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Screening for Inborn Errors of Amino Acid Metabolism*

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ABSTRACT

Early diagnosis and treatment may prevent brain damage and mental retardation in young infants with inborn errors of amino acid metabolism. The abnormal blood and urinary amino acids and their metabolites are listed in two separate tables in association with each disorder to aid laboratories in making a diagnosis during screening. Because of recent developments and discoveries, more detailed descriptions and diagnostic approaches in phenylketonuria (PKU) variants and urea cycle deficiencies are also presented.

The test procedures routinely used for screening inherited metabolic disorders are also described. These include five simple chemical tests to detect excessive metabolites and amino acids; a one dimensional thin layer chromatography (TLC) to screen urine for abnormal amino acid patterns; a two-dimensional TLC for semiquantitative identification of amino acids in both urine and blood; and a high performance liquid chromatographic (HPLC) method for quantitative identification of amino acids. In addition, both one- and two-dimensional chromatographies run on small thin layer cellulose plates, are introduced, modifications which save a great deal of time, labor, and reagents. A new automated HPLC system is introduced for the quantitation of both primary and secondary amino acids; the sensitivity and speed of this system is especially useful for screening large numbers of physiological fluids. It is recommended that both the urine and blood from the same patient be screened to ensure that a diagnosis is not overlooked.

Introduction

Inborn errors of metabolism are caused by mutant genes that produce abnormal proteins. The affected protein may be altered in quantity, structure and/or function. If the protein is an enzyme, the enzyme catalyzed reaction may be completely or partially inactivated. If the affected protein is responsible for transporting amino acids across a membrane, amino acids may accumulate

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on one side of the membrane. As a result, the pathogenesis may involve a deficiency of metabolites owing to impaired synthesis or transportation or, in the majority of cases, a harmful accumulation of metabolites. These metabolites may be amino acids, sugars, organic acids, glycosaminoglycans, lipids, etc.[2] In the case of inborn errors of amino acid metabolism, it is the metabolic pathways involving amino acids that are being blocked, resulting in altered concentrations of amino acids and their metabolites. Therefore, the diagnosis of such disorders can be made by the measurement of amino acids and their metabolites; and an early detection, followed by an early treatment, may prevent irreversible damages from occurring.[2,14,21]

Abnormal Urinary Amino Acids[6]

In table I are listed the increased amino acids and metabolites for each disorder of inborn errors of amino acid metabolism. When increased concentrations of certain amino acids are detected in the urine, one can simply refer to the table and look up the associated disorder. Any abnormalities in blood amino acids are also described in the table, which may be helpful for the differentiation of minor variants having the same abnormal urinary amino acid patterns.

It should be noted that aminoaciduria may be hereditary or acquired; furthermore, not every aminoaciduria is pathologic. Increased urinary amino acids may be found in normal newborns because the renal tubular absorptive function is often not fully developed. Urine from normal newborns always contains increased concentrations of proline, hydroxyproline, glycine, and slightly increased concentrations of threonine and serine. The newborn baby pattern should not be confused with inherited disorders. For primary aminoacidurias, one or more amino acids accumulate both in the urine and blood or even in other body fluids, such as cerebrospinal fluid. These disorders are usually the consequence of a single inherited defect in either an enzyme mediating metabolism or a protein involved in the transportation of amino acids. It should also be noted that aminoaciduria is nonspecific and may be transient. Aminoaciduria is frequently due to a secondary manifestation of other diseases, such as liver or renal diseases. Damage to the renal absorption of amino acids is commonly observed by heavy metals, burns, galactosemia, and antibiotics. The primary aminoacidurias may be further divided into three main groups[6]

(1) Overflow Aminoaciduria

Increased urinary amino acids are caused by an overflow of elevated blood amino acids into the urine. Elevation of blood amino acids is due to the presence of inactive enzymes.

(2) No-threshold Aminoaciduria

There is no normal renal mechanism for the reabsorption of this group of amino acids. Blood amino acids accumulate owing to impaired enzyme activity and are excreted immediately into the urine. Therefore, blood amino acids could be normal or slightly increased while the urinary amino acids are elevated. Examples have been found in phosphoethanolamine, β-aminoisobutyric acid, homocystine, cystathionine and argininosuccinic acid. Therefore, a diagnosis of these disorders is made more accurately by examining the urine than blood.

(3) Renal-(transport) Aminoaciduria

This is caused by a defective carrier protein in the renal tubule responsible for the reabsorption of amino acids. In these cases, the blood concentrations of amino acids may be normal or low. Consequently, these disorders can only be diagnosed by examination of the urine.

Frequent elevation of multiple urinary amino acids can be found with renal tubule damage, because the same transport protein is responsible for the transportation of several amino acids.

Abnormal Blood Amino Acids

In table II are listed the abnormal plasma amino acids associated with specific disorders of inborn errors of amino acid metabolism. This table facilitates the diagnosis of specific disorders based on laboratory findings in blood amino acids. Detection of abnormal blood amino acids is usually indicative of certain inborn errors of amino acid metabolism. When blood amino acids are ordered following an urine metabolic screening, table II is used to confirm the diagnosis made by urine screening. When certain amino acids are elevated in the urine but not in the blood, this suggests that the elevation of urinary amino acids is due to impairment of renal reabsorptive functions and not to a metabolic defect, except those of no-threshold aminoaciduria. If drug induced renal toxicity is suspected, a second urine specimen should be screened one week after cessation of medication.

Note in the tables that not all increased blood amino acids are accompanied by elevated urinary amino acids, and vice versa. Therefore, screening urine or blood amino acids alone may overlook the diagnosis of certain disorders. For asymptomatic infants, both urine and blood should be screened.

Phenylketonuria

Hyperphenylalaninemia is clinically and biochemically heterogeneous. Several alleles at different gene loci are involved in the expression of various phenotypes.[11,18,19] Classical phenylketonuria (PKU) is only one of the phenotypes caused by inactive phenylalanine hydroxylase. It is important to differentiate among various subtypes because different treatments are required. For example, those with benign hyperphenylalaninemia do not necessarily require treatment, but adjuncts to conventional dietary management are required for PKU variants with tetrahydrobiopterin (BH_4) deficiencies.

As shown in figure 1, PKU can be detected by measuring the metabolites, such as phenylpyruvic, phenyllactic, and phenylacetic acids, and by direct measurement of blood phenylalanine. It has been well established that early institution of a diet low in phenylalanine is effective in preventing retardation from PKU. However, there were patients with elevated phenylalanine whose neurological symptoms persisted even when hyperphenylalaninemia was controlled by a low phenylalanine diet. It was later discovered that the primary defect in PKU variants is not related to the protein moiety of the phenylalanine hydroxylase, but to the regeneration and synthesis of the cofactor (BH_4)[9] which is essential for enzyme activity (figure 2). As indicated in figure 2, a defect in any of these three enzymes (I, II, III) will cause the concentration of phenylalanine to increase in the blood and urine. "Enzyme I" represents inactive phenylalanine hydroxylase, which raises the phenylalanine concentration by blocking the conversion of phenylalanine to tyrosine; enzymes II and III represent dihydropteridine reductase and dihydrobiopterin synthetase, respectively. Because of their involvement in the regeneration and synthesis of BH_4, their deficiencies will indirectly raise phenylalanine concentration since phenylalanine hydroxylase requires BH_4 for activity. The accumulation of phenylalanine in PKU variants, however, is less than that observed in the classic cases.

