proposed selected method

A Screening Procedure and a Specific Quantitative Method for UDPglucose:α-D-galactose-1-phosphate Uridylyltransferase (EC 2.7.7.12)

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Introduction

Deficiencies of two different enzymes can produce the inherited disease referred to as galactosemia: galactokinase (ATP:D-galactose 1-phosphotransferase, EC 2.7.1.6) deficiency and UDPglucose: α-D-galactose-1-phosphate uridylyltransferase (EC 2.7.7.12) deficiency. This paper is concerned only with the detection and measurement of the second of these enzymes.

Transferase-deficient galactosemia occurs rarely, the gene frequency as determined by examination of 4074 blood samples lying between 0.0035 and 0.0041 (*1*), or a prevalence of one galactosemic infant per 81 600 to 59 500 births. The combined results of several studies from various countries, involving an aggregate of more than 3 000 000 neonates, indicate that the prevalence, which varies with geographic area, is in fact 1/75 000 overall (*2*). Potentially, there may be a discrepancy between the prevalence anticipated from gene frequency estimates and that found in screening programs that rely on the detection of individuals with clinically evident galactosemia. This difference is attributable to the fact that severely affected fetuses may die before they are born, while of those who survive birth, some perish before the diagnosis is made (*3*). The results of testing 223 326 five-day-old babies in New Zealand yielded five cases—a rate of 1/44 600 (*4*). Severe symptoms are noted in the newborn, usually within a few days after it starts taking milk. Because effective treatment is available and a definitive laboratory test can be undertaken during the newborn period (even cord blood is a suitable specimen), there is a demand for a rapid screening procedure. At least one community has elected to examine every newborn for the presence of galactosemia (*3, 5*).

The selected screening method described below, which is based on the methods of Beutler and Baluda (*6, 7*), is not specific for galactose-1-phosphate uridylyltransferase deficiency and under certain conditions yields falsely abnormal results.

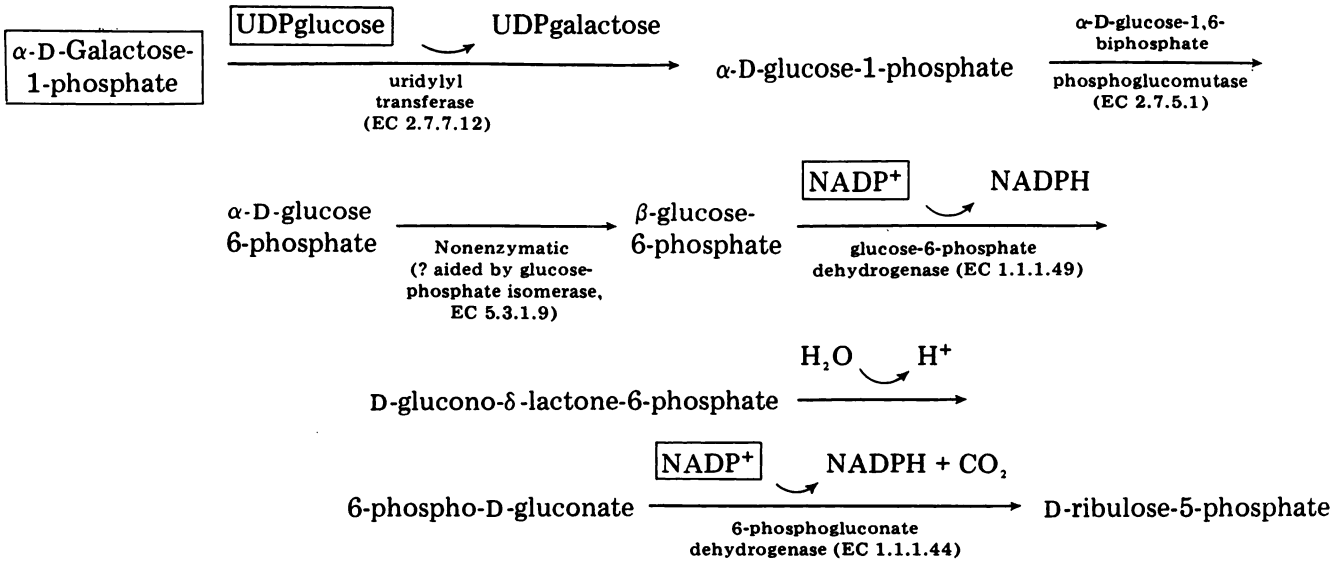
Note: Evaluator E.B. states that spuriously abnormal results caused by deficiencies of other enzymes or cofactors are extremely rare. However, one case of phosphoglucomutase (PGM_1) deficiency was detected because of the use of this screening test (*8* and personal communication to E.B.).

