methods in nutrition

Plasma amino acids: screening, quantitation, and interpretation[1,2]

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Information about the steady-state concentration of amino acids in human plasma has accumulated steadily in recent years, partly as the result of new methodology, but also because of the relevance of this information to human health and disease. There are two areas in particular that have both served and benefited from the growth and application of this knowledge. One is the field of nutrition; the other pertains to hereditary and acquired diseases that affect amino acid metabolism.

A number of simple qualitative or semiquantitative methods have been developed to detect perturbation of the normal amino acid pattern in plasma. As a result, it has been possible to detect, with mass screening early in life, the few subjects with abnormal amino acid metabolism among the many who are normal (1, 2). Nonetheless, abnormal patterns and their exact significance can be recognized only when the normal is known. Consequently, there have been many studies, often using the newer semiautomatic elution chromatographic methods of quantitative analysis, to define the normal interindividual variation in steady-state distributions of amino acids in human plasma. When these parameters are known, variation more subtle than that generated by inborn errors of metabolism can also be identified. Hence we know that circadian rhythms and nutritional factors generate intraindividual variation in the steady-state levels of amino acids in plasma.

It is our intention to describe the methods that have been widely used to study plasma amino acids in man. We will also discuss the advantages and shortcomings of the methods with which we are familiar. The mean, standard deviation, and range of values for the principal amino acids in the plasma of normal human subjects from birth to maturity are compiled from the literature so that this report may assist the clinical investigator.

The plasma free amino acid pool; a steady state?

Measurements of plasma amino acids are usually made at a single point in time, and it is upon such measurements that we must often decide whether a subject manifests "normal" or "abnormal" amino acid metabolism, even though the dynamics of amino acid metabolism have not been evaluated. Christensen (3) has discussed the factors that regulate the apparent steady-state levels of amino acid in plasma; these are indicated in Fig. 1. The equilibrium of amino acids in plasma is a reflection of their inflow (dietary intake, intestinal absorption, release from endogenous protein stores, and net endogenous synthesis of nonessential amino acids), and their outflow (uptake into liver and nonhepatic tissues, endogenous protein synthesis, and catabolism). The fact that plasma levels fluctuate remarkably little, indeed usually less than $\pm 50\%$, despite intermittent dietary influxes that may be tenfold greater than the

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[2] Supported by the Department of National Health and Welfare Grant 604-7-643, Medical Research Council Grant MT-1085, and the National Genetics Foundation, New York.

The American Journal of Clinical Nutrition 24: JULY 1971, pp. 876–890. Printed in U.S.A.

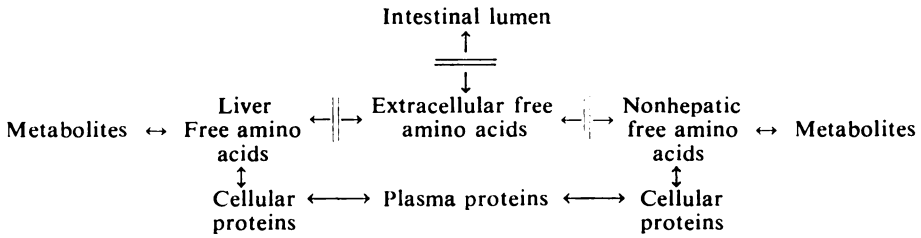

FIG. 1. Equilibrium system for free amino acids in extracellular space, including plasma. —||— Indicates flux across cell membranes.

amounts of amino acid present initially in the extracellular space, indicates the importance of tissue uptake and metabolism in the control of amino acid levels in plasma.

Membrane transport. Uptake of amino acids into cells against a tissue:plasma gradient is achieved by conjugate forces in the membrane, which utilizes ionic interactions and energy to regulate the flux of the chemically unmodified substrate (4, 5). Genetic events can alter amino acid transport in man (6, 7) and this, coupled with evidence for specificity in the selection of the substrate by the transport process, indicates that the membrane possesses transport proteins (8). Uptake of amino acids by human cells is evidently constitutive and operates independently of metabolic events that may affect the size of the intracellular pool. For example, the renal transport of an amino acid, the catabolism of which is blocked, is no different in the "blocked catabolic" mutant than in the normal person. Thus, one can conclude that transport regulates plasma steady-state levels to some extent by allowing amino acids to equilibrate with the intracellular pools that are many times larger than the extracellular pool.

Periodicity of control systems. The presence of circadian periodicity produces measurable intraindividual variation (9–11), indicating that there are subtle regulatory mechanisms that control steady-state levels of amino acids in plasma. Analysis of the periodicity of plasma amino acid levels indicates that it is partly dependent on the exogenous input of the amino acids themselves, but that it is also modulated by other nutrients and by hormonal events that influence cellular uptake of amino acids. Periodicity of enzyme activities that control entry of amino acids into catabolic pathways further determines the intracellular fate of these substrates. Awareness of circadian periodicity may be important when the investigator uses the concentration of an amino acid in plasma to define, for example, the heterozygous phenotype of an aminoacidopathy (12).

