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Title: Vitamin B-6 Status, Energy and Protein Intakes, and Amino Acids in the Diets and Plasma of School-Aged Patients with Phenylketonuria: Implications for an Improved Nutritional Treatment

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Dietary intake data of 15 treated patients with phenylketonuria (PKU) (7-17 years) and six control siblings (6-14 years) were used to evaluate the effectiveness of medical foods to balance energy, protein, vitamin B-6, and individual amino acids from natural foods and to evaluate vitamin B-6 status. Four-day diet records were computer-analyzed and the contribution of medical foods and natural foods to the total diets of the patients was determined. A fasting blood sample and two 24-hour urine collections were obtained from each subject.

In eight patients consuming a strict diet, natural foods provided 0.9 g protein and 39 mg phenylalanine (phe)/100 calories, a significant reduction from control intakes of 3.3 g protein and 153 mg phe/100 calories. However, plasma phe levels were above the acceptable treatment range. Medical foods were consumed in less than

the recommended quantities to meet approximately 120% of the Recommended Dietary Allowances (RDAs) for protein. These foods contributed 73% of the total protein RDAs but only met 22% of the energy RDAs of patients consuming the strict diet. Taste qualities of certain L-amino acids (L-AAs; L-methionine, L-glutamic and L-aspartic acids), which constitute the protein in elemental medical foods (EMFs) for school-aged patients, make acceptance of these products difficult. Intakes of each of these L-AAs were above nutritional standards and suggested that their levels could be safely lowered in EMFs.

Natural foods and medical foods provided 0.057 and 0.046 mg vitamin B-6/g protein, respectively, above the 0.020 RDA standard and the mean control intake of 0.018 mg vitamin B-6/g protein. The mean plasma pyridoxal 5'-phosphate (PLP) concentration for the patients with PKU was over twice that of the mean control concentration and above literature values. However, more than half of the patients excreted less than 30% of their vitamin B-6 as 4-pyridoxic acid; values below the criterion suggested for inadequate status.

This work provides data for a better understanding of vitamin B-6 metabolism and status in PKU and supports the design and testing of a new EMF to balance energy, protein, and amino acids from natural foods and which may improve dietary adherence.

Vitamin B-6 Status, Energy and Protein Intakes, and Amino Acids in the Diets and Plasma of School-Aged Patients with Phenylketonuria: Implications for an Improved Nutritional Treatment

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INTRODUCTION

A number of genetic defects of amino acid metabolism call for a restricted natural protein intake to decrease the accumulation of one or more particular amino acids. Phenylketonuria (PKU) is such a disorder, in which affected patients have a near total deficiency of phenylhydroxylase (PAH) activity (Scriver, Kaufman, & Woo, 1989), which interferes with their ability to metabolize the amino acid phenylalanine (phe). In healthy children and adolescents, the metabolism of phe is partitioned between three major pathways: hydroxylation to tyrosine (primary), transamination to phenylpyruvate (secondary), and incorporation into synthesis of proteins (essential) (Kaufman, 1977). In PKU, the failure to metabolize phe through its primary pathway to tyrosine leads to a considerable increase in the plasma concentration of phe once the needs for protein synthesis are exceeded and, less consistently, to a reduction in plasma levels of tyrosine (Brouwer, de Bree, van Sprang, & Wadman, 1977; Koepp & Held, 1977). If not treated within approximately three months of birth, irreversible mental retardation and neurologic damage occur (Bickel, Gerrard, & Hickmans, 1953). Fortunately, there is a reasonably effective method of treatment. A strict therapeutic diet, sufficiently low

in natural protein to reduce phe to its essential requirement and supplemented with a special amino acid mixture, prevents the damage in early infancy and childhood (Dobson, Williamson, Azen, & Koch, 1977). And, thus, as pointed out by Scriver and Clow, "PKU is a disease only when the mutant allele is expressed in an obligatory environment containing an abundance of phenylalanine" (Scriver & Clow, 1980, p. 164). Because all natural proteins contain approximately 2-9% phe by weight (Elsas & Acosta, 1988), the special amino acid mixtures known as medical foods[1] are necessary to supply the majority of the protein intakes of patients with PKU. Two types of medical foods are available: mixtures of amino acids and peptides formed by the hydrolysis of natural proteins (e.g., casein or beef serum) from which almost all of the phe has been removed, or "elemental" mixtures of free amino acids other than phe, known as elemental medical foods (EMFs). The current medical foods or EMFs are intended to supply 100% or more of the 1980 Recommended Dietary Allowances (RDAs) and the Estimated Safe and Adequate Daily Dietary Intakes (Food and Nutrition Board [FNB], 1980) of vitamins and minerals, as well as a portion of the protein and energy

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[1] Medical foods are specially formulated products with defined composition intended for use under medical supervision in the nutritional support of patients with various diseases or conditions (Committee on Nutrition, American Academy of Pediatrics [CON-AAP], 1987).

requirements (Hunt, Berry, & White, 1985). They differ in their nutrient content, relative to the age-specific 1980 RDAs (FNB, 1980). Thus, there are medical foods and EMFs designed for use in infants (< 2 years); younger children (2-8 years); and older children, adolescents, and adults.

Medical foods and EMFs have been shown to be safe and efficacious up to school-age, based on outcome variables such as normal physical growth (Chang, Weisberg, & Fisch, 1984), and mental development (Dobson, Williamson, Azen, & Koch, 1977), and a reduction of plasma phe and increased levels of the other plasma amino acids (Berry, Sutherland, Hunt, Fogelson, & O'Grady, 1976). Nutrient intakes have been found to be adequate in treated infants (Acosta, Wenz, & Williamson, 1977) and, using current medical foods and EMFs in prescribed dosages, projected to be adequate for children and adolescents with PKU (Hunt et al., 1985). However, these products have strong, unpalatable tastes and odors associated with the modified protein (Schuett, Brown, & Michals, 1985; Committee On Nutrition, American Academy of Pediatrics [CON-AAP], 1976) which makes continued use of the products in the recommended amounts difficult. At school age when children become more socially aware of their diets (Matalon & Matalon, 1989), continued adherence to the strict diet becomes more difficult (Acosta, 1989). When the regimen is relaxed, protein from natural foods replaces some of the protein from medical foods, and as a

result total protein and amino acid intakes decrease and plasma phe levels increase as the phe tolerance[2] is exceeded.

The recent literature suggests very little interest in improving treatment. Elucidation of the pathogenic mechanism(s) of PKU continues to be the most challenging topic in the PKU field. The currently accepted theory is that the excess phe interferes with the uptake into the brain of the other competing neutral amino acids (Kaufman, 1989). The rather simplistic quote by Scriver and Clow emphasized a reduction of exogenous phe, but failed to mention the importance of an adequate intake of the other amino acids with which phe competes for transport and tissue uptake. Presumably, the medical foods and EMFs were designed to create an appropriate nutritional environment for PKU, reduced in phe and increased in the other amino acids. Interestingly, neither these foods nor the natural foods which are recommended for patients with PKU have been evaluated for their content of those nutrients which are of greatest importance in an amino acid-modified diet: energy, total protein and individual amino acids, and

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[2] "Tolerance" refers to the quantity of phe in the diet (input) which is necessary to maintain phe homeostasis in the plasma. Input will expand the plasma phe pool unless there is compensating incorporation, oxidation, or metabolic conversion (runout). In PKU, due to the blocked runout, tolerance is estimated to be 200-500 mg phe/day in the infant and young child and not more than 1.5 times greater than these values in older children (Scriver et al., 1989).

vitamin B-6.

A reduction in the adherence of patients to the strict diet at school age has been associated with adverse clinical effects on behavior and learning capacities (Smith et al., 1978; Matthews, Barabas, Cusack, & Ferrari, 1986; Michals, Dominik, Schuett, Brown, & Matalon, 1985). In an animalmodel for PKU, Loo and Ritman (1967) reported impairments in learning of rats fed a diet high in both phe and vitamin B-6. In the only human study of vitamin B-6 status in patients with PKU, plasma levels of the active coenzyme B-6 vitamer, pyridoxal 5'-phosphate (PLP), were found to be above control values (Anderson, 1986). Phe is known to be an <u>in vitro</u> inhibitor of alkaline phosphatase. Alkaline phosphatase hydrolyzes PLP to pyridoxal, one of the B-6 vitamers that is taken up by the liver and other tissues. In animals, the level of vitamin B-6 in the liver is directly proportional to the percent protein in the diet (Sheppard & McHenry, 1946). Miller, Leklem, and Shultz (1985) deomonstrated in human subjects that the level of dietary protein influences the concentrations of plasma and urinary vitamin B-6 compounds generally used to assess vitamin B-6 status. In patients with PKU, protein and vitamin B-6 intakes are influenced by the degree of adherence to the therapeutic diet and the consumption of natural and medical foods. Furthermore, dietary adherence is an important factor in the plasma phe concentrations of

patients.

