

**Galactose-1-phosphate uridylyltransferase and galactokinase activity in cultured human diploid fibroblasts and peripheral blood leukocytes: *I. Analysis of transferase genotypes by the ratio of the activities of the two enzymes***

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*J Clin Invest.* 1969;48(12):2390-2397. <https://doi.org/10.1172/JCI106205>.

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The kinetic properties of fibroblast galactokinase were examined. Galactose-1-phosphate inhibits galactokinase activity in both normal and galactosemic cell strains, whereas other glycolytic intermediates have no effect.

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# Galactose-1-Phosphate Uridyltransferase and Galactokinase Activity in Cultured Human Diploid Fibroblasts and Peripheral Blood Leukocytes

## I. ANALYSIS OF TRANSFERASE GENOTYPES BY THE RATIO OF THE ACTIVITIES OF THE TWO ENZYMES

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**ABSTRACT** The specific activities of galactokinase and galactose-1-phosphate uridyltransferase were determined in peripheral blood leukocytes directly after separation from whole blood, and in cultured skin fibroblasts at various times during the subculture growth period. Growth curves were obtained for fibroblasts based on three different parameters: direct cell counts, total protein, and total deoxyribonucleic acid (DNA) content. At the time in culture when the specific activity of both enzymes was maximal and least variable, the ratio of transferase to galactokinase correlated well with the transferase genotypes of the original tissue donors. Leukocyte transferase: galactokinase ratios gave a similar distribution pattern.

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The kinetic properties of fibroblast galactokinase were examined. Galactose-1-phosphate inhibits galactokinase activity in both normal and galactosemic cell strains, whereas other glycolytic intermediates have no effect.

There was no detectable transferase activity in eight galactosemic ( $Gt^g/Gt^g$ ) cell strains when transferase activity was maximal in cell strains of other transferase genotypes. Inhibitors responsible for the absence of transferase activity could not be demonstrated. In ad-

dition, transferase activity in galactosemic cell lysates was not observed in cells during logarithmic growth; measurable uridine diphosphate galactose (UDPgal) pyrophosphorylase activity was found in human diploid fibroblast cultures, as well as significant levels of endogenous uridine triphosphate (UTP) in lysates of fibroblast cultures.

## INTRODUCTION

Galactokinase catalyzes the conversion of galactose to galactose-1-phosphate (gal-1-P), while galactose-1-phosphate uridyltransferase (transferase) forms uridine diphosphate galactose (UDPgal) and glucose-1-phosphate (glu-1-P) from galactose-1-phosphate and uridine diphosphate glucose (UDPglu). Transferase activity is lacking in galactosemia, a metabolic disorder in man that conforms to autosomal mendelian inheritance (1, 2). The two transferase alleles appear to display dosage effects in human cells; activity is quantitatively expressed as the summation of two genes, either normal or mutant. Two mutant alleles are recognized: the galactosemic allele ( $Gt^g$ ) has no detectable transferase activity, and the Duarte variant allele ( $Gt^D$ ), described by Beutler, Baluda, Sturgeon, and Day (3), has approximately 1/3 the activity of the normal allele (4). Thus, with support from family studies, there are three alleles and six genotypes which can be identified by quantitation of transferase activity (5).

This report establishes the use of cultured human diploid fibroblasts as an experimental tool for investi-

*Received for publication 7 July 1969 and in revised form 10 August 1969.*

gating the nature of the defect in galactosemia by demonstrating that cell strains reflect their individual donors at the transferase locus, as determined by transferase: galactokinase ratios. Supplemental data suggest that a mechanism does exist in cultured fibroblast lysates for the nontransferase conversion of gal-1-P to UDPgal.

## METHODS

### Reagents

Adenosine triphosphate (ATP), nicotinamide-adenine dinucleotide (NAD), nicotinamide-adenine dinucleotide phosphate (NADP), gal-1-P, glu-1-P, UDPglu, UDPgal, UDPglu dehydrogenase, phosphoglucomutase, and glucose-6-phosphate (glu-6-P) dehydrogenase were all obtained from Sigma Chemical Co. Dithiothreitol (DTT) was from Calbiochem. D-galactose-1-<sup>14</sup>C (SA 8.15  $\mu\text{C}/\mu\text{mole}$ ) was obtained from New England Nuclear Corp. Whatman DE81 was used as diethylaminoethyl cellulose (DEAE-cellulose) anion exchange paper.