Nutritional state. The state of nutrition must also be considered when interpreting interindividual and intraindividual variation in amino acid levels in plasma. Obese subjects have a particular pattern of hyperaminoacidemia indicative of the insulin ineffectiveness characteristic of obesity (13). Although pre- and postprandial sampling is likely to witness only subtle changes in the plasma pattern when the normal subject is eating a normal diet (14), prolonged fasting in obese and nonobese subjects may modulate the concentrations of plasma amino acids to a much greater extent. For example, in the initial few days there may be a two- to threefold increase in the plasma concentration of branched-chain amino acids, whereas an equivalent increase in glycine and threonine appears only after this initial period; as fasting continues, a dramatic fall in α-alanine occurs. The initial rise in amino acid levels presumably reflects a fall in plasma insulin activity, whereas the latter changes reflect, in turn, increased peripheral release (threonine and glycine) and increased splanchnic utilization (α-alanine) of amino acids.

These observations may seem extreme and irrelevant. However, many patients with aminoacidopathies are first seen when acutely ill, often at times of diminished food intake. Under such conditions, it will be necessary to segregate the amino acid imbalance caused by secondary illness from that associated with the primary disease. Furthermore, if

screening programs are extended to countries where malnutrition is common, as may indeed be the case (2), the investigator will need to distinguish the amino acid imbalance due to primary malnutrition (17, 18) from the primary inherited aminoacidopathy. Finally, the investigator should remember that selective modification of protein intake may affect plasma amino acids (14, 19); this may become significant, for example, when patients with hyperammoniogenic aminoacidopathies are treated with low protein diets. Many additional viewpoints (20) on the influence of protein nutrition on the steady state of free amino acids in plasma are available for the interested reader.

Qualitative methods of amino acid analysis

The adaptation of simple partition chromatographic methods to the analysis of amino acids has provided an opportunity to evaluate the normal amino acid pattern in plasma or whole blood and its variations at birth and thereafter. Consequently abnormalities representing hereditary or acquired variation in amino acid metabolism can now be easily recognized in mass screening programs.

Two qualitative methods in particular (21, 22) have been widely used to survey the human plasma amino acid profile; these are indicated in Fig. 2. Both techniques combine deproteinization of the sample directly on the filter paper, with development of the chromatogram in an *n*-butanol, acetic acid, water solvent (12:3:5) (23); the use of various location reagents increases the sensitivity with which various amino acids can be detected. The ease with which samples in large numbers may be processed with these filter paper methods, makes them preferable to thin-layer methods for purposes of mass screening.

Plasma method. A sample of whole blood is drawn from a skin puncture into heparinized microcapillary tubes (75 mm x 1.4 mm, outer diameter) (21). After sealing one end of the tube with plasticine, the plasma is separated from the cells by centrifugation in an International microcapillary centrifuge (Model MB). A 10-μl aliquot of plasma[3] is applied directly to a line drawn 2.5 cm from one edge of a 25.4-cm square, corner-punched Whatman No. 3 MM filter paper (A pattern). Eleven samples can be applied at about 2-cm intervals to one sheet of filter paper. Transfer of plasma is simplified if a cut segment of tube, the length of which is equivalent to 10 μl plasma, is held in the vertical position against the paper and the plasma is allowed to flow onto the paper in a single application. The mean error in obtaining a 10-μl volume by this method is $\pm 3\%$ (24). Approximately 1 min is required to process one sample completely. The filter papers are then mounted on "universal" frames with spacers (25). They are then developed overnight by ascending partition chromatography in a freshly prepared *n*-butanol, acetic acid, water mixture (12:3:5). In the morning, the papers are removed from the solvent, dried for 1 hr at room temperature in a stream of air, then removed from the frame and stained in a mixture of ninhydrin (0.25% w/v), isatin (0.01% w/v), and 2,6-lutidine (1% v/v) in acetone. The chromatograms are dried at 80 C for 15 min and then viewed with combined transmitted and reflected light upon a lighted X-ray viewing screen. Significant elevation or diminution of amino acid content is readily visible, and the sensitivity of the method has been evaluated (21). The upper three-fourths of the paper (above the glutamic acid spot) may then be overstained with Ehrlich's reagent (*p*-dimethylaminobenzaldehyde) to identify hydroxyproline, citrulline, and homocitrulline. If the lower portion of the chromatogram is stained with Pauly's reagent (diazotized sulfanilic acid), the histidine spot will be selectively identified.

Whole blood method. Whole blood is transferred from a skin puncture onto Schleicher and Schuell (Keene, New Hampshire) paper No. 903; the paper must be soaked through on both surfaces (22). The air-dried sample is then dry-autoclaved at 250 F for 3 min. A $\frac{3}{16}$-inch punch (equivalent to about 7 μl of blood) or $\frac{1}{4}$-inch punch of the blood spot is then withdrawn and inserted into holes of equivalent size in a

[3] Aliquots of 20 μl applied in two lots can also be analyzed by this method without sacrificing resolution. The sensitivity of the method can thus be increased.

