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INSIGHTS INTO THE PATHOGENESIS OF GALACTOSEMIA

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Key Words mouse model, galactose, galactitol, galactonate, UDP-galactose

■ **Abstract** In humans, the absence of galactose-1-phosphate uridyltransferase (GALT) leads to significant neonatal morbidity and mortality which are dependent on galactose ingestion, as well as long-term complications of primary ovarian failure and cognitive dysfunction, which are diet independent. The creation of a knockout mouse model for GALT deficiency was aimed at providing an organism in which metabolic challenges and gene manipulation could address the enigmatic pathophysiologic questions raised by humans with galactosemia. Instead, the mouse represents a biochemical phenotype without evidence of clinical morbidity. The similarities and differences between mice and humans with galactosemia are explored from metabolite, enzyme, and process points of view. The mouse both produces and oxidizes galactose in a manner similar to humans. It differs in brain accumulation of galactitol. Future directions for exploration of this enigmatic condition are discussed.

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INTRODUCTION

Galactose-1-phosphate uridyl transferase (GALT) (19), the second enzyme in the Leloir pathway (Figure 1), is essential in human infants consuming lactose as their primary carbohydrate source. Nursing infants must move large amounts of galactose through this pathway in order to utilize the carbon skeletons for energy. Studies of galactose utilization in dogs and rats suggest that galactose may indeed be the preferred carbon source in mammalian neonates, since it is incorporated

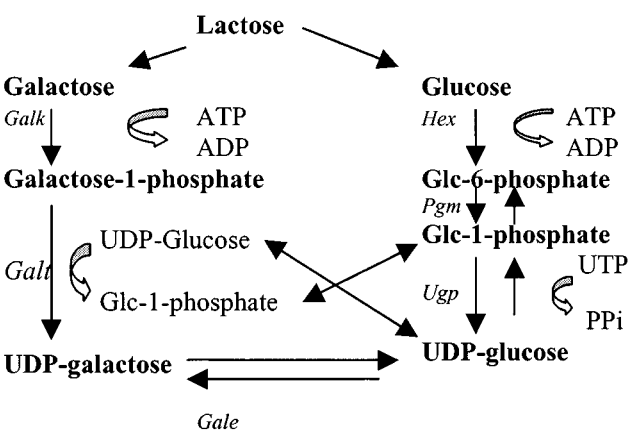

Figure 1 Composite diagram of the Leloir pathway and uridine diphosphate (UDP)-glucose pyrophosphorylase pathway. *Galk*, galactokinase; *galt*, galactose-1-phosphate uridyltransferase; *Gale*, UDP-galactose 4-epimerase; *Hex*, hexokinase; *Pgm*, phosphoglucomutase; *Ugp*, UDP-glucose pyrophosphorylase.

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into glycogen more efficiently than is glucose (30). In infants with classical galactosemia, who have near total absence of GALT activity, exposure to dietary galactose results in acute deterioration of multiple organ systems, including liver dysfunction, coagulopathy, poor feeding and weight loss, renal tubular dysfunction, cerebral edema, vitreous hemorrhage, and *Escherichia coli* sepsis. This "neonatal toxicity syndrome" can be reversed by withdrawal of dietary galactose. Most state screening programs have included galactosemia in their newborn screening programs, anticipating that early detection and intervention would prevent long-term complications such as mental retardation, premature ovarian failure, and speech delay. Unfortunately, these expectations have not been borne out by experience. A wide variety of specific and global deficits have been observed in patients who have been studied with sensitive neuropsychological assessment tools (26). Clinically evident speech delay (36) and cerebellar signs are more frequent than other findings. Abnormal white matter signal is found in most subjects, but this abnormality does not correlate with cognitive outcome (37). Premature ovarian failure is nearly universal in females with galactosemia (28), although the age of onset is quite variable.

The difficulties in designing adequate studies to examine neonatal, hepatic, brain, and ovarian processes in humans with galactosemia created an impetus for the development of a mammalian GALT-deficient model. When homologous recombination and embryonic stem cell technology were used, a mouse with undetectable GALT activity was produced by replacing exons 6–8, the coding region for the active catalytic site, with a hypoxanthine phosphoribosyl transferase coding sequence. These GALT-deficient animals (34, 40) have no obvious evidence of neonatal toxicity during the suckling period. Extending the period of galactose exposure by weaning the animals to a high-galactose diet for four weeks results in prolonged derangement of steady state galactose metabolite levels (Figure 2), but no obvious phenotype (39). Animals kept for long periods show no difference in longevity from normal and are fertile to the same age as normal mice. GALT deficiency has been bred into several mouse strains without producing a galactosemic phenotype (N.D. Leslie, unpublished observation).

The discrepancy between biochemical and clinical phenotype in these animals provides impetus to develop alternative hypotheses regarding the pathophysiology of galactosemia. Obviously, the biochemical phenotype cannot be identical to that of human, or there would be no difference in cellular response to GALT gene deletion. Yet the typical parameters used to study humans with galactosemia have not yielded the needed clues to the discrepancy. In this review, the metabolites, enzymes, and processes involved in normal and deranged galactose metabolism are reviewed for clues to the enigma of galactosemia.