TABLE I

Abnormal Urinary Amino Acids and Metabolites and Their Associated Disorders

Abnormal Urinary Amino Acids (Metabolites)	*Abnormal Blood Amino Acids*	*Disorder*	*Overflow*	*Threshold*	*Renal*
Alanine (pyruvate)	Alanine and pyruvate	Hyperalaninemia			
β–alanine, taurine, β–aminoisobutyric acid, γ–aminobutyric acid	β–alanine, γ–aminobutyric acid	β–alaninemia			
Phenylalanine (o–hydroxyphenyl acetic acid, o–hydroxyphenyl–pyruvic acid	Phenylalanine	Phenylketonuria	Yes		
Tyrosine (p–hydroxyphenylacids, succinyl acetone, succinyl–acetoacetone)	Tyrosine	Tyrosinemia	Yes		
Histidine (imidazolepyruvic acid, lactic acid)	Histidine	Histidinemia	Yes		
Histidine	Normal histidine	Histidinuria			Yes
Urocanic acid (histidine and its metabolites may be slightly increased		Urocanic aciduria (urocanase of liver biopsy is defective)			
Camosine, anserine without the presence of 1–methylhistidine	Camosine	Camosinemia		Yes	
Leucine, isoleucine, valine, alloiso–leucine (branched chain δ–ketoacids)	Valine, leucine, isoleucine, alloisoleucine	Maple–syrup urine disease	Yes		
Valine	Valine	Hypervalinemia	Yes		
Glycine	Glycine (also leucine if on high protein diet	Nonketotic hyperglycinemia	Yes		
Glycine (propionic acid)	Glycine	Ketotic hyperglycinemia			Yes
Glycine, proline, hydroxyproline		Iminoglycinuria (asymptomatic)			Yes

TABLE I (continued)

Abnormal Urinary Amino Acids and Metabolites and Their Associated Disorders

Abnormal Urinary Amino Acids (Metabolites)	*Abnormal Blood Amino Acids*	*Disorder*	*Overflow*	*Threshold*	*Renal*
Methionine and small amount of valine, leucine, isoleucine, tyrosine, and phenylalanine (δ–hydroxybutyric acid)		Methionine malabsorption syndrome			Yes
Neutral amino acids including alanine, threonine, serine, glutamine, histidine, branched chain amino acids, phenylalanine, tyrosine, & tryptophan are increased. Proline hydroxyproline and glycine are normal (indole derivatives)		Hartnup disease			Yes
Generalized hyperaminoaciduria (glucose)		Fanconi or Lowe syndrome			Yes
Citrulline	Ciruulline and ammonia	Citrullinemia			
Lysine, arginine, ornithine, cystine (pipecolic acid)	Lysine, homoarginine	Hyperlysinemia		Yes	
Lysine, homocitrulline, citrulline, homoarginine, saccharopine (aminoadipic acid)	Saccharopine, lysine, homocitrulline, citrulline	Saccharopinuria			
Dibasic amino acids (arginine, lysine ornithine)	Normal blood amino acids	Hyperdibasic aminoaciduria			Yes
Lysine is markedly increased and slightly increased in arginine		Familial protein intolerance			
Arginine, cystine, cysteine–homocysteine, ornithine	Arginine and ammonia	Hyperagininemia		Yes	
Proline, hydroxyproline and glycine in great excess (up to 3 g/day)	Proline	Hyperprolinemia, type I			Yes
Same as previous plus Δ–pyrroline–5–carboxylate	Proline	Hyperprolinemia, type II			

(continued)

Since BH_4 is required for tyrosine and tryptophane hydroxylases to synthesize neurotransmitters, such as catecholamines, serotonin, and 5-hydroxyindole acetic acid (5-HIAA) to maintain normal neurological function (figure 3), simply lowering phenylalanine without providing BH_4 will not relieve patients from neurological symptoms. It is important to recognize PKU variants by laboratory tests because almost all patients of PKU variants die before their seventh year. Listed in table III are the laboratory tests that will help to differentiate classical PKU from PKU variants. In PKU variants, urinary 5-hydroxyindole acetic acid (5-HIAA), homovanillic acid (HVA), vanillylmandelic acid (VMA), catecholamines, and serotonin are reduced in concentration. Increased urinary neopterin indicates a deficiency in biopterin-synthetase, whereas an increase in both neopterin and BH_4 would suggest a deficiency in dihydropteridine-reductase.[5]

Urea Cycle Deficiencies

The urea cycle is the major route for the body to remove excess toxic ammonia in the form of urea (figures 4 and 5), and five inherited enzyme deficiencies involving urea cycle have been identified. It is extremely important to make a correct and prompt diagnosis since all these deficiencies are fatal within the first two weeks of life and are treatable.[4,12]

As shown in figure 5, all these enzyme defects will raise the ammonia levels. Elevation in ammonia is frequently associated with elevated glutamine and alanine (figure 4), which may be detected during amino acid screening. Once urea cycle deficiencies are suspected, additional amino acids should be measured to identify the specific enzyme defect. In figure 5, the various metabolic blocks related to all four major deficiencies are high-lighted. Amino acids and metabolites that may be elevated as a consequence of these inactive enzymes are also emphasized. Various biochemical changes among the different defects are also summarized in figure 6. Conceivably, a differential diagnosis could be made by simply measuring citrulline, orotic acid, pH, and the anion gap. It should be noted that the normal concentration of citrulline is low and is usualy not detectable by one and two-dimensional TLC. Consequently, a slight increase in citrulline concentration may be missed by TLC. Therefore, a sensitive HPLC procedure (or amino acid analyzer) should be used to measure citrulline quantitatively for the diagnosis of urea cycle deficiencies.

Newborn Screening

Phenylketonuria is the only inborn error of amino acid metabolism that is screened routinely by the majority of state laboratories for newborns.[17] Measurements of blood phenylalanine concentrations are more reliable than measurements of metabolites such, as phenylpyruvic acid in the urine, because the enzyme responsible for converting phenylalanine to its metabolites may not be matured in newborn babies. Many PKU babies do not show positive ferric chloride tests because insufficient phenylpyruvic acid is produced even when the blood concentrations of phenylalanine are elevated. It should be realized that at birth the phenylalanine concentrations of PKU babies do not always rise above the usual cutoff concentration. A second blood specimen should be obtained for the measurement of phenylalanine two weeks after birth, after protein intake (such as milk feeding), either to confirm the diagnosis made by the first specimen or to rule out transient hyperphenylalaninemia.