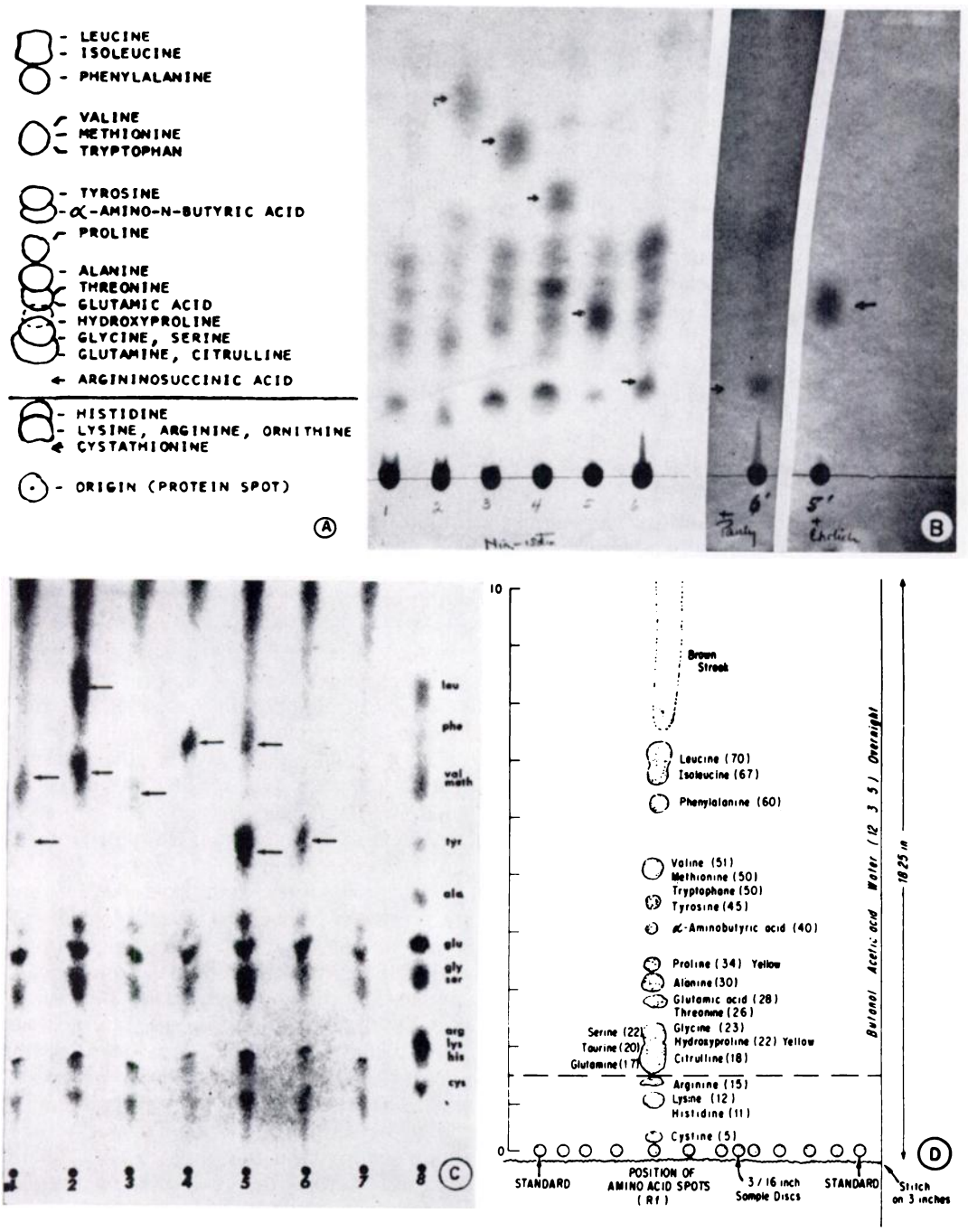

FIG. 2. Examples of partition chromatographic methods for screening on plasma amino acids (see text for details of development and staining). A) Map of the spots for "plasma method" (21). B) Examples of actual samples examined by "plasma method" showing, from left to right: *1)* normal plasma; *2)* hyperphenylalaninemia (in PKU); *3)* hypermethioninemia; *4)* transient neonatal hypertyrosinemia; *5)* hypercitrullinemia (in citrullinemia); *6)* hyperhistidinemia (in histidinemia). The next channel (6^1) shows Pauly overstain of sample 6 and the last channel (5^1) shows Ehrlich overstain to confirm citrulline. (Reproduced from Scriver (52).) C) Examples of actual samples examined by "whole blood method" (22) showing from left to right: *1)* "homocystinuria; *2)* "maple syrup urine disease;" *3)* transient hypermethioninemia; *4)* "phenylketonuria;" *5)* "neonatal tyrosinemia;" *6)* hypertyrosinemia; *7)* normal plasma; and *8)* standard amino acid mixture. (Reproduced from Levy et al. (29).) D) Map of the spots for whole blood method. (Taken from Efron et al. (22).)

"carrier" sheet of filter paper (Whatman No. 3 MM); the discs should be handled with forceps to avoid contamination (26). Friction holds the discs in place satisfactorily. The carrier sheet, which may be charged with 25 discs set 2.5 cm from one edge, is developed overnight by ascending chromatography (paper size, 63 x 30 cm, cut from 30-cm width rolls) or by descending chromatography (paper size, 57 x 33 cm with a wick, 57 x 7-6 cm, sewn in with zigzag stitching (22)), in the *n*-butanol, acetic acid, water solvent (12:3:5). Staining and location of amino acids is performed as described above.