Lactose Synthesis

Lactose is the predominant carbohydrate in mammalian milk, but its major role is that of an osmole which assures the production of a fluid milk (55). In mouse models

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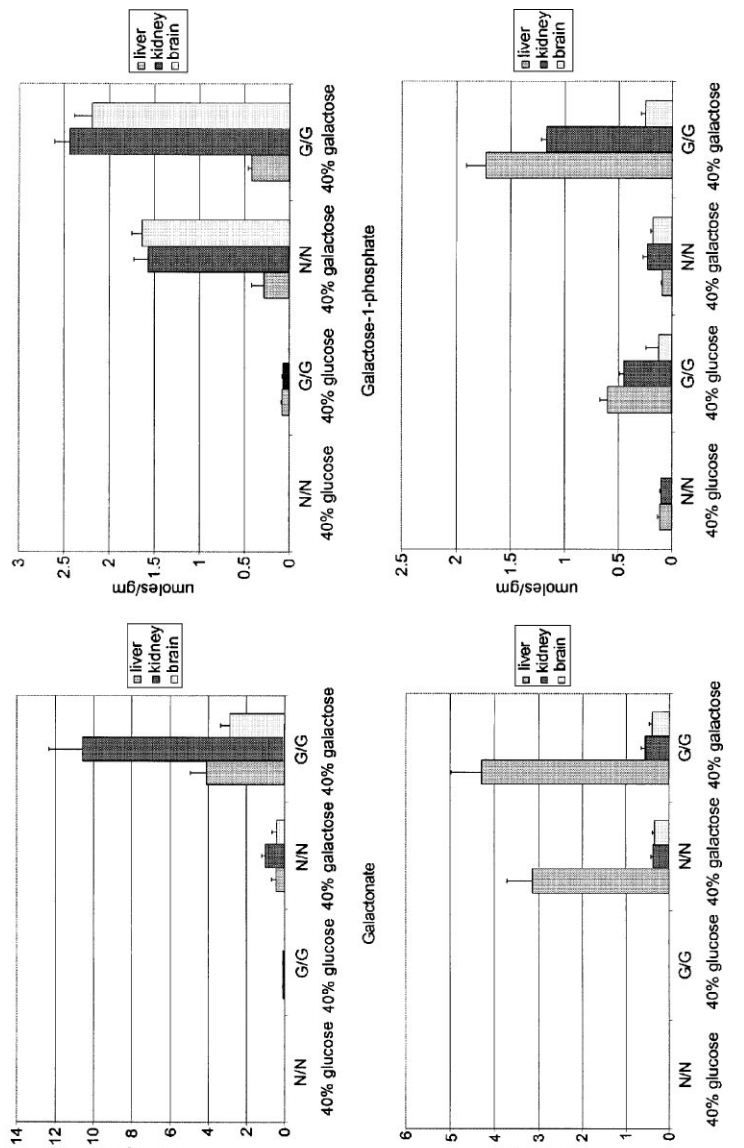

Figure 2 Steady state galactose and galactose metabolite levels in adult mice fed defined diets containing 40% glucose or galactose. G/G, GALT deficient; N/N, normal.

which lack the ability to produce lactose, the resulting milk is viscous and the pups die of inanition. There is variation in lactose content among mammalian milks, with mice having less lactose per ml (20–25 mg/ml) in comparison to humans (66 mg/ml). However, the amount is sufficient to raise metabolite levels significantly in GALT-deficient pups (34). As expected, milk production, which is dependent on uridine diphosphate (UDP)-galactose production, is completely normal in GALT-deficient mice, as it is in the few human females with galactosemia who have been able to conceive.

Galactose Production

The ability of the human with galactosemia to "self-intoxicate" with galactose and its metabolites has been long suspected because elevated levels of erythrocyte galactose-1-phosphate have been measured in infants who have never received dietary galactose (21). These suspicions were confirmed with elegant stable isotope measurements, which suggested that the endogenous production of circulating free galactose in adults with GALT deficiency ranged from 0.53 to 1.05 mg/kg/hr (6, 38). Although direct measurement of endogenous production has not been measured in infants, the indirect evidence suggests that it occurs, and the magnitude is suspected to be higher per kilogram than in adults. Where does all this galactose come from? UDP-galactose can be synthesized from glucose through UDP-glucose pyrophosphorylase and UDP-galactose 4-epimerase (the *right side* and *bottom* of Figure 1), from which free galactose can be released by the reverse reaction of pyrophosphorylase. The galactose residues which are appropriately transferred to complex molecules will eventually be released by lysosomal hydrolysis. Is this amount of synthesized or released galactose toxic to cells? Could it be that mice don't synthesize galactose and it is lack of production that tips the balance in favor of better cellular tolerance to GALT deficiency? The answer to the former question is unknown, at least in humans. However, mice certainly accumulate galactose and its metabolites even when fed a glucose-based chow (Figure 2), making it likely that the observed galactose metabolites are newly synthesized or released. Furthermore, galactose loading of suckling or adult GALT-deficient mice increases liver and kidney levels of free galactose and galactose-1-phosphate 20–40 fold over those observed in similar mice fed glucose-based chow, yet still show no clinical toxicity (Figure 2) (39).

Oxidation to CO_2

The clearest biochemical parameter correlated with clinical outcome and GALT genotype is that of galactose oxidation to carbon dioxide (5, 7). The early studies of Segal & Cuatrecasas (49) on patients with the African-American variant [now characterized as S135L (32)] of galactosemia showed both augmented CO_2 production from radiolabeled galactose and residual GALT activity demonstrable on liver biopsy. The ability to determine oxidation with orally administered

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stable-isotope labeled galactose (7) has made this a practical way of determining whole-body galactose oxidation in an outpatient setting.