The specific aim of this project was to assess the vitamin B-6 status of treated school-aged patients with PKU. One advantage associated with a study of treated patients at school age was that adherence to the strict diet was not homogeneous. This situation lent itself to a study designed to: 1) describe school-aged patients on the dietary and biochemical variables that might affect a vitamin B-6 status assessment, and 2) test for significant differences in vitamin B-6 status indices, between the overall patient group and a group of controls and within subgroups of patients.

CHAPTER I: REVIEW OF THE LITERATURE

A. THE CURRENT NUTRITIONAL TREATMENT FOR SCHOOL-AGED PATIENTS

PKU is the best known inborn error of metabolism for which the response to nutritional treatment has been extensively evaluated (CON-AAP, 1976). As recently as 10 years ago, it was common practice to take patients off the treatment at 5 to 6 years of age, when it was considered safe to expose the mature nervous system to excess phe (Schuett, Gurda, & Brown, 1980). Subsequent reports which have described deteriorations in intelligence (Smith et al., 1978), behavior (Matthews et al., 1986), and school performance (Michals et al., 1985) associated with this practice, suggested that some element(s) of the neurotoxicity of the untreated disorder extended beyond early childhood. The current theory contends that this is preventable if the strict diet is maintained (Michals, Azen, Acosta, Koch, & Matalon, 1988) and even reversible if the therapy is re-established (Bruhl, Arneson, & Bruhl, 1965).

In support of these findings, the Writing Committee of the U.S. National Collaborative Study of Children Treated for PKU has advocated adherence to the strict diet for all patients at least through adolescence (Fishler, Azen, & Henderson, 1987). A 1982 national survey (Schuett & Brown, 1984) found that 40% of U.S. PKU treatment centers were unwilling to recommend relaxation or termination of the

strict diet at any age. In a more recent (1988) follow-up survey (Schuett, 1990), 61% and 77% of clinics recommended indefinite strict diet continuation for males and females, respectively (some clinics had a more conservative policy for females due to the concern for the "Maternal PKU Syndrome"[3]). Diet discontinuation in 1988, for the few clinics that routinely considered it in individual cases, was allowed at an average age of nearly 13 years (Schuett, 1990), compared to 7 years in the 1982 survey (Schuett & Brown, 1984). While some differences of opinion exist, most U.S. clinics try to maintain patients on a strict diet for as long as possible and there is little disagreement that continuing the strict diet control of PKU indefinitely is the best assurance that patients will fulfill their true developmental potential (Schuett, 1990).

However, a group of school-aged patients who, deliberately or de facto, are no longer adhering to the strict diet continues to emerge. Schuett (1990) found in the 1988 survey that only 33% of early-treated U.S. patients over 10 years of age were still on diet.[4] In this

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[3] The Maternal PKU Syndrome refers to the condition in which infants born to females with PKU have a greater than 90% chance of fetal damage unless the mother's blood phe levels are controlled by a strict diet during the entire pregnancy and, ideally, prior to conception (Lenke & Levy, 1980).

[4] For the purposes of the survey, on diet persons were defined as those who were consuming a diet considered to be significantly restricted in phe, regardless of the level of plasma phe (Schuett, 1990).

study, 55% of the 4,000 U.S. patients with PKU were at least 10 years old (Schuett, 1990). Previous diet discontinuation practices by clinics have contributed to some of these off diet school-aged patients. Once the strict diet is relaxed, subsequent attempts to reinstate it have proved to be largely unsuccessful (Michals et al., 1985; Schuett et al., 1985).

Data were not reported on the number of these patients who were still consuming a strict diet (defined by 95% of programs as resulting in an upper limit of blood phe $\leq$ 10 mg/dL, the equivalent of $\leq$ 605 umol/L). It would be safe to speculate that: 1) Of the 33% of patients over 10 years of age, not all of them were still consuming a strict diet; and 2) More early-treated patients will continue to join the off diet group, or a group not on a strict diet, as they reach school-age, which will in turn create a greater magnitude of problems.

In addition to age, a second factor which plays a role in adherence to the strict diet is the nutritional treatment itself. There are three components which have been described as particularly difficult for school-aged patients (Hogan, Gates, MacDonald, & Clarke, 1986):

1. Restriction of high phe foods. Estimates of the phe tolerance of school children range from 200-500 mg/day, thereby reducing natural protein to 5-15 g/day (Matalon & Matalon, 1989; Acosta,

1989), which eliminates the meat and milk food groups and severely restricts foods from the breads and cereals group.

2. Lack of variety of low phe foods. Allowed foods are limited to fruits; vegetables; baked products made from specially modified low-protein baking and bread mixes, gelatins, and pastas; and pure fats and sugars (Acosta, 1989).
3. Poor organoleptic qualities of medical foods and EMFs. The current amino acid mixtures have objectionable tastes and odors attributed to several low molecular weight peptides in the protein hydrolysates (Fujimaki, Arai, & Yamashita, 1977) and to some of the free L-amino acids (L-AAs) in the EMFs (Schiffman & Gagnon, 1981).

The relative importance of each of these three barriers to the dietary management of patients with PKU has not been previously examined. Therefore, in preliminary studies a mail questionnaire was designed for this purpose. Twenty-five caregivers of the 15 school-aged patients who participated in the nutritional and biochemical studies completed the survey. The tastes and dosages of medical foods and EMFs were rated as the most serious problems. The consumption of adequate low phe foods (to meet energy needs) was reported as the next most difficult part of the

nutritional treatment, with the restriction of high phe foods rated as a lesser problem. These findings suggested that improving the tastes of medical foods and ensuring an adequate energy supply from medical foods, EMFs, or other low phe foods are priorities in the improvement of and adherence to the strict diet.

The energy, protein, amino acids, and vitamin B-6 levels in the EMFs designed for school-aged patients have not been previously evaluated. The protein:energy densities, the amino acid profiles, and the vitamin B-6:protein ratios of these products vary widely and there are no reports as to the rationale for these differences, nor which describe the energy, protein, amino acid, or vitamin B-6 intakes of school-aged patients. Based on this lack of information and in order to evaluate the nutritional and biochemical bases for the current dietary management approaches which patients reject at school age (Acosta, 1989; Matalon & Matalon, 1989), it was necessary to develop an understanding of the quantitative and qualitative aspects of human protein, amino acid, and vitamin B-6 metabolism and their relationships to one another and to the dietary supply of energy, protein, amino acids, and vitamin B-6. The literature, especially that body relative to children and adolescents with PKU, is reviewed in this first chapter.

B. PROTEIN AND AMINO ACID METABOLISM AND FUNCTION

1. Synthesis and catabolism

There are 28 substances that are classified as amino acids and are found in blood and intracellular tissue water in the free form. Of these, 20 are also found in mammalian proteins and are used in protein synthesis (Abumrad & Miller, 1983). Conceptually, each of the 20 amino acids should be available at the ribosomal level for optimal intracellular protein synthesis (reviewed in Zlotkin, Stallings, & Pencharz, 1985). If, during protein synthesis, one or more of the amino acids is unavailable in adequate quantities to meet the needs of the organism, protein synthesis will be impaired.

Amino acid availability to an organism depends on a supply of endogenously produced amino acids released during the breakdown of body protein, plus the amino acids supplied in the diet (reviewed in Zlotkin et al., 1985). Humans can synthesize approximately half of the 20 amino acids required; the remainder, which must therefore be supplied in the diet, have historically been termed the nutritionally essential amino acids (shortened generally to essential amino acids, EAAs). Those amino acids that humans can synthesize are termed the nutritionally nonessential amino acids (NEAAs). One of the more traditional classifications of human amino acid

requirements is shown in Table I.1 (from Scriver & Rosenthal, 1973). The existence of such requirements suggests that dependence on an external supply [the diet] of a required intermediate can be of greater survival value than the ability to manufacture it.

