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Pathways of galactose metabolism by galactosemics: Evidence for galactose conversion to hepatic UDPglucose

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Abstract

To determine if classic galactosemics have residual galactose-1-phosphate uridyltransferase (GALT) activity to explain their considerable ability to oxidize galactose over 24 h, we devised a method for assessing their ability to form hepatic UDPglucose (UDPglu), an intermediate in the normal Leloir pathway of galactose metabolism. The protocol involved the single oral administration of 7 mg/kg [2-^{13}C]galactose concomitant with multiple small doses of acetaminophen with measurement of the extent of labeling of urinary acetaminophen glucuronide, the glucuronide moiety being formed from hepatic UDPglu. We performed the study lasting 24 h in two normal subjects and three classic galactosemics, two homozygous for the Q188R mutation and one compound for the Q188R/K258N mutation. The labeling and total excretion of acetaminophen glucuronide was measured in urine by nuclear magnetic resonance techniques. Concomitant with determination of label in the glucuronide measurement was made of galactose oxidation to $^{13}CO_2$ and the ^{13}C enrichment of plasma glucose. All of the galactosemic patients formed ^{13}C enriched acetaminophen glucuronide indicating that they had converted the labeled galactose to [^{13}C]UDPglu and that residual GALT or another pathway that forms UDPglu is present in hepatic tissue. Compared to the normal whose glucuronide labeling was rapid and short-lived that of the galactosemics was delayed and extended for a long period over 10 h. The extent of isotopic enrichment of glucuronide by galactosemics was comparable to the normals, resulting in a much greater conversion of galactose to UDPglu by the galactosemics. The labeling of the UDPglu pool was reflected by the rate of $^{13}CO_2$ formation being rapid in the normal with peak labeling at 2–3 h with total oxidation of over 70% in 24 h. The oxidation of the galactosemics was slow with a broad peak of $^{13}CO_2$ at 10 h and a total excretion of 25–39% of the [^{13}C]galactose administered. The normal subjects formed highly enriched plasma glucose within 30 min while no enrichment of plasma glucose was detected until after 300 min in galactosemics. The exact pathway(s) of galactose metabolism by galactosemics to UDPglu remain to be determined. Their delineation may contribute to new approaches to therapeutic strategies for this enigmatic disorder.

Keywords: Galactosemia; Galactose-1-phosphate uridyltransferase; UDPgalactose; UDPglucose; Nuclear magnetic resonance; Acetaminophen

Introduction

Galactosemia due to galactose-1-phosphate uridyltransferase (GALT)[1] deficiency is an enigma. Long-term complications of cognitive impairment, speech defects, neurologic ataxias, cerebellar atrophy and ovarian failure occur despite early inception of a galactose-restricted diet [1,2]. Indeed, a comprehension of the pathobiochemical basis of the disorder has remained elusive. We now know that galactosemic patients form significant amounts of galactose endogenously which is age-dependent [3,4]. Adults can have a galactose appearance rate of a gram or more per day. However, the galactosemic patient does not accumulate galactose and, on a galactose-restricted diet, has a plasma

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[1] *Abbreviations used:* GALT, galactose-1-phosphate uridyltransferase, gal-1-P, galactose-1-phosphate; glu-1-P, glucose-1-phosphate; glu-6-P, glucose-6-phosphate; UDPgal, UDPgalactose; UDPglu, UDPglu; RBC, red blood cells.

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galactose level of 0.1–3 μM not much different from normals [5]. Galactosemics appear to be in a steady-state, albeit, with constantly elevated red blood cell (RBC) galactose-1-phosphate (gal-1-P) [6], galactitol and galactonate [7], plasma galactitol [5], and markedly increased urinary excretion of galactitol [8,9] and galactonate [10,11] from the function of alternate metabolic pathways. Urinary galactitol excretion is estimated to account for about 20% of the endogenous galactose production [12]. Since there is no further metabolism of galactitol, other pathways must be involved in disposition of the endogenous load.

Evidence has mounted that oxidation to CO_2 accounts for much of the handling of the endogenously formed galactose as well as the small dietary galactose intake. Although quantitation of the conversion of administered [1-^{14}C] and [1-^{13}C]galactose to labeled CO_2 by galactosemics over short periods of time revealed limited oxidation [13–15], longer term studies have showed otherwise. In studies lasting up to 10 h after giving radiolabeled galactose, some patients oxidized 20% of [1-^{14}C]galactose to $^{14}CO_2$ [16]. Recent studies show that Q188R homozygous as well as other classic patients can oxidize up to 50% of the dose in 24 h [17].

The metabolic pathway(s) mediating galactose oxidation remain to be clarified. Segal and Cuatrecasas [16] reported that galactosemics oxidized greater amounts of administered [1-^{14}C]galactose to $^{14}CO_2$ than [2-^{14}C]galactose and postulated that the difference was due to the direct oxidation of the C-1 carbon to CO_2 via a pathway through galactonate [18] while the C-2 oxidation represented residual GALT activity. That a patient with a GALT deletion can oxidize [1-^{13}C]galactose to $^{13}CO_2$ over a 24-h period provides evidence for an alternate pathway of galactose utilization in the absence of any possible GALT mediation [19].

Although when expressed in COS cells, the Q188R mutated GALT has 10% activity [20], the use of yeast [21], and bacterial expression systems [22] revealed no activity of the mutant protein. This has been the accepted explanation of the severity in patients with the Q188R mutation. Recently we reported that lymphoblasts with this mutation when incubated with [^{13}C]galactose formed some UDPgalactose (UDPgal) and UDPglu (UDPglu) while cells from patients with the deleted GALT gene did not, suggesting the cells with the Q188R mutation, indeed, have residual GALT activity [23].