What do the whole-body galactose oxidation studies tell us? First, the absence of a significant 2–5 hour peak of galactose oxidation is observed in humans with genotypes associated with a severe neonatal course in infants exposed to dietary galactose (7). Second, in humans, both Q188R homozygous and gene deletion homozygous patients show similar oxidation curves (4), implying that the slow oxidation rate is not dependent on micro amounts of residual GALT activity, but must depend on another pathway. Third, both gene deletion humans and gene deletion mice (40) have similar overall curves (Figure 3), despite vastly different clinical phenotypes, suggesting that the phenotypic resistance of mice to large galactose loads is not due to the presence of an alternative rapid oxidation pathway. If galactosemic humans both produce galactose and consume small amounts in a typical galactose-limited diet, then it follows that they must have some means to dispose of it or they would over time accumulate larger and larger amounts of galactose in tissues, or excrete large amounts in urine. Neither of these phenomena can be demonstrated; therefore it is reasonable to assume that production and consumption probably equal slow oxidation, at least in older individuals. However, the exact nature of this disposal pathway has yet to be elucidated.

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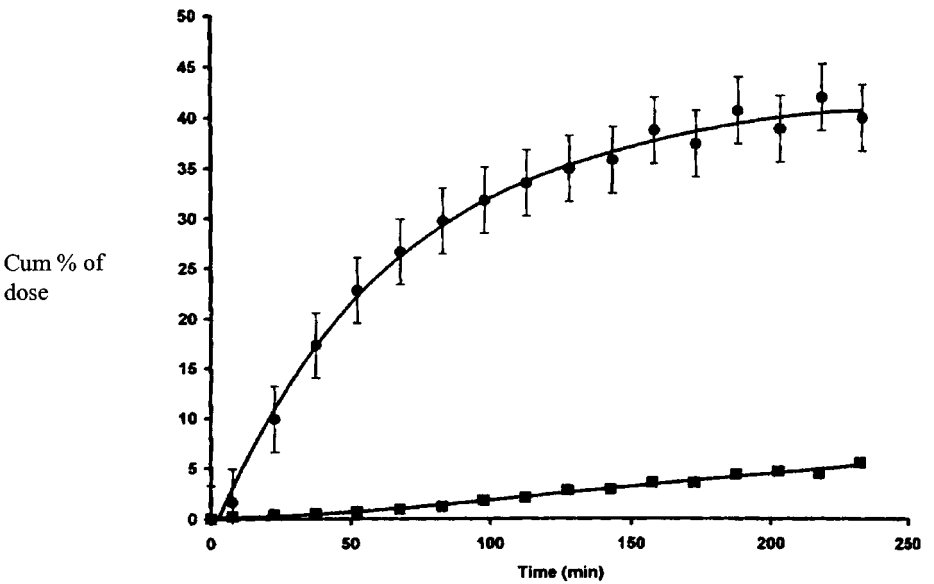

Figure 3 Galactose oxidation in adult mice given 0.5 uCi 1-^{14}C-galactose intraperitoneally as a single dose. Expired $^{14}CO_2$ was collected at 15-minute intervals and cumulative percentage of the dose was plotted versus time. Percent of dose expired was constant over a dose range of 10–100 mg/kg. GALT deficient, ■; normal, ●.

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THE METABOLITES

Galactitol

Free galactose may be reduced to galactitol through the action of aldose reductase (61) (Figure 4). Galactitol production is a dead-end pathway, and the product is poorly diffusable. Because of this, it is likely that significant tissue accumulations of galactitol would be derived from galactose already in the cell. Indeed, galactitol accumulation has been demonstrated in the brain of toxic neonates with classical galactosemia (3, 46, 59). The amounts of galactitol measured directly in GALT-deficient mice are lower (2 mM) than levels detected by magnetic resonance spectroscopy in human subjects (8 mM) (3, 57).

Galactose-1-Phosphate

Free galactose is transported into cells through a variety of transporters that are members of the glucose transporter (GLUT) family of transporters. Deficient transport of galactose into hepatocytes is evident in human patients with Fanconi-Bickel syndrome, in which the GLUT 2 transporter is defective (9). Once inside, galactose is readily trapped as galactose-1-phosphate in cells that express galactokinase. The accumulation of galactose-1-phosphate in red cells is exploited diagnostically in both newborn screening for galactosemia as well as in confirmatory studies and follow-up monitoring of patients (11, 21). Before the creation of the GALT-deficient mouse, it was widely believed that galactose-1-phosphate was the toxic metabolite responsible for the galactosemia phenotype. Indeed, in neither galactokinase deficiency nor epimerase deficiency, in which galactose-1-phosphate accumulation is either blocked or at best modestly elevated, is there a comparable devastating neonatal phenotype, nor is there a comparable chronic ovarian or brain

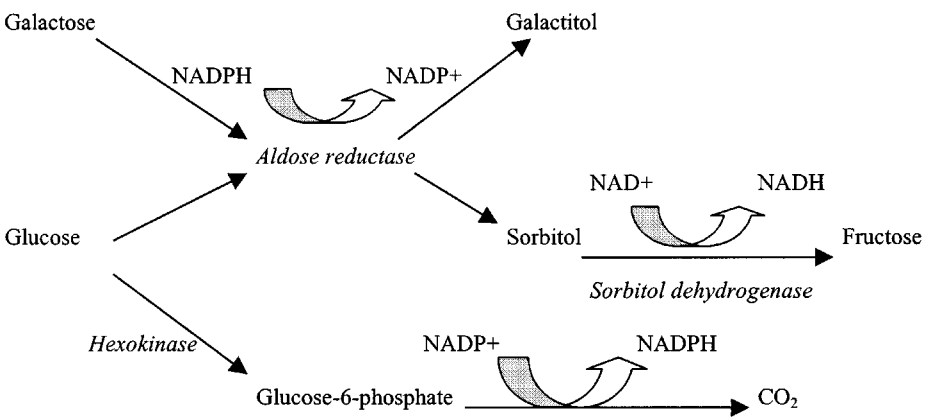

Figure 4 Metabolism of glucose and galactose through the polyol pathway.

phenotype. However, mice with GALT deficiency accumulate large amounts of galactose-1-phosphate in all tissues studied (34, 39, 40). Actual quantification of galactose-1-phosphate in target tissues of humans is currently impossible by non-invasive means, making erythrocytes the only tissue in which direct comparison can be made between mice and humans. In erythrocytes, mice can be observed to accumulate large amounts of galactose-1-phosphate, and under loading conditions, these levels can be sustained at high levels for weeks at a time.