This method is advantageous in its ease of collection and storage of samples. However, streaking of amino acid spots on the developed chromatogram, because of hemoglobin tailing, may be a problem. Two modifications avoid this problem:

1) The "tandem chromatography technique." Szeinberg and colleagues (27) use 25-cm square Whatman No. 3 MM filter paper (see plasma method) as the carrier sheet for the $\frac{3}{16}$-inch Schleicher and Schuell discs containing the whole blood sample. The chromatogram is developed first by ascending chromatography for 6 hr in an isopropanol, water mixture (3:1). The paper is then dried for 1 hr at room temperature in a stream of air, after which the discs, which retain the hemoglobin, are pushed out. The chromatogram is then replaced in the same direction, in a second solvent mixture of *n*-butanol, acetic acid, water (12:3:5) solvent and developed overnight; the developed chromatogram is dried and stained as described above.

2) The "elution technique." Blood-soaked discs (12-mm diameter, Schleicher and Schuell filter paper No. 2992 containing about 40 μl whole blood) are eluted overnight in 100 μl of an ethanol and water mixture (60:40) in capped conical microcentrifuge tubes (0.4-ml capacity) (28). Forty microliters of the hemoglobin-free eluate is then spotted, using several applications, onto Whatman No. 3 MM paper (25 x 25 cm) and developed by ascending chromatography as described above (21). An additional spot, probably glutathione, is found between the "dibasic" amino acids and the origin. The sensitivity of this modification is equivalent to the plasma method (21). The size of the disc and the volume of the alcohol mixture could probably be reduced to diminish the number of transfers of eluate to the filter paper.

Performance. Both basic methods have also been used satisfactorily for semiquantitative analysis. As a result, monitoring of plasma amino acid concentrations in patients who are being treated for an aminoacidopathy is greatly facilitated. Present experience indicates that this relaxed approach is compatible with good clinical control of most aminoacidopathies.

Large field trials have been performed with both methods. Mass screening of newborn infants has been reported on about 150,000 subjects (24, 29–31) and it is evident that the methods perform well for this purpose. A wide variety of normal transient patterns of hyperaminoacidemia has been observed (24, 29–31). Many of the aminoacidopathies associated with a specific hyperaminoacidemia have also been recognized in the newborn period with these methods (see Table 1). Partington (32) has evaluated the sensitivity and specificity of the plasma method (21) for the specific detection of hyperphenylalaninemia; he found it to be equivalent to the inhibition assay of Guthrie and Susi in reliability.

Ninhydrin-positive aminoacidopathies that are presently known and could be detected by chromatographic screening of plasma and whole blood are listed in Table 1; aminoacidopathies still unknown might also be discovered by these methods in the future. The above-mentioned methods can be used for preventive community screening, if personnel are trained to interpret the chromatograms and to be aware of the significance of the amino acid patterns that they observe. There must also be facilities to locate, retest, and follow all subjects with abnormal initial tests (24).

Quantitative methods of amino acid analysis

A little over a decade ago, very few investigators were able to perform more than one complete analysis per week, i.e., of the free amino acids in physiological fluids.

TABLE 1
Aminoacidopathies that can be detected by plasma or whole blood screening

Condition or disease	Amino acids affected	Abnormal enzyme	Comment
Group I: Common perinatal (adaptive) traits			
Neonatal hyperphenylalaninemia[a]	Phenylalanine	Phenylalanine hydroxylating system (?)	Benign; may respond to folic acid; often occurs with tyrosinemia.
Neonatal tyrosinemia[a]	Tyrosine	*p*-Hydroxyphenyl pyruvic acid hydroxylase (EC 1.14.2.2)	Benign; responds to ascorbic acid and reduced protein intake.
Hypermethioninemia[a]	Methionine	ATP: L-methionine S-adenosyltransferase (?) (EC 2.5.1.6)	Benign; usually found with high protein intake.
Hyperhistidinemia[a]	Histidine	L-Histidine ammonia lyase (?) (EC 4.3.1.3)	Benign; related to high protein intake.
Group II: Inherited aminoacidopathies			
1. The hyperphenylalaninemias			
i) Classical phenylketonuria[a]	Phenylalanine	L-Phenylalanine tetrahydropteridine: oxygen oxidoreductase (4-hydroxylating) (EC 1.14.3.1)	i) Plasma phenylalanine >16 mg/100 ml; causes mental retardation. When treated, L-phenylalanine tolerance in diet is 250–500 mg phe/day.
ii) Atypical phenylketonuria[a]	Phenylalanine	L-Phenylalaninetetrahydropteridine: oxygen oxidoreductase (4-hydroxylating) (EC 1.14.3.1)	ii) Plasma phenylalanine >16 mg/100 ml; similar to i) but dietary tolerance for L-phenylalanine is >500 mg/day
iii) Transient phenylketonuria[a]	Phenylalanine	L-Phenylalanine tetrahydropteridine: oxygen oxidoreductase (4-hydroxylating) (EC 1.14.3.1) (?)	iii) Plasma phenylalanine >16 mg/100 ml; change in status to iv), or normal, several months or years after birth.
iv) Benign hyperphenylalaninemia[a]	Phenylalanine	L-Phenylalanine tetrahydropteridine: oxygen oxidoreductase (4-hydroxylating) (EC 1.14.3.1)	iv) Plasma phenylalanine <16 mg/100 ml; on normal diet. Benign trait.
2. The hypertyrosinemias			
i) Tyrosinosis (Medes)	Tyrosine	L-Tyrosine: pyruvate aminotransferase (?) (EC 2.6.1.20)	One case known; myasthenia gravis, probably incidental finding.
ii) "Super tyrosinemia" (Oregon)[a]	Tyrosine	Soluble (cytoplasmic) isozyme of tyrosine aminotransferase	One case; associated mental retardation.
iii) Hereditary tyrosinemia[a]	Tyrosine (and methionine)	*p*-HHPA hydroxylase (EC 1.14.2.2) Primary or secondary defect?	Hepatic cirrhosis, and renal tubular failure, eventually fatal; responds to tyrosine restriction.
3. The hyperhistidinemias			
i) Classical form[a]	Histidine (alanine in some cases)	L-Histidine ammonia lyase (EC 4.3.1.3) (liver and skin)	Usually associated with mental retardation and speech defect.
ii) Variant form	Histidine	L-Histidine ammonia lyase (EC 4.3.1.3) (liver only)	As above.