Biosynthesis involves only those amino acids which are nutritionally nonessential (except under unusual circumstances which affect the EAAs) (Scriver & Rosenthal, 1973). For the purposes of this discussion, histidine and arginine (considered to be essential for humans because they are synthesized at rates inadequate to support growth, especially of children), and tyrosine (considered to be essential for patients with PKU because of the metabolic block in the normal conversion of phe to tyrosine) are viewed as EAAs. Biosynthesis requires a reduced form of nitrogen and a carbon skeleton to which the nitrogen moiety is attached. The sources of the carbon atoms of the amino acids generally considered to be nutritionally nonessential are listed in Table I.2 (Scriver & Rosenthal, 1973). Three important routes of entry for these carbon chains in NEAA synthesis are α-ketoglutarate, pyruvate, and oxaloacetate. The reactions used to synthesize the NEAAs from these three carbon chain sources are summarized in Figure I.1. (Scriver & Rosenthal, 1973). PLP is the required coenzyme.

The traditional view of the EAAs has undergone further

TABLE I.1 Nutritionally essential amino acids (EAAs) and nonessential amino acids (NEAAs)

EAAs	NEAAs
arginine[1]	alanine
histidine[1]	aspartate
threonine	asparagine
methionine	glutamate
lysine	glutamine
isoleucine	proline
valine	serine
leucine	glycine
tyrosine[2]	cysteine
phenylalanine	
tryptophan	

[1] Considered to be "nutritionally semiessential" in humans because they are synthesized at rates inadequate to support growth, especially of children.

[2] Considered to be nutritionally essential in PKU (see text).

TABLE I.2 Sources of the carbon atoms for the nonessential amino acids synthesized by humans[1, 2]

amino acid	sources of carbon-atoms
alanine	pyruvate
aspartic acid, asparagine	oxaloacetate
cysteine	pyruvate via serine
glutamic acid, glutamine	α-ketoglutarate
glycine	pyruvate via serine
proline	glutamate via α-ketoglutarate
serine	pyruvate via phosphoglycerate

[1] From Scriver & Rosenthal, 1973.

[2] Arginine, histidine, and tyrosine are not included as NEAAs for the previously discussed reasons (see text).

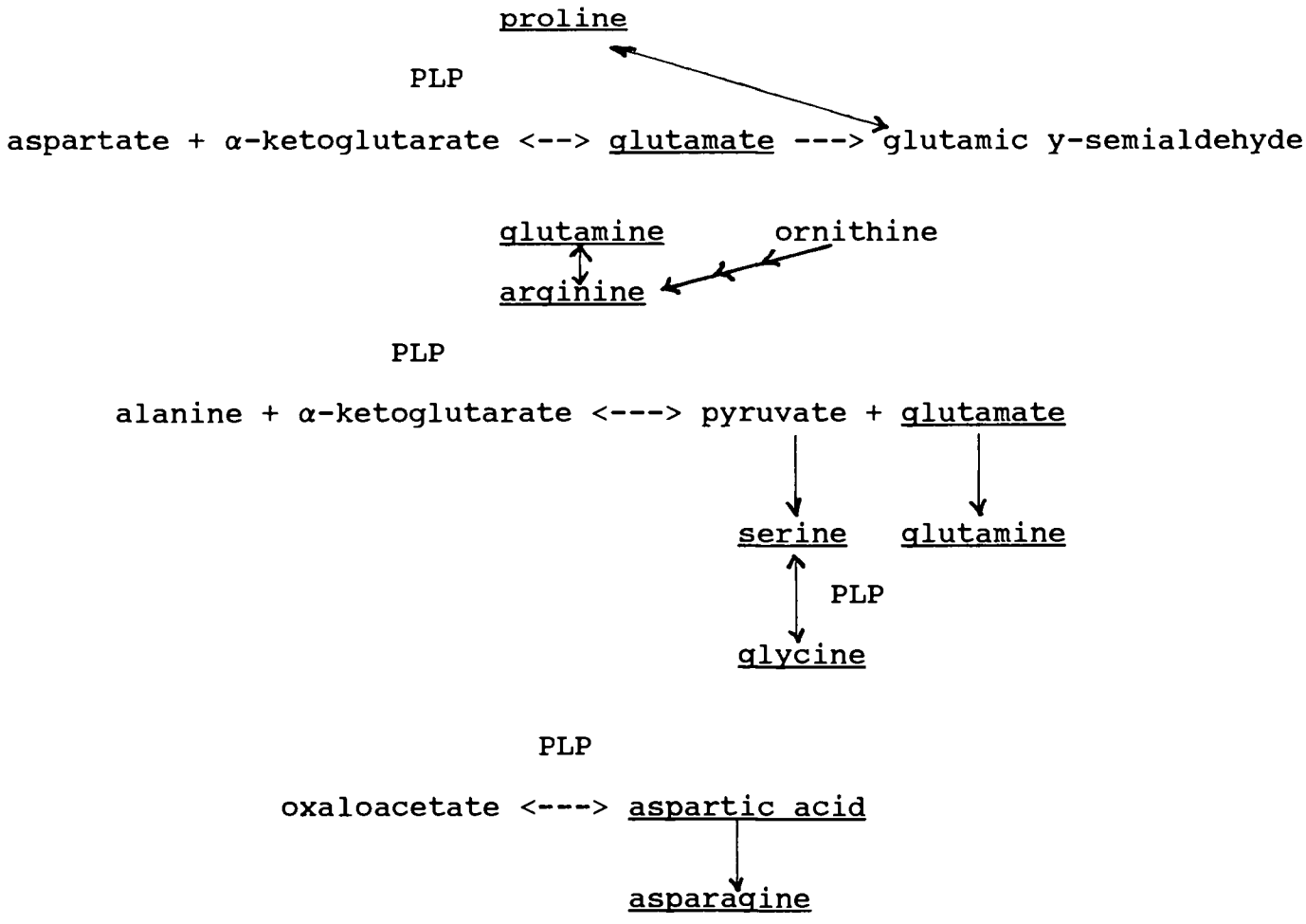

FIGURE I.1. Interrelationships of the synthesis of the nonessential amino acids and pyridoxal 5'-phosphate (PLP) as a coenzyme.

revisions over the years. Under certain conditions, indispensable nitrogen requirements can be met instead by the corresponding keto acids (referred to in Laidlaw & Kopple, 1987). An EAA is one which cannot be synthesized endogenously because of an irreversible [or blocked] step in the normal degradative pathway (Scriver & Rosenthal, 1973). For example, in phe catabolism, the first hydroxylating step whereby PAH converts phe to tyrosine, is effectively irreversible [or blocked in PKU] and in the catabolism of the branched-chain amino acids (valine, isoleucine, and leucine), it is the second step, involving oxidative decarboxylation of the equivalent keto acids. However, in both cases, the keto acids are in reversible equilibrium with their corresponding amino acids. In the presence of a nitrogen source, aminotransferase enzyme, and PLP as the coenzyme, it should be possible to meet amino acid needs by feeding the keto acid (Scriver & Rosenthal, 1973). Early studies by Rose (1938) were the first to question whether it was the entire amino acid or only the carbon skeleton that was indispensable.

Jackson (1983) re-examined this question and amino acid classification from a biochemistry/metabolism perspective. He suggested a classification system based on the ability/ inability of the human to synthesize all or part of the amino acid: 1) those amino acids with carbon skeletons that cannot be synthesized <u>in vivo</u> nor formed by transamination

reactions (lysine, threonine); 2) those amino acids with carbon skeletons that cannot be synthesized in vivo but can be synthesized from their keto or hydroxy acid analogues by transamination (histidine, leucine, isoleucine, valine, methionine, phe, and tryptophan); 3) those amino acids with carbon skeletons that can be readily synthesized but at a low rate of transamination (glycine, serine); and 4) those amino acids with carbon skeletons that are readily synthesized and transaminated (alanine, glutamate, and aspartate).