**Equipment.** Spectrophotometric determinations were performed with either a Hitachi Perkin-Elmer model 139 or a Gifford converted Beckman spectrophotometer. Fluorimetric determinations were performed with an Eppendorff fluorimeter containing a 313 + 366  $m\mu$  excitation filter and a 400–3000  $m\mu$  output filter. The fluorimeter was equipped with a zero suppression, scale-expander unit (Registrieradapter 2134) and a Honeywell recorder. All radioactive counting was done in a Packard model 3320 scintillation spectrometer. All centrifugation was performed at 4°C with either an International model PR-2, or a Sorvall model RC2-B centrifuge.

### Growth and maintenance of human diploid cell cultures

Primary cultures were started from skin biopsies obtained with the drill described by Davidson, Brusilow, and Nitowsky (6) and cell strains were propagated as described by Hayflick and Moorhead (7). The medium used was basal medium Eagle (BME) with twice the concentrations of amino acids and vitamins in Earle's balanced salt solution, supplemented with fetal calf serum (10% v/v), penicillin (60 U/ml), streptomycin (60 U/ml), and aureomycin (50  $\mu\text{g}/\text{ml}$ ). Growth experiments were performed in either 160 ml milk dilution or in 1-liter Blake bottles inoculated with  $0.2\text{--}1.0 \times 10^6$  cells. These cultures had a complete change of medium every 3–4 days. Cells were harvested, counted, and DNA was determined as previously described (8). Total cell protein was determined by the method of Lowry, Rosebrough, Farr, and Randall (9). The 18 control cell lines used in these studies were from donors who were identified as nonmutants at the transferase locus (Gt<sup>+</sup>/Gt<sup>+</sup>). These donors were both healthy and diseased; the diseases of the donors had no apparent effect on either cell growth or the galactokinase or transferase activities of their cell lines.

### Preparations of lysates

After harvesting by trypsinization, cultured fibroblasts were centrifuged and resuspended in water at  $20 \times 10^6$  cells/ml. The suspensions were rapidly freeze-thawed four times, then centrifuged at 20,000 *g* for 10 min. The supernatant solutions were used in the enzyme assays.

Peripheral blood leukocytes were obtained from dextran-sedimented heparinized whole blood and prepared as pre-

viously described (10). Leukocyte lysates were prepared at a concentration of  $50 \times 10^6$  cells/ml in a manner similar to that described for fibroblasts. Cell counts, DNA, and protein determinations were as described for fibroblasts.

### Enzyme assays

**Galactokinase.** The assay procedure was essentially the Cuatrecasas and Segal (11) modification of the Sherman and Adler (12) method. The incubation mixture contained the following reactants in the concentrations stated and in a total volume of 0.2 ml: 0.2 M Tris-HCl, pH 7.6; 5 mM DTT; 6 mM ATP; 5 mM NaF; 0.4 mM 4-galactose-1-<sup>14</sup>C; and 50  $\mu\text{l}$  of enzyme preparation. Incubations were carried out at 37°C and reactions were run for either 15 or 30 min or both. Preincubation of buffered enzyme preparations with DTT was found to be necessary for optimal activity determinations. Galactose-<sup>14</sup>C was routinely added to start the reactions and the reactions were stopped by placing a 10  $\mu\text{l}$  aliquot of the reaction mixture on DEAE-cellulose anion exchange paper. The procedures for chromatography and scintillation counting were as described (11).

**Galactose-1-phosphate uridylyltransferase.** Assays by the UDPglu consumption method were as previously described (13). Fibroblast and leukocyte lysates were first buffered and then preincubated with DTT in order to minimize non-transferase UDPglu consumption (14).

The rate reaction method described by Isselbacher (15) which couples the transferase reaction with phosphoglucomutase and glu-6-P dehydrogenase was also used. This method was used in a final reaction volume of 0.5 ml and the reaction rates at 27°C were recorded with an Eppendorff fluorimeter.