For this reason, a specific and quantitative method also is described for measuring the activity of this transferase enzyme, for use in confirming abnormal findings obtained in the screening procedure.

A. Proposed Selected Screening Procedure

Principle

The objective of this screening procedure is to demonstrate whether or not galactose-1-phosphate uridylyltransferase activity is present in the patient's blood. To do this, the following series of coupled reactions is used:



(Compounds shown in boxes are supplied in the incubation mixture.)

Measurement of the fluorescence of one of the end products, NADPH, is used as an indirect measure of enzyme action.

The compounds shown in boxes are those added to the incubation mixture. The enzymes phosphoglucomutase (EC 2.7.5.1), glucose-6-phosphate dehydrogenase (EC 1.1.1.49), and 6-phosphogluconate dehydrogenase (EC 1.1.1.44) are assumed to be already present in the patient's blood sample.

Materials and Method

Reagents

1. *Uridyldiphosphoglucose, 9.5 × 10^{-3} mol/liter.* Dissolve 59.7 mg of disodium UDPglucose monohydrate, or equivalent, in water and dilute to 10 ml. Store at −20 °C.

2. *Galactose-1-phosphate, 2.7 × 10^{-2} mol/liter.* Dissolve 100.5 mg of dipotassium α-D-galactose-1-phosphate dihydrate, or equivalent, in water and dilute to 10 ml. Store at −20 °C.

3. *NADP+, 6.6 × 10^{-3} mol/liter.* Dissolve 52.1 mg of disodium NADP+ in water and dilute to 10 ml. Store at −20 °C.

4. *EDTA, 2.7 × 10^{-2} mol/liter.* Dissolve 100.4 mg of disodium ethylenediaminetetraacetate dihydrate in water and dilute to 10 ml. Store at room temperature.

5. *Digitonin, saturated solution in water.* Add about 0.5 g of digitonin to 100 ml of water. Shake thoroughly, and store at room temperature. Use the supernatant solution.

6. *Tris acetate buffer, pH 8.0, 0.75 mol/liter.* Dissolve 9 g of tris(hydroxymethyl)methylamine in about 60 ml of water. Add glacial acetic acid dropwise until the pH of the mixture is 8.0 at 25 °C, measured with a pH meter. Dilute to 100 ml with water and store at 4 °C.

7. *Incubation mixture.* Make a mixture containing the above reagents in the following volumes:

UDPG	0.4 ml
Galactose-1-phosphate	0.8 ml
NADP+	1.2 ml
Tris acetate buffer	4.0 ml
Digitonin	1.6 ml
EDTA	60 μl
Water	3.94 ml
Total volume	12.00 ml

Dispense 0.25-ml aliquots of the mixture into small test tubes. Stopper the tubes and store them at −20 °C, a temperature at which the reagents in the incubation mixture are stable for at least two months (*6*).

Note: Evaluator D.S. has confirmed this stability statement.

The reaction mixture also can be lyophilized if the temperature does not exceed 20 °C during the process and the concentration of UDPglucose is doubled to compensate for loss during lyophilization (*6*).

Obtaining and Storing Specimens

Either capillary or venous blood may be used. When collected into either acid-citrate–dextrose solution (ACD),[1] Na_2EDTA (1 g/liter of blood), or heparin (10^3 USP units/liter of blood), blood samples may be maintained at 25 ± 2 °C for at least five days or at 4 °C for not less than 20 days with a loss of enzyme activity that does not exceed 10% (*7*).