TABLE II

Abnormal Blood Amino Acids and Their Associated Disorders

Increased Amino Acids in Blood	*Abnormal Metabolites in Urine*	*Disorder*	*Corresponding Defected Enzyme (Source)*
Phenylalanine	o–Hydroxyphenyl acetic acid; phenylpyruvic phenylacetic, and phenyllactic acid	Phenylketonuria	Phenylalanine hydroxylases (liver)
Valine, leucine, isoleucine, alloisoleucine	Excessive branched–chain keto acids	Maple syrup urine disease	Branched–chain keto acid decarboxylase (leukocytes)
Valine		Hypervalinemia	Valine δ–ketoisovaleric acid transaminase (leukocytes)
Methionine; homocystine slightly increased	Homocystine in excess	Homocystinuria	Cystathionine synthetase (liver)
Tryptophan		Tryptophanemia	Tryptophan pyrrolase or formylase
Lysine	Ornithine, r–aminobytyric acid and ethanolamine	Hyperlysinemia	Not known
Lysine, arginine		Congenital lysine intolerance	Lysine dehydrogenase (liver)
Tyrosine (methionine and other amino acids may also be elevated)	Succinylacetone, succinylacetoacetate generalized aminoaciduria. P–OH–phenylacids, tyrosine (64–150 times normal)	Tyrosinemia I (hereditary tyrosinemia or tyrosinosis)	Fumarylacetoacetate hydroxylase p–OH–phenylpyruvic acid (liver)
Normal plasma tyrosine 2.39±0.6 mM (0–7 yr); 1.27±0.29 mM (8–55 yr)	p–Hydroxyphenylpyruvic acid acetic and lactic acids; tyrosine, N–acetyltyrosine, tyramine (88 – 170 times normal), and methionine may be increased	Tyrosinemia II (20–100 times normal)	Hepatic tyrosine aminotransferase
Cystathionine slightly elevated	Cystathionine (may be > 1 g per day)	Cystathioninuria	Cystathioninase
Homocystine and methionine and low in cystine	Homocystine (30–300 mg per day)	Homocystinuria	Cystathionine synthetase (liver, brain)

(continued)

TABLE II (continued)

Abnormal Blood Amino Acids and Their Associated Disorders

Increased Amino Acids in Blood	*Abnormal Metabolites in Urine*	*Disorder*	*Corresponding Defected Enzyme (Source)*
Glycine (some other amino acids)	Acetone	Hyperglycinemia, severe infantile	Not known
Glycine	Decreased urinary oxalate	Hyperglycinemia with hypooxaluria	Glycine oxidase ?
Argininosuccinic acid (about 4 mg/100 ml)	Argininosuccinic acid (2.5–9 g/day)	Argininosuccinic aciduria	Argininosuccinase (liver, erythrocytes)
Citrulline		Citrullinemia	Argininosuccinic acid synthetase (liver)
Ornithine	Ornithine may be normal	Ornithinemia	Ornithine transcarbamylase
Histidine (alanine may be elevated)	Alanine may be elevated; imidazolepyruvic, acetic, and lactic acids	Histidinemia (1:10,000)	Histidase (skin)
	Carnosine (20–100 mg/day)	Carnosinuria	Carnosinase
β–alanine, γ–aminobutyric acid	β–aminoisobutyric acid, γ–aminobutyric acid, and taurine in excess	Hyperbeta–alaninemia	β–alanine, δ–ketoglutarate transaminase
Proline	Hydroxyproline, glycine	Hyperprolinemia type I	Proline oxidase (liver)
Proline	Δ–pyrroline–5–carboxylate, hydroxyproline, glycine	Hyperprolinemia type II dehydrogenase	Δ–pyrroline–5–carboxylate
Hydroxyproline	No excretion of Δ–pyrroline–3–hydroxy–5–carboxylate or γ–hydroxyglutamic acid after hydroxyproline load	Hydroxyprolinemia	Hydroxyproline oxidase
Sarcosine, ethanolamine		Sarcosinemia	Sarcosine dehydrogenase
Phosphoethanolamine slightly increased (0.4 mg/100 ml)	Phosphoethanolamine (up to 150 mg/day)	Hypophosphatasia	Alkaline phosphatase (serum)

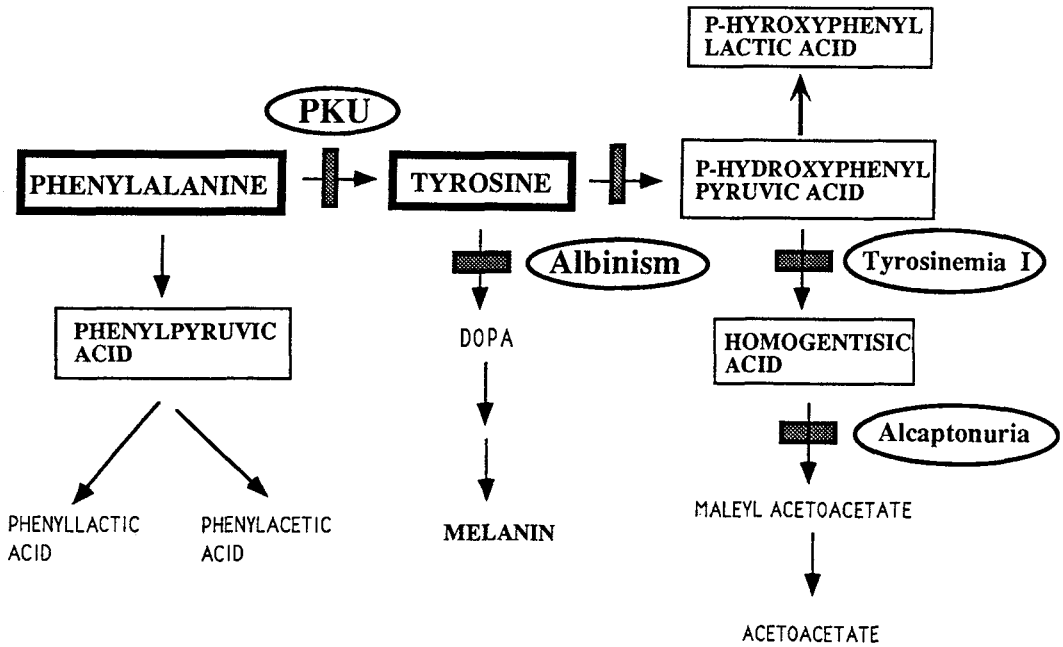

FIGURE 1. Metabolic pathways showing locations of various metabolic blocks, their associated disorders, and elevated key amino acids and metabolites.

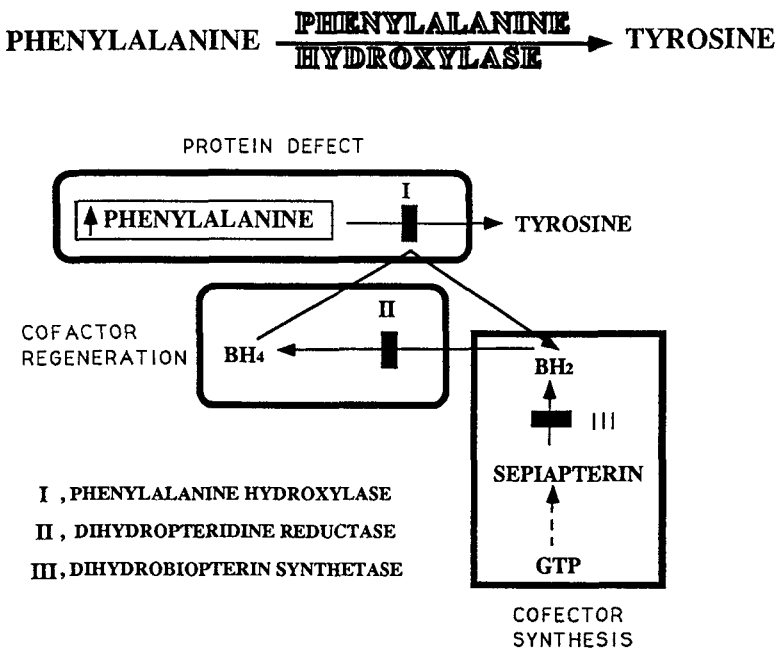

FIGURE 2. The relationship of three enzyme defects (I, II, III, blocks) with hyperphenylalaninemia and the difference between classical phenylketonuria (PKU) (protein defect) and PKU variants (lack of cofactor regeneration or synthesis).