TABLE 1—*Continued*

Condition or disease	Amino acids affected	Abnormal enzyme	Comment
4. The branched-chain hyperaminoacidemias			
i) Classical "maple syrup urine disease" [a]	Leucine, isoleucine, valine	Branched-chain α-keto acid oxidase(s)	Postnatal collapse, mental retardation in survivors; diet therapy can be effective.
ii) Intermittent form	Leucine, isoleucine, valine	Branched-chain α-keto acid oxidase(s) (partial activity)	Intermittent symptoms; development may be otherwise normal.
iii) Mild form	Same	Same	Unremittent; milder than i).
iv) Thiamin-responsive	Same	Same	Mild form; B_1-responsive.
v) Hypervalinemia	Valine	Valine aminotransferase (EC 2.6.1.–)	Retarded development and vomiting; responds to diet.
5. Sulfuraminoacidemias			
i) Homocystinuria[a]	Methionine and homocystine	L-Serine hydrolyase (deaminating); ("cystathionine synthetase") (EC 4.2.1.13)	Usually associated with thrombo-embolic disease, mental retardation, and Marfan phenotype.
ii) Cystathioninuria[b]	Cystathionine	L-Homoserine hydrolyase (deaminating) (EC 4.2.1.15)	Probably benign trait; vitamin B_6 corrects biochemical trait in most patients.
6. The hyperglycinemias			
i) Ketotic form[a]	Glycine and other glucogenic amino acids	Propionyl-CoA:carbondioxide ligase(ADP) (EC 6.4.1.3)	Ketosis, neutropenia, mental retardation; often fatal.
ii) Non-ketotic form[a]	Glycine	Same or "glycine decarboxylase" (?)	Milder form of trait.
7. Sarcosinemia[b]	Sarcosine (ethanolamine)	Sarcosine:oxygen oxidoreductase (demethylating) (EC 1.5.3.1)	Benign trait (probably).
8. The hyperprolinemias			
i) Type I[a]	Proline	L-Proline:NAD(P) 5-oxidoreductase ("proline oxidase") (EC 1.5.1.2)	Benign trait, which is sometimes associated with hereditary nephritis.
ii) Type II	Proline	"Δ^1-pyrroline-5-carboxylate dehydrogenase"	Possibly benign trait, sometimes associated with CNS disease; ΔPC excreted in urine; proline concentration greater than in Type I.
9. Hydroxyprolinemia	Hydroxyproline	"Hydroxyproline oxidase"	Two cases, associated with CNS disease; others normal
10. The hyperlysinemias			
i) Type I	Lysine (and glutamine)	Lysine:α-ketoglutarate: triphosphopyridine nucleotide (TPNH), oxidoreductase (ε-N-[L-glutaryl-2]-L-lysine forming)	Associated with mental retardation and hypotonia.
ii) Type II	Lysine, arginine, (NH_3)	(Partial defect of above [10, i]) or different enzyme?	Hyperammonemia symptoms, related to protein intake.
iii) Saccharopinuria[b]	Lysine, saccharopine, citrulline	"Saccharopinase"(?)	One case; associated with mental retardation and short stature.

TABLE 1—*Continued*

Condition or disease	Amino acids affected	Abnormal enzyme	Comment
11. Pipecolicacidemia[b]	Pipecolicacid	"Pipecolate oxidase" (?)	Hepatomegaly and mental retardation.
12. The hyperammonemias			
i) Type I	Glycine, glutamine	ATP: carbamate phosphotransferase (EC 2.7.2.2)	A group of diseases that shows ammonia intoxication, protein intolerance, hepatomegaly, vomiting, et cetera. ASAuria also has trichorrhexis nodosa.
ii) Type II	Glutamine	Carbamoylphosphate: L-ornithine carbamoyltransferase (EC 2.1.3.3)	
iii) Hyperornithinemia	Ornithine	Ornithine-ketoacid aminotransferase	
iv) Citrullinemia[a]	Citrulline	L-Citrulline: L-aspartase ligase (AMP) (EC 6.3.4.5)	
v) Argininosuccinicaciduria[b]	ASA	L-Argininosuccinate arginine-lyase (EC 4.3.2.1)	
vi) (See 10, Type II)			
Group III. Nutritional and other diseases which may affect amino acids in plasma			
Protein-calorie malnutrition	Tryptophan/leucine/isoleucine/valine ↓; Tyrosine/glycine/proline ↑		Severity of change related to severity of malnutrition.
Prolonged fasting	Alanine ↓; threonine; glycine ↑		See text.
Obesity	Leucine/isoleucine/valine/phenylalanine/tyrosine ↑; Glycine ↓		Reflects insulin insensitivity.
Hepatitis	Methionine/tyrosine ↑		Reflects severity of liver disease.

