaired patient alleles of human GALT in a null-background strain of yeast (27) and defining the galactose sensitivity of the resulting strains. Our results demonstrated a clear inverse relationship between the level of residual GALT activity and sensitivity of the corresponding strains. Furthermore, it appeared that human GALT alleles associated with greater than about 10% wild-type human GALT activity, corresponding to about 3% yeast GAL7 activity, were indistinguishable from wild-type cells.

Here we have addressed the quantitative relationship between GALE impairment and galactose metabolism and sensitivity in yeast. Although conceptually parallel to our earlier work, the results of these studies demonstrate both important similarities and important contrasts to GALT. In particular, we have asked three questions. 1) Is GALE rate-limiting for galactose metabolism in otherwise Leloir wild-type yeast and, if so, over what range? 2) What is the relationship between GALE activity and galactose-dependent growth impairment in yeast? 3) What is the relationship between GALE activity and the abnormal accumulation of galactose metabolites in yeast? With this last question we are seeking to know if some galactose metabolites may correlate more closely with the degree of galactose sensitivity than do others, perhaps implicating a role for these compounds in the mechanism of sensitivity.

To ask these questions we inserted a doxycycline-regulated promoter just upstream of the GAL10 open reading frame in otherwise Leloir wild-type yeast, rendering the encoded GALE enzyme doxycycline repressible. We then grew these cells under conditions of differential expression of gal10-null yeast served as positive and negative controls, respectively.

Our results demonstrated that the answer to the first question was, yes. There was a clear linear relationship between residual GALE activity and galactose metabolism in otherwise Leloir wild-type yeast over a range from 0 to about 5% wild-type GALE. Above about 5% GALE was no longer limiting. The answer to the second question was that although all of the yeast grew similarly in the absence of galactose, in the presence of galactose there was a clear step relationship between growth rate and GALE activity, with a threshold for growth at about 5% wild-type GALE. Finally, in answer to the third question, GALE showed an inverse, near linear relationship with accumulation of abnormal levels of gal-1P and UDP-gal, again up to a threshold of about 5% wild-type GALE. Above 6% GALE appeared to be in excess for all of the outcomes measured. Together, these data both quantify the role of GALE and define key similarities and distinctions between GALT and GALE as mediators of galactose metabolism and sensitivity in yeast.

**EXPERIMENTAL PROCEDURES**

**Yeast Strains and Manipulation**—All yeast manipulations were performed according to standard protocols (30) using haploid strains derived from W303 (MATa ade2–1 his3–11,15 leu2–3,112 ura3–1 trp1–1 can1–100 RADS+), which was the kind gift of Dr. Rodney Rothstein, Columbia University, New York, NY. To enable the expression of endogenous yeast GAL genes in both the presence and absence of galactose, all strains were rendered gal80-null by one-step gene replacement, as described previously (31). The negative control strain JFy4931 was also rendered gal10-null by the same method (31).

The dox.GAL10 yeast strain (JFy4828) was constructed by insertion of a cassette containing a doxycycline-repressible promoter (32, 33) just upstream of the endogenous GAL10 open reading frame. This manipulation was achieved by one-step gene replacement using a PCR-generated fragment (from plasmid CM225) amplified to carry flanking sequences homologous to the desired insertion site. To tighten regulatory control over the doxycycline-repressible promoter, a second cassette encoding a repressor moiety (from plasmid CM244) was also integrated into the LELI2 locus, as described elsewhere (32, 33). All integrations were confirmed by genomic PCR of the resulting strains.