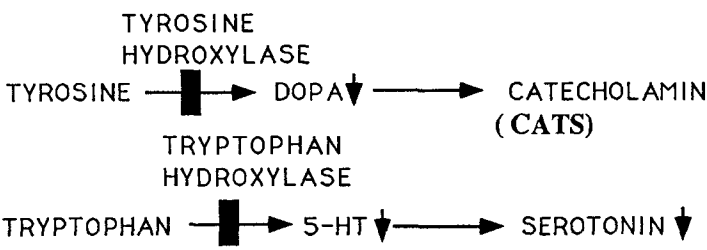

FIGURE 3. Neuropathy owing to reduced concentration of catecholamines and serotonin, etc. in phenylketonuria (PKU) variants are the consequence of inactive tyrosine and tryptophane hydroxylases, which require cofactor tetrahydrobiopterin (BH_4) for activity.

The Guthrie bacterial inhibition assay for phenylalanine in blood specimens collected on filter paper has been used for newborn screenings for PKU. Because this technique is based upon the inhibition of normal bacterial growth by β-2-thienylalanine included in the agar medium and the extent of reversal by phenylalanine contained in the filter paper, the assay may yield false-negatives and is not as accurate or precise as the fluorometric method of McCaman and Robins.[13] On the other hand, this fluorometric procedure requires a large aliquot of plasma or serum and lacks the sensitivity to measure the phenylalanine of the filter paper specimens which are routinely collected from newborn infants. A modified manual procedure with improved sensitivity has been developed by us which allows the measurement of phenylalanine for blood specimens on filter paper.[26] The modified procedure is suitable both for screening newborns for PKU and for monitoring blood phenylalanine during dietary therapy.

Metabolic Screening[2,13]

While most newborn screening is carried out by state laboratories, clinical laboratories are becoming increasingly involved in the screening for inborn errors of metabolism in infants showing growth and developmental retardation, mental problems, convulsions, seizures, comas, intolerance to protein feed, etc. Because the majority of inborn errors of amino acid metabolism are inherited as autosomal recessive traits, it is important to make the correct diagnosis and to identify heterozygotes in the family for counseling and for future family planning, regardless of whether or not treatment is effective for the homozygotes.

Urine is usually the first specimen received for metabolic screening from an symptomatic infants. However, a complete diagnosis of inborn metabolic errors should include (a) screening for increased concentrations of metabolites and amino acids in the urine, (b) blood amino acid determination, (c) identifica-

TABLE III

Laboratory Tests Useful for the Differentiation of Phenylketonuria from Phenylketonuria Variants

Analytes	*Diagnosis*
Elevated phenylalanine	Phenylketonuria
Decreased urinary 5-hydroxyindoleacetic acid, vanillylmandelic acid, homovanillic acid, serotonin, catecholamines	Phenylketonuria variants
Elevated neopterin but normal biopterin	Biopterin synthetase defect III
Elevated biopterin and neopterin	Dihydropteridine-reductase defect II

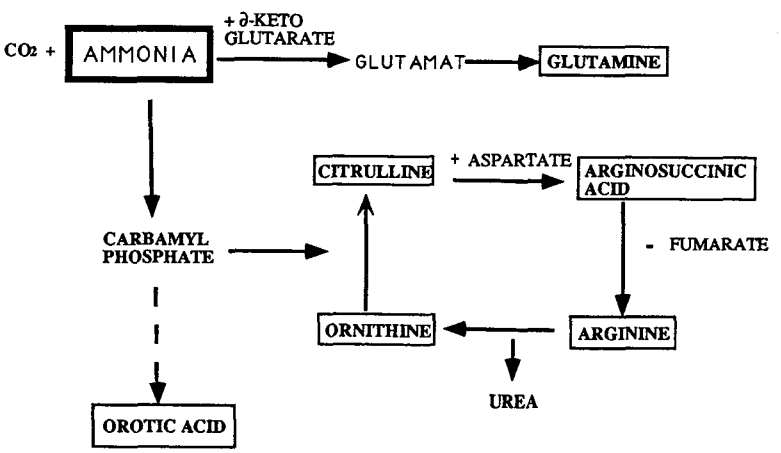

FIGURE 4. Key amino acids and metabolites involved in a normal urea cycle.

tion of the specific enzyme defect, and ideally (d) identification of the gene mutations (figure 7). Clinical laboratories usually are not involved in specific enzyme identification and gene analysis because both analyses require either tissue biopsy specimens or tissue cell cultures. Most of these low volume, specialized tests are performed in a specialized laboratory.

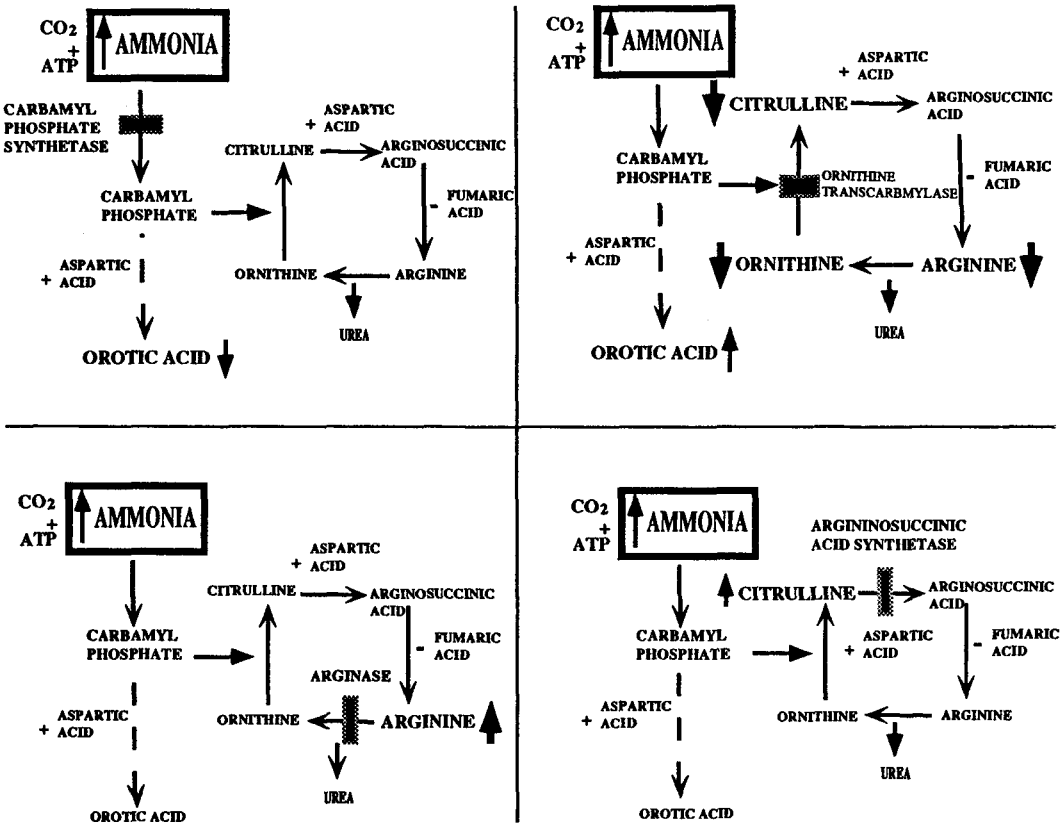

FIGURE 5. Four different disorders (caused by four different inactive enzymes) involving the urea cycle.