**UDPglu and UDPgal pyrophosphorylase.** These activities were measured fluorimetrically at 27°C in a reaction system coupled with added UDPglu dehydrogenase. The reaction system contained the following reactants in the concentrations stated and in a final volume of 0.5 ml: 0.1 M glycylglycine, pH 8.7; 4.0 mM MgCl<sub>2</sub>; 10 mM NAD; 100 U of UDPglu dehydrogenase; 0.5 mM uridine triphosphate (UTP); and 0.6 mM glu-1-P or gal-1-P. The reactions were started by the addition of hexose-1-P. Controls were run with UTP deleted and with hexose-1-P deleted. In the presence of excess UDPglu dehydrogenase endogenous UDPgal-4-epimerase quantitatively converts UDPgal to UDPglu so that the same reaction system can be used to measure the rate of both UDPglu and UDPgal formation.

All enzyme assays were performed under conditions of maximal enzyme kinetics as tested for by linearity of reaction with both time and enzyme concentration.

### Measurement of endogenous UTP

Endogenous UTP was estimated with the UDPglu pyrophosphorylase reaction by deleting UTP from the reaction. The total change in fluorescence was proportional to the quantity of lysate in the cuvette after the addition of glu-1-P. Calculations were based on comparison with the fluorescence produced by appropriate increments of standard UTP.

## RESULTS

**Growth experiments.** The increase in cell population as measured by direct cell count, total cell protein and total DNA content is shown in Fig. 1. With an inoculum of  $1 \times 10^6$  cells into Blake-type culture bottles,

cell numbers were consistently maximal 10 days later. This increase represents four to five cell doublings. The curve in Fig. 1 is representative of the cell strains used in this study.

*Properties of fibroblast and leukocyte galactokinase.* The pH optimum for both fibroblasts and leukocytes was similar. Maximal activity was obtained at pH 7.5 with the following relative activities expressed as per cent of maximum: leukocytes, pH 6.5, 40.4%; 7.0, 85.8%; 7.5, 100%; 8.0, 61.4%; 8.5, 37.3%; fibroblasts, pH 6.5, 50%; 7.0, 91.2%; 7.5, 100%; 8.0, 55.2%; 8.5, 21.0%.

The  $K_m$  for ATP at 0.4 mM galactose was 0.2 mmole/liter for fibroblasts and 0.52 mmole/liter for leukocytes.

The  $K_m$  for galactose at 0.6 mM ATP in fibroblast preparations was found to be similar to that described for rat liver (0.15–0.91 mmole/liter) (11) with a range of 0.18–0.42 mmole/liter in six studies of three different cell strains. The galactose  $K_m$  of a leukocyte preparation was 0.17 mmole/liter.

Galactose-1-phosphate inhibition of galactokinase was observed in three control and three galactosemic cell strains. At 4.5 mM gal-1-P the three controls had 40, 50, and 53% inhibition while the three galactosemic strains had 41, 43, and 46% inhibition. In one experiment, 2.25, 4.5, and 9.0 mM gal-1-P caused 33, 48, and 62% inhibition of galactokinase activity respectively. One of the galactosemic cell strains was from a Negro male adult with "atypical" galactosemia (16) and the other two were from children of European origin.

The following compounds were tested as inhibitors of galactokinase at the concentrations indicated: 50 and 100 mM glucose; 1 and 5 mM glu-1-P; 1 and 5 mM glu-6-P; 1, 5, and 10 mM fructose-6-P; 5 mM pyruvate; 5 and 10 mM NADH. None of these had a significant effect on galactokinase activity.  $K_2HPO_4$ , 5 and 10 mmoles/liter, was added to reaction systems containing 5 mM

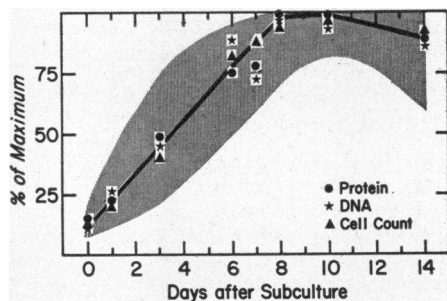


FIGURE 1 The increase in cell population after subculture measured by direct cell count, total protein, and DNA content. The points and curve represent the means of eight individual experiments. The shaded area shows the range of the individual determinations. Individual data were converted to per cent of maximum for each experiment.  $10^6$  cells = 12  $\mu$ g DNA = 300  $\mu$ g protein.