**Yeast Culture Conditions**—All yeast were cultured at 30 °C in yeast-rich growth medium containing 2% glycerol, 2% ethanol (YPGE) unless otherwise indicated, as in Fig. 3 and Supplemental Fig. 2, for which yeast were cultured in synthetic medium containing 2% glycerol, 2% ethanol (SGE). Cells were exposed to doxycycline as follows. On day 1, a given colony was inoculated into medium containing 3 μg/ml doxycycline to completely shut down expression of the dox.GAL10 allele. After growth overnight cells were harvested by centrifugation, washed 3 times with sterile water to remove residual drug, and resuspended to an OD600 of ~0.05 in medium containing the desired final concentration of doxycycline (e.g. 0–80 ng/ml). Cells were grown in this medium to an OD600 of ~1.5, at which point they were diluted into fresh medium containing the same level of drug and allowed to grow for another approximately eight doublings under these conditions. Relevant doxycycline concentrations are indicated for each experiment.

**Leloir Enzyme Activity Assays from Soluble Yeast Lysates**—Soluble yeast protein lysates were prepared as follows. Cell pellets were washed with water and resuspended in lysis buffer (20 mM Hepes/KOH (pH 7.5), 1 mM dithiothreitol, and 0.3 mg bovine serum albumin/ml) supplemented with protease inhibitors (complete mini protease inhibitor mixture, Roche Applied Science). Lysis was carried out by vigorous agitation with 0.5-mm acid-washed glass beads at 4 °C. Lysates were clarified by centrifugation at high speed in an Eppendorf microcentrifuge for 10 min at 4 °C. To remove small metabolites, supernatants were passed through Bio-Spin 30 columns (Bio-Rad) before protein quantification. Protein concentration was then determined using the Bio-Rad protein reagent, as recommended by the manufacturer, with bovine serum albumin as the standard. Samples were stored at ~85 °C until use.

Galactokinase activity was evaluated in samples by measuring the conversion of galactose to gal-1P, essentially as described previously (28). Assays were stopped by the addition of 450 μl of ice-cold water followed immediately by filtration through 0.2-μm nylon micro-spin columns (Corning) to remove proteins and particulates before high performance liquid chromatography analysis.
GALT activity was evaluated by measuring the conversion of gal-1P into glucose 1-phosphate, essentially as described previously (28). Assays were stopped by the addition of 450 μl of ice-cold water followed immediately by filtration as described above.

GALE activity was evaluated in samples by monitoring the conversion of UDP-gal to UDP-glc, essentially as described previously (28). Assays were stopped by the addition of 237.5 μl of ice-cold water followed immediately by filtration as described above.

**RESULTS**

**Survey of Galactokinase Activity, GALT, and GALE Enzyme Activities in Wild-type and dox.GAL10 Yeast Cultured in the Presence and Absence of Doxycycline**

Values are the mean ± S.E., n = 3, except where indicated.

| Enzyme | Treatment | Wild-type strain | dox.GAL10 strain |
|--------|-----------|-----------------|-----------------|
|        |           | μmol/mg prot/min | μmol/mg protein/min |
| Galactokinase | No drug | 2.66 ± 0.12 | 2.39 ± 0.05 (n = 2) |
| GALT | 3 μg/ml doxycycline | 1.49 ± 0.52 | 2.96 ± 0.17 |
| GALE | 3 μg/ml doxycycline | 1.56 ± 0.04 | 0 ± 0.00* |

* Statistical significance.

As illustrated in Table 1, Galactokinase and GALT activities in wild-type and dox.GAL10 yeast were unaffected by the presence or absence of drug. In contrast, GALE in wild-type and dox.GAL10 strains were statistically indistinguishable regardless of the presence or absence of doxycycline. In the absence of drug, GALE activity was undetectable in the presence of doxycycline, whereas in the presence of 25 ng/ml doxycycline, cells cultured in the presence of 35–50 ng/ml drug demonstrated 10% GALE activity. Finally, as described under “Experimental Procedures,” initial cultures were exposed to 3 μg/ml doxycycline to completely repress GALE expression, after which point cells were cultured in the presence of as little as 0.002% galactose (26, 28). Consistent with that result, we observed here that in the absence of doxycycline (Fig. 2, left panel), both wild-type and dox.GAL10 yeast grew well in both the absence and presence of galactose. In the presence of 3 μg/ml doxycycline (Fig. 2, right panel), however, only the wild-type strain grew well in both the presence and absence of galactose; both the dox.GAL10 and gal10-null yeast completely arrested growth upon the addition of galactose to the medium. Together, these data corroborate the in vitro GALE activity assay results described above and further demonstrate that 26% wild-type GALE activity is sufficient to confer galactose resistance in yeast.