To determine whether patients with the Q188R mutation form UDPglu from galactose, which occurs if the normal Leloir pathway is functioning, via GALT and subsequent UDPgal-4-epimerase activity, we have devised a protocol termed a "metabolic liver biopsy." We have taken advantage of the fact that individuals over the age of 10 year excrete administered acetaminophen primarily as a glucuronide adduct in urine [24] and that the glucuronide is formed in liver as a result of the oxidation of UDPglu [25]. Measurements have been made of the ^{13}C labeling of acetaminophen glucuronide following the administration of [2-^{13}C]galactose. The extent of stable isotope enrichment in acetaminophen glucuronide is thus an indicator of galactose metabolism to UDPglu and the extent of labeling of the UDPglu pool. The results of performing this study in two normal subjects and three classic galactosemics is the subject of this report.

Materials and methods

Materials

[2-^{13}C]Galactose, 99% APE, was purchased from Omicron Chemicals, South Bend, IN. Purity and enrichment were confirmed by NMR and GC–MS analysis. The acetaminophen was given as a pediatric, Tylenol preparation.

Subjects

Two normal male subjects, aged 23 and 25, with normal RBC GALT activity and liver function tests, as well as three galactosemic males, one aged 20 and two aged 40. The galactosemics had absent or barely detectable RBC GALT activity. The two older subjects were homozygous for the Q188R mutation while the younger patient was a compound of Q188R and K258N mutations. The normal controls were on regular diets prior to the study while the galactosemics were on galactose-restricted diets with RBC gal-1-P, galactitol and galactonate and urinary galactitol and galactonate in the appropriate range for diet-managed patients. The metabolic characterization of the subjects at the start of the study is shown in Table 1.

The study was approved by the Institutional Review Board of the University of Pennsylvania and performed in the Clinical Research Center of the Hospital of the University of Pennsylvania after approval by its Advisory Committee.

Protocol

The protocol involves the collection of expired air, urine, and blood samples at intervals for 24 h after the oral administration of 7 mg/kg of [2-^{13}C]galactose dissolved in water to subjects who were fasting overnight. Four hours prior to the galactose administration the subjects were started at 7 AM on the oral administration of 100 mg acetaminophen every 2 h for a total dose of 1.2 g over the 24-h period. Single breaths of expired air were collected through a one-way valve into an expandable bag from which air was transferred to a glass vacuum tube for analysis of $^{13}CO_2$ as described previously [17]. Urine samples were obtained ad libitum but especially at 2–4 h intervals. Blood was obtained from a forearm vein through an indwelling catheter. The subjects' fast continued for 8 h after the galactose was administered at which time a galactose-restricted meal was given. The subjects lay in bed or sat in chairs but were ambulatory to urinate.

Table 1
Metabolic characterization of subjects

Subject (Age)	Genotype	GALT U/gr Hb/hr	RBC			Plasma			Urine	
			gal-1-P (mg/dL)	Galactitol (μM)	Galactonate (μM)	Galactose (μM)	Galactitol (μM)	Galactonate (μM)	Galactitol (mmol/mole) creatinine	Galactonate (mmol/mole) creatinine
Normal										
#1 (25 yr)		22.88	nd[a]	0.38	3.80	0.35	nd	nd	2	4
#2 (23 yr)		25.23	nd	1.63	15.57	0.26	nd	nd	4	6
Galactosemic										
#1 (40 yr)	Q188R/Q188R	0	2.95	10.60	5.93	1.58	24.00	4.61	132	31
#2 (40 yr)	Q188R/Q188R	0	4.72	17.84	13.60	1.36	39.20	nd	176	45
#3 (20 yr)	Q188R/K258N	0.41	3.43	11.35	11.10	1.05	29.40	nd	143	19

[a] nd = non-detectable.

Tubes containing air samples were kept at room temperature until analyzed. Urine and blood samples were refrigerated until urine volumes were measured and aliquots frozen. Blood was centrifuged to separate red cells and plasma which were then frozen at −80° until analyzed.

Determination of acetaminophen by ^{13}C and ^{1}H NMR

A combination of proton and carbon NMR techniques enables the determination of the amount of acetaminophen glucuronide formed and its isotopic enrichment, the total excretion of acetaminophen and partition of its adducts [26]. The NMR analyses were performed on a Bruker Advance 400 wide bore instrument. A 5-mm inverse proton probe and a 5-mm $^{1}H/^{13}C$ dual probe were used for proton and carbon spectra, respectively.

Quantitative proton NMR of intact urine allowed the measurement of the amount of acetaminophen glucuronide in each urine sample as well as the distribution of acetaminophen excretion products, glucuronide, sulfate and *N*-acetylcysteinyl adducts. The assignment of NMR resonances to each of these metabolites was done according to Bales et al. [26]. Proton spectra of intact urine were obtained with the following conditions: 0.6 ml of intact urine was introduced into a 5-mm NMR tube together with a sealed capillary of trimethylsilylpropionic acid (TSP) solution in D_2O. The capillary had been previously calibrated against citrate solutions of known concentration. This external standard has a triple function: the deuterium resonance is used for the field-frequency lock, the TSP resonance serves as chemical shift reference and allows for quantitation of the metabolites. Standard quantitative acquisition conditions were as follows: 45° pulse width (PW), 8.8 s repetition time (TR), water saturation during the relaxation delay, spectral width (SW) 6775 Hz, 64K data points (TD), 0.103 Hz/Pt digital resolution, 64–128 scans i.e., 10–20 min of data collection. Spectra were Fourier transformed without line broadening. The integral routine of the XWINNMR Bruker spectroscopy software was used to integrate each signal after careful baseline correction. Fig. 1 shows the aromatic region (8–7 ppm) and the acetyl region (2.15 ppm) of a urine proton spectrum 11 h after the start of acetaminophen ingestion. The resonances of acetaminophen glucuronide are easily identified and