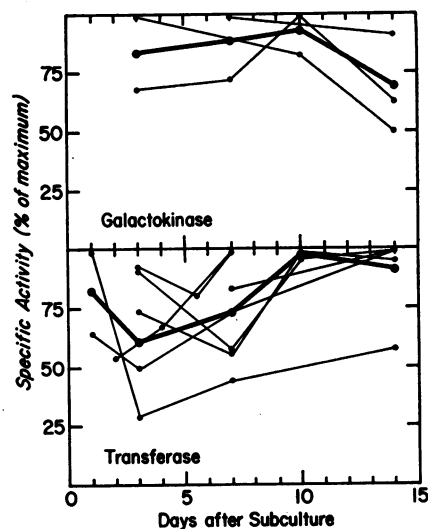


FIGURE 2 The specific activity of galactokinase and transferase at various times after subculture for control cell lines. The data were converted to per cent of maximum activity in each experiment. Bold points and lines represent the averages of individual experiments (fine points and lines).

gal-1-P to test the possibility that phosphate may reverse gal-1-P inhibition. Unlike the effect of phosphate on reversing the inhibitory effect of glu-6-P in the hexokinase reaction (17), phosphate has no effect on the inhibition of galactokinase by gal-1-P.

*Galactokinase and transferase activities during the culture cycle.* The specific activities of galactokinase and transferase were determined at various times after subculture as illustrated in Fig. 2. For the experiments depicted in Fig. 2, control cell lines were used. Both enzyme activities were consistently near maximal and least variable 10 days after subculture. Consequently, transferase:galactokinase ratios for all cell strains were based on assays with cells harvested 10 days after subculture. The cumulative data from our laboratory of galactokinase and transferase assays are recorded in Tables I and II respectively. Galactokinase activity observed for the three cell types (Table I) was not influenced by transferase genotype: 17 of 35 fibroblast lines, 15 of 34 leukocyte donors, and 16 of 42 erythrocyte donors were the Gt mutants used in this study. The transferase data are separated into the six Gt genotypes ( $Gt^+/Gt^+$ ;  $Gt^+/Gt^D$ ;  $Gt^+/Gt^G$ ;  $Gt^D/Gt^D$ ;  $Gt^D/Gt^G$ ;  $Gt^G/Gt^G$ ). The tables also contain assays of the same enzymes in leukocytes and erythrocytes for comparison.

*Relationship of transferase genotype to transferase:galactokinase ratio.* Cultures derived from 12 normals ( $Gt^+/Gt^+$ ), one Duarte variant heterozygote ( $Gt^+/Gt^D$ ), seven galactosemic heterozygotes (parents of galactosemics  $Gt^+/Gt^G$ ), two Duarte homozygotes ( $Gt^D/Gt^D$ ), one

TABLE I  
Galactokinase Activities in Human Fibroblast Cultures, Leukocytes, and Erythrocytes

Cell type	n	Mean	SD	Specific activity
Fibroblasts	35 (94)*	1.33 23.30	±0.70 ±10.84	μmoles gal-1-P formed/hr per mg DNA μmoles gal-1-P formed/hr per 10 <sup>9</sup> cells
Leukocytes	34	0.52 5.85	±0.29 ±4.01	μmoles gal-1-P formed/hr per mg DNA μmoles gal-1-P formed/hr per 10 <sup>8</sup> cells
Erythrocytes	42	0.281 0.035	±0.08	μmoles gal-1-P formed/hr per ml cells μmoles gal-1-P formed/hr per 10 <sup>8</sup> cells†

\* n = number of individuals or cell lines tested (includes Gt mutants). Number in brackets represents number of repeat determinations.

† Based on assumption that 1 ml packed RBC = 8 × 10<sup>8</sup> cells.

Duarte-galactosemic heterozygote (Gt<sup>D</sup>/Gt<sup>o</sup>), and eight galactosemics (Gt<sup>o</sup>/Gt<sup>o</sup>) were tested for transferase and galactokinase activity 10 days after subculture under the conditions described above.