**Loss of Epimerase Activity, Not Mutarotase Activity, Mediates Galactose Sensitivity of gal10-Null Yeast**—Unlike human GALE, the protein product of the yeast GAL10 gene, Gal10p, is a fusion protein that exhibits both UDP-galactose 4-epimerase activity and also galactose mutarotase activity (35, 36). Deletion or repression of GAL10, therefore, results in the loss of both activities. To test whether the galactose-dependent growth arrest observed in gal10-null yeast reflected loss of epimerase, mutarotase, or both activities, we tested the galactose sensitivity of gal10-null yeast engineered to express human GALE, which complements the loss of epimerase activity but not the loss of mutarotase activity. As illustrated in Fig. 3, human GALE fully complemented the growth arrest of gal10-null yeast exposed to galactose, thereby demonstrating that it is loss of epimerase and not loss of mutarotase activity that underlies the galactose sensitivity of gal10-null yeast.

**Titration of GALE Activity in dox.GAL10 Yeast**—To investigate the quantitative relationship between GALE and galactose sensitivity in yeast, we first defined the relationship between doxycycline exposure and GALE activity in our dox.GAL10 strain. As described under “Experimental Procedures,” initial cultures were exposed to 3 μg/ml doxycycline to completely repress GALE expression, after which point cells were diluted into fresh medium containing the desired level of drug (0–80 ng/ml). After ~40 h of continuous culture at the desired level of doxycycline, at which point GALE activity levels had stabilized, yeast were harvested and assayed for GALE activity relative to a wild-type control.

As illustrated in Fig. 4, there was a clear and reproducible inverse relationship between the level of drug exposure and the level of GALE activity in the dox.GAL10 yeast. In particular, cells cultured in the absence of drug demonstrated 26% GALE activity, cells cultured in the presence of 25 ng/ml demonstrated about 12% GALE, cells cultured in the presence of 35–50 ng/ml drug demonstrated <10% GALE, and cells cultured in >55 ng/ml drug demonstrated <5% GALE.
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**FIGURE 2.** Effect of doxycycline on the growth rate of yeast in the presence and absence of galactose. The indicated strains were inoculated into YPGE medium in 96-well plates in the presence or absence of 3 μg/ml doxycycline and also in the presence or absence of 0.025% galactose as indicated. Open symbols connected by dashed lines represent yeast grown in the absence of galactose; closed symbols connected by solid lines represent yeast grown in the presence of galactose. Values represent the mean ± S.E., n = 4. WT, wild type.

**FIGURE 3.** Human GALE complements the galactose-dependent growth impairment of gal10-null yeast. Wild-type (WT), gal10-null, and gal10-null yeast engineered to express wild-type human GALE (hGALE: JFy4346) were grown in YPGE medium ± 0.025% galactose. Values plotted represent the means ± S.E., n = 3.

**FIGURE 4.** Titration of GALE activity by doxycycline in dox.GAL10 yeast. Soluble lysates prepared from dox.GAL10 yeast cultured in the indicated levels of doxycycline were assayed for GALE activity, as described under “Experimental Procedures.” Values plotted represent the mean ± S.E., n = 3 for each strain normalized to growth of the same strain in the absence of doxycycline. Values listed above each bar represent the mean value for that sample relative to the level of GALE activity detected in wild-type yeast.