Note: Evaluator D.S. reports the following stability data obtained in his laboratory: blood samples were collected into heparin and into EDTA from 20 subjects with no family history of galactosemia, two homozygous galactosemic individuals, and three parents of galactosemic subjects. These specimens were tested within 4 h of collection. Aliquots were then stored at 25, 4, and −20 °C and tested after 1, 2, 3, 5, and 10 days of storage at the designated temperatures. The samples from normal and heterozygous individuals stored at 25 °C gave the expect-

[1] Acid-citrate–dextrose solution is prepared as follows: weigh out 7.3 g of citric acid ($C_6H_8O_7$), 22.0 g of sodium citrate ($C_6H_5O_7Na_3 \cdot 2H_2O$) and 24.5 g of glucose. Dissolve in water and dilute to 1 liter.

ed results for up to two days but at five days showed no fluorescence by the screening test. Acceptable results were obtained for samples stored at 4 °C for as long as five days, but at 10 days they produced little or no fluorescence. Storage at −20 °C was satisfactory for the test period of 10 days. The samples from the homozygous galactosemic individuals gave no fluorescence under any conditions.

EDTA stimulates fluorescence at the concentration recommended for the incubation mixture but is inhibitory at concentrations exceeding 0.1 g/liter. For this reason, when EDTA is used as the anticoagulant it should be omitted from the incubation mixture (*6*).

Note: Evaluator D.S. performed the test with reagent prepared with and without EDTA on normal blood samples collected separately into EDTA and heparin. He observed no difference in the fluorescence produced and thus was unable to confirm this inhibitory effect of EDTA.

Blood samples also may be collected on filter paper: Whatman No. 4[2] (*9*) and Schleicher and Schuell No. 903[3] (*10*) both have been recommended. Enzyme activity was stable for five days when specimens were collected on Whatman No. 4 filter paper and stored in the dark at room temperature (*9*). Samples collected on Schleicher and Schuell No. 903 filter paper retained activity for at least a week and possibly for much longer (*10*). In another study (3), filter paper preparations were found satisfactory for at least 10 days unless subjected to heat, when deterioration was rapid.

Note: Data obtained by Evaluator D.S. indicate that blood from 20 normal and from three heterozygous individuals, collected on Schleicher and Schuell No. 903 filter paper and stored at 25 °C, was stable for two days and that the results given by blood from heterozygous subjects were distinguishable from those from homozygotes at this time. When tested on the fifth day of storage, samples from the heterozygous subjects produced no fluorescence; blood from the non-galactosemic individuals gave weak reactions after five and 10 days of storage when compared to those obtained originally. Blood from two homozygous galactosemic patients gave no fluorescence at any time.

Procedure

1. Thaw a sufficient number of tubes of incubation mixture, re-mix their contents, and place them in an ice bath.

2. (*a*) With a disposable microliter-scale pipette, deliver 25 μl of heparinized blood into the incubation mixture in one of the tubes and mix. Insert the pipette into the tube, replace the stopper, and return the tube to the ice bath until ready to proceed with a batch of tests. (Parafilm may be used instead of a stopper.)

or

(*b*) Punch a 6-mm (one-quarter inch) disk with an office paper punch from a dried blood spot and immerse it in the incubation mixture in one of the tubes, replace the stopper, and return the tube to the ice bath until ready to proceed with a batch of tests. (Parafilm may be used instead of a stopper.) Include one sample from a normal individual, as a control.

3. Place the tubes in a 37 °C water bath and note the time.

4. At the end of 1 h, withdraw from each tube a few microliters of the incubation mixture and spot it onto a Whatman No. 1 filter paper to form a circle 4 to 10 mm in diameter, and allow it to dry spontaneously.

5. At the end of 2 h at 37 °C, withdraw another portion, spot it onto filter paper, and allow it to dry.

6. Examine the two dried spots with a long-wave ultraviolet light source in a darkened room. (The following lamp was found suitable: Model UVL-56, "Blak-Ray", principle radiation 366 nm.)[4]

Interpretation of Results

(*a*) *Using liquid blood:*

Normal: Both the 1- and the 2-h spots show a bright fluorescence.