Galactonate

The pathway of galactose metabolism to D-xylulose (Figure 5), then through the pentose phosphate pathway, described by Cuatrecasas & Segal (14–16) deserves further exploration. Large amounts of galactonate are formed in the liver of galactose-fed animals, indicating the presence of an alternate pathway for galactose disposition (39). In humans with GALT deficiency, there is ample evidence that this pathway is functional, including galactonate excretion and by the formation of large amounts of galactonate in human red blood cells incubated with galactose (48, 58). The oxidation of galactonate preferentially releases the first carbon of galactose as CO_2, and therefore one would expect a more rapid accumulation of labeled carbon appearing if a portion is produced through this pathway. Indeed, such differential oxidation can be demonstrated (48), showing that the galactonate pathway accounts for a portion, but certainly not all, of the galactose oxidation demonstrated in humans with galactosemia. GALT-sufficient humans do not excrete any significant amounts of galactonate under basal conditions, but under conditions of loading, urine galactonate can be detected (58).

Mice form galactonate in liver and excrete significant amounts in the urine under conditions of galactose loading (Figure 2), and these metabolites are found in both GALT-deficient mice and wild-type mice, suggesting that the pathway is activated by excessive loading rather than just by a blocked Leloir pathway (39).

UDP-Galactose and UDP-Glucose

UDP-galactose is necessary for appropriate glycosylation of galactoproteins and galactolipids. A UDP-gal transporter facilitates the delivery of this metabolite to the Golgi, where specific modification of the target molecules proceeds.

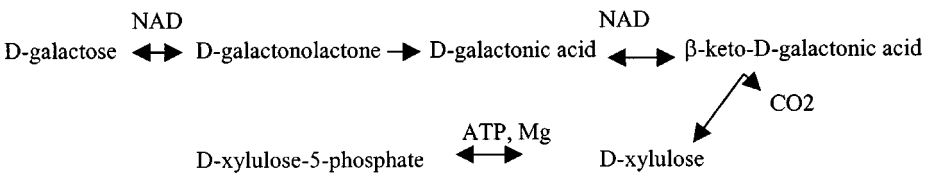

Figure 5 Direct oxidation of galactose through the galactonate pathway.

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Cellular deficiency of UDP-gal has been proposed as a mechanism for the pathophysiology of GALT deficiency in humans (47), but the evidence accumulated is conflicting and controversial. From a common-sense standpoint, UDP-galactose should be produced as long as epimerase can convert UDP-glu to UDP-gal (lower portion of Figure 1). In cellulo, the deficiency of epimerase shows clear alteration of function when UDP-gal production through the Leloir pathway is limited by galactose withdrawal. Low density lipoprotein (LDL) receptors formed under these conditions by epimerase-deficient Chinese hamster ovary (CHO) cells do not function appropriately. The phenotype of epimerase deficiency in humans is less clear (56). A partial or "peripheral" defect is common in African Americans, and appears to be a benign biochemical polymorphism. Several families with more severe epimerase deficiency have been reported, but most are from highly consanguineous families, and there does not appear to be a consistent phenotype which can clearly point to the epimerase deficiency as causal. Indeed, since there is no such thing as a galactose-free diet (except in those few individuals consuming a completely synthetic diet) or complete galactose deficiency with no synthesis, the conditions observed in CHO cells would rarely be observed in epimerase-deficient humans. No mouse model for complete epimerase deficiency exists, much less a double mutant with both GALT and UDP-galactose-4-epimerase (GALE) deficiencies.

Is there "relative" UDP-gal deficiency in humans with GALT deficiency? Demonstration of nucleotide sugar levels requires careful analytic methodology using HPLC (20, 29, 51). Lower UDP-gal levels in erythrocytes can indeed be demonstrated in individuals with GALT deficiency, but similar levels are seen in patients with phenylketonuria (PKU) who consume low levels of dietary lactose, and these are corrected when the diet is supplemented with galactose (20). (It can be pointed out that both galactosemia and PKU have unexplained cognitive deficits, casting concerns over the choice of PKU patients as a "control" group. However, patients on defined metabolic diets represent a small fraction of individuals consuming low-lactose diets, most of whom have no cognitive issues whatsoever.) Therefore, steady state erythrocyte UDP-gal levels appear to be dependent on flux through the Leloir pathway, but decreased UDP-gal levels are not unique to patients with GALT deficiency. A threshold effect in target tissues not amenable to study remains a possibility. However, the only trial which has addressed the possibility that oral uridine supplementation could correct the phenotype of GALT deficiency showed no effect on cognitive outcome (35).

Defective galactosylation remains a possible explanation for some of the galactosemia phenotype (43, 47). Ornstein et al. (42) and Dobbie et al. (18) found evidence of unoccupied galactose acceptor sites in cultured fibroblasts from GALT-deficient patients. Prestoz et al. (45) documented a charge alteration in follicle-stimulating hormone (FSH) isoforms circulating in women with galactosemia and premature ovarian failure. Although this work does not document whether the patients studied had been treated with exogenous estrogen, the shift in pI goes in the opposite direction of that observed in women with estrogen deficiency. On the

other hand, Kaufman et al. (28) demonstrated normal bioactivity of gonadotropins isolated from the urine of women with galactosemia and there is no published report of treatment of infertility with recombinant FSH. Carbohydrate-deficient transferrin is increased in infants with galactosemia, an additional piece of evidence suggesting defective galactosylation of target proteins (10). GALT-deficient fibroblasts isolated from human patients will not grow in galactose as a sole carbon source unless inosine is added to the culture medium, providing a ribose source for nucleotide synthesis (44). In addition, galactose induces a state of uridine auxotrophy in cells lacking a competent oxidative phosphorylation pathway. These are artificial states—no human will be limited to galactose as a sole carbon source—but they do highlight an interaction between the Leloir pathway and the uridyl sugars required for appropriate protein and lipid processing.