Laidlaw and Kopple more recently (1987) expanded this system to take into account clinical and therapeutic considerations of the nutritionally essential or indispensable amino acids (Table I.3). Data from human subjects with conditions that resulted in nutritional amino acid requirements outside the normal healthy adult ranges used by Jackson supported their approach. When viewed in this way, cysteine, tyrosine, arginine, and citrulline are conditionally indispensable amino acids. For example, in PKU the conversion of phe to tyrosine is abnormally reduced as a result of the low PAH enzyme activity. Because tyrosine synthesis proceeds exclusively through this metabolic pathway, in PKU tyrosine is conditionally indispensable and if not supplied in the diet of patients with PKU in adequate quantities, protein synthesis will

TABLE I.3 Jackson's[1] classification system of amino acid based on biochemistry and metabolism with clinical and therapeutic considerations[2] (continued)

degree of indispensability (ID)	amino acids	conditions, substitutes	effects if deleted from the diet
acquired ID		may become ID when:	
	tyrosine, cysteine	synthetic processes are immature (premature infant)	adverse, hyperammonemia
	arginine, citrulline tyrosine, cysteine	genetic disorders (PKU), chronic liver failure, Reyes Syndrome	
	arginine	large amino acid intake (intravenous nutrition)	
dispensable	alanine, glutamate, aspartate	carbon skeleton readily synthesized and transaminated	none (?)

1 From Jackson, 1983.

2 From Laidlaw & Kopple, 1987.

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	arginine	large amino acid intake (intravenous nutrition)	
dispensable	alanine, glutamate, aspartate	carbon skeleton readily synthesized and transaminated	none (?)

1 From Jackson, 1983.

2 From Laidlaw & Kopple, 1987.

become nutrient-restricted (as referred to in Scriver & Rosenthal, 1973).

Regardless of their source (or degree of dietary essentiality/indispensability), amino acids that are not immediately incorporated into new protein are rapidly degraded; there is no storage form per se for excess amino acids. Protein catabolism and subsequent amino acid degradation involve the biosynthesis of urea, which begins with aminotransferase enzyme reactions (transaminations) and, therefore, requires PLP. Two of these enzymes, alanine-pyruvate transaminase and glutamate α-ketoglutarate transaminase, catalyze the transfer of amino groups from most amino acids to form alanine (from pyruvate) or glutamate (from α-ketoglutarate) (see Figure I.1.). Since the equilibrium constant for most aminotransferase reactions is close to unity, transamination is a freely reversible process which permits aminotransferase enzymes and PLP to function both in amino acid synthesis and catabolism.

Free amino acids play a key role in overall protein metabolism; it is in this form that most of the metabolically active nitrogen is absorbed from the gut and transported to various organs and tissues. Changes in free amino acid concentrations in plasma have been used as indices of dietary and systemic protein and amino acid status and metabolism. There are, however, reports which caution against this approach. Abumrad et al. (1989)

suggested that plasma free amino acid concentrations may be influenced by many variables which include: the hormonal mileau and its effects on the intracellular turnover (synthesis and breakdown) of the protein and amino acid pools and the extent of intracellular recycling of amino acids, organ and tissue fluxes, the volume of distribution, and the rates of amino acid utilization by various organs and tissues. Frame (1958) as well as Yearick and Nadeau (1967) provided experimental support for this caution and suggested that the pattern of amino acids in plasma is unrelated to nutritional status via the dietary intake pattern; either because of the influence of tissue uptake and release of amino acids (supported by Miller, 1962) or because ingested protein and amino acids are mixed with several times their mass of endogenous protein and amino acids (Nasset, 1965). However, Adibi and Mercer (1973) and Silk et al. (1979) reported significant correlations between amino acid intakes and subsequent increments in the plasma amino acid concentrations. The current understanding of the influence of diet and other factors on protein and amino acid metabolism in vivo has been reviewed by Abumrad et al. (1989) using an inter-organ approach.

2. Interorgan exchanges

In general, the maintenance of free amino acid concentrations in plasma is dependent upon the net balance

between the intake, synthesis, and release of amino acids from endogenous protein stores and utilization by various tissues (Felig, 1975). Because skeletal muscle accounts for more than half of the total body pool of free amino acids (Munro, 1970), and the liver is the primary site of the enzymes necessary for amino acid catabolism and removal of the nitrogen component, these two organs play major roles in the regulation of the circulating plasma levels of free amino acids. The flux of amino acids between these various compartments (liver, plasma, muscle) is represented schematically in Figure I.2. (Abumrad et al., 1989). The rates and directions of the free amino acid movements are influenced by the hormonal mileau and in response to diet. The differences between the opposing rates (the net flow of amino acids) are the result of either stimulation of protein synthesis or inhibition of protein breakdown. The direction of flow in humans in the post-absorptive state (i.e. the condition that exists after a 10-12 hour fast) has been determined by the simultaneous sampling of arterial (A) and venous (V) blood across various organs and measuring blood flow (as reviewed in Felig, 1975). By this arteriovenous (A-V) catheterization technique, it has been demonstrated that there is a net release of amino acids from muscle tissue to the liver and the extrahepatic tissues (splanchnic bed) (Abumrad, Rabin, Wise, & Lacy, 1982). Complimenting this negative balance of amino acids in muscle is the uptake

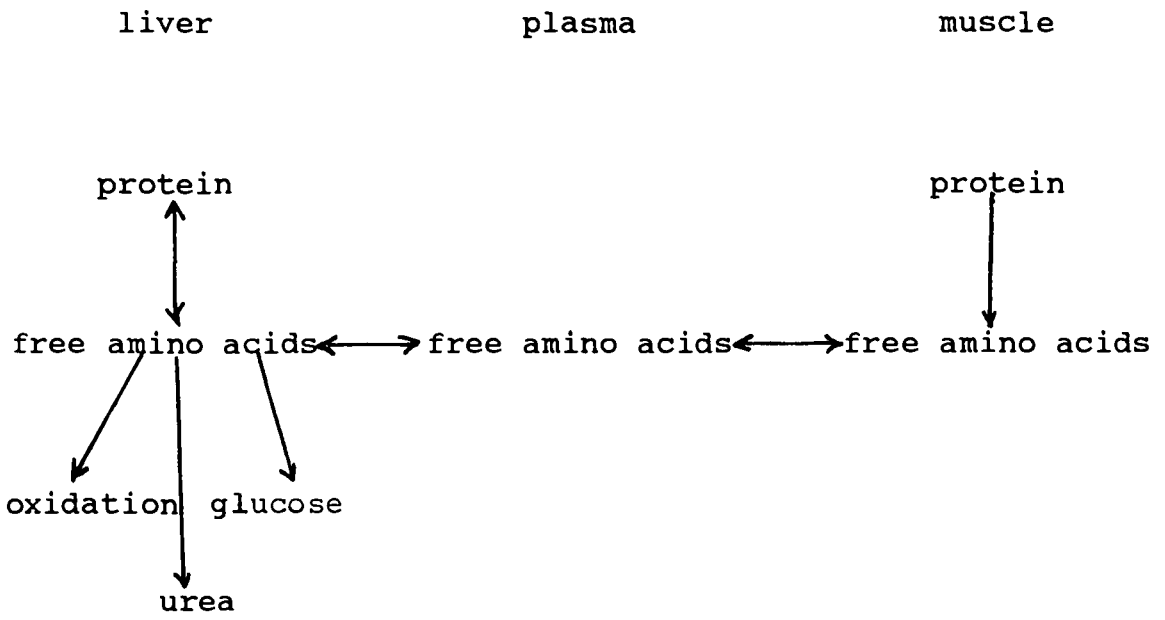


FIGURE I.2. Schematic representation of amino acid flux between the intracellular (liver, muscle) and extracellular (plasma) compartments.[1]

[1] From Abumrad et al., 1989.