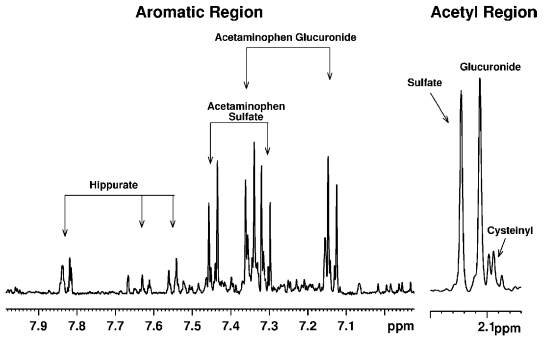

Fig. 1. Proton NMR spectrum of a urine sample 11 h after the start of acetaminophen ingestion. The resonances of each acetaminophen adduct are clearly identified and quantified.

quantified as well as those of the sulfate and cysteinyl derivative. Fig. 2A shows the cumulative excretion of acetaminophen in a galactosemic patient over 24 h with administration of 100 mg acetaminophen every 2 h and the excretion rate (mg/h) which remains constant from the time of galactose administration until the end of the experiment. The distribution of acetaminophen adducts is given in Fig. 2B. The acetaminophen sulfate is the first metabolite to appear. After 2–3 h an equilibrium is reached in which the proportions of glucuronide and sulfate are of the same order of magnitude but the *N*-acetylcysteinyl derivative remains a minor component.

Carbon spectra of concentrated urine provided a rapid way of measuring the ^{13}C enrichment of acetaminophen glucuronide. Indeed, in the carbon spectrum of a solution of synthetic acetaminophen glucuronide recorded under approximately fully relaxed conditions and broadband proton decoupling, the intensity per protonated carbon is essentially the same for all carbons in the molecule. This implies that the nuclear Overhauser effect (NOE) is the same within experimental error for all protonated carbons. However, in a urine sample 7 h after [2-^{13}C]galactose administration (Fig. 3), the intensity of carbon-2 of the glucuronide moiety is significantly higher due to ^{13}C enrichment compared to that of other carbons of the same molecule at natural abundance. The relative increase of the C-2 carbon is a measure of the ^{13}C enrichment. For the sake of clarity, we chose a numbering of the glucuronide carbons

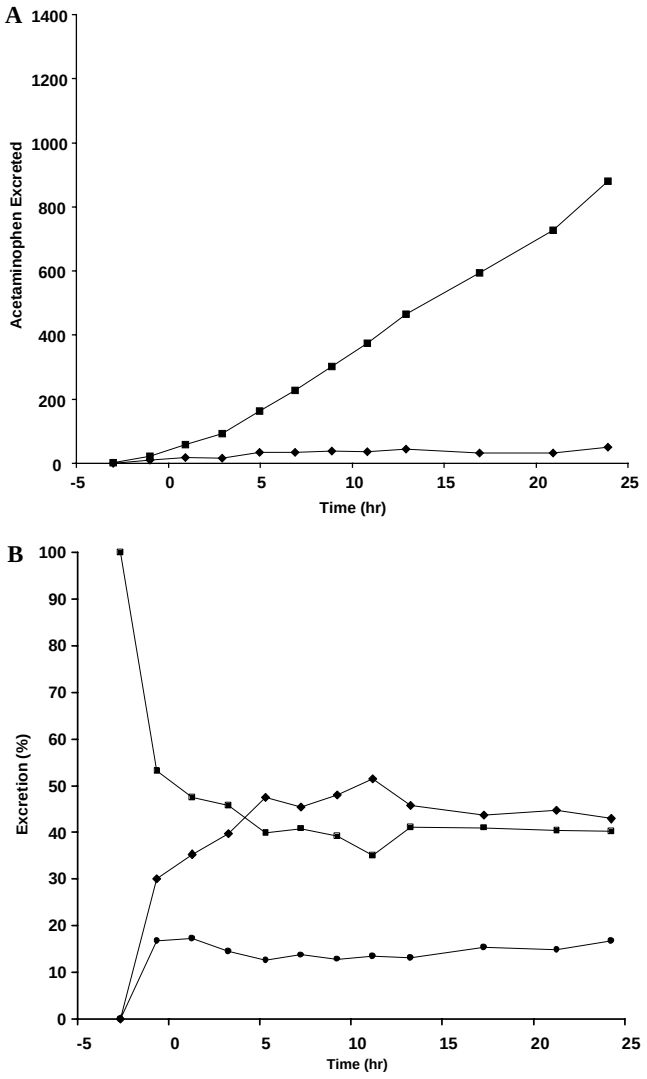

Fig. 2. (A) Total acetaminophen excretion by a galactosemic patient over the course of the 24-h experiment. ■, Cumulative excretion (mg); ◆, excretion rate in mg/h. (B) Excretion (in % of total) of the three major acetaminophen metabolites over the course of the 24-h experiment. ■, sulfate; ◆, glucuronide; and ●, *N*-acetylcysteinyl.