Mice with GALT deficiency typically consume a chow diet with 1.75% bioavailable galactose, as do most laboratory mice. Adult mice on such a diet were studied to determine levels of UDP sugars in the liver (40). GALT-deficient mice did not differ from wild-type mice in steady state UDP-glu levels, UDP-gal levels, or their ratio. No similar data are available for human liver tissue.

Summary

Both mice and humans with GALT deficiency produce and oxidize some galactose, through incompletely characterized pathways. The relative flux through these metabolic paths, estimated from steady state metabolite levels, is shown in Figure 6. The biggest known difference between humans and mice is in tissue galactitol levels, best measured in brain. Whether these levels account for differences in pathology between mice and humans is not yet known but certainly provides a fertile ground for further exploration.

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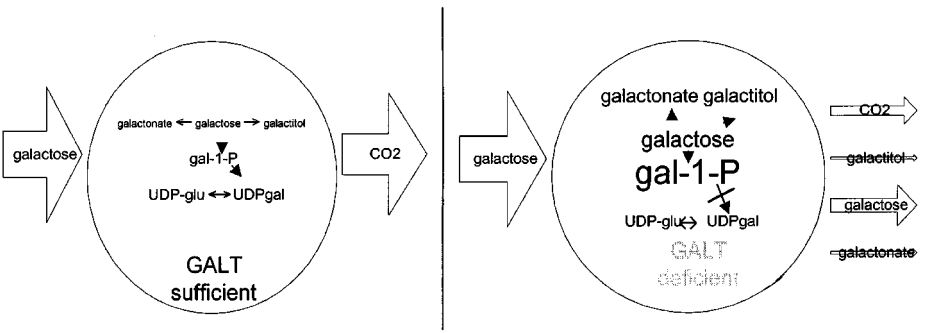

Figure 6 Estimated disposal of a galactose load in normal or GALT-deficient mice, based on oxidation and excretion data observed in adult mice.

THE ENZYMES

Galactose-1-Phosphate Uridyltransferase (EC 2.7.7.10)

GALT is a member of the histidine triad (HIT) family of enzymes (8), so named because of a hisϕhisϕhis$\phi\phi$ motif (ϕ referring to a hydrophobic amino acid). In this family of nucleotide hydrolases and transferases, GALT has a stable catalytic intermediate with a covalently bound uridyl moiety (23).

$$\text{UDP-glc} + \text{his-GALT} \rightarrow \text{UMP-hisGALT} + \text{glc-1-P}$$
$$\text{UMP-hisGALT} + \text{gal-1-P} \rightarrow \text{UDP gal} + \text{hisGALT}$$

Although GALT mutants which affect the active site (such as Q188R) severely affect catalytic throughput, slow uridylation and deuridylation rates can still be estimated, which may confer measurable amounts of direct labeling of uridyl sugar from a labeled sugar phosphate substrate.

GALT is expressed ubiquitously (52) but retains some elements of tissue specificity and developmental regulation. Studies of GALT activity in rats show high expression in liver, kidney, brain, and cardiac muscle (50). Liver GALT activity rises over the first week of life, then decreases to low adult levels. Northern analysis of GALT mRNA in rats parallels the enzymologic data (22), suggesting that increased transcription may be a factor in the regulation of GALT activity. Retention of residual GALT activity in liver of subjects with the African American variant of galactosemia (genotype S135L) is correlated with residual whole-body galactose oxidation and paucity of clinical symptoms (7). These associations suggest that GALT presence in an organ correlates with biological need, and therefore GALT deficiency will lead to pathology. However, analysis of endogenous GALT mRNA in mice placed on high- or low-galactose diets show no modification of expression in adult tissues (33). Furthermore, studies of a GALT promoter/luciferase reporter construct in transgenic mice show no upregulation of promoter activity in either mice with a GALT gene deletion or in mice loaded with galactose. These studies suggest that, in contrast to yeast and bacteria, GALT is not directly modulated by galactose and its metabolites. These data give support to the alternative viewpoint that GALT activity in tissues such as brain and ovary may play a greater role in the supply side of the Leloir pathway.

Galactokinase (EC 2.7.1.6)

Galactokinase is a polypeptide of 392 amino acids, encoded by the *GK1* gene on human chromosome 17q21-22. The enzyme catalyzes a bidirectional reaction, but the equilibrium favors formation of galactose-1-phosphate. Such high levels of product inhibit the forward reaction, and in the presence of a blocked Leloir pathway there is sufficient GALK inhibition to allow accumulation of free galactose,

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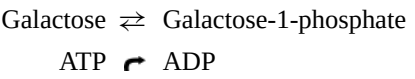
which is detected in plasma (41) and urine, as well as to supply substrate to the aldose reductase pathway.



A second galactokinase gene, *GK2*, maps to chromosome 15. The expression patterns of the two are quite different, with GK1 expressed primarily in liver and fetal liver, while GK2 has a much more heterogeneous expression pattern, including myelogenous cell lines (52). In liver, galactokinase can efficiently trap galactose absorbed from the gut and transported with GLUT 2. Although one would predict a strong hepatic phenotype for GALK deficiency under such conditions of loading, no hepatic pathology is known for either humans or mice with GALK deficiency.