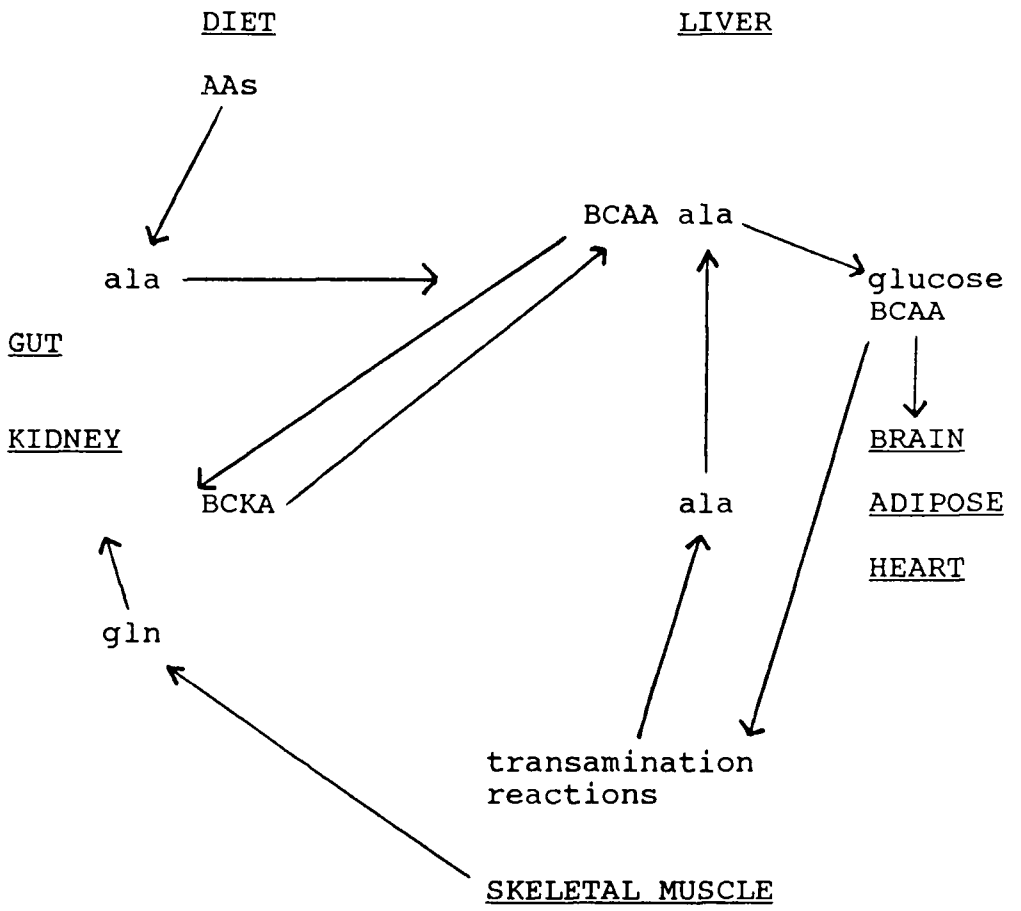
of amino acids across the splanchnic bed.

Both the quantity and quality of these free amino acid transfers and pools have been studied extensively. Several isotopic studies have suggested that 2-3% of the total body protein pool turns over daily (Matthews et al., 1982; Tessari, Tsalikian, Schwenk, Nissen, & Hammond, 1985). In a healthy adult, this represents approximately 500-600 g of free amino acids which are transported daily via the plasma pool (Munro, 1970), of which the typical diet would constitute no more than 25% (Munro, 1972). In general, the intracellular concentration of free nutritionally essential or indispensable amino acids (IDAAs) is low compared with that of the nutritionally non-essential or dispensable amino acids (DAAs). Alanine, glutamine, glutamic acid, and glycine (all DAAs) account for nearly 80% of the total free amino acid nitrogen (Munro, 1970). Although alanine and glutamine constitute 60-70% of the free amino acids released by skeletal muscle, they account for less than 15% of the total skeletal muscle protein (Kominz, Hough, Symonds, & Laki, 1954). These data suggest that alanine and glutamine are synthesized de novo in skeletal muscle (Goldberg & Chang, 1978). Other amino acids released from muscle to plasma in significant amounts are glycine, threonine, phe, and tyrosine (Abumrad, Patrick, Rannels, & Lacy, 1981). Conversely, the branched-chain amino acids (BCAAs; valine, isoleucine, and leucine) are present in the skeletal muscle

in greater amounts than in the plasma (Felig, 1975). These findings have continued to support an earlier suggestion of Miller (1962) that the BCAAs are uniquely and primarily metabolized in the skeletal muscle. The current understanding of the interorgan exchanges of free amino acids is shown in Figure I.3. (Harper, Miller, & Block, 1984). More recent studies of Abumrad (referred to in Abumrad et al., 1989) indicate that the gut is also important in the utilization of the BCAAs.

Diet influences the interorgan exchanges of free amino acids and, thereby, the levels of total and individual free amino acids in the plasma compartment (Abumrad et al., 1989). Following initial digestion in the stomach, protein is presented to the small intestine where it is hydrolyzed to oligopeptides and, at the brush border, to free amino acids, di- and tripeptides. Approximately 30% of an intact protein meal is absorbed as di-and tripeptides (Adibi, 1987).

In studies of peptide transport using <u>in vivo</u> perfusion techniques, there has been a consistent finding that on a molar basis amino acid residues are absorbed faster when presented to the intestinal mucosa as peptides rather than as the free form (Adibi, Morse, Masilamani, & Amin, 1975). Silk et al. (1979) compared the rate and extent of absorption of peptides and free amino acids in six healthy subjects. Two isonitrogenous test meals, one containing 50



DIET: 20-30% glu/gln, asp

GUT: primarily releases ala to liver

LIVER: synthesizes protein, glucose from AAs

PLASMA: 80% glu/gln, ala, gly

MUSCLE: 15% ala; releases 60-70% ala, gln to plasma, kidney

FIGURE I.3. Interorgan transfers of free amino acids.[1]

[1] From Harper, Miller and Block, 1984.

g of a fish protein hydrolysate in which 80% of the nitrogen content was present as small peptides, and the other as a mixture of free amino acids (the composition and molar pattern of which simulated that of the peptide meal), were administered on separate occasions to the subjects who were intubated with a triple lumen tube. Comparisons were made of the plasma free amino acid profiles, and the intestinal free and peptide bound amino acid profiles, in response to ingestion of the two meals. Both meals contained unlabelled polyethylene glycol so that it was also possible to estimate the extent of the amino acid absorption from the two meals. The total sum of the individual free plasma amino acid increments over the time course of the experiments (before and at 30 minutes, one, two and three hours after meal ingestion) are shown in Figure I.4. Greater plasma amino acid increments occurred 30 minutes and one hour after ingestion of the peptide meal compared to the amino acid test meal. There were significant correlations at 30 minutes and one hour between the amino acid profiles of each meal and the increments in the plasma amino acids. By three hours, however, there was no significant correlation for either meal and the plasma levels. There were no significant differences at any time period between the fractional absorption of the amino acid residues from the two meals at any of the three intestinal locations examined (proximal, middle, and distal small intestine) by the triple

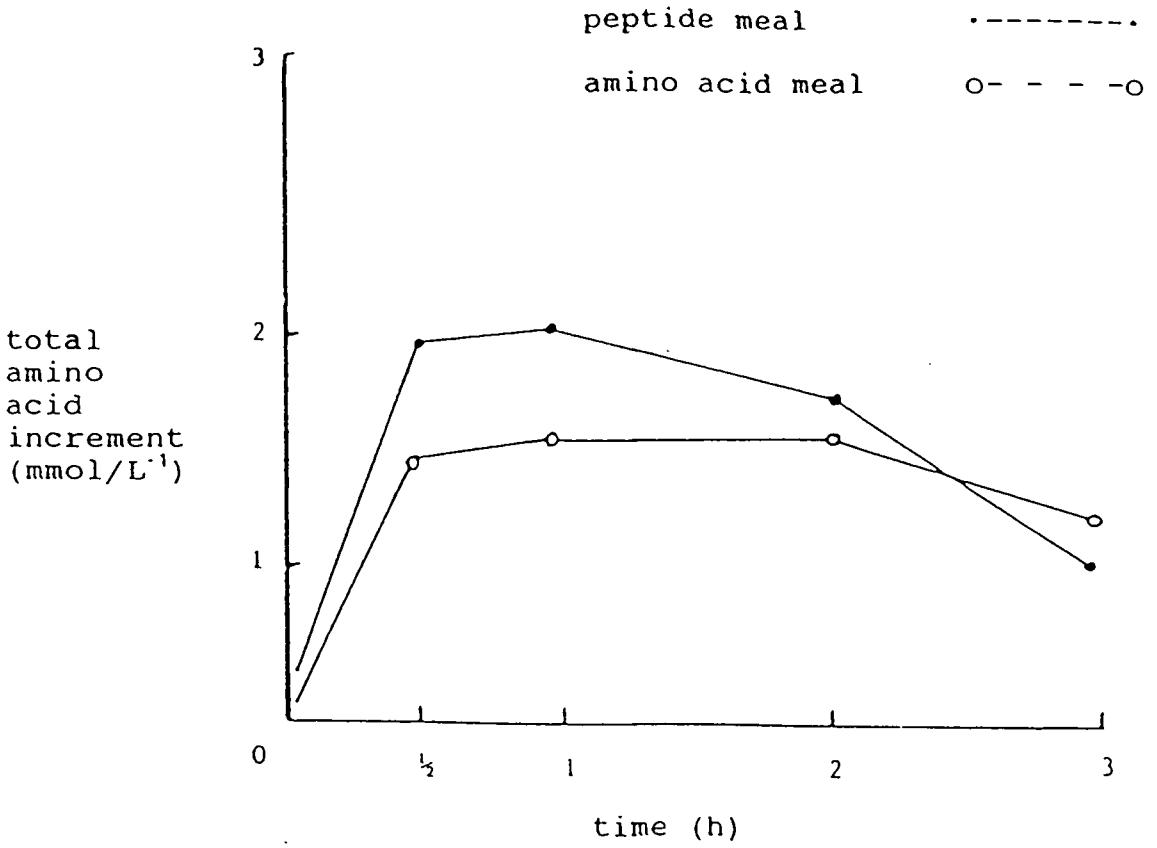


FIGURE I.4 Total mean increments in plasma amino acids of six healthy subjects fed peptide and free amino acid meals[1]

[1]From Silk et al., 1979

lumen technique. In summary, these studies suggest that in healthy subjects free amino acids and peptides are equally well-absorbed over a three hour period. They do not support a need for increased amounts of total protein when free amino acids are consumed as the protein source.