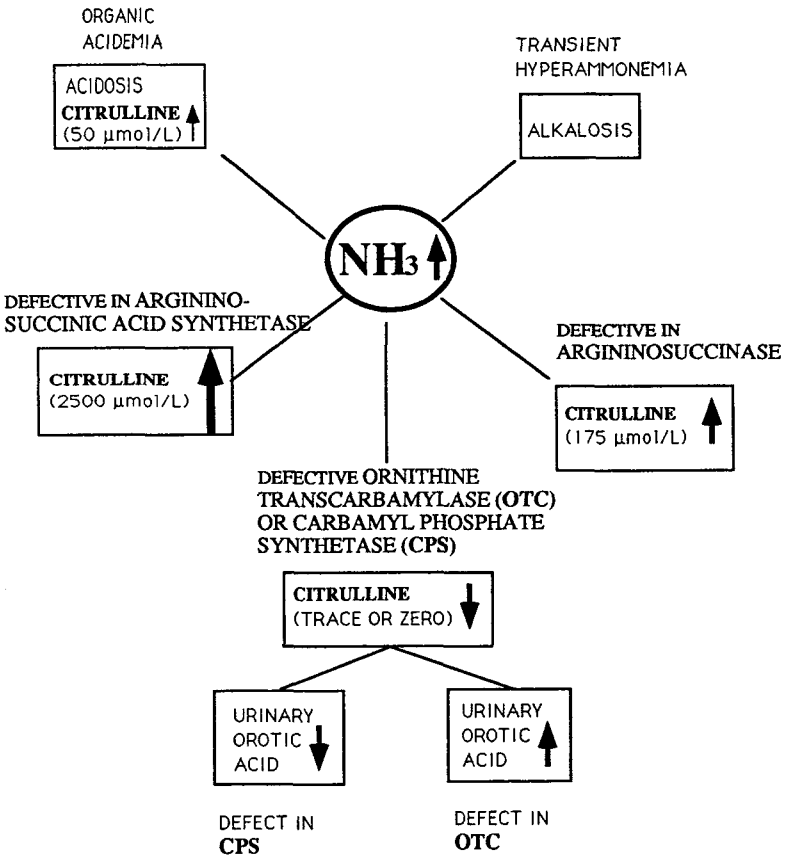

FIGURE 6. Differential diagnosis among various urea cycle deficiencies by measuring citrulline and orotic acid in addition to ammonia.

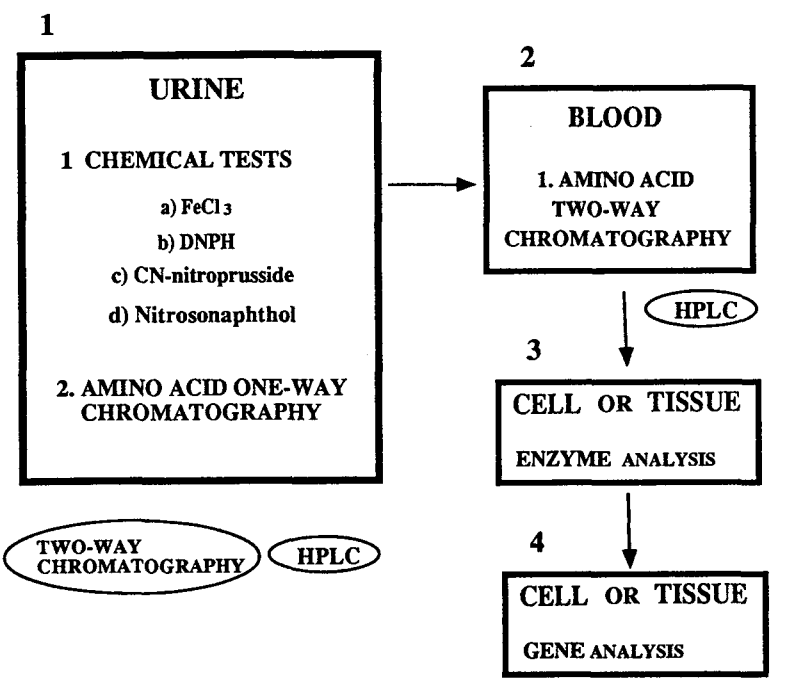

FIGURE 7. Specimens and test procedures normally employed in the sequence of steps for the diagnosis of inborn errors of amino acid metabolism. Techniques listed inside an elipse are complementary procedures to be used for confirmation of results obtained by routinely used procedures listed inside the rectangular boxes.

Laboratory Techniques[14,21]

The tests described are used frequently in our clinical laboratories for the screening for inborn errors of amino acid metabolism.

Chemical Tests

The following chemical tests are simple colorimetric procedures which use random urine specimens. Most of the tests are designed to screen for excess metabolites derived from elevated amino acids due to inborn errors of amino acid metabolism. Five chemical tests are routinely used to screen urine for inborn errors of amino acid metabolism.

(1). Ferric Chloride Test: A green color indicates positive for PKU. A fading green color indicates the presence of p-hydroxyphenylpyruvic acid for tyrosinemia. The test gives various colors with a variety of drugs including a purple color with salicylates.

(2). 2,4-Dinitrophenylhydrazine (DNPH) Test: The appearance of a yellow precipitate 10 min after the addition of DNPH reagent indicates the presence of excess of alpha-keto acids. A positive DNPH test confirms the diagnosis of PKU by ferric chloride test. A greenish gray color suggests maple syrup urine disease.

(3). Nitrosonaphthol Test (Millon Reaction): The appearance of an orange-red color indicates the presence of excessive amounts of tyrosine derivatives.

(4). Cyanide-nitroprusside Test: The appearance of a magenta color indicates the presence of excessive cystine, or homocystine, or β-mercaptolacetate-cysteine disulfide.

(5). Silver-nitroprusside Test: The appearance of a magenta color indicates the presence of homocystine. It is used to differentiate between homocystine and cystine when there is a positive reaction in the cyanide-nitroprusside test.

These screening tests are sensitive, but not specific. Therefore, positive results should be confirmed by more definitive assays for amino acids, such as two-dimensional TLC. These clinical tests also complement the method of TLC because the two methods measure different analytes derived from the same enzyme defect, whereas spots on TLC plates could be produced by interferences present in the urine specimens.