Aldose Reductase (EC 1.1.1.21)

Aldose reductase (61) is the first enzyme in the sorbitol pathway (Figure 4) and catalyzes the reduction of sugars to alcohols, particularly glucose to sorbitol and galactose to galactitol. Although sorbitol can be further metabolized, the production of galactitol is a dead-end pathway.

Human aldose reductase is expressed in heart, kidney, brain, and skeletal muscle, as well as other tissues, and is transcriptionally upregulated in respose to hypertonicity and to the inflammatory cytokine, TNF-α. Overexpression of aldose reductase in hamster pancreatic beta cells alters the redox potential via the intracellular NADPH/NADP+ ratio. The relative paucity of active aldose reductase in mice has been a source of frustration for diabetes researchers, who found the contrast in phenotypic manifestations in response to galactose loading or experimental diabetes a disappointment when compared with rat models. Indeed, in a relevant model of cataracts induced by GALK deficiency (2), the phenotype is expressed only when aldose reductase is overexpressed by a transgene (see Cataracts, below). On the other hand, aldose reductase indeed plays a functional role even in mice, since mice homozygous for aldose reductase deletion express a phenotype of nephrogenic diabetes insipidus (24). Of all the organs examined in GALT-deficient mice, it is kidney that has the highest levels of galactitol accumulation (Figure 2), suggesting that aldose reductase is expressed and active in this organ but that galactitol accumulation or NADPH consumption do not cause a renal phenotype in mice.

UDP-Glucose Pyrophosphorylase (E.C. 2.7.7.9)

UDP-glucose pyrophosphorylase (UTP: α-D-glucose-1-phosphate uridyltransferase) and UDP-galactose pyrophosphorylase (UTP: α-D-galactose-1-phosphate uridyltransferase) were thought at one time to be separate proteins, although many attempts to separate the two activities failed (1). Pyrophosphorylase has figured importantly in the literature on galactosemia, since it represented an alternative pathway for the disposal of galactose-1-phosphate and, through epimerase, the eventual

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synthesis of UDP-galactose. However, the actual contribution of pyrophosphorylase to galactose metabolism in liver has been doubtful since the 1960s, when it was demonstrated that the contribution of pyrophosphorylase to galactose metabolism was less than 1% of that due to GALT itself. The catalytic activities of UDP-gal pyrophosphorylase and UDP-glu pyrophosphorylase indeed reside in the same protein.

$$\text{UTP} + \text{glucose-1-phosphate} \Leftrightarrow \text{UDP-glucose} + \text{PPi}$$
$$\text{UTP} + \text{galactose-1-phosphate} \Leftrightarrow \text{UDP-galactose} + \text{PPi}$$

Rekindling of an interest in the role of pyrophosphorylase (17) was provided when Lai & Elsas (31) showed that GALT-deficient yeast which reverted in their ability to grow on galactose had highly enhanced expression of uridyl diphosphate glucose pyrophosphorylase (UGP), and that transfection of GALT-deficient yeast with the human UGP restored the ability to grow on galactose. Could such a mechanism account for the mild clinical phenotype of the GALT-deficient mouse? The results of the whole-body oxidation studies suggested that enhanced pyrophosphorylase expression and/or activity could not be the answer, since substantial activity would be expected to deliver the carbon skeletons of labeled galactose-1-phosphate through pyrophosphorylase, epimerase, and then through the glycogen pathway and ultimate conversion to labeled CO_2. Direct analysis of mouse liver has shown that UDP-gal pyrophosphorylase activity is negligible in GALT-deficient animals and could not be increased by prior exposure to high-galactose chow (N.D. Leslie, C.L. Yager & S. Segal, manuscript in preparation). In animals with intact GALT and GALE, apparently high levels of conversion of gal-1-P to UDP-gal were observed, but inactivation of GALE by extensive dialysis to remove NAD reduced the apparent pyrophosphorylase activity to the same levels as seen in GALT-deficient animals. UGP message was not increased in GALT-less mice relative to wild-type controls (N.D. Leslie, unpublished). Therefore, although it is indeed likely that pyrophosphorylase contributes to the slow phase of galactose oxidation seen in patients and mice with absent GALT activity, there is no support for a role in rapid disposal of galactose-1-phosphate in mouse liver. Since the oxidation profiles between mouse and human are similar, a large role for pyrophosphorylase in humans with galactosemia must return to the "probably not" category.

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UDP-Galactose-4-Epimerase (E.C. 5.1.3.2)

UDP-galactose-4-epimerase (GALE) catalyzes the interconversion of UDP-glu and UDP-gal as well as the interconversion of UDP-gluNAc and UDP-galNac.

$$\begin{array}{rcl} \text{UDP-glucose} & \Leftrightarrow & \text{UDP-galactose} \\ \text{UDP-GluNAc} & \Leftrightarrow & \text{UDP-GalNac} \end{array}$$

NAD

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In gene expression studies, GALE is expressed in most cells and tissues, with greatest expression in human placenta and lung, as well as in several transformed cells lines (52). A "peripheral" form of epimerase deficiency, in which the defect is confined to the erythrocytes, is commonly ascertained in newborn screening programs using galactose as a primary analyte. This form is considered to be clinically benign. More severe forms of epimerase deficiency have been described in a handful of patients, many of whom come from highly consanguineous families (56). In these patients, poor feeding and modest accumulation of galactose metabolites have been observed during the neonatal period, and continued poor growth and hypotonia are features of both probands and early-treated individuals. Ovarian failure was not a feature of the two oldest female patients who have been reported. In two patients reported by Walter et al. (56), abnormal serum transferrins were observed, with a pattern similar to that observed in classical galactosemia, providing evidence of deranged glycosylation.