Frame (1958) showed that, following absorption of a protein meal, the outflow of amino acids from the intestine into the portal vein was characterized by a predominance of alanine. In contrast, glutamic and aspartic acids, which constituted 20-30% of the dietary protein, were absent. This first suggested that glutamic and aspartic acids (as well as glutamine) were primarily metabolized by the small intestine into alanine. More recent studies have confirmed these findings and shown that amino acid utilization by the liver is enhanced following protein ingestion, resulting in rapid hepatic protein synthesis and formation of albumin (Miller, 1962). The nitrogen in the excess amino acids is converted to urea, with the carbon skeletons contributing to the formation of glucose, glycogen, and fatty acids which provide energy for a variety of biosynthetic processes (Cherrington et al., 1987). This rapid adaptation of the liver buffers the tissues from excessive changes in the free amino acid concentrations as a result of large intakes of dietary proteins (Munro, 1972).

Diets that are deficient in protein and/or energy to meet the body's needs result in significant changes in the

type of amino acids released into the plasma compartment. A characteristic pattern develops in the plasma amino acid concentrations with chronically deficient protein intakes: a depression of the IDAAs and an elevation of the DAAs (Swendseid, Umezawa, & Drenick, 1969).

These alterations in the plasma aminogram have been used in field studies, especially for evaluation of the nutritional status of children in developing countries (Whitehead, 1964). Specifically, "Whitehead's quotient" is computed (i.e. the ratio of four DAAs -- taurine, glycine, serine, and glutamine -- to four IDAAs -- methionine, leucine, isoleucine, and valine). Whitehead's quotient has been shown to increase in response to a lowered dietary protein intake (Whitehead, 1964; Holmgren, 1974). Based on an extrapolation from studies in experimental animals (reviewed in Munro, 1970), these plasma amino acid changes may be reflective of reduced levels of amino acids in some tissues such as muscle and liver (Young & Scrimshaw, 1977).

In addition to the changes in the concentration of amino acids in the plasma, muscle, and liver compartments in response to inadequate amino acid intakes, enzymes of amino acid metabolism adapt with changes in the nutritional environment. Studies by Swenseid and Kopple (1973) with ^{14}C-labeled amino acids in adults have demonstrated reduced oxidation of certain amino acids when their dietary supply is low. Others have reported a reduction in amino acid

transamination in response to chronically deficient protein intakes (Lunn, Whitehead, & Baker, 1976). Conceptually, if a reduced amino acid intake leads to lowered plasma amino acid concentrations and uptake by the tissues, the amount of enzymes involved in the catabolic processes and interconversions of amino acids (i.e. oxidation and transamination) would be expected to fall (Young & Scrimshaw, 1977).

3. Plasma amino acid concentrations in PKU

Measurements of the concentrations of amino acids (other than phe) in the plasma (or serum) of treated patients with PKU over the past 25 years have generally been made by investigators interested in comparing the effect(s) of one amino acid mixture against another (Smith, Francis, Clayton, & Wolff, 1975; Held, Koepp, Plettner, & Gruttner, 1983) or as one measure of the safety and efficacy of an amino acid mixture (Berry et al., 1976; Link & Wachtel, 1984; Brouwer et al., 1977; Koepp & Held, 1977). Findings have been conflicting; some of the studies have shown reductions in the concentrations in plasma of one or more amino acids in patients, compared to normal reference values (Brouwer et al., 1977); Held, Koepp, Plettner, & Gruttner, 1983). These studies and others have shown increased concentrations of one or more amino acids (Held et al., 1983; Link & Wachtel, 1984), and several reports have found

all amino acids measured (except phe) to be within the normal range or slightly increased (Berry et al., 1976; Koepp & Held, 1977).

A more consistent finding, first reported more than 20 years ago and which has received little attention since, is a characteristic reduction in plasma amino acids of untreated patients with PKU (Efron, Kang, Visakorpi, & Fellers, 1969). Only recently has this subject been re-examined by Christensen (1987), who was one of the original investigators of the reduced plasma amino acids in untreated patients (Christensen, 1953). Christensen's original prediction (1953) was that the phe accumulation in the plasma of patients with PKU would handicap the flow of some amino acids into the brain. Thirty-five years later (1987), Christensen offered an explanation as to why the developing brain might be particularly sensitive to this phenomenon. The most recent hypothesis developed from the earlier observations that certain amino acids are characteristically lowered in the plasma of untreated patients with PKU. Specifically, threonine, glutamine, asparagine, proline, glycine, alanine, and leucine were found to be reduced (Efron et al., 1969). Christensen proposed that this was a result of the phe accumulation in the plasma which reduced the transport of some amino acids out of the tissues and into the plasma. Christensen's perception, regarded as central to the interpretation of amino acid distribution [in

PKU], was stated as follows (Christensen, 1987):

> An amino acid accumulated in excess can inhibit either the net tissue uptake or the net tissue release of another amino acid, depending in each case on the net directional role of the transport system or systems suffering the larger competitive influence. The directional role will not be the same for every pair of interacting amino acids ... which may furthermore not be uniform from one tissue to another.(p. 195).

This principle, derived from an understanding of interorgan exchanges and transport systems in animals and humans, has received support from kinetic studies of transport inhibition across the blood brain barriers (BBB) (reviewed by Pratt, 1982).

Since measurement of the kinetic parameters of transport across the BBB in patients is impractical for technical and ethical reasons, kinetic data derived from animal studies have been extrapolated to humans. Fortunately, these results are sufficiently similar in a number of different species to suggest that the basic pattern of carrier-mediated transport across the BBB is much the same in all mammalian species (reviewed in Pratt, 1982). From these studies, Pratt (1980) has proposed that the phe accumulation in the plasma of patients with PKU monopolizes the transport carrier that normally takes a number of amino acids across the BBB. Thus it prevents the brain cells from receiving enough of the other amino acids needed for cerebral protein synthesis.

Phe competes with other bulky dipolar amino acids for transport: tyrosine, tryptophan, valine, isoleucine, leucine, and methionine. Both the plasma concentrations (Berry et al., 1976) and the brain content (McKean & Boggs, 1968) of several of these have been found to be lowered in untreated patients with PKU. Pratt has suggested that the rate at which any of these amino acids enter the brain increases with its plasma concentration, but is reduced by competition from the others (Pratt, 1979). Therefore, Pratt has proposed that there are two reasons why the influx of some of these amino acids into the brain in PKU is low: 1) The plasma levels of competing amino acids are low; and 2) The high plasma phe concentration competes with these amino acids for transport.

Based upon plasma amino acid concentrations of untreated patients with PKU (Efron et al., 1969; Smith et al., 1975), kinetic constants for the transport of each amino acid across the BBB obtained from animal experimental work were used to calculate the probable influx of each amino acid into the brain of patients (Pratt, 1980). Estimates were also made of the effect on the influx of each amino acid into the human brain, of raising its level in the blood by a standard amount and reducing the level of phe in the blood by a standard amount (to simulate a strict low phe diet). Using these data, Pratt proposed a new approach to the nutritional treatment whereby amino acid supplements

would be added to the medical foods or EMFs to result in desirable plasma concentrations and, thus, improved transport across the BBB (Pratt, 1980). In this report, Pratt provided the desirable plasma concentrations of the 11 neutral amino acids found to undergo transport inhibition by phe. The data are compared to the normal reference ranges for these amino acids found in fasting healthy children and adolescents (Armstrong & Stave, 1973) in Table I.4. As pointed out by Pratt (1980), the desirable ranges are above the reference ranges. Although phe does not directly inhibit the transport of the dibasic amino acids, arginine and lysine, Pratt also recommends dietary supplements of these two amino acids and that higher than normal plasma concentrations be maintained, because of their essential nature.