One and Two-Dimensional Thin Layer Chromatography

One-dimensional TLC is usually carried out on the identical urine specimens with chemical tests (figure 7). The arrangement for sample application and the distribution of various amino acids on the chromatogram are shown in figure 8. Because the amino acids are not completely separated, the one-dimensional TLC is used mainly to confirm results obtained by the chemical tests and to identify abnormal amino acid patterns. When the one-dimensional, urinary amino acid pattern is abnormal, a two-dimensional TLC should be performed immediately on the same specimen in order to identify all abnormal amino acids specifically (figures 9 and 10). For both one- and two-dimensional TLC, duplicate plates are made so that one plate may be stained with ninhydrin and one with isatin. Because of the lower sensitivity of the isatin reagent, the plate stained with isatin should contain twice the amount of specimen. Isatin is used not only to identify proline and hydroxyproline, it also produces different colors for several amino acids which are helpful for their identification. These amino acids include phenylalanine, tyrosine, cystine, ornithine and carnosine, etc. Isatin will also help to identify drug

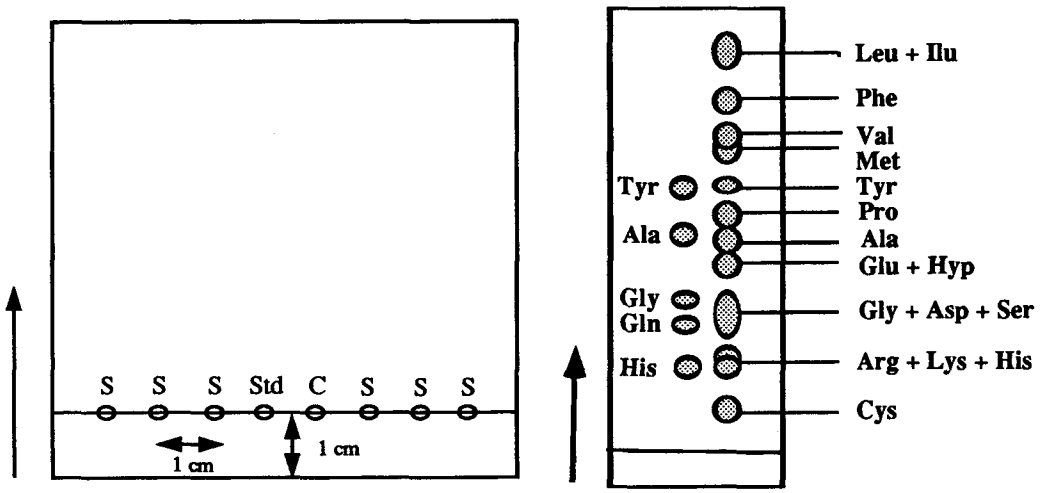

Plate: Cellulose, 9.2 x 9.2 cm
Tank: Alltech, 17 x 15 x 8 cm, cat # 7580
Solvent: Butanol/acetic acid/water, 12/3/5 by volume
Sample (S): Urine containing 0.5 mg creatinie (for ninhydrin) urine containing 1 mg creatinine (for isatin)

Control (C): Pooled normal urines containing 0.5 (for ninhydrin) or 1 mg (for isatine) creatinine
Amino acid standard (Std): Apply 2 µL of 4 times diluted standard Sigma AA-S-18

FIGURE 8. One dimensional amino acid chromatography. Arrangement for sample application and distribution of various amino acids after chromatography.

interferences, such as ampicillin, amoxicillin, and gentamycin.[28] Ampicillin, in particular, is frequently used in pediatric patients. Ampicillin and its metabolites migrate in the region of leucine, isoleucine, and valine in one dimensional TLC and give ninhydrin-positive spots, similar to what may be found in maple syrup urine diseases. When isatin is used, all these antibiotics and their metabolites

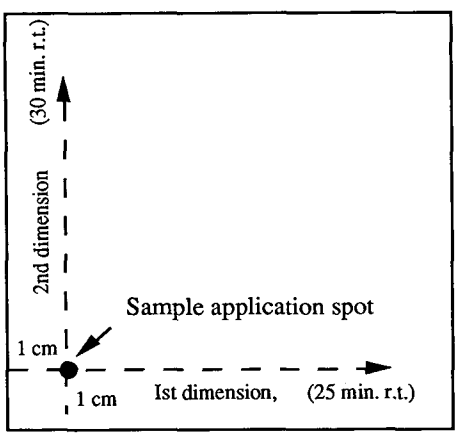

Plate: Cellulose sheet, 7 x 7 cm
Tank: Kodak, 3.5 x 3.5 x 1.5 inches, cat. #136.6269
Solvent: First, pyridine/acetone/NH_4OH/water, 4.5/3/0.5/2, by volume.
second, isopropanol/formic acid/water, 7.5/1.25/1.25, by volume.
Urine: Containing 0.5 mg creatinine (for ninhydrin), or 1 mg creatinine (for isatin)
Plasma extract: 5 µL(ninhydrin), 10 µL (isatin)
Standard: 2 µL of 4 times diluted Sigma standard, AA-S-18
Control: Pooled normal urines containing 0.5 mg (ninhyrin) or 1 mg (isatin) creatinine

FIGURE 9. Conditions and arrangement for two-dimensional amino aicds after chromatography.