In yeast with a null background of epimerase activity, growth in medium with galactose providing the sole carbon source is unmeasurable, although growth in glucose-containing medium is normal (60). This implies that gal-1-P accumulation is toxic to yeast, because the alternative explanation—that UDP-gal is essential—doesn't work in this scenario, or the yeast wouldn't grow in glucose alone. In Chinese hamster ovary (CHO) cells with defective epimerase activity, deficient O-linked glycosylation and action of low density lipoprotein (LDL) receptors can be demonstrated in cells grown without supplemental galactose. In the CHO cell model, the provision of exogenous galactose and an intact Leloir pathway through GALT are able to supply UDP-galactose needed for glycosylation. In humans with epimerase deficiency, galactose is never the sole carbon source, the Leloir pathway is intact through GALT, and UDP-glucose can be synthesized normally through the pyrophosphorylase pathway. Under such conditions, why should there be any phenotype? Three possibilities exist: (*a*) gal-1-P is directly toxic (except that documented levels in red cells are low compared with classical GALT-deficient patients as well as variant GALT-deficient patients); (*b*) UDP-gal and UDP-glu must be balanced (the usual ratio is 3 UDP-glu per UDP-gal), and having two separate synthetic pathways with no rebalancing makes this suboptimal; (*c*) the enzymes of the Leloir pathway function best as a "metabolon," handing substrates directly from one step to another, and then to the UDP-gal transporter, without re-equilibrating with the remainder of the cytoplasm. Indirect evidence for such aggregation has been demonstrated in yeast expression studies (12).

In patients with "severe" epimerase deficiency characterized thus far, partial flux from UDP-gal to UDP-glu can be demonstrated. In elegant experiments, the most common mutation, V94M, has been characterized by crystallography. The action of epimerase depends on the binding of substrate, reduction of carbon through a bound NAD cofactor, followed by rotation of substrate within the active site and positioning of the new hydroxyl group on the opposite side. The mutant enzyme puts fewer steric constraints on rotation, so that actual chemical transformation occurs less readily, much as an engine performs with the clutch pushed in. The

alternative substrate, UDP-GalNac, is bulkier and has less free rotation within the active site, which is likely to account for the relative preservation of catalytic activity toward this substrate (53, 54). The role of UDP-GalNac conversion in GALE deficiency has not been well studied, but data available thus far suggest a lesser role in the pathophysiology of known patients. On the other hand, most patients with GALE deficiency would not be ascertained by ordinary clinical testing if the only defect were that of the UDP-GalNac conversion.

THE PATHOPHYSIOLOGIC PROCESSES

Cataracts

The pathophysiology of cataracts is one of the few well-characterized aspects of galactosemia. Cataracts can be seen in the neonatal period in infants with classical GALT-deficient galactosemia, and may reoccur in older patients who have poor dietary compliance. Cataracts are also found in galactokinase deficiency, and similarly, may be prevented by dietary galactose restriction. Cataracts are not seen in mice with either GALT or GALK deficiency, although their appearance can be induced in GALK deficiency by overexpressing a gene for aldose reductase in lens tissue (2). Similarly, cataracts are uncommon in mice with experimental diabetes, although rats may develop them either in experimental diabetes or with dietary galactose loading. These findings lead to the conclusion that lens tissue accumulates galactose but not galactitol under conditions of either GALK or GALT deficiency, and the phenotypic expression of cataracts requires the activity of aldose reductase. Galactitol acts as an osmole and results in lens swelling, in a similar manner to sorbitol's action in diabetes. Inhibition of aldose reductase with inhibitors such as tolrestat is known to prevent or reverse cataract formation. Presumably aldose reductase inhibitors would have a similar therapeutic effect in GALK or GALT deficiency, but concerns about exacerbation of problems not convincingly due to galactitol production have diminished enthusiasm for its use.

Neonatal Toxicity

The acute neonatal "diet-dependent" presentation of galactosemia is by far the best understood in terms of therapeutic efficacy, but the exact reason for such drastic deterioration in humans with complete or near-complete GALT deficiency is unclear. There is a large discrepancy in acute phenotype between infants with Q188R homozygosity who have limited galactose oxidation and "variant" individuals who may similarly lack erythrocyte GALT activity but still have substantial or near-normal galactose oxidation ability (7). This suggests that the threshold for hepatic toxicity in the neonatal period is very low, and the large amount of consumed galactose easily overwhelms it. However, the lack of a similar oxidation pattern in GALT-deficient mice remains a puzzle. Mice are known to be

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more resistant to the adverse phenotypic effects of uridine trapping agents such as galactosamine—perhaps this is part of the explanation.

Ovarian Failure

Hypergonadotropic hypogonadism in females with classical GALT deficiency occurs almost universally, although the rapidity and severity of this problem varies widely (28). Women with breath test oxidation results which approximate normal kinetics are much less likely to have ovarian failure, suggesting that the threshold for ovarian failure is close to zero GALT activity. Prenatal identification and treatment do not prevent this complication. Mice with GALT deficiency have no evident infertility, even with advanced age or with galactose loading. Although rats with intact transferase may produce infertile pups if fed large amounts of galactose during gestation, GALT-deficient mice are able to conceive and produce small litters, and the female offspring from such experiments are able to conceive and produce litters (N.D. Leslie, unpublished). The galactose diet fed to these mice has appropriate amounts of protein, so that malnutrition is not likely to be the cause of small litters, but mice on these diets are quite polyuric. This seems to stress the animal considerably, since glucose-based chow has the same effect on litter size. These findings suggest that the effect of GALT deficiency on the ovary may be local and not mediated by diet or galactose exposure in the newborn period. Exactly why this happens is uncertain and is limited to females, suggesting a peculiar susceptibility of the ovarian follicle to GALT deficiency. Although several studies have been published suggesting a relationship between variants in the GALT gene with ovarian cancer, endometriosis, or Mullerian aplasia, a convincing dose-response effect between partial GALT deficiency and female reproductive disease has never been demonstrated and several recent studies debunk the existence of any relationship (13).