[a] These diseases have been detected in newborn patients by the screening tests described in this paper.
[b] These traits may not be detectable by screening tests because plasma elevation is too small; since the renal clearance of the relevant substances is high, urine testing would be preferable. Other traits in this class are phosphoethanolaminuria (in the hypophosphatasias), hyper-β-alaninemia, and β-aminoisobutyricacidemia.

Moreover, a large sample was often required to obtain reliable results. It is now possible, by virtue of better resins and instrumentation, to perform (by elution chromatography on ion exchange resins) several analyses daily of very small samples of plasma or serum. We and others (33–35) have found that attention paid to details in handling the samples is important (Table 2), and when certain artifacts are avoided the investigator can obtain accurate analysis of the free amino acids in plasma samples.

1) The effect of venipuncture. The concentration of taurine and glutamic acid in plasma falls about 30 min after venipuncture and returns to normal in about 1 hr (36). This artifact should be distinguished from physiological changes in studies requiring multiple sequential venipunctures.

2) Plasma versus serum, and choice of anticoagulant. In general, plasma is easier to prepare for application to the resin column. Plasma may be deproteinized immediately after the blood cells are separated by centrifugation, whereas serum may experience changes in its amino acid composition upon standing at room temperature during clot retraction.

The choice of anticoagulant can be important. Most investigators use heparin; how-

TABLE 2
Artifacts in plasma amino acid composition related to technique in handling samples

Procedure	Effect on particular amino acids	
	Concentration in sample decreases	Concentration in sample increases
1) Repeated venipuncture	Taurine, glutamic acid	
2) Clotting of serum standing at room temperature	Glutamine, asparagine	Taurine, aspartic acid, glutamic acid
3) Delay in deproteinization	Cystine, homocystine, and mixed disulfides	
Picric acid method[a]	Citrulline, homocitrulline, tryptophan	
4) Contamination		
Platelets and WBC		Taurine, aspartic acid, glutamic acid
RBC (hemolysis)	Arginine, cystine	Glutathione, ornithine
"Fingerprints" (sweat)		Many amino acids
5) Storage		
Temperature above −68 C	Glutamine, asparagine, tryptophan	Glutamic acid, aspartic acid
Handling and elution at temperature >40 C	Glutamine, asparagine	Glutamic acid, aspartic acid

[a] Losses occur during step when picric acid is removed on Dowex 1 or 2 resin.

ever, an excess of this anticoagulant may cause hemolysis leading to artifacts related to constituents of the red blood cells. Impurities in some batches of ethylenediaminetetraacetic acid (EDTA), which is sometimes used as an anticoagulant, can produce ninhydrin-positive peaks in the elution chromatogram (33).

3) Deproteinization. Many investigators prefer to deproteinize plasma with 3% sulfosalicylic acid (35) (1 vol plasma:5 vol 3% sulfosalicylic acid), or with direct addition of 30 mg sulfosalicylic acid powder/ml plasma. After high speed centrifugation (21,000 × *g*) for 10 min, the supernatant may be stored or applied directly to the ion exchange column.

The other popular method of deproteinization (37) uses picric acid, which because of its yellow color, must be removed on Dowex 1 or 2 resin before the deproteinized sample can be analyzed for taurine and urea. The sulfosalicylic acid method of deproteinization also prevents substantial losses of tryptophan, citrulline, and homocitrulline that occur when picric acid is removed from deproteinized samples (35). The arguments are numerous, both for and against sulfosalicylic acid versus picric acid as the agent of choice for deproteinization in various situations, and the numerous discussions on this matter (33, 35, 38, 39) should be consulted by the reader.

Immediate removal of protein from the sample is important so as to avoid significant losses of disulfide amino acids that will bind to plasma proteins on standing at room temperature or in the refrigerator (33, 35, 37, 40). Rapid deproteinization is necessary in field studies, or in those situations in which shipment of samples is required,[4] or when the treatment of patients with disorders of sulfur amino acid metabolism is being monitored.

[4] Homocystine was not found in plasma samples obtained from the original cases of homocystinuria (41). A delay in deproteinization accounted for this artifact.

4) Contamination. Hamilton (26) has mentioned that contamination of glassware with sweat from a fingertip can jeopardize the reliability of analyses obtained by some high sensitivity methods.

Platelets and leukocytes contain large amounts of taurine and dicarboxylic amino acids (33, 36, 42). Therefore, plasma or serum samples can be contaminated with these substances released from the cellular constituents in the buffy coat. Plasma should be drawn off from the cell–plasma interface with care to avoid disturbing the buffy coat.

Hemolysis of red blood cells may also distort the apparent plasma pattern. Glutathione (reduced and oxidized) is present in red cells (36); release of arginase from erythrocytes may diminish arginine and increase ornithine in plasma; and plasma cystine may be lowered, either by dilution with an intracellular pool that is low in cystine, or by binding to protein in the hemolysate. Other amino acids are not significantly altered, as their concentration in mature red cells is similar to that of plasma (42, 43).