As illustrated in Fig. 5A, there was a clear inverse relationship between galactose-dependent growth impairment and GALE activity. In particular, cultures exhibiting less than 3.7% GALE activity completely arrested growth, whereas cultures exhibiting between 4.7 and 5.2% GALE activity were partially growth-impaired, and cultures exhibiting greater than 6.4% GALE activity grew indistinguishably from cells cultured in the absence of drug. Wild-type cultures exposed to comparable levels of doxycycline showed no impact on growth, confirming that the growth impairment observed in the dox.GAL10 cultures resulted from GALE repression and not from some unexpected side effect of drug treatment (data not shown). An alternate view of the results from Fig. 5A is presented in Fig. 5B, which plots the slopes of individual growth curves in the interval from 15–17 h as a function of GALE activity. In the absence of galactose, the relationship is essentially flat (left panel), but in the presence of 0.025% galactose there is a sharp “step” from no or low growth at ≤5% GALE activity to essentially wild-type growth at greater than ~6% GALE activity (right panel).

**Relationship between GALE Activity and Galactose Metabolism in dox.GAL10 Yeast**—As a first step to explore the quantitative relationship between GALE activity and galactose metabolism in vivo, we tested the ability of dox.GAL10 yeast to deplete their medium of galactose in the presence versus absence of 3 μg/ml doxycycline. Wild-type and gal10-null strains were analyzed in parallel as positive and negative controls, respectively. Previously, we have demonstrated that gal10-null yeast are unable to deplete galactose from their medium and that upon exposure to galactose these cells accumulate abnormally high intracellular levels of galactose, gal-1P, and UDP-gal and abnormally low levels of UDP-glc (28).

As illustrated in Supplemental Fig. 1, in the absence of doxycycline (shaded bars), the dox.GAL10 strain depleted external galactose and accumulated internal metabolites to levels indistinguishable from the wild-type strain. In the presence of 3 μg/ml doxycycline, however, this same dox.GAL10 strain failed to deplete external galactose and accumulated internal metabolites to levels indistinguishable from the gal10-null strain.

In the absence of added galactose, abnormal levels of UDP-gal were detected only in the gal10-null strain and in the dox.GAL10 strain treated with drug, and these levels were transient, returning almost to

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
background within 16 h of continuous culture in the same medium (Supplemental Fig. 2, panel C). Previously, we reported an abnormal accumulation of UDP-gal in gal10-null yeast only upon the addition of galactose to the medium (28). To uncover the basis for this apparent discrepancy, we tested the possibility that culture medium could account for the difference; rich medium (YPGE) was used for the studies reported here, whereas the earlier studies were performed using synthetic medium (SGE). As illustrated in Supplemental Fig. 2, panel E, the difference in medium indeed accounted for the discrepancy; the dox.GAL10 and gal10-null strains both accumulated abnormally high levels of UDP-gal only in the YPGE medium and not in the SGE medium. This difference presumably reflects the presence of hidden sources of galactose in the rich medium. It is also interesting to note that although UDP-gal levels in the gal10-null and dox.GAL10 yeast were sensitive to this cryptic galactose, gal-1P levels were not (Supplemental Fig. 2, panel B).

To define the quantitative relationship between GALE activity and galactose metabolism in yeast, we next studied dox.GAL10 cells cultured in levels of drug selected to repress GALE into a closely spaced range from 0 to 26% of wild-type activity. As illustrated in Fig. 6, panel A, cells demonstrating 5% or less of wild-type GALE activity were significantly impaired with regard to their ability to deplete external galactose, especially at the 6-h time point, whereas cells exhibiting >6% GALE activity were only marginally impaired, if at all. Indeed, by the 16-h time point there appeared to be a near-linear relationship ($r^2 = 0.89$) between the percentage of external galactose remaining in the culture medium and GALE activity, up to a threshold of ~6% wild-type GALE, above which all cultures fully depleted their medium of galactose.

Internal metabolites followed a similar pattern, such that elevated levels of intracellular galactose, gal-1P, and UDP-gal also demonstrated a near-linear inverse relationship with GALE activity ($r^2 = 0.70, 0.79$, and $0.55$, respectively), up to a threshold of about 5–6% wild-type GALE, above which point metabolites essentially normalized. Finally, abnormal depletion of UDP-glc in the presence of galactose was reproducibly seen only at the very lowest levels of GALE activity (e.g. gal10-null strain and dox.GAL10 strain in the presence of drug).