Heterozygotes for galactosemia and homozygotes for the Duarte variant: At 1 h, fluorescence is considerably less than normal, while the 2-h spot appears normal.

Congenital galactosemic: No fluorescence at 1 or 2 h. The appearance of a homozygote or heterozygote galactosemic result can be simulated by withdrawing a portion of incubation mixture from any normal blood sample at zero time and at 10–15 min.

(*b*) *Using blood from a spot on filter paper:*

Normal: The 2-h spot shows a bright fluorescence. Homozygotes and heterozygotes for congenital galactosemia and homozygotes for the Duarte variant show no or barely detectable fluorescence at 2 h. When dried blood is used it is not possible to distinguish between the heterozygous and homozygous states of congenital galactosemia.

Discussion

It can be seen from the reactions diagrammed in the section on *Principle* that even though this procedure is designed to detect galactose-1-phosphate uridylyltransferase activity, a normal result—the generation of fluorescent NADPH—can be obtained only if phosphoglucomutase and glucose-6-phosphate dehydrogenase are also present in nonlimiting quantities.

In an investigation to assess the probability of obtaining falsely negative results, samples from individuals with various degrees of glucose-6-phosphate dehydrogenase deficiency were found to produce fluorescence at normal or nearly normal rates. However, blood from cases of congenital nonspherocytic hemolytic anemia associated with an extreme deficiency of this enzyme failed to give fluorescence in this test (*6*). In addition to the requirement that phosphoglucomutase be present, a sufficiency of its cofactor, glucose-1,6-biphosphate, is also necessary. All of these assumptions emphasize that the method detailed above

[2] Whatman Inc., 9 Bridewell Place, Clifton, N. J. 07014.

[3] Schleicher and Schuell, Inc., 543 Washington St., Keene, N. H. 03431.

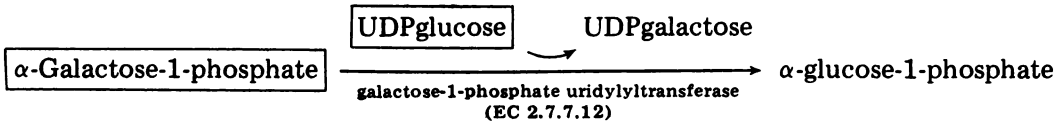
[4] Ultra-Violet Products, Inc., 5100 Walnut Grove, San Gabriel, Calif. 91778.

is intended only for screening and that any abnormal result must be checked by a specific and preferably quantitative measure of the transferase enzyme. A quantitative version of this screening method has been developed (*11*). It requires access to a filter fluorometer—and acceptance of the assumptions involved in the procedure just described.

B. Proposed Selected Specific Quantitative Method

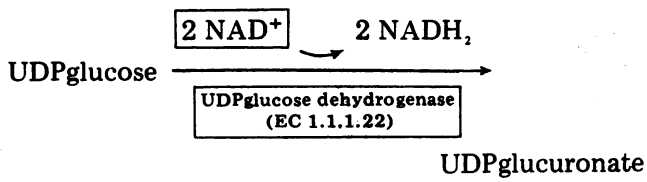
Principle

The enzyme that is deficient in congenital galactosemia catalyzes the transformation:



(Compounds shown in boxes are supplied in the incubation mixture.)

In the quantitative assay, which is based on a modification (*12*) of the original publication (*13*), a preparation of the patient's erythrocytes is incubated with galactose-1-phosphate and a controlled excess of UDPglucose. After a fixed period of incubation the reaction is halted and the quantity of UDPglucose transformed is measured by use of the following system:



(Compounds shown in boxes are supplied in the incubation mixture.)

The amount of NAD^+ that is reduced, when appropriately corrected for blank and for deviation from linearity (*12*), is commensurate with the quantity of UDPglucose transferred from the original incubation mixture and thus is inversely proportional to the transferase activity in the patient's erythrocytes.