in acetaminophen glucuronide that parallels the numbering of the carbons in galactose. Assignments of the glucuronide carbons were based on a pH variation assuming the carbons closest to the acid function are the most shifted by the pH change. Carbon-13 spectra of the various urine samples were obtained in the following way. Samples of 5–10 ml of intact urine were lyophilized to dryness and in turn dissolved in either 0.25 or 0.5 ml of D_2O. The 250 µl samples were introduced into a Shigemi tube whereas for the 500-µl samples a regular 5 mm NMR sample tube was used. Carbon spectra were acquired under the following acquisition conditions: PW 45°, TR 2 s, SW 25 KHz, TD 64K. Depending on the concentration of the sample, the number of scans varied from 1800 to 25,000, which corresponds to 1 h to an overnight acquisition. Spectra were Fourier transformed with a gaussian window (LB −1 Hz; GB 0.1). Signals were integrated using the integral routine of the XWINNMR

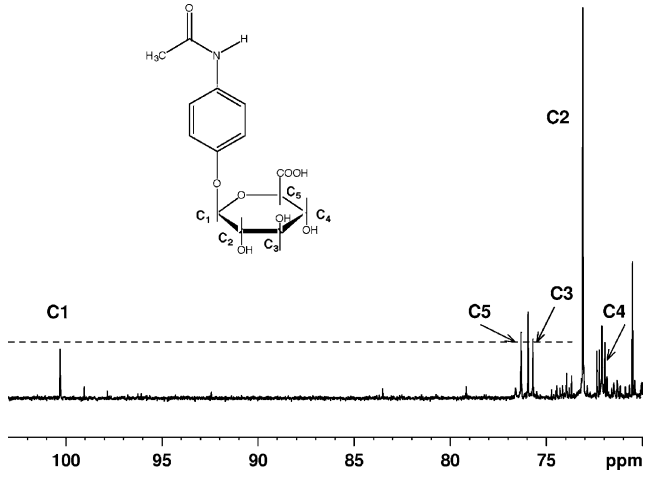

Fig. 3. Partial carbon spectrum of an urine sample 7 h after [2-^{13}C]galactose ingestion showing the signals of the acetaminophen glucuronide carbohydrate carbons. The significantly increased intensity of carbon C-2 relative to the other carbons of the same molecule results from enrichment in ^{13}C.

Bruker spectroscopy software after careful baseline correction. To calculate the average intensity per carbon at natural abundance, we used the carbon signal ortho to the carbon bearing the oxygen on the aromatic ring (δ 117.3 ppm; 2 carbons) and the anomeric carbon (δ 100.5 ppm; 1 carbon). Other carbon signals from the glucuronide ring (C-3; C-4; and C-5) overlap with signals of unknown carbohydrates or hydroxylated compounds and their respective intensities are randomly increased. It is therefore not possible to include these signals in the calculation of the average intensity per carbon. Knowing the amount of acetaminophen glucuronide in each urine sample and its enrichment, it is possible to calculate the amount of [^{13}C]galactose metabolized during the time elapsed between urine collections.

Other analyses

The measurement of $^{13}CO_2$ in expired air of each breath sample was measured by automated gas-isotope ratio mass spectrometry by Metabolic Solutions, Nashua, NH, and the production rate of $^{13}CO_2$ and quantitation of cumulative excretion over 24 h calculated as described previously [17]. Enrichment of ^{13}C in plasma glucose was determined by GC–MS employing the aldononitrile pentaacetate derivative [5]. Galactose metabolites in RBC and urine were determined by isotope dilution GC–MS methods [7,27].

Results

Oxidation to $^{13}CO_2$

The oxidation of [2-^{13}C]galactose to $^{13}CO_2$ over 24 h is shown in Fig. 4. Fig. 4A shows that the rate of $^{13}CO_2$ formation in the two normals is rapid and reaches a peak at about 2–3 h while the three galactosemic patients slowly

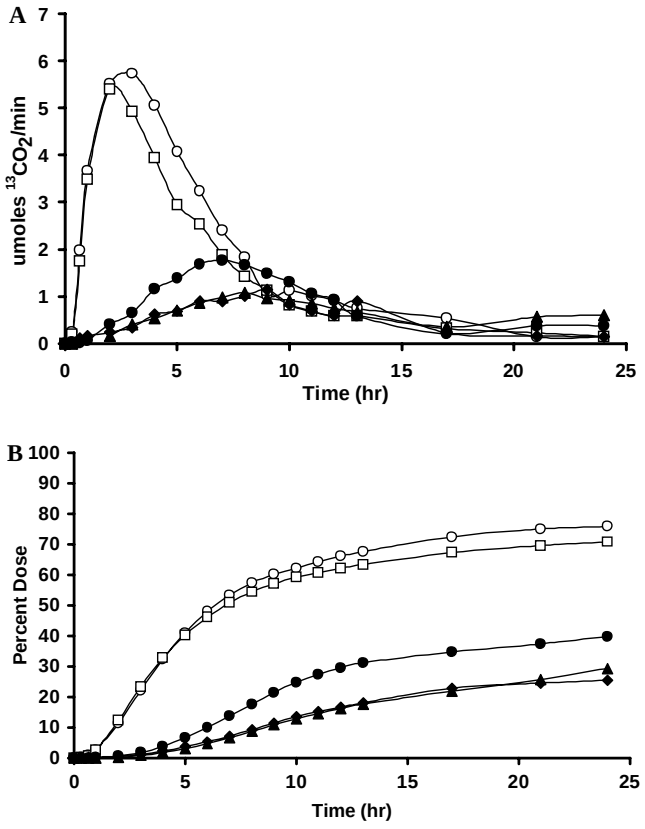

Fig. 4. The oxidation of [2-^{13}C]galactose to $^{13}CO_2$. (A) Rate of $^{13}CO_2$ expired per minute. (B) The percent of the dose excreted as $^{13}CO_2$ over the period of study. ○, Normal subject #1; □, normal subject #2; ▲, galactosemic patient #1; ◆, galactosemic patient #2; and ●, galactosemic patient #3.

oxidize galactose after a lag with a broad peak at 6–10 h. The rate of $^{13}CO_2$ formation by the galactosemic subject is about 10% that of the normal after the lag period. The time curve of cumulative $^{13}CO_2$ excretion over 24 h of the two normals and three galactosemics can be seen in Fig. 4B with cumulative values in Table 2. Whereas the normals excreted 76.7 and 71.7% of the dose, the 40-year-old galactosemics with the Q188R/Q188R genotype oxidized 29.3 and 25.4% of the dose while the 20-year-old with the Q188R/K258N genotype oxidized 39.8% of the dose.