5) Storage. The concentrations of glutamic acid and aspartic acid rise slowly, and glutamine and asparagine fall equivalently in samples stored for long periods at −20 C. These changes are minimized by storage at −68 C and enhanced at −4 C, or at room temperature (35, 38). Evaporating and drying of samples or elution of amino acids from ion exchange columns at temperatures above 40 C will reduce the glutamine and asparagine content of plasma samples.

Methods of quantitative analysis. There are now many reports in the literature that describe technical modifications in the basic methods for semiautomated elution chromatography of amino acids on ion exchange resin columns. These modifications, in general, provide improved speed, resolution, and automation of analysis; a later article in this Journal will discuss these developments. We will mention only two reports, which provide the investigator with a portfolio of simple operating programs, and which will allow him to investigate specific aminoacidopathies without sacrificing column time.

Shih and colleagues (44) described a series of protocols for operation of the single column (45, 46) type of analyzer. They were able to obtain within 1 to 2 hr quantitative analysis of particular groups of amino acid from short resin columns. The protocols are relevant for the investigation of many of the aminoacidopathies listed in Table 1.

Our group (47) described methods for rapid operation of the two-column analyzer system (48) as well as the simultaneous use of two columns. Recoveries and reproducibility of the methods compare well with the parent techniques (48).

Experience with these modified techniques has demonstrated their value in monitoring patients under treatment for hereditary aminoacidopathies and in performing investigations that require analysis of many samples for a particular amino acid.

Normal values. Values for amino acid concentration in plasma or serum of healthy human subjects are presented in Table 3. These data represent many studies performed by investigators using reasonable precautions for the handling and analysis of the samples of plasma or serum.[5] Several different techniques for amino acid analysis by elution chromatography on ion exchange resin were used to obtain the results presented in Table 3 and yet the data are reasonably homogeneous. One observes that essential amino acids in plasma are lower during the period of rapid somatic growth (49, 50) despite the higher protein intakes on the basis of body weight at such times. Dickinson et al. (51) showed that amino acid concentrations in plasma fall after the first day of life, with particular reference to nonessential amino acids. Nonetheless, despite undoubted variation in diet of the subjects of the various reports, the interindividual variation in human subjects is relatively modest, reflecting presumably the remarkable control exerted upon the steady-state free amino acid pattern in plasma.

Comment

It has been several years since the merits of mass screening for hereditary metabolic diseases (52) became a popular topic for discussion. Although the rate of discovery of "new" aminoacidopathies has continued to

[5] Artifacts, with regard to glutamine and glutamate, are noticeable in some of the older studies.

TABLE 3

Amino acid concentration in plasma or serum, micromoles/liter

Amino Acid	Prematures[a] Mean ± SD		Neonates[b] Mean ± SD		Infants[c] Mean ± SD		Children[d] Mean ± SD		Children[e]		Children[f]		Adults[g, h]		Adults[i]		
									Mean	Range	Mean	Range	Mean[g]	Range[h]	Mean ± SD		Range
Taurine	180	75	141	40					80	57–115	49	19–91	66	27–168	59	12	41–78
Hydroxyproline	40	40	32							25							
Aspartic acid	10	10	8	4	19	2	16	3	10	4–20	2	0–9	16	0–24			trace–5
Threonine	215	60	217	21	177	36	145	16	76	42–95	60	33–128	162	79–246	138	31	75–189
Serine	270	75[j]	163	34	131	27[j]	121	14[j]	94	79–112	92	24–172	112	67–193	99	19	67–129
Asparagine + Glutamine[k]	905	250[l]	759	136					295	57–467	135	46–290	603	413–690	696	73[m]	554–824[m]
Proline	230	75	183	32	193	52	176	33	106	68–148	115	51–185	233	100–442	185	48	90–270
Glutamic acid	65	35	52	25					110	23–250			58	14–192	24	12	10–67
Glycine	460	275	343	69	213	35	219	33	166	117–223	170	56–308	231	120–553	234	44	162–335
Alanine	375	50	329	55	292	53	271	36	234	137–305	219	99–313	344	209–659	360	71	205–496
Valine	130	50	136	39	161	38	181	20	162	128–283	127	57–262	169	116–315	225	40	151–302
Half cystine	65	10	62	13	42	9	44	7	60	45–77			74	48–141	49	9	34–67
Methionine	35	5	29	8	18	3	16	3	14	11–16	21	3–29	21	6–39	21	4	13–32
Isoleucine	40	20	39	8	39	8	44	6	43	28–84	44	26–94	54	35–97	60	12	38–83
Leucine	70	25	72	17	77	21	90	13	85	56–178	75	45–155	100	71–175	115	23	77–162
Tyrosine	120	100	69	16	54	21	45	8	43	31–71	45	11–122	50	21–87	54	11	40–80
Phenylalanine	90	20	78	14	55	10	47	5	42	26–61	40	23–69	57	37–115	48	7	37–61
Ornithine	90	20	91	25	50	11	46	8	33	27–86	40	10–107	69	29–125	58	14	32–88
Lysine	190	60	200	46	135	28	130	20	111	71–151	87	45–144	173	82–236	186	36	99–249
Histidine	50	20	77	16	78	14	80	13	55	24–85	64	24–112	79	31–106	88	16	65–119
Arginine	50	20	54	17	62	9	85	15	53	23–86	31	11–65	81	21–137	82	17	53–115
Tryptophan	30	15	32	17											31	6	19–45
β-Alanine			14.5											25–73			

Values are mean ± SD except where indicated otherwise. All data obtained by elution chromatography on ion exchange resin columns.