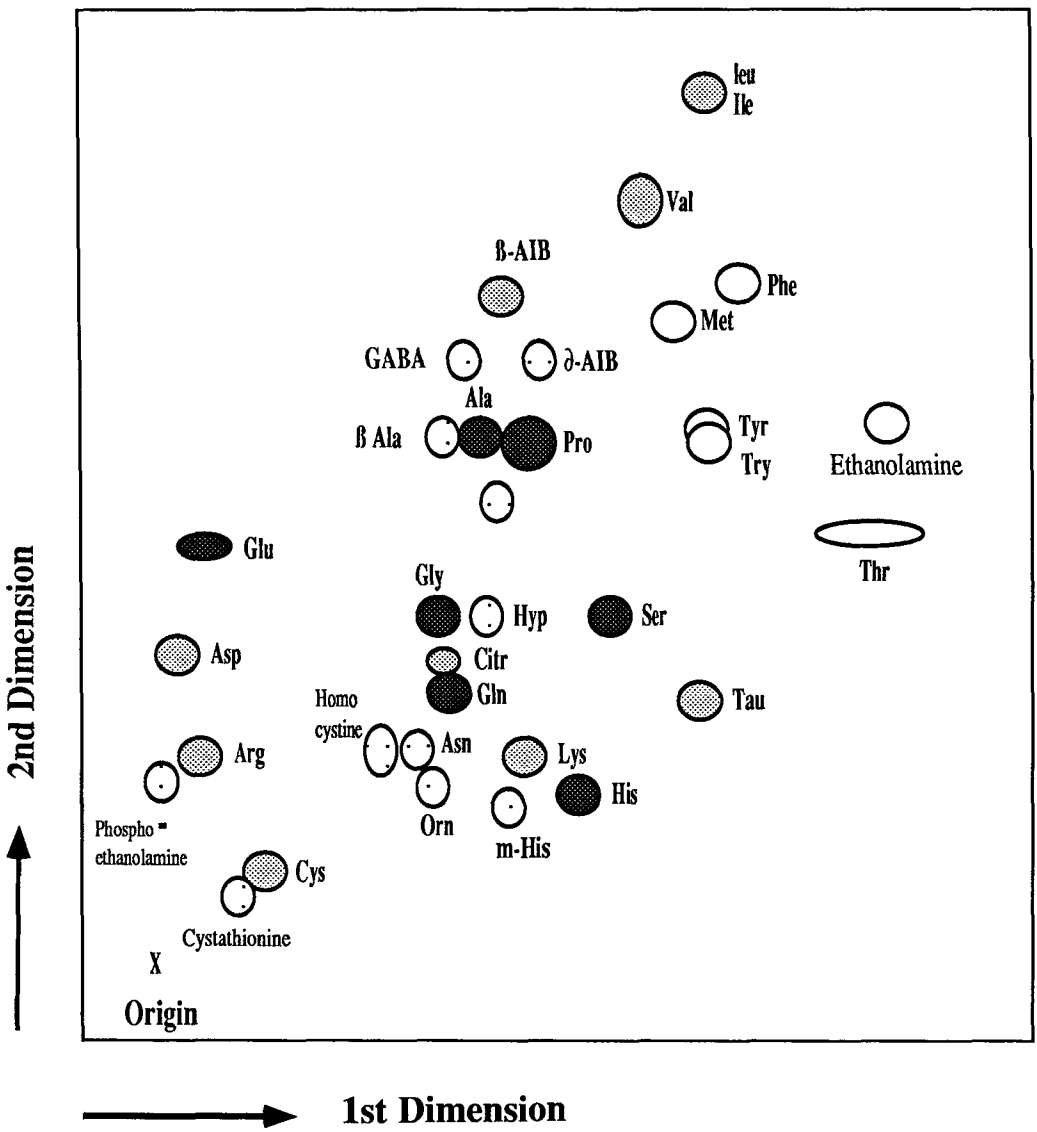

FIGURE 10. Chromatogram showing the distribution of various amino acids after two-dimensional thin layer chromatography.

give a bright yellow color, distinctively different from the pink color produced by most amino acids.

Thin layer chromotography is usually a labor intensive and time-consuming process. By using a smaller plate it was found that chromatography was greatly shortened, and much less time was also required for spotting because of the smaller sample volume. Moreover, using smaller plates does not sacrifice the resolution among amino acids.[27] For one-dimensional chromatography, as many as six urine specimens (S) can be analyzed on a single plate (figure 8). Every plate includes one amino acid standard (Std) mixture and one urine control (C). The amino acid standard mixture is used to check for the resolution of chromatography, the color reagent, and to the positions of some key amino acids. The urine control helps to distinguish abnormal from normal urinary amino acid patterns. The urine control is pooled from

newborn to one month old infants. Since young infants contain much higher concentrations of urinary amino acids, using pooled urine from young infants as a control avoids the reporting of false positives for young infants.

A small plate is also used for two-dimensional amino acid TLC. The modification allows rapid semiquantitative analysis of amino acids within a few hours (figure 9). Because proteins and peptides do not migrate on thin layer cellulose plates, two-dimensional TLC helps to identify interferences caused by peptides in the urine when other methods are employed. When abnormal urinary amino acid(s) are identified by two-dimensional chromatography,[25] a plasma specimen from the same patient should be obtained, and the abnormal amino acid(s) should be confirmed and quantified by HPLC analysis.

Amino Acids by High Performance Liquid Chromatography

Quantitative measurements of both urinary and blood amino acids can now be made readily by HPLC. Quantitation is important not only for a more definitive diagnosis but is useful for following patients undergoing dietary therapy. The quantification of amino acids by automatic amino acid analyzers has been largely replaced by HPLC in the clinical laboratories in recent years because of its speed and sensitivity. Though the most popular HPLC method for amino acid determination is based on a precolumn derivatization of amino acids with O-phthaladehyde (OPA), this method is limited to primary amino acids. Recently, another new HPLC procedure, using color reagents OPA and 9-fluorenylmethyl-chloroformate (FMOC), has been developed[3,20] to react with amino acids in a sequence and detects both primary and secondary amino acids (figure 11). An automated precolumn sample preparation system is needed to carry out a two-step precisely time-controlled, derivatization procedure. During derivatization, all primary amino acids react with OPA, and all secondary amino acids with FMOC. The system is totally automated by Hewlett Packard including an autosampler so that large number of specimens can be run unattended. In addition to the automation, the short turn-around time also makes this new HPLC system most suitable for clinical laboratory use. It should be noted, however, that an electrochemical detector is required for quantitative measurement of cystine and homocystine. A sensitive and quantitative chemical procedure has been developed to allow measurement of cystine and homocystine and to differentiate between them.[29] Because of the simplicity of this new method, the same procedure is also used for the routine screening of cystinuria.

Miscellaneous Analyses

Serotinin, hydroxyindoleacetic acid (HIAA), urinary catacholamines, vanillylmandelic acid (VMA), and homovanillic acid (HVA), routinely measured in most clinical laboratories, are useful for the identification of PKU variants. They are usually determined by HPLC and TLC procedures.[1,3,8] A chemical method available for measuring urinary orotic acid by reacting random urine with bromine water and measuring a color product at 480 nm[14,16] is helpful for the differentiation between defective ornithine transcarbamylase (OTC) and defective carbamyl phosphate synthetase (CPS) in urea cycle deficiencies (table III). As an adjunct test to confirm the diagnosis of tyrosinemia made by amino acid determinations, the measurement of delta-aminolevulinic acid can be made either quantitatively by HPLC[23] or qualitatively[22] (figure 12). The increased concentration of succinylacetone in tyrosinemia inhibits the enzyme delta-aminolevulinic acid dehydrase and produces an elevation of delta-amino levulinic acid in the urine.