^{13}C in acetaminophen glucuronide

In normal controls acetaminophen glucuronide was rapidly enriched as shown in Fig. 5A, one subject having a peak enrichment of over 5% at 2 h after [2-^{13}C]galactose was ingested, the other with a peak enrichment of 3.9% at 3 h. All three galactosemic patients incorporated isotope from galactose indicating that they too had converted galactose to UDPglu. The incorporation of label into glucuronide by the galactosemic subjects was slower than normal with a broad peak enrichment 5–10 h after galactose was administered. The two 40-year-old Q188R/Q188R patients reached over 5% enrichment while the 20-year-old Q188R/K258N patient had over 7% enrichment of the glucuronide with an earlier sharper peak than the others. The time curves of the ^{13}C labeling of the glucuronide paralleled the curves of the excretion of $^{13}CO_2$ in both normals and galactosemics as shown in Fig. 4.

Fig. 5B shows the cumulative excretion of [^{13}C]galactose as glucuronide. Table 2 enumerates the total amount of acetaminophen glucuronide excreted, the amount of galactose converted to glucuronide and the percent of the dose of galactose that was converted to glucuronide. The total mmol of acetaminophen excreted as glucuronide was similar in the normals and galactosemics, 3.16 and 3.32 mmol in the normals and 3.81, 4.08, and 2.20 in the galactosemics. The amount of galactose converted to glucuronide was markedly different in the normals and galactosemics being 4.98 and 4.63 mg in the normals and 12.6, 17.1, and 20.9 in the galactosemics which represented 0.9% of the galactose dose in the normal and 2.4, 3.3, and 4.3% in the galactosemic patients.

Galactose oxidized to CO_2 and incorporated into glucuronide

There was a marked difference in the ratio of the amount of galactose oxidized and the amount excreted

Table 2
Metabolic disposition of galactose

Subject	Galactose dose (mg)	Recovered in				Total recovered (%)	Ratio (air/urine)	Total acetaminophen glucuronide (mmol)
		Air[a]		Urine[b]				
		mg	%	mg	%			
Normal								
#1	571	432.8	75.8	5.0	0.9	76.7	86.6	3.32
#2	506	358.2	70.8	4.6	0.9	71.7	77.9	3.16
Galactosemic								
#1	525	153.8	29.3	12.6	2.4	31.7	12.2	2.20
#2	522	132.6	25.4	17.1	3.3	28.7	7.8	4.08
#3	490	195.0	39.8	20.9	4.3	44.1	9.3	3.81

[a] [^{13}C]Galactose converted to $^{13}CO_2$.
[b] [^{13}C]Galactose converted to labeled acetaminophen glucuronide.

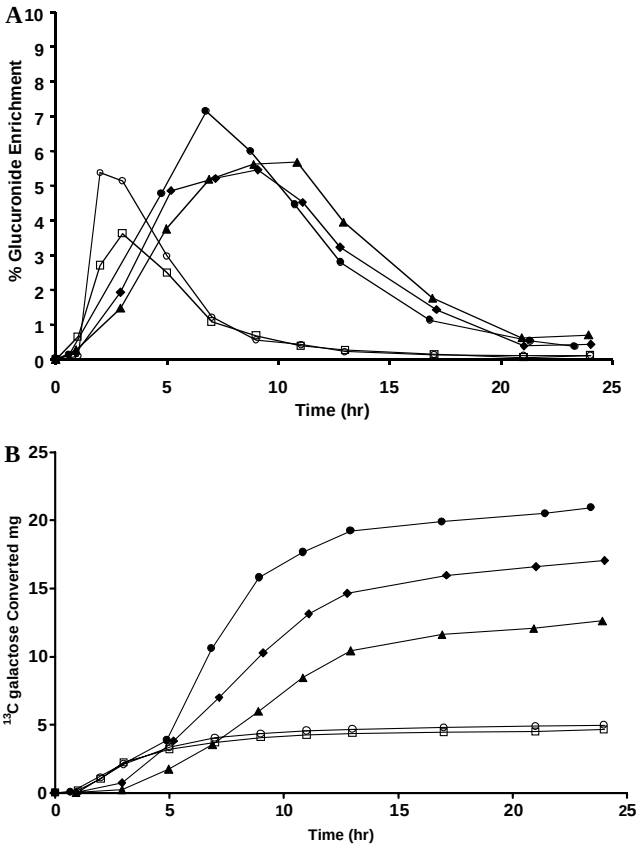

Fig. 5. (A) Acetaminophen glucuronide enrichment after ingestion of [2-^{13}C]galactose over a period of 24 h in normal urines and in galactosemic urines. (B) Total amount of galactose converted to acetaminophen glucuronide. ○, Normal subject #1; □, normal subject #2; ▲, galactosemic patient #1; ◆, galactosemic patient #2; and ●, galactosemic patient #3.

as glucuronide in the normals, 86.6 and 77.9 vs 12.2, 7.8 and 9.3 in the galactosemics. Table 2. The total amount of the dose recovered by these two routes averaged 74.2% in the normals. It was 31.7 and 28.9% in the two 40-year-old Q188R homozygous galactosemics and 44.1% of the dose by the compound Q188R/K258N patient. Compared to the total metabolized by the two routes in the two normals (74.2% of the dose) the two Q188R/Q188R patients metabolized 42.7 and 38.7% while the Q188R/K258N patient metabolized 59.4% of the normal capacity.