The frequency distribution of transferase:galactokinase ratios for fibroblasts is shown in Fig. 3 A. The ratios of fibroblasts of mutant transferase genotypes are well separated from the normal genotype. One cell strain, originally obtained as a control and assumed to be normal for transferase (Gt<sup>+</sup>/Gt<sup>+</sup>), on repeated assay fell into the heterozygote range (Gt<sup>+</sup>/Gt<sup>o</sup>). A family study of this individual's parental red blood cell (RBC) transferase activity revealed that both the parents had activity consistent with the Duarte variant heterozygote (Gt<sup>+</sup>/Gt<sup>D</sup>). Two of four children in this family had normal activity and the other two had the red cell transferase activities of Duarte homozygotes (Gt<sup>D</sup>/Gt<sup>D</sup>). (See Fig. 4.)

With the exception of a few normals, the donors of biopsies for the fibroblast cultures described above were

also assayed for leukocyte transferase:galactokinase ratios. This frequency distribution also reveals a clear separation of the mutant and normal transferase genotypes as shown in Fig. 3 B, and is nearly identical with that of the fibroblast distribution. It is interesting that the leukocyte ratio of 3 to 6 (Gt<sup>+</sup>/Gt<sup>+</sup>) is significantly different from the fibroblast ratio of 1 to 2, suggesting the possibility of tissue specificity with regard to this ratio. Table III lists the mean transferase:galactokinase ratios of the various transferase genotypes for both fibroblast and leukocyte preparations. The standard deviations of the ratios for the various Gt genotypes are considerably less than those of the absolute transferase activities as noted in Table II.

*Family studies.* We have previously reported the erythrocyte transferase activities and family studies for the various transferase mutants used in this study (5). We have also previously reported on a particular Negro family with two galactosemic children, one of whom was asymptomatic (18). Studies based on this family suggested that it might be possible to discriminate two galactosemic genotypes (19) with parental leukocyte

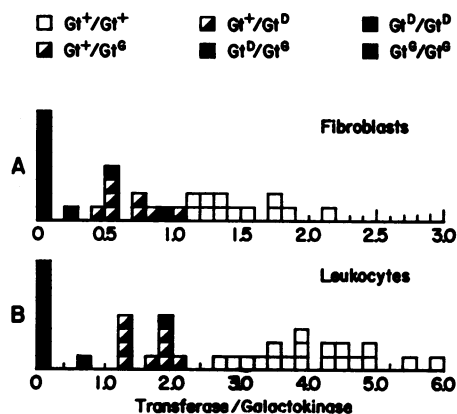


FIGURE 3 The distribution of transferase genotypes expressed as the ratio of transferase:galactokinase activity for cultured fibroblasts (A) and peripheral blood leukocytes (B). The units for the activity of each enzyme are given in Tables I and II.

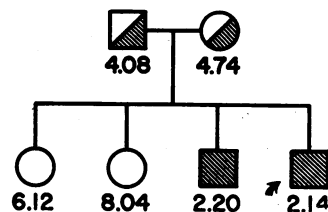


FIGURE 4 The pedigree of a child whose skin fibroblast culture was initially used as a "control" in these studies, and assumed to have a normal transferase genotype (Gt<sup>+</sup>/Gt<sup>+</sup>). Transferase:galactokinase ratios in this cell strain were repeatedly lower than normal. The erythrocyte transferase activities (μmoles UDPG consumed/hr per ml packed RBC's) are the numbers shown in the figure. Both parents have Duarte heterozygote (Gt<sup>+</sup>/Gt<sup>D</sup>) activity. Two of the four children have normal (Gt<sup>+</sup>/Gt<sup>+</sup>) activity; the biopsy donor and another child have Duarte homozygote (Gt<sup>D</sup>/Gt<sup>D</sup>) activity.

TABLE II  
Transferase Activities in Human Fibroblast Cultures,

Cell type	Gt <sup>+</sup> /Gt <sup>+</sup>			Gt <sup>+</sup> /Gt <sup>D</sup>			Gt <sup>D</sup> /Gt <sup>D</sup>		
	n*	Mean	SD	n	Mean	SD	n	Mean	SD
Fibroblasts	18 (85)	2.17 36.99	1.33 19.12	1 (3)	1.41 30.60		2 (6)	1.04 16.63	0.09 5.46
Leukocytes	33	1.93 23.96	0.89 13.79	2 (4)	1.05 10.83		1 (4)	0.800 12.03	
Erythrocytes	209 (233)	5.99 0.75	0.98	21 (50)	4.20 0.53	0.47	3 (20)	2.50 0.31	0.42

\* n = number of individuals or cell lines tested. Numbers in brackets represents number of repeat determinations.