Chronic Brain Effects

The most vexing problem facing patients with classical galactosemia is the effect of GALT deficiency on brain development and function (27). Although there are very specific deficits in some patients with GALT deficiency, including developmental speech dyspraxia and tremor, the typical individual with galactosemia may have globally decreased IQ and/or learning disabilities. These problems often become more evident as individuals progress in school or attempt to establish independent adult lives. It is clear that prevention of an acute neonatal toxic state benefits the infant as far as mortality and morbidity in the early weeks of life, but it is also clear that avoidance of such events in subsequent siblings does not prevent long-term neurologic morbidity. What is most uncertain is whether these deficits are initiated in early development, perhaps even prenatally, and unmasked as more complex brain function is required, or whether they represent true neurodegenerative processes compounded by dietary exposure and endogenous production of "intoxicants."

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FUTURE DIRECTIONS

From knowledge gained in the studies detailed above, additional pathways toward gaining insight are evident.

Can We Express Aldose Reductase in the Relevant Cells and Determine the Effect on Phenotype?

An obvious direction in which to go is to recreate the scenario of an aldose reductase–expressing mouse with concommitant GALT deficiency, an analogous experiment to the GALK−/− mouse model. Unfortunately, the aldose reductase–expressing transgenic that was bred with GALK mice expressed the enzyme only in lens, and a suitable animal with aldose reductase expression in liver, kidney, or brain is not available. Could such a model recreate the finding of cerebral edema seen in toxic neonates with increased galactitol demonstrated by magnetic resonance spectroscopy? This is certainly plausible, but the bigger question is whether such a mouse would have similar long-term CNS pathology. In the diabetes literature, arguments are found that aldose reductase serves a protective effect. Certainly, there is no clinical phenotype to protect in the GALT-deficient mouse, but a deleterious and unexpected outcome should also be kept in mind in evaluating such a model.

What is the Effect of Uridine or UDP-Gal Depletion on Overall Phenotype?

The "uridine controversy" (25) revolved around the idea that deficient UDP-galactose was the underlying feature of GALT deficiency and that oral uridine supplements could ameliorate it. Although a clinical trial showed no cognitive improvement in patients consuming uridine, it may have been that the timing, outcome measures, and intervention all missed the mark. It is quite convincing that red cell UDP-gal levels do not reflect any underlying defect in uridyl-sugar balance where it really counts: transport into the Golgi and subsequent utilization for glycosylation. Oral uridine is poorly absorbed—a more proper intervention might be to facilitate synthesis or salvage in the target cell. And finally, the outcome measures may be too insensitive or unchanging to see much difference, or perhaps the wrong time frame was assessed. Newer information about congenital disorders of glycosylation and their effect on a chronic brain phenotype as well as on defects of nucleotide metabolism and/or salvage on cellular function suggest that the uridine controversy should be re-examined with better tools.

If the Mouse Has a Protector Gene, Can We Find a Modifier Gene Within Human Sibling Pairs?

It is evident from the collective experience of many clinicians that within sibling pairs concordant for galactosemia, the second sibling often benefits by not experiencing an acute episode of neonatal toxicity, but by no means is that individual

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guaranteed to have a better long-term outcome. Such sibling pairs would constitute a resource for a search for a candidate modifier gene. Currently there is no registry or database from which to draw such patients, and achieving an appropriate sample size would likely require a national or perhaps international collaborative effort, as well as well-defined evaluative tools.

What is the Magnitude of Clinical Effect of Consumed Galactose in Humans?

From a patient point of view, a vexing question is, What can I safely eat? Since the demonstration that the fruits and vegetables most often used in a galactosemia diet may contain significant amounts of free and complex galactose, the definition of "well treated" has been uncertain. Biochemical measures of "control," including erythrocyte galactose-1-phosphate levels, have not proved terribly useful even in carefully controlled clinical research settings. Robust and reproducible tools for assessment of treatment and, more importantly, demonstration of clinical utility in affecting long-term effects of the disease are needed.

Must GALT Deficiency Be Corrected in the Target Organs, or Would Expression in Liver Be Sufficient?

The role of hepatic GALT in trapping and converting consumed galactose into glucose and eventually into carbon dioxide could easily be addressed in mice by creating a liver-expressing transgenic line. However, with no phenotype in the whole-body knockout and abundant metabolites in target tissues such as brain and neonatal liver, the ability of such a model to answer the key questions is limited. On the other hand, if a faithful phenotypic mouse model could be developed, this is a reasonable question. The ability to reconstitute hepatic GALT activity in humans is likely to be possible in the future. It is likely that such a therapy would relieve some of the burdens of dietary restriction but might not ameliorate the real pathology.

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SUMMARY

The importance of the Leloir pathway in changing the stereochemistry of a single carbon of galactose (19) is demonstrated by the conservation of the pathway through evolution. Experiments in simple organisms have shown the importance of regulated metabolism in efficient utilization of limited carbon sources, but mammals have co-opted these pathways for more complex post-translational modifications necessary for reproduction and higher cognitive functions. The insights gained over the past 10 years of research have been humbling, in that dogma about the role of various pathways and metabolites has been challenged. However, the sophisticated tools which have been developed may now be applied to solving the enigma of galactosemia.

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