Enrichment of blood glucose

The blood glucose of both normal controls showed rapid enrichment of plasma glucose with a maximum at 30 min after [^{13}C]galactose administration of 2.21 and 2.75% with an exponential decline to no detectable enrichment at 300 min, Fig. 6. The galactosemic patients had no detectable enrichment of plasma glucose for 300 min. Enrichment was detected subsequently at a peak level of between 0.15 and 0.16% in the 40-year-old Q188R homozygous patients and 0.31% in the Q188R/K258N 20-year-old and remained detectable for about 3 h, Fig. 6. The timing of the glucose enrichment by the galactosemics

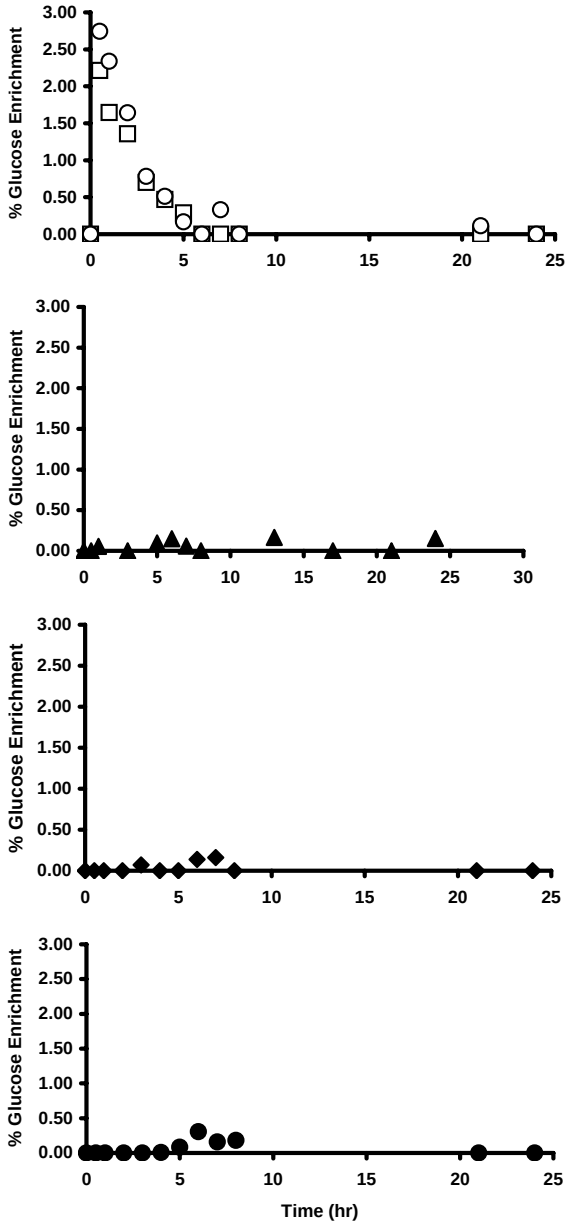

Fig. 6. The ^{13}C enrichment of plasma glucose after [2^{13}C]galactose administration to normal subjects ○, subject #1; □, subject #2 and to galactosemic patients. ▲, Patient #1; ◆, patient #2; and ●, patient #3.

corresponded to the increasing rate of oxidation of galactose to $^{13}CO_2$ and preceded the peak in enrichment of the urine glucuronide. The peak glucose enrichment in the normals at 30 min also preceded the peak in the excretion of $^{13}CO_2$ in expired air as seen in Fig. 4A.

Discussion

We have demonstrated for the first time by what we have termed a "metabolic liver biopsy" that galactosemic subjects homozygous for the Q188R mutation or with a compound heterozygosity for its Q188R/K258N form labeled UDPglu from orally administered [2-^{13}C]galactose. The data imply that the classic galactosemic possesses residual

GALT activity or some other pathway for forming UDPglu from galactose. In addition to showing the time dependent labeling of the hepatic UDPglu pool, we have correlated the pattern with the oxidation of the isotope to $^{13}CO_2$ and the ^{13}C enrichment of circulating glucose. The comparison of how galactosemics metabolize galactose compared to normal subjects provides insight into the pathway(s) involved.

The labeling of the hepatic UDPglu pool was determined by the concomitant administration of [2-^{13}C]galactose and acetaminophen and a combination of proton and carbon NMR techniques to quantify and assess the isotope enrichment of urinary acetaminophen glucuronide. The protocol employed was based on the work of others [25,28–30] who validated the turnover of the hepatic UDPglu pool with constant infusions of tracer amounts of either ^{14}C or [^{13}C]galactose and the administration of acetaminophen to form acetaminophen glucuronide. The rationale for this is shown in Fig. 7, where UDPglu is oxidized in the liver by UDPglu dehydrogenase to form UDP glucuronic acid. The acetaminophen is handled in the liver for urinary excretion by conjugation with glucuronide by the enzyme UDPglucuronyltransferase. The excretion of acetaminophen glucuronide studied by proton NMR is well established [26]. Glucuronide formation accounts for about 60% of the handling in subjects over age 10 while sulfation is the primary excretion product in younger children [24]. The use of NMR to detect ^{13}C enrichment and quantitate acetaminophen glucuronide is a new application of NMR for our study. There is great advantage to the NMR technique in that no sample preparation is required and urine is used without derivatization. NMR permits the quantitation of the acetaminophen metabolites, the enrichment of ^{13}C acetaminophen glucuronide, the total excretion of the compound and the calculation of administered galactose converted to glucuronide. The NMR study of ^{13}C enrichment is best served when a single carbon is labeled. 1 or [2-^{13}C]galactose are the best substrates. [2-^{13}C]Galactose was selected for this study since oxidation to $^{13}CO_2$ was also assessed and we wished to eliminate direct oxidation of carbon 1 that could occur via the galactonate pathway. The use of a single labeled carbon also enables the determination of metabolic labeling of other carbons in glucuronide as a result of other metabolic pathways. In our study, the acetaminophen glucuronide was only labeled in the position corresponding to [2-^{13}C]galactose throughout the 24-h study suggesting that there was no breakdown and resynthesis of UDPglu that would result in scrambling of the labeled carbon.