Materials and Method

Reagents

1. *Tris acetate buffer, 0.2 mol/liter, pH 8.7.* Dissolve 2.4 g of tris(hydroxymethyl)methylamine in about 80 ml of water. Electrometrically adjust the pH to 8.7 at 25 °C with glacial acetic acid. Dilute to 100 ml with water. Store at 4 °C.

2. *UDPglucose, 7 mmol/liter.* Dissolve 75 mg of disodium UDPglucose·$4H_2O$ in 10 ml of water. Prepare a 100-fold dilution of this solution in water and measure its absorbance at 260 nm. Based on this reading, dilute the remainder of the solution so that a 100-fold dilution would read 0.70 ± 0.01 *A*. Store at −20 °C.

3. *Galactose-1-phosphate, 8 mmol/liter.* Dissolve 3 mg of dipotassium galactose-1-phosphate·$2H_2O$ in 1 ml of water. Store at −20 °C.

4. *NAD^+, 13.6 mmol/liter.* Dissolve 50 mg of nicotinamide adenine dinucleotide tetrahydrate in 4 ml of water. Adjust the pH to 7.0 with 0.2 mol/liter NaOH (pH meter). Do not overtitrate, because NAD^+ is unstable in alkaline solution. A 300-fold dilution should have an absorbance at 260 nm of 0.815. Adjust the concentration of the stronger solution if necessary.

5. *UDPglucose dehydrogenase (EC 1.1.1.22).* This enzyme is dissolved in a volume of water such that the activity is 32 U/liter. Store at −20 °C in convenient volumes.

6. *NaCl solution, 9 g/liter.* Physiological saline.

Apparatus

A photometer is required that can operate at 340 nm and that gives the established molar absorptivity of 6.31×10^3 liter mol^{-1} cm^{-1} for solutions of NADH. Because it is necessary to derive the absorbance of the reaction mixture at zero time by extrapolation, a recording spectrophotometer offers considerable convenience. Nevertheless, instruments that can give a printout at accurately timed intervals are acceptable, as indeed are completely manually operated spectrophotometers. The only essential criterion is that reliable readings can be made at 0.5-min intervals.

Obtaining and Storing Specimens

Venous blood samples collected into acid-citrate–dextrose solution, EDTA, or heparin (see above) retain up to 90% of activity when stored for five days at 25 ± 2 °C or 20 days at 4 °C as whole blood. Washed, lysed cells stored at 4 and −25 °C for two days showed small losses in activity (*12*).

Note: Evaluator H.K.B. strongly recommends preincubation of the hemolysate with dithiothreitol to completely restore activity to lysates that have been stored even for long periods (*13*). This reactivation procedure is described in the section below on the UDPglucose consumption assay.

Procedure

(a) Preparation of blood sample and determination of hemoglobin.

1. Cool the heparinized blood in an ice bath. Centrifuge at 4 °C at 1000 × *g* in a clinical centrifuge. With

a Pasteur pipette, aspirate and discard the plasma and the buffy coat (leukocytes).

2. Wash the erythrocytes twice with two volumes of the cold saline solution, centrifuging between each washing as in Step 1. Remove the saline as completely as possible, together with the uppermost layer of erythrocytes and any remaining buffy coat.

3. Hemolyze the washed erythrocytes by adding an equal volume of cold distilled water and stirring with a glass rod. Freeze in a solid CO_2/acetone bath and then thaw.

4. Place the hemolysate in a 37 °C water bath for 10 min, then store at 4 or −20 °C until assayed.

5. Determine the hemoglobin concentration of the hemolysate by the usual cyanmethemoglobin procedure.

(*b*) *UDPglucose consumption procedure.*

1. Freshly prepare a mixture consisting of 1 ml of buffer (Reagent 1) and 0.25 ml of UDPglucose solution (Reagent 2).

2. Into four 12-ml heavy-duty Pyrex centrifuge tubes, pipette:

	Tube No.	Buffered UDPglucose, μl	Gal-1-phosphate, μl	Water, μl
Blank	1	100	—	50
Blank	2	100	—	50
Test	3	100	50	—
Test	4	100	50	—

Place all four tubes in a water bath at 37 °C for 5 min.