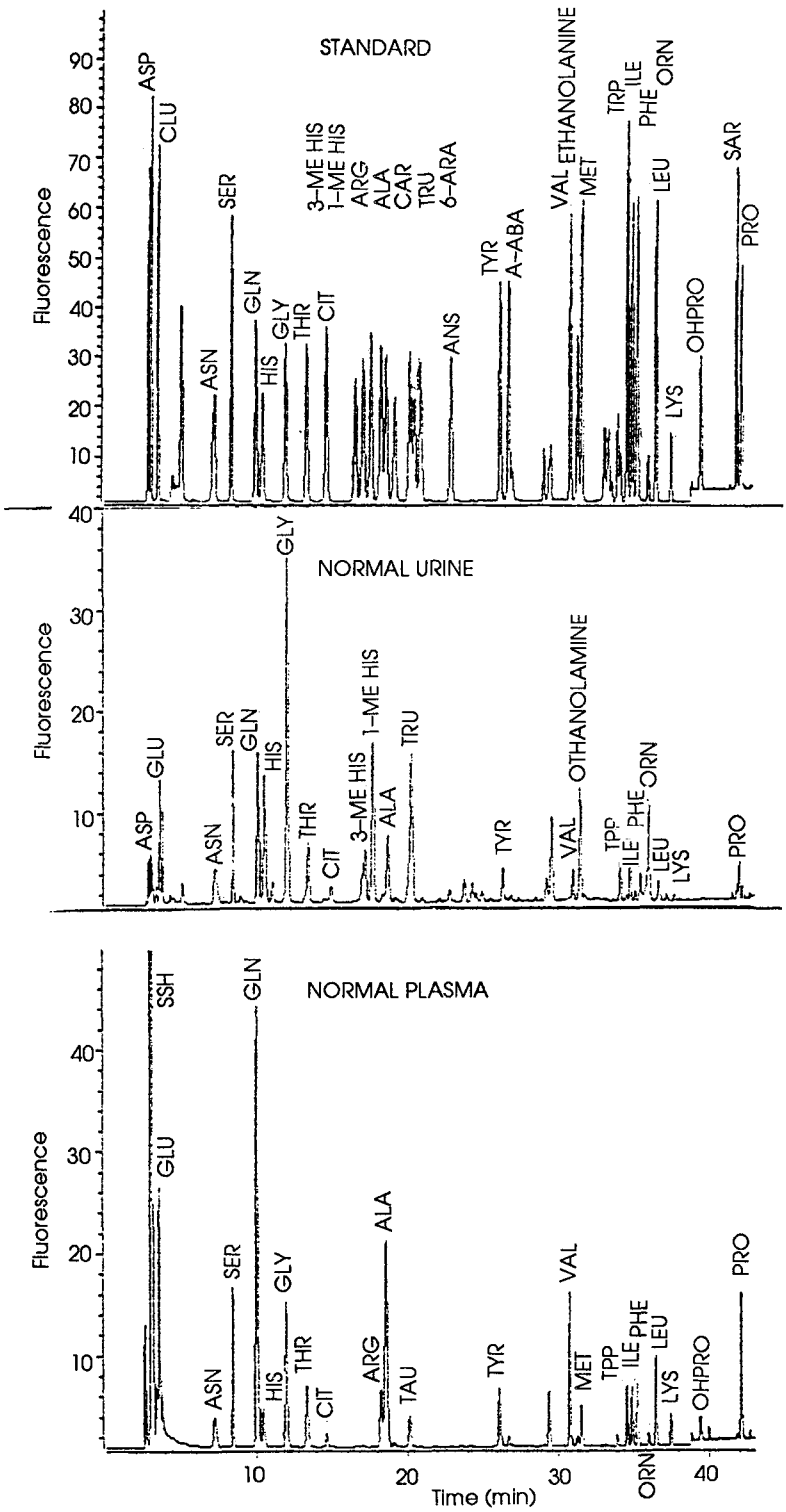

FIGURE 11. Amino acid profiles of a standard amino acid mixture, a normal plasma extract by sulfosalicylic acid (SSA) and a normal urine by an automated precolumn derivatization with both 0-phthaladehyde (OPA) and 9-fluorenylmethylchloroformate (FOMC) and high performance liquid chromatography.

Sample: urine, 1 µl containing 0.2 g per L creatining; plasma, 1 µl of deproteinized plasma; amino acid standard, 1 µl containing 250 µmol per L of every amino acid. *Instrument*: HP 1090 series M liquid chromatographs. *Column*: 200 × 4.6 mm Hypersil-ODS, 5 µm. *Mobile phase*: A = 0.03 M NaAc + 0.25% THF + 0.1 mM NaN_3 (pH 7.5); B = CH_3CN/).1 M NaAc (4:1). *Flow*: 1 mL per min. *Gradient*: 3 percent to 40 percent B in 20 min. *Reaction Temperature*: Ambient. *Column Temperature*: 46°C. *Detection*: HP 1046 A Fluorescence Detector. Excitation was at 230 nm and emission at 455 nm. After 16 min. change excitation to 266 nm and emission to 315 nm.

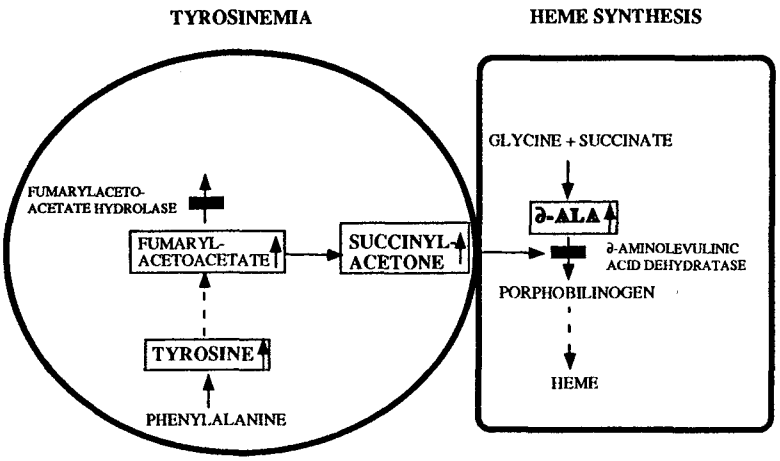

FIGURE 12. Metabolic pathways indicating why the measurements of delta-aminolevulinic acid is useful for confirming the diagnosis of tyrosinemia.

Screening for Organic Aciduria[14,21]

As shown in figure 1, amino acids such as phenylalanine and tyrosine, accumulated owing to enzyme blockage, will convert to various metabolites and raise their concentrations in the blood. In the case of PKU, it is the alpha-keto acids that become increased in the blood (figures 1). However, a variety of organic acids may also be elevated in inborn errors of amino acid metabolism following a similar mechanism. Consequently, the screening of organic acids in urine is complementary to the amino acid determination for the diagnosis of these amino acid disorders. If table I is examined carefully, it will be found that the measurement of pyruvic acid, O-hydroxyphenylacids, and p-hydroxyphenylacids provide support for the diagnosis of hyperalaninemia, PKU, and tyrosinemia, respectively. Measurements of imidazolepyruvic acid and lactic acid will help to distinguish histidinemia from histidinuria, just as propionic acid helps to distinguish ketotic hyperglycinemia from nonketotic hyperglycinemia, and methylmalonic acid to distinguish the homocystinuria of B_{12} coenzyme metabolic defect from that owing to cystathionine synthase deficiency. Organic acids are traditionally measured and identified by a capillary gas chromatograph-mass spectrometer. However, recent developments in liquid chromatograph-mass spectrometry could make organic acid determinations easier than before since sample derivization is not required.

Summary

Background information describing techniques and current procedures has been provided for laboratories interested in establishing programs that screen for inborn errors of amino acid metabolism. The normal sequence of investigations has been discussed, as well as the type of specimens used, and the techniques and procedures employed during the various steps of screening. Several new procedures introduced here—manual fluorometric procedures for PKU, small thin layer chromatographies for the semiquantitative measurement of amino acids, and automated HPLC systems for the precise quantification of amino acids—should be helpful in improving the overall amino acid screenings.

Interpretation of results obtained from urine and blood screenings and the different effects of inborn errors and renal absorptive function on the results of urinary amino acids are noted. In view of the recent findings in PKU variants and urea cycle deficiencies, strategies for differential diagnosis of PKU variants from classic PKU and differentiation among various urea cycle deficiencies were also suggested.

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