Our first study was performed in a normal control whose enriched UDPglu reached over 5% APE and we wondered if there was a small amount of residual GALT whether the enrichment in UDPglu would be detectable. To our surprise the galactosemic patients not only showed comparable enrichment, albeit more slowly, but sustained it over a longer period of time. Three to four times as much galactose was incorporated into the hepatic UDPglu pool by galactosemics than by normals. This was not a reflection of the same amount of galactose entering a smaller than normal UDPglu pool in galactosemics. The UDPglu levels of RBC of galactosemics has been shown to be normal [31,32] and the hepatic UDPglu content of GALT deficient mice does not differ from normal [33]. We assume that the lesser incorporation in the normal is a reflection of the rapid rate of galactose metabolism.

There are two possible pathway explanations for the conversion of labeled galactose to hepatic UDPglu. The first is that there is residual GALT activity in the Leloir route which slowly labels UDPglu. The second is that there is an ancillary pathway by which galactose is converted to UDPglu, namely, the activity of UDPglu pyrophosphorylase to convert gal-1-P by reacting with UTP to form UDPgal which is then epimerized to UDPglu. Indeed, a combination of these possibilities may exist. The expected fate of the [2-^{13}C]galactose provides an indication of how the molecule traverses the normal Leloir pathway of galactose metabolism shown in Fig. 8A. After phosphorylation of galactose, ^{13}C gal-1-P reacts with UDPglu to form UDPgal and glucose-1-phosphate (glu-1-P), [^{13}C]UDPgal is converted to [^{13}C]UDPglu which becomes labeled. The

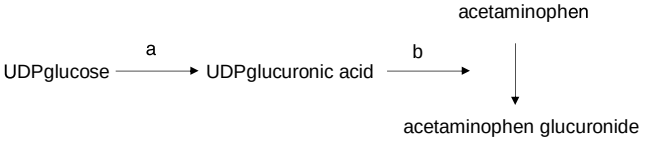

Fig. 7. The pathway of acetaminophen metabolism in the liver by formation of acetaminophen glucuronide for urinary excretion. (a) UDPglu dehydrogenase reaction, UDPglu plus 2NAD to form UDPglucuronic acid + 2 NADH; (b) glucuronyl transferase.

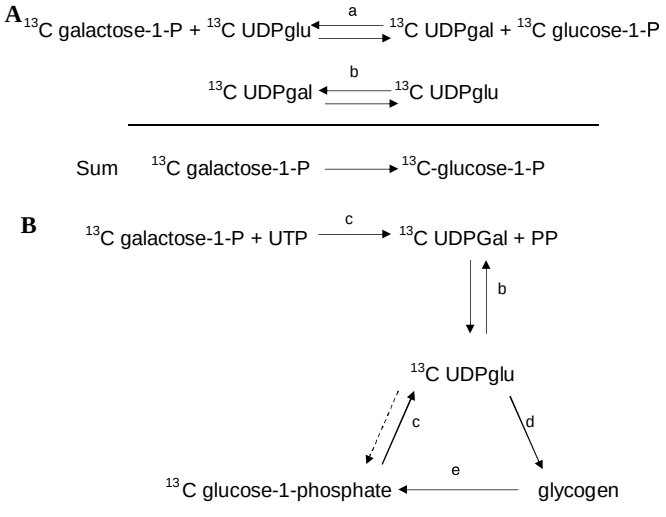

Fig. 8. (A) The Leloir pathway of galactose metabolism. (a) GALT; (b) UDPgal-4-epimerase. (B) The "UDPgal pyrophosphorylase" pathway and routes of glu-1-P formation. (b) UDPgal-4-epimerase; (c) the action of UDPglu pyrophosphorylase reacting with gal-1-P and UTP to form UDPgal or with glu-1-P to form UDPglu; (d) glycogen synthase; and (e) phosphorylase.

[^{13}C]UDPglu then reacts with labeled gal-1-P to form labeled glu-1-P. Eventually, all of the compounds become labeled with ^{13}C as shown in Fig. 8A. The overall sum of the series is that labeled gal-1-P is converted to labeled glu-1-P. The latter is converted to glucose-6-P which is hydrolyzed to glucose. Our data are consistent with this in normal subjects in that at 30 min the plasma glucose is highly enriched. With a plasma glucose of 80 mg/dL and assuming a glucose space of 20% body weight, we can estimate that, with an enrichment of glucose of 2.2 and 2.7, 50% of administered galactose had been converted to circulating glucose at 30 min. The rapid oxidation of galactose to $^{13}CO_2$ by normals results from the oxidation of labeled plasma glucose. It has been known that administration of large galactose loads results in a transient rise in blood glucose [34] and that the curves of oxidation of ^{14}C labeled galactose to $^{14}CO_2$ are similar to the resulting curves when ^{14}C labeled glucose is given [35,36]. Apparently, with the rapid traverse of galactose through the pathway the UDPglu is rapidly labeled but to a small extent since the pool turns over rapidly and the galactose is rapidly oxidized to $^{13}CO_2$ via the resulting glu-1-P and glu-6-P conversion to glucose.