**DISCUSSION**

We have addressed three fundamental questions regarding the role of GALE in galactose metabolism and sensitivity in yeast. We have asked the following. 1) Is GALE rate-limiting for galactose metabolism in otherwise Leloir-wild-type yeast and, if so, over what range of activity? 2) What is the relationship between GALE activity and yeast growth rate in the presence of galactose? 3) What is the relationship between GALE activity and the abnormal accumulation of galactose metabolites in yeast? In answering these questions we have both delineated the role of GALE in galactose sensitivity in yeast and also contrasted this role with that of GALT. In addition, we have further set the stage for future studies in mammalian cells aimed at exploring the role of GALE as a mediator of pathophysiology and patient outcome in patients with galactosemia.

**Role of GALE in Galactose Metabolism in Yeast**—We found that the ability of yeast to deplete their medium of galactose was rate-limited by GALE over a range from 0–5% wild-type activity, and this relationship was remarkably linear ($r^2 = 0.89$). Yeast expressing GALE activity levels above 6% depleted environmental galactose as quickly and completely as did their wild-type counterparts. Although the precise threshold is likely a function of the amount of galactose added, among other factors, the important conclusion is that, like GALT (27), GALE is expressed in marked excess of Leloir requirements in normal yeast.

**Relationship between GALE Activity and Growth Impairment in Yeast**—A comparison of strains cultured in YPGE medium spiked with 0.025% galactose and different levels of doxycycline demonstrated an inverse step function relationship between growth rate and GALE activity. At less than 4% GALE, growth rates were almost 0; between 4 and 5% GALE, cultures demonstrated a long lag, after which some growth became evident; above 6% GALE, growth rates were normal. It is interesting to note that the lag in growth of the 4–5% GALE cultures corre-
sponded precisely with the time frame of external galactose depletion for these cells. These data suggest that whereas galactose metabolism may be a linear function of GALE activity within a limiting range (0 to 5%), the growth response of yeast to that metabolic block is not linear.

These data are strikingly similar, at least in qualitative terms, to our earlier data with GALT (27). In particular, we previously observed that yeast expressing 1% human GALT activity, corresponding to 0.3% yeast GALT activity, could not grow in the presence of galactose, whereas yeast expressing 5% human GALT, corresponding to 2% yeast GALT activity, demonstrated a long lag followed by some growth, and finally that yeast expressing 10% human GALT activity, corresponding to 3% yeast GALT activity, grew indistinguishably from the wild-type control. Especially considering that the level of galactose used in the GALT experiments (0.05%) was twice the level used here (0.025%), these data suggest that whereas the patterns may be qualitatively similar, yeast are quantitatively hypersensitive to the loss of GALE relative to the loss of GALT. These data are fully consistent with our earlier results demonstrating that GALE-null yeast growth arrest upon exposure to galactose levels 10-fold lower than do their GALT-null counterparts (28).

Relationship between GALE Activity and Abnormal Galactose Metabolite Levels—One obvious result of impaired GALE activity in yeast exposed to environmental galactose is the accumulation of abnormal levels of intracellular galactose metabolites, including galactose, gal-1P, and UDP-gal (28). We explored the possibility that one or more of these metabolites might correlate with the step-function growth restriction of GALE-impaired yeast but found that within the key range of 0–5% enzyme activity, each measured metabolite demonstrated a clear linear inverse relationship with GALE. Furthermore, only yeast with very low GALE activity levels (<1%) showed a decreased abundance of UDP-glc in the presence of galactose.

These data suggest that if abnormal accumulation of the metabolites measured indeed underlies the galactose-dependent growth restriction observed, either the impact of individual metabolites must be synergistic, producing an apparent step function in growth, or else the sensitivity must follow a steep threshold such that even the slightest accumulation of gal-1P or UDP-gal results in growth arrest. Future experiments that
manipulate the accumulation of individual metabolites independently will be required to distinguish between these possibilities.

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