Upon review of the reports which have described plasma amino acid concentrations in patients with PKU compared to control subjects, the results are conflicting and may be related to the differences in techniques surrounding the data collections. Factors known to influence plasma amino acid concentrations include circadian periodicity (Feigin, Klainer, & Beisel, 1967), age (Armstrong & Stave, 1973), sex (Armstrong & Stave, 1973), and protein intake (Holmgren, 1974). Besides these, many methodological problems influence the accuracy (both validity and reliability) of plasma free amino acid measurements. Cysteine can be

TABLE I.4 Pratt's desirable plasma amino acid concentrations for patients with PKU[1]

	Pratt proposed	Armstrong & Stave
thr	200-400	75-203[2]
ser	150-300	70-178
gly	400-800	160-304
ala	400-800	185-537
val	200-400	161-285
met	75-150	17-37
ile	100-200	41-93
leu	150-300	85-169
tyr	100-200	45-89
his	125-250	65-105
trp	80-160	31-80
lys[3]	200-400	114-226
arg[3]	100-200	49-129

[1] From Pratt, 1980. The values, umol/L plasma, proposed by Pratt are compared to the healthy reference ranges of Armstrong and Stave, 1973.

[2] Reference range includes $\pm$ 2 standard deviations about the reference mean (Armstrong & Stave, 1973).

[3] Lys and arg, dibasic amino acids, do not compete for transport with phenylalanine but are monitored in plasma due to their essential nature (Pratt, 1980).

estimated only with great difficulty because of its rapid oxidation to cystine and other compounds; cystine and other disulphides are quickly bound to proteins and then become inaccessible for analysis. Therefore, the time interval between blood removal and deproteinization of the plasma sample becomes important. Even following deproteinization, the value for glutamine in stored filtrates decreases, while the value for glutamic acid increases (Dickinson, Rosenblum, & Hamilton, 1965). These changes can be prevented if plasma is deproteinized almost immediately following collection, and the filtrate is stored at -68° C (Dickinson et al., 1965). To avoid a faulty evaluation of the plasma amino acid concentrations, it is necessary to have some degree of knowledge of these factors and problems (and, ideally, of the background protein intake), both for the individual's (or group of individuals') values of interest, and for the reference values. Reference values are usually obtained from groups of individuals who are considered to be normal.

On the subject of normal values, Bremer, Duran, Kamerling, Przyrembel and Wadman, stated (1981):

> For recognizing abnormalities it is necessary to know what is normal. Because normality in biology can only be an approach to a fictive state, normal values reflect coincidental events, methodological problems, and the degree of knowledge of the respective variables. Therefore, only rarely is a state approached that satisfies investigators for a long period ... all published normal values are only approaches to an ideal state; some are

> better and some are less well adapted to the problem (p. 181).

These authors presented the reference values for fasting plasma amino acids, by age and sex, which they considered to be the most reliable. They chose the values for school-aged children and adolescents (6-18 years of age) of Armstrong and Stave (1973). The mean values and reference ranges, based on $\pm$ 1 SD or $\pm$ 2 SD about the mean, for each of the 24 amino acids are shown in Table I.5. It should be noted that platelet-rich plasma was used for taurine, aspartic and glutamic acid, resulting in high values for these amino acids. Although nearly 20 years old, these reference ranges continue to be used in clinical laboratories and are referenced in literature reports in the U.S. They represent the largest U.S. sample of school-aged children (80-136 specimens constitute the mean value for each of 24 amino acids) and the conditions surrounding the collections are well-described. Samples were collected at 8:30-9:30 a.m., following an overnight fast. Plasma was removed and deproteinized within 30 minutes and samples were frozen at -68° C until analysis by an automated amino acid analyzer. The background protein intakes of the children were not examined; the subjects were reported to be normal and healthy. According to the 1977-78 Food Consumption Survey (USDA, 1984), the reference range for protein intakes of children in this age range would be 1.7-2.7 g/kg body weight

TABLE I.5 Reference values for fasting plasma amino acid concentrations in school-aged children and adolescents[1]

	Mean[2] ± S.D.	<------- range -------> ± 1 S.D.[3]	± 2 S.D.[4]
tau[5]	109 ± 63	46 - 172	0 - 235
3 Hyp	23 ± 12	11 - 35	0 - 47
asp	6 ± 4	2 - 10	0 - 14
thr	139 ± 32	107 - 171	75 - 203
ser	124 ± 27	97 - 151	70 - 178
asn[5]	48 ± 7	41 - 55	34 - 62
gln	596 ± 66	530 - 662	464 - 728
glu[5]	36 ± 14	22 - 50	8 - 64
pro	187 ± 58	129 - 245	71 - 303
citr	35 ± 8	27 - 43	19 - 51
gly	232 ± 36	196 - 268	160 - 304
ala	361 ± 88	273 - 449	185 - 537
aba[6]	23 ± 7	16 - 30	9 - 37
1/2-cys[7]	93 ± 19	74 - 112	55 - 131
cys-cys	---	---	---
val	223 ± 31	192 - 254	161 - 285
met	27 ± 5	22 - 32	17 - 37
ile	67 ± 13	54 - 80	41 - 93
leu	127 ± 21	106 - 148	85 - 169
tyr	67 ± 11	56 - 78	45 - 89
phe	58 ± 8	50 - 66	42 - 74
orn	49 ± 14	35 - 63	21 - 77
lys	170 ± 28	142 - 198	114 - 226
his	85 ± 10	75 - 95	65 - 105
trp	55 ± 12	43 - 67	31 - 80
arg	89 ± 20	69 - 109	49 - 129
sum of 24	3,029		

[1] From Armstrong and Stave (1973). Subjects were 6-18 years; values are in umol/L plasma.
[2] Mean values derived from 80-136 samples for each amino acid.
[3] ± 1 standard deviation from the mean includes approximately 66% of all cases.
[4] ± 2 standard deviations from the mean includes approximately 95% of all cases.
[5] Platelet-rich plasma was used for these analyses, resulting in high values.
[6] α-amino-n-butyric acid.
[7] half-cystine; values for cystine (cys-cys) not available.

(177-206% of protein RDAs).

Few reports have quantitated a complete plasma amino acid profile in treated patients with PKU. Berry and co-workers compared the plasma amino acid concentrations of two groups of treated patients with PKU, 0-3 years of age (Berry et al., 1976). Four patients consumed 1.8-2.6 g protein/kg, primarily from a casein protein hydrolysate (Lofenalac®, Mead Johnson Nutritional Division, Bristol-Meyers Co., Evansville, Indiana), and five received a similar protein intake from an animal serum protein hydrolysate (Albumaid X-P®, Scientific Hospital Supplies, Liverpool, England). Compared to values for 20 amino acids obtained in 18 normal children (ages and diets not described), 11 amino acids in the plasma of patients fed Lofenalac® and six amino acids in the plasma of those receiving Albumaid X-P® were increased to levels more than two SD above the normal mean values (Table I.6). The sum of the mean concentrations was approximately 40% higher with Albumaid X-P® and 75% higher with Lofenalac®, compared to the sum of the normal mean values. Plasma levels of seven amino acids in the Lofenalac® group were within the range of concentrations for the 11 amino acids proposed by Pratt (1980) (Table I.4).