The pattern of [^{13}C]galactose disposition by galactosemics has similar elements to that in normals but with a time shift. There is a slower rate of oxidation to $^{13}CO_2$ peaking about 10 h with labeling of the UDPglu pool parallel to the oxidation and a small extent of plasma glucose labeling somewhat preceding the increasing oxidation to $^{13}CO_2$. This could be explained by a low residual activity of GALT slowly handling the [^{13}C]galactose administration. On the other hand, it could be explained by the formation of UDPgal by the catalytic activity of "UDPgal pyrophosphorylase" with gal-1-P and UTP to form UDPgal which would circumvent the loss of GALT in the Leloir pathway (Fig. 8B) and with subsequent formation of UDPglu. Isselbacher [37] demonstrated this reaction in rat liver as an alternate pathway of galactose disposition. Abraham and Howell [38] and Shin et al. [39] found it to be of minute activity in human liver. Koop and Hanson [40] found that crystalline UDPglu pyrophosphorylase whose prime substrate is glu-1-P has about 5% activity with gal-1-P. Although of minute activity by in vitro assay of liver extract Leslie et al. [41] estimated that when amplified to the whole organ activity it could explain the limited ability of the GALT knock-out mouse to oxidize galactose.

The big difference of the "UDPgal pyrophosphorylase" activity compared with that of GALT and the Leloir pathway is that in the latter gal-1-P is converted to glu-1-P which readily forms circulating glucose while the UDPgal pyrophosphorylase reaction converts gal-1-P to UDPgal and thus UDPglu without the direct formation of glu-1-P. The oxidation of galactose to CO_2 in the pathway depends on the metabolism of UDPglu and its possible breakdown to glu-1-P. As shown in Fig. 8 although UDPglu pyrophosphorylase is an equilibrium reaction in which UDPglu could react with PP to form glu-1-P, thermodynamically with low cellular PP due to PP hydrolysis the reaction is thought to be in favor of UDPglu synthesis [42] as it does with gal-1-P which is the alternative to GALT. If this be the case, the fate of UDPglu formed from UDPgal via pyrophosphorylase is conversion to glycogen. Glycogen hydrolyzed forms glu-1-P (Fig. 8B). This round about route to glucose formation and oxidation might explain the delayed conversion of 2-^{13}C gal to $^{13}CO_2$.

As far as we know, the only route of [2-^{13}C]galactose oxidation is via the oxidation of labeled glucose to which it is converted. 2-^{13}C gal was chosen for this study instead of [1-^{13}C]galactose which has been the substrate in previous assessments of the ability of galactosemics to oxidize galactose to eliminate the possible direct oxidation of C-1 via the galactonate pathway described by Cuatrecasas and Segal [18]. This would overestimate the oxidation and underestimate the amount of galactose converted to UDP glucuronide. It is possible that with the oxidation of C-1 of the [2-^{13}C]galactose the resulting [1-^{13}C]xylulose could be converted to 1,3^{13}C labeled glucose by the hexosemonophosphate pathway of glucose metabolism, thus contributing to the oxidation to labeled $^{13}CO_2$ and glucuronide. This seems unlikely since throughout the study, the isotope in glucuronide remained in the C-2 position with no evidence of carbon recycling.

The impetus for this study was the fact that lymphoblasts from galactosemic subjects homozygous for the Q188R mutation were able to form labeled UDPgal and UDPglu when incubated with [^{13}C]galactose [23]. The supposition that these cells possessed some GALT activity resulted from the fact that cells from galactosemics with deletion of the GALT gene did not have the same ability. The interpretation of the pathway(s) involved in our study of galactosemic subjects should be resolved by examination of patients with deletion of the GALT gene for acetaminophen glucuronide labeling since they would not have residual GALT activity. The fact that a patient with GALT deletion is able to oxidize galactose to CO_2 suggests that they do possess an alternate route of galactose metabolism not related to GALT activity [19]. Should future studies reveal that galactosemics have residual GALT activity, it would suggest that the expression of the Q188R mutation in human liver differs from the expression in yeast [21] and bacterial [22] systems.

There are other unanswered questions. Is there a variation in the pathway(s) depending on the GALT mutation? The patient heterozygous for Q188R/K258N oxidized more galactose and formed more labeled UDPglu than the Q188R homozygous. Is there a variation within patients with the homozygous Q188R mutation and do African American galactosemics with the S135L mutation who do oxidize galactose almost normally have a pattern of metabolism similar to the normal? The study of more galactosemics with various genotypes would, indeed, be important. The delineation of these pathways in galactosemic patients may pave the way for new therapeutic strategies in the treatment of this enigmatic disease.

The application of the protocol and techniques described here may be applied to the study of another defect in

galactose metabolism, UDP Gal-4-epimerase deficiency [43,44]. It would be useful in answering the question of how patients with UDP Gal-4-epimerase deficiency who cannot convert UDPgal to UDPglu handle galactose and if there is a genotype correlation with ability to metabolize galactose. The study of the ability of the liver to form UDPglucose from labeled galactose should answer the question of the extent of hepatic involvement in relation to the metabolic defect seen in RBC and lymphoblasts which has been a vexing problem in comprehending the epimerase deficiency disorder, its systemic [45,46] and peripheral nature [47].

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