† Based on assumption that 1 ml packed RBC =  $8 \times 10^9$  cells.

transferase assays. This family is included in this study; here we have found no difference between galactosemic heterozygotes of Negro and European origin, as determined by transferase:galactokinase ratios of both leukocyte and fibroblast preparations (See Fig. 5).

**Galactosemic cell strains.** None of eight galactosemic cell strains tested had any detectable transferase activity. Two cell strains were tested at 3 days after subculture when rapid cell division was in progress and all eight were tested at 10 days as described above. One cell lysate was concentrated by lyophilization and prepared at 10 times the concentration normally used in our assay. No enzyme activity was detectable in this preparation while heterozygote and normal cell lysates prepared in the same way showed the expected increase in activity.

**UDPglu and UDPgal pyrophosphorylase activity.** UDPglu pyrophosphorylase activity in fibroblast lysates has a range of 6–172  $\mu$ moles/hr per mg DNA based on 10 lysates with a mean of 51.6. Activity with gal-1-P can be demonstrated in this system at a rate which is 0.6–1.0% of the activity of glu-1-P. The specific activity with time in culture shows an increase to maximum at about 7–10 days after subculture and then declines. The activity with gal-1-P has now been demonstrated with three cell strains, one of which is galactosemic. All three show similar ratios of UDPglu:UDPgal pyrophosphorylase activity (mean: 141).

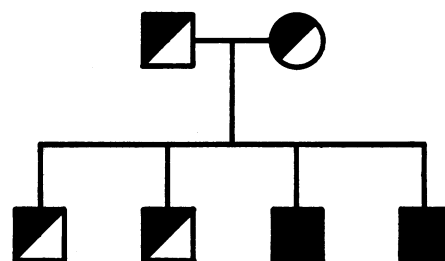
**Estimation of endogenous UTP.** Since gal-1-P does function in the pyrophosphorylase system, the conversion of gal-1-P by this pathway in the absence of added UTP would depend upon the availability of endogenous UTP as a substrate for this reaction. Seven lysates thus far tested have a mean UTP content of 2.14  $m\mu$ moles/ $10^6$  cells with a range of 1.18–3.66.

**Tests for activators or inhibitors.** Mixtures of galactosemic and normal fibroblast lysates have failed to yield any evidence for the existence of either activators or

transferase which might be absent from galactosemic cells or inhibitors which might be present. This is consistent with the fact that the normal allele in the galactosemic heterozygote (Gt<sup>+</sup>/Gt<sup>D</sup>) expresses a predictable activity based on the dosage of a single functional gene, apparently unaffected by the presence of a presumably functionless galactosemic allele.

## DISCUSSION

Human fibroblast cultures established from biopsies of individuals of the various galactose-1-phosphate uridyltransferase genotypes reflect the genotype of their individual donors with respect to transferase activity.



	Transferase			Transferase: galactokinase		
	RBC*	WBC†	CC†	RBC	WBC	CC
	SA					
Father	2.96	0.68	0.72	14.6	1.94	0.59
Mother	2.60	0.73	0.82	10.6	1.35	0.45
Normal (Gt <sup>+</sup> /Gt <sup>+</sup> )	5.99	1.93	2.17	20.3	4.14	1.61

\*  $\mu$ moles/hr per ml packed RBC.

†  $\mu$ moles/hr per mg DNA.

FIGURE 5 A Negro family with two galactosemic children. The table below the pedigree lists erythrocyte, leukocyte, and cultured fibroblast transferase activities as well as the transferase:galactokinase ratios of the parents. The normal values given are the means for Gt<sup>+</sup>/Gt<sup>+</sup> cells.