[a] Adapted from Dickinson, Rosenblum and Hamilton. (51) Data are for first day of life from 10 infants and with birth weights less than 2,500 grams. [b] Recalculated from Dickinson et al. (35); 25 infants (more than 2,500 grams) studied before first feeding. [c] Recalculated from Brodehl et al. (49); 12 infants, 16 days to 4 months of age, studied after 6 to 8-hr fast. [d] Recalculated from Brodehl et al. (49); 12 children, 2 to 12 years of age, studied after overnight fast. [e] Scriver and Davies (50); 9 children, 3 to 10 years of age, studied after overnight fast. [f] Soupart (42); 20 children, 9 months to 2 years of age, studied after overnight fast. [g] Recalculated from Dickinson et al. (35); 8 adults. [h] Data on 76 adults compiled from 9 sources by Dickinson et al. (35); includes variation recorded by Soupart (42). [i] Data on 10 men and 10 women (age range 33 to 56 years), from Perry and Hansen (33). [j] Includes asparagine. [k] Asparagine and glutamine, as combined amounts. [l] Signifies glutamine alone. [m] Signifies pooled asparagine and glutamine. The individual values for mean, SD, and range are: glutamine 640, 58, 520 to 742; asparagine, 56, 15, 34 to 82.

burgeon (53), a clearer interphase has developed between the use of mass screening methods and the recognition and the understanding of many acquired and inherited diseases of amino acid metabolism. There is little doubt that the proper use of screening methods, such as those discussed in this article, benefits the student of health and disease and his patient. Yet, as more use is made of mass screening programs, there must be equally wide awareness of the limitations inherent in these methods and in the interpretation of their findings. Today's advances in diagnosis will further burden those facilities that must provide medical care to patients with disordered amino acid metabolism; it is clear that the methods described here can also be used to facilitate the investigation and treatment of patients with such problems. It must be a matter of some gratification for the pioneers of partition and elution chromatography to see their little bits of filter paper and their batches of resin performing yeoman service in the mainstream of human biology today.

Addendum

An unfortunate delay between receipt of this solicited manuscript by the editors and its publication by the Journal can be offset to some extent with the following selected material:

Transport and metabolism

Brodehl and colleagues (54) studied tubular reabsorption of phenylalanine in classical phenylketonuric patients. Transport is normal even though endogenous oxidation of the substrate is completely blocked. It is now known that phenylalanine is oxidized to a significant extent in mammalian kidney (55, 56). Similar observations on the independence of transport of a substrate from its intracellular fate were obtained by Glorieux et al. (57), studying sarcosine transport in normal and sarcosinemic subjects.

Circadian variation

Güttler, Olesen and Wamberg (58) observed a reversal of the normal circadian rhythm for plasma phenylalanine in phenylketonuric patients. This finding further emphasizes the need to standardize conditions for discrimination of normal subjects and heterozygotes for phenylketonuria. Burghen et al. (59) discovered that chloramphenicol therapy altered normal periodicity and the initial steady-state concentration of amino acids in plasma.

Nutrition and plasma amino acids

Relationships between feeding and plasma amino acids continue to be of interest. Adibi and colleagues (60) showed that starvation leading to changes in plasma insulin could not solely explain changes in branched chain amino acid levels. Alanine was clearly sensitive to endogenous carbohydrate metabolism.

Synderman's group continues to publish data on the effect of protein intake on the plasma amino acids (61). Restricted protein intake (2 g/kg per day) in the premature infant depresses plasma lysine and elevates glycine. High protein intakes (9 g/kg per day) increase proline, phenylalanine, tyrosine, methionine, valine, leucine, and isoleucine in plasma. The RBC:plasma distribution ratio for most free amino acids is not significantly altered by widely differing protein intakes. Leucine feeding depresses isoleucine and valine in plasma (20, 62), presumably by influencing catabolism of the affected amino acid specifically. Whitehead's group in Uganda (63) reported that fasting serum amino acids provide a useful monitor of the progress of kwashiorkor and its treatment. Stegink and Baker (64) reported relationships between malnutrition and plasma amino acid levels. Eskimo and Caucasian children had similar postprandial amino acid levels in serum despite different diets.

The so-called "alanine cycle" in man has received considerable attention (65). Alanine flux from muscle to liver serves gluconeogenesis in the latter organ, assuming net alanine synthesis from pyruvate in muscle. The cycle might also serve urea synthesis in the transfer and removal of ammonia from peripheral tissues. Formation and release of alanine in muscle may be the rate limiting

step in gluconeogenesis in human starvation. Hypoalaninemia may play a pivotal role in the pathogenesis of ketotic hypoglycemia of childhood (66). Cahill's group (67) has shown that glutamate is taken up by muscle, whereas glutamine is released; the latter is taken up across the splanchnic bed in man. It is possible that this other "shunt" may be an important source of urinary ammonia.

We wish to thank Mrs. Eveline Zifkin for her assistance in the preparation of this manuscript.

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