3. At 1-min intervals, add 100 μl of cold (4 °C) hemolysate to each tube. Mix, and note the time.

4. When each tube has incubated 15 min at 37 °C, add 500 μl of the cold saline, transfer the tube immediately to a boiling water bath for 2 min, stir the contents of the tube with a glass rod to fragment the coagulum, and place the tubes in an ice bath.

5. Centrifuge all tubes at 4 °C for 20 min. Transfer the clear supernate to another tube. It may then be stored at −20 °C overnight if necessary, although about 5% UDPglucose may be lost.

Note: Evaluator H.K.B. recommends the following procedure for the UDPglucose assay: Transfer 50 μl of dithiothreitol (0.13 mol/liter, prepared by dissolving 20 mg of dithiothreitol in 1 ml of water) into each test and blank tube. Then add 100 μl of the hemolysate, followed by 100 μl of UDPglucose solution. Incubate at 37 °C for 5 min and then start the reaction by adding 50 μl of galactose-1-phosphate reagent. Continue incubation for 15 min, at which time the reaction is terminated by adding 450 μl of saline and the tubes are placed in the boiling water bath as described in Step 4.

(*c*) *Assay of residual UDPglucose.*

It is an advantage, but is by no means essential, to use a recording spectrophotometer for this determination.

1. Into 50 × 10 × 4.3 mm cuvets pipette 450 μl of water, 150 μl of buffer (Reagent 1), 100 μl of NAD^+ (Reagent 4), and 200 μl of the supernatant fluid from the above procedure.

2. Start the chart recorder at 2.5 cm (1 in.)/min. Set the wavelength of the spectrophotometer at 340 nm.

3. Add 100 μl of UDPglucose dehydrogenase (Reagent 5) to the first cuvet, mix (using Parafilm), mark the chart to indicate the instant of mixing, and place the cuvet in the light path. Repeat with the remaining cuvets.

4. Three minutes after the last cuvet has been placed in the light path, reduce the chart speed to 0.6 cm (¼ in.)/min and allow recording to continue until the readings have become stable (20–30 min).

Note: When a nonrecording spectrophotometer is used, read the tests vs. a water blank at 0.5, 1, 1.5, and 2 min after adding the UDPglucose dehydrogenase. Thereafter, read at 4-min intervals until the absorbance readings reach a stable maximum.

Calculation

1. Extrapolate the linear portion of the record for each cuvet back to zero time and note the absorbance reading at that time.

2. Read from the chart the maximum absorbance achieved 20–30 min after the reaction was begun.

Note: If the reading declined gradually after the maximum, use the maximum reading. If, after the reaction appears complete, there is a slow continuous rise in absorbance, take the reading when the rate of increase becomes constant.

3. Subtract the absorbance at zero time from the final absorbance for each cuvet.

Average the two readings for the blank:

$$\frac{\Delta A_1 + \Delta A_2}{2} = \Delta A \text{ blank}$$

and for the test:

$$\frac{\Delta A_3 + \Delta A_4}{2} = \Delta A \text{ test}$$

4. Activity of the enzyme, in IUB units (U) is the number of micromoles of UDPG consumed per minute (at 37 °C) per kilogram of hemoglobin:

Galactose 1-phosphate uridylyltransferase, U/kg Hb =

$$\frac{(\Delta A_{blank} - \Delta A_{test}) \times 1 \times 0.75 \times 100 \times 1000 \times F}{6.31 \times 2 \times 15 \times 0.2 \times 0.1 \times Hb}$$

$$= \frac{19810\, F\, (\Delta A_{blank} - \Delta A_{test})}{Hb}$$

Where:

6.31 = absorbance of 1 mmol of NADH/liter
2 = factor to convert number of moles of NADH generated to number of moles of UDPglucose transformed
15 = factor to convert to a reaction time of 1 min
1 = volume (ml) of incubation mixture for assay of residual UDPglucose
0.2 = volume (ml) of supernatant fluid from UDPglucose consumption procedure

0.75 = volume (ml) of incubation mixture for UDPglucose consumption procedure
0.1 = volume (ml) of hemolysate used for UDPglucose consumption procedure
100 = factor to express activity per 100 ml of hemolysate
Hb = concentration of hemoglobin in grams per 100 ml of hemolysate
1000 = factor to express activity per 1000 g of hemoglobin
F = factor from Table below to correct for nonlinearity of UDPG consumption assay (see *Discussion*).