*Leukocytes, and Erythrocytes of Various Gt Types*

Gt <sup>+</sup> /Gt <sup>a</sup>			Gt <sup>D</sup> /Gt <sup>a</sup>			Gt <sup>a</sup> /Gt <sup>a</sup>			Specific activity
n	Mean	SD	n	Mean	SD	n	Mean	SD	
7 (19)	0.86	0.57	1 (2)	0.48		8 (11)	0	—	μmoles/hr per mg DNA
	16.88	9.48		8.68		—	—	—	μmoles/hr per 10 <sup>9</sup> cells
17 (17)	0.803	0.28	1 (3)	0.300		8 (8)	0	—	μmoles/hr per mg DNA
	9.55	5.92		3.92		—	—	—	μmoles/hr per 10 <sup>9</sup> cells
29 (41)	3.09	0.54	1 (6)	0.88	0.25	10 (10)	0	—	μmoles/hr per ml packed RBC
	0.39			0.11		—	—	—	μmoles/hr per 10 <sup>9</sup> cells†

Studies of galactose metabolism by Krooth and Weinberg (20) and transferase assays by Russell and DeMars (21) have demonstrated the quantitative relationship of transferase activity to genotype for normal (Gt<sup>+</sup>/Gt<sup>+</sup>), galactosemic heterozygote (Gt<sup>+</sup>/Gt<sup>a</sup>), and galactosemic (Gt<sup>a</sup>/Gt<sup>a</sup>) fibroblast cultures. Our data, based on transferase:galactokinase ratios, confirm these findings and include the Duarte variant genotypes. The ratio of the activities of these two enzymes appears to be the best determinant of transferase genotype expression in uncultured leukocyte lysates as well as cultured fibroblast lysates. From an examination of the data for the Gt<sup>+</sup>/Gt<sup>+</sup> and Gt<sup>+</sup>/Gt<sup>a</sup> groups presented in Tables II and III, it is apparent that there is greater within-group variability of the absolute transferase data than of the transferase:kinase ratios of the same groups. The ratio of the two activities compensates for the variation usually observed with leukocytes and cultured fibroblasts, since these two enzymes appear to fluctuate simultaneously under varying physiologic conditions.

A previous preliminary report from our laboratory suggested that certain Negro heterozygous parents of galactosemics had the expected heterozygote activity in their erythrocytes, and normal activity in their leukocytes (19). This family is included in the present study where both leukocyte and cultured fibroblast transferase:galactokinase ratios place them clearly in the galactosemic heterozygote range (Fig. 5).

The data presented in this report provide evidence to substantiate the use of cultured human diploid fibroblasts as experimental material for the investigation of the nature of the defect in galactosemia. The observation that human leukocytes and cultured fibroblasts have different transferase:galactokinase ratios is noteworthy. This observation may explain why certain tissues in individuals with transferase mutations might be more susceptible to galactose toxicity than others.

The data in Table III on simultaneous assays of galactokinase and transferase activities in fibroblast preparations suggest that transferase may well be the rate-limiting step in the utilization of galactose by cells with mutant transferase genotypes such as the galactosemic heterozygote (Gt<sup>+</sup>/Gt<sup>a</sup>), the Duarte variant homozygote (Gt<sup>D</sup>/Gt<sup>D</sup>), and the mixed heterozygote (Gt<sup>a</sup>/Gt<sup>D</sup>).

In leukocytes of the Gt<sup>+</sup>/Gt<sup>+</sup> transferase genotype the transferase:galactokinase ratio is approximately 4:1 and in cultured fibroblasts the ratio is 1.6:1. It is evident that in these two cell types transferase may limit the rate of galactose utilization in most of the mutant transferase genotypes. The pH optima of galactokinase and transferase should also be considered here. Galactokinase has a pH optimum in the physiologic range (about 7.5), whereas transferase has a pH optimum near 8.5. At physiologic pH's transferase may be limiting even for cells of normal transferase genotype. In contrast, human erythrocytes of normal transferase genotype (Gt<sup>+</sup>/Gt<sup>+</sup>) have a transferase:galactokinase ratio of about 20:1. This ratio suggests that galactokinase may be the rate-limiting step in galactose utilization by red cells even when transferase activity is reduced to about 15% of normal as in the Gt<sup>a</sup>/Gt<sup>D</sup> geno-

TABLE III  
*Transferase:Kinase Ratios in Human Fibroblast Cell Cultures and Leukocytes of Various Gt Types*