Table of Correction Factors

$(A_{blank} - A_{test})$	*F*	$(A_{blank} - A_{test})$	*F*
<0.200	1.000	0.265	1.132
0.200	1.010	0.270	1.144
0.205	1.020	0.275	1.160
0.210	1.029	0.280	1.175
0.215	1.037	0.285	1.189
0.220	1.045	0.290	1.203
0.225	1.053	0.295	1.220
0.230	1.061	0.300	1.243
0.235	1.072	0.305	1.269
0.240	1.083	0.310	1.294
0.245	1.094	0.315	1.317
0.250	1.100	0.320	1.353
0.255	1.110	0.325	1.412
0.260	1.119	0.330	1.479

Discussion

The above procedure is based upon that given in the original publication (*14*) as subsequently modified (*7, 12*). The pH optimum for the transferase enzyme is about 8.7. Chloride ion is inhibitory; therefore glycine acetate and Tris acetate are acceptable buffers but Tris·HCl is not.

The relationship between transferase activity and rate of transformation of UDPglucose shows some departure from linearity (*12*). This appears to be referrable to the inhibitory effect of one product of the reaction, UDPgalactose (*7*), and to the concentration of UDPglucose, which is insufficient in the latter portion of the incubation period to saturate the transferase so that the reaction rate declines. Increased concentrations of UDPglucose improve linearity but have the disadvantage of producing high absorbance values for the tests and blanks. The enzyme activity is then represented by relatively small differences between two large numbers. A detailed discussion of the nonlinearity problem associated with the UDPglucose consumption method has been published (*15*).

The hemolysate is heated at 37 °C to destroy NAD^+ and so inactivate UDPglucose 4-epimerase (EC 5.1.3.2). Should this enzyme remain active, a pathway would exist for the conversion of UDPgalactose back to UDPglucose, thus interfering with the test. Although NAD^+ nucleosidase (EC 3.2.2.5) activity is usually present in erythrocytes, this may not be true of cells from very young infants. Therefore, when blood samples are being examined from infants who are younger than three months, 0.025 volume of a solution containing 630 U of NAD nucleosidase per liter should be added to each volume of hemolysate before it is incubated at 37 °C (*7*).

Interpretation of Results

The results of assays of erythrocytic enzymes can be expressed in various ways, such as enzyme activity (*a*) per selected number of erythrocytes, (*b*) per milliliter of packed erythrocytes, or (*c*) per selected quantity of hemoglobin. Differences in size and hemoglobin content of the erythrocytes from various individuals, especially if they are anemic, together with variations in the thoroughness with which cells are centrifuged before a hemolysate is prepared are some of the factors making it difficult to decide which is the most nearly satisfactory expression. The analytically most reliable and practicable reference is the hemoglobin concentration of the hemolysate, because enzyme and reference assays are performed on the same solution. More detailed discussions of this problem have been published (*7, 16*).

The normal and abnormal values given below, taken from publications by Beutler (*7*) and Beutler and Baluda (*12*), have been converted to IUB units (U) as defined above.

The values found by this procedure for normal persons lie in the range 304–468 U/kg of hemoglobin. Individuals who are heterozygous for congenital galactosemia give results between 140 and 222 U/kg of hemoglobin; activities for homozygotes for the disease are <8 U/kg of hemoglobin.

Note: Evaluator D.S. reported the following data (in U/kg of hemoglobin): nongalactosemic (10): 266[5]-453; nongalactosemic, cord blood (2): 375 and 342; heterozygote (1): 223; homozygote (2): <10 (Numbers in parentheses are number of subjects tested.)

Four electrophoretically distinguishable variants of the transferase now have been recognized and are known as Duarte (*17–20*), Los Angeles (*21, 22*), Rennes (*23*), and Indiana (*24*). The Duarte variant, first reported in 1965 and not associated with any clinical disorder, occurs with a higher frequency than does the gene for classical galactosemia. In one study, the homozygote frequency was estimated as 0.30%, the

[5] The assignment of a lower limit for the normal range is complicated by the frequency with which the Duarte variant heterozygote occurs (*14*). The establishment or confirmation of the normal range requires that these individuals with the Duarte variant be identified, for example, by electrophoretic examination of the enzyme, and excluded.

heterozygote frequency 10.4% (*18*). Enzyme activities in blood from homozygotes for this variant are in the same range as those obtained for heterozygotes for congenital transferase deficiency galactosemia: 140–222 U/kg of hemoglobin. Subjects who have inherited one normal allele and one for the variant allele show values in the range 222–304 U/kg of hemoglobin; those who are doubly anomalous and possess one gene for the variant and one for congenital galactosemia have an enzyme activity ranging from 57 to 140 U/kg of hemoglobin (*7*). Depending upon the particular starch-gel electrophoresis system used, the Duarte variant shows one band of activity migrating faster than the usual enzyme (*19*) or one band in the same position as that from normal persons plus two faster-moving bands (*20*).

The Los Angeles variant, first described in 1971 (*21*), also is not linked to any clinical disorder. In contrast to the Duarte variant, the occurrence of the Los Angeles enzyme variant is associated with greater than normal transferase activity. A study of 418 subjects (*22*) uncovered two individual homozygous for this variant, while 4.5% were found to be heterozygous for Los Angeles and normal enzymes. On starch-gel electrophoresis, three bands of activity have been demonstrated with mobilities similar to those of the Duarte variant, but in this case all three bands are well marked although the two faster bands are the more intense.

The variant named Indiana (*24*) is characterized by decreased mobility on starch-gel electrophoresis as compared with the usual enzyme, and by being distinctly unstable on storage. Blood from individuals who are heterozygous for this variant showed transferase activities in the same range as heterozygotes for the usual gene, but almost half of this activity was lost on refrigeration of heparinized blood for 72 h at 4 °C. The proband was a child with clinical galactosemia but with an erythrocyte transferase activity approximately 35% of normal.

Two galactosemic siblings demonstrated incomplete deficiencies of transferase. On electrophoresis on starch gel, enzyme activity was demonstrated, but with slower mobility than the normal enzyme (*23*). This variant has been termed "Rennes," the birthplace of the patients.

One case of atypical galactosemia now has been reported (*25*) in which the erythrocyte transferase activity was decreased by 70%. This enzyme was stable in blood for 72 h and was associated with recessively inherited galactosemia, the clinical symptoms of which were relieved by galactose-free diet.

Precision

The within-run precision of the quantitative method was assessed on six aliquots of a single normal blood sample (*12*). The mean value was 318 (1 SD = 17.3) U/kg of hemoglobin.

Note: Evaluator D.S. submitted data on the within-run precision of the quantitative method. One sample run five times gave a mean value of 358 (1 SD = 17.7) U/kg of hemoglobin.

Note: Evaluator H.K.B. states that in her laboratory the mean value for 10 determinations done on the same specimen during two years was 301 ± 22 U/kg of hemoglobin. Six determinations carried out on another specimen over a period of a year gave a mean value of 323 ± 16.8 U/kg of hemoglobin. There was no trend toward loss of activity over the period when dithiothreitol was used.

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Editor's note: The reader is reminded that Selected Methods do not bear the official imprimatur of the Association. They are methods that seem durable and generally useful, and that have been checked by several evaluators. As detailed elsewhere [Clin. Chem. **19**, 1207 (1973)], *these methods are offered here for criticism by the world community of users, and will be revised appropriately before being collected into a bound volume,* Selected Methods of Clinical Chemistry.

No reprints of these papers will be available, because they are not regarded as necessarily being final versions.