Gt genotype	Fibroblast			Leukocytes		
	n	Mean	SD	n	Mean	SD
Gt <sup>+</sup> /Gt <sup>+</sup>	12	1.61	0.53	19	4.14	0.99
Gt <sup>+</sup> /Gt <sup>D</sup>	1	0.94	—	—	—	—
Gt <sup>D</sup> /Gt <sup>D</sup>	2	0.79	—	1	1.88	—
Gt <sup>a</sup> /Gt <sup>+</sup>	7	0.64	0.19	9	1.66	0.33
Gt <sup>a</sup> /Gt <sup>D</sup>	1	0.21	—	1	0.65	—
Gt <sup>a</sup> /Gt <sup>a</sup>	8	0	—	8	0	—

type. In the fetal and newborn periods when red cell galactokinase has been noted to be three to four times that of adult levels (22), this may not be so. Gitzelmann, Poley, and Prader have identified an infant of the Gt<sup>p</sup>/Gt<sup>e</sup> genotype with transient hemolytic disease in the newborn period which may have been due to the higher galactokinase activity of newborn erythrocytes (23).

Although the evidence for rate-limiting steps in metabolism from this type of data is only suggestive, the cell culture system provides a model for directly testing the significance of the conclusions suggested by relative enzyme activities. Direct information on the rate of galactose metabolism and the rate and degree of gal-1-P accumulation by cells of various transferase genotypes after incubation in galactose for standard periods of time needs to be obtained.

We have observed that galactose does not seem to be toxic to galactosemic cells. When such cells are incubated with galactose in the presence of adequate amounts of glucose, galactose does not appear to alter their growth or survival. When glucose is removed from the medium there is little difference in the survival of galactosemic cells in the presence or absence of galactose. In contrast, normal cells utilize galactose, grow, and thrive when galactose is substituted for glucose in the medium. If galactosemic cells are found by direct measurement to accumulate gal-1-P in the presence of glucose without apparent toxicity, this would provide a further challenge to the contention that gal-1-P is the toxic factor in galactosemia. Recent evidence that glycogen accumulates in the presence of glucose and is mobilized in the absence of glucose (24) has provided an additional system for testing the toxicity of galactose in the cell culture system. One might expect that glycogen would not be mobilized normally if glycolysis were inhibited by gal-1-P accumulation.

The report (21) of transferase activity in galactosemic fibroblasts under certain culture conditions as well as the ability of such cells to incorporate galactose-<sup>14</sup>C can be explained in two ways: (a) the galactosemic cell does have transferase activity that is due to an unstable enzyme protein, and (b) there exists an alternate mechanism for the transfer of gal-1-P into the UDP-hexose pool. We have been unable to demonstrate any transferase activity with two galactosemic cell strains in the logarithmic and eight in the stationary growth phase.

The possibility that an alternate pathway for galactose metabolism is functional in cultured fibroblasts is currently under investigation. We have considered the possibility that activity for the conversion of gal-1-P to UDPgal in fibroblast lysates might be due to the utilization of gal-1-P by UDPglu pyrophosphorylase, an en-

zyme which is very active in human fibroblast lysates. We have therefore investigated UDPgal pyrophosphorylase activity in fibroblast lysates, and the availability of endogenous UTP as a substrate for this reaction. Isselbacher (25) previously reported UDPgal pyrophosphorylase activity in rat and bovine liver, and Abraham and Howell (26) have characterized human hepatic UDPgal pyrophosphorylase. This enzyme catalyzes the reaction



The report by Ting and Hansen (27) that UDPglu and UDPgal pyrophosphorylase activities cannot be separated in the purification of the calf liver enzymes suggests that UDPglu pyrophosphorylase is most likely degenerate in its substrate specificity and can utilize gal-1-P, albeit less efficiently than glu-1-P. The relative activities of glu-1-P and gal-1-P in the human fibroblast pyrophosphorylase system are 141:1 as compared to 70:1 reported by Ting and Hansen for calf liver. Although estimates of endogenous UTP in fibroblast lysates are inadequate for maximal kinetics they are probably sufficient to permit activity of UDPgal pyrophosphorylase. Whether this UTP is available for pyrophosphorylase activity with gal-1-P in intact cultures remains to be answered.

#### ACKNOWLEDGMENTS

The following physicians generously referred patients and their families included in this study: Doctors Angelo DiGeorge, Lester Baker, David Cornfeld, and Stanton Segal. This work was supported by U. S. Public Health Service Grants HD 00588, HD 15545, FR 40, and FR 240.

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