Link and Wachtel (1984) studied 13 treated patients (1-16 years) receiving the L-amino acid mixture PKU-2® (Milupa Corporation, Friedrichsdorf, Germany). Plasma amino acid concentrations were presented in aminogram figures, compared

TABLE I.6 Mean plasma amino acid concentrations in treated patients with PKU compared to normal children[1]

	normal children (n=18)	treated patients Albumaid X-P (n=5)	Lofenalac (n=4)
tau	---	---	---
3 Hyp	---	---	---
asp	28 ± 13	26 ± 8	26 ± 9
thr	93 ± 39	175 ± 63##	237 ± 69##
ser	157 ± 34	219 ± 77#	288 ± 33##
asn	---	---	---
gln	367 ± 97	477 ± 119	498 ± 200#
glu	147 ± 54	110 ± 38	75 ± 43*
pro	204 ± 54	190 ± 30	309 ± 13#
citr	25 ± 8	59 ± 9#	34 ± 4
gly	229 ± 60	296 ± 13#	349 ± 93##
ala	349 ± 116	430 ± 70	579 ± 125#
cys-cys	---	---	---
val	183 ± 51	296 ± 63##	479 ± 62##
met	17 ± 4	16 ± 3	44 ± 8##
ile	55 ± 18	50 ± 20	102 ± 18##
leu	109 ± 32	125 ± 38	200 ± 21##
tyr	56 ± 13	85 ± 35##	49 ± 9
phe	62 ± 12	402 ± 135##	581 ± 347##
orn	81 ± 24	94 ± 11	106 ± 25#
lys	145 ± 38	178 ± 28	336 ± 77##
his	45 ± 14	80 ± 21##	84 ± 13##
trp	37 ± 14	58 ± 24##	69 ± 10##
arg	79 ± 34	89 ± 40	68 ± 24
sum of 20	2,434	3,455	4,213

[1] From Berry et al., 1976. The patients were 0-3 years of age and were consuming Lofenalac, a casein hydrolysate and free L-amino acid mixture, or Albumaid X-P®, an animal serum protein hydrolysate and free L-amino acid mixture. The values are in umol/L plasma, mean ± SD.

--- Dashed lines indicate no data available.
* Mean value is ≤ 1 SD below the mean value of the normal children.
** Mean value is ≤ 2 SD below the mean value of the normal children.
Mean value is ≥ 2 SD above the mean value of the normal children.

to normal reference values (mean ± 1 SD) in 1-18 year old children. Estimating from these figures, the mean concentrations of the normal children are compared to the estimated mean values of the patients in Table I.7. Recognizing that these estimates are not the exact umol quantities, the sum of the mean concentrations for the patients was approximately 24% above the sum of the mean concentrations for the normal children.

These studies show a trend toward higher plasma amino acid concentrations in treated infants and children with PKU. Unfortunately, the previously discussed factors that are known to affect amino acids in the plasma (i.e. age of subjects, protein intakes, fasting vs non-fasting state, and treatment of the blood once collected) are only poorly described. Several reports have examined more closely the ages and dietary backgrounds of treated patients, relative to their plasma amino acid values. One of the few reports in which the conditions surrounding the sample collections were standardized and described was a study by Held, Koepp, Plettner, & Gruttner (1983). Amino acids were measured in the plasma of 41 treated patients with PKU from samples drawn at noon, 4-6 hours after a meal. The patients were grouped by age: 2-6 months (n=6), 7-18 months (n=10), 19 months - 5 years (n=10), 6-10 years (n=10), and 11-15 years (n=5). All patients received a protein hydrolysate (Albumaid X-P® or Aponti-40®) up to 12 months of age; this

TABLE I.7 Mean plasma amino acid concentrations in treated patients with PKU compared to normal children[1]

	normal children (n=?)	treated patients receiving PKU-2® (n=13)
tau	105	90
3 Hyp	---	---
asp	30	20
thr	90	125
ser	130	140
asn	40	35
gln	440	340
glu	80	180
pro	280	240
citr	---	---
gly	240	320
ala	350	500
cys-cys	20	10
val	210	260
met	20	20
ile	50	60
leu	110	120
tyr	---	---
phe	50	340
orn	55	105
lys	140	195
his	80	110
trp	---	---
arg	90	30
sum of 20	2,610	3,240

--- Dashed lines indicate no data provided.

[1] From Link and Wachtel, 1984. The values for the patients, 1-16 years, were estimated from the aminogram figures provided in the report. The normal children were 1-18 years. Values are in umol/L plasma. PKU-2® is a free amino acid mixture.

was replaced by an amino acid mixture (PAM®) in the older patients. The amount of medical food or EMF was prescribed at 2.5 g/kg (2-6 months), 2.2 g/kg (7 months - 3 years), 2.0 g/kg (4-6 years), 1.8 g/kg (7-9 years), and 1.5 g/kg (10-15 years); levels which range from 20-50% above the age-respective protein RDAs, and do not include protein intakes from natural foods. Thirty-one normal healthy children, age-matched for each group, served as controls, resulting in 5-10 control subjects in each age range. The data were presented in plasma aminogram figures (actual values were not given); reference means ± 1 and 2 standard deviations were depicted on the figures and were used to compute the data in Table I.8. This report suggests that the desirable elevations in the neutral and dibasic amino acids recommended by Pratt are more readily achieved in older patients, in spite of slightly higher phe concentrations and lower EMF amino acid intakes on a per kg weight basis.

These findings conflict to some extent with those of Antonozzi, Carducci, Vestri, Manzari and Dominici (1987), in which amino acids in 15 treated patients, aged 5-12 years, were measured in the fasting state (9:00 a.m.). Protein intakes ranged from 1.7-2.0 g/kg (similar to the previous study). All plasma amino acids were reported to be within the reference ranges of Armstrong and Stave (1973) with the exception of phe. Because this reference range (Armstrong & Stave, 1973) includes ± 2 standard deviations about the

TABLE I.8 Plasma amino acids of treated patients with PKU, grouped by age, compared to levels of age-matched control children[1]

	2-18 months	19 months to 5 yrs	7-10 years	11-15 years
≥ 1 SD above the age-matched reference mean	ser	his	ser, his lys, gly	ser, his, val, gly, orn, arg, glu
≥ 2 SD above the age-matched reference mean	phe (360)[2]	phe (665)[2]	phe (720)[2]	phe (720)[2] lys, leu, met
≤ 1 SD below the age-matched reference mean		arg, tyr		

[1] From Held et al., 1983. Values were computed from aminogram figures and compared to normal references means ± 1, 2 DS.

[2] Values are in umol/L plasma.

mean, it is not possible to tell from the report of Antonozzi et al. if they observed amino acids more than ± 1 standard deviation of the mean (but less than 2) as Held et al. reported for six amino acids in the plasma of their treated patients. Antonozzi et al. did report the mean values for valine, isoleucine, and leucine, all of which were within 1 standard deviation of their respective mean reference values; the mean tyrosine value was 45 umol/L which is equal to 2 standard deviations below its mean reference value.

These differences may be explained partly by diurnal periodicity. Lowered amino acid levels after an overnight fast and relatively higher values at noon have been described in both normal healthy children and in patients with PKU (reviewed in Bremer et al., 1981). All amino acids show a tendency to periodicity, but the degree and time of fluctuation differ and are influenced by the amount of protein administered (Wurtman, Rose, Chou, & Larin, 1968). This rhythm is not affected by the amount of protein intake, although the intake does affect the absolute concentrations (Wurtman, Chou, & Rose, 1967). Circadian rhythmicity is especially accentuated for tyrosine, phe, and tryptophan levels (Bremer et al., 1981).

Two reports in treated patients with PKU support potential differences in plasma tyrosine concentrations, when blood is withdrawn from patients at different times of

the day. Brouwer et al. (1977) reported consistently low fasting plasma tyrosine values in their treated patients with PKU. In response to this report, Koepp and Held (1977) presented data which showed that approximately 80% of their treated patients had normal tyrosine values when blood was obtained in the fasting state at noon.

In summary, much attention in the literature has been given to regulating the plasma phe levels of treated patients with PKU. The primary objective of dietary treatment is to reduce and maintain the plasma phe level to a restricted acceptable range between 120 and 605 umol/L. However, as reviewed by Pratt (1982) and supported by others (Christensen, 1953; McKean & Boggs, 1968; Oldendorf, 1973; Christensen, 1987), regulation of the plasma levels of the other amino acids might be important. Problems to be considered are whether an abnormally high phe concentration will lead to a reduction in the flux of other amino acids into the brain which is of a sufficient magnitude to interfere with cerebral protein synthesis. Additionally, there is no <u>in vivo</u> work to support that the plasma levels suggested by Pratt (1980) are truly desirable for patients with PKU. Further data on plasma amino acid patterns during treatment of PKU, with blood samples carefully collected, analyzed, and compared to intakes of amino acids, are needed so as to establish more clearly any potential inverse relationship between the effectiveness of treatment with the

currently available amino acid mixtures (i.e. medical foods and EMFs) and plasma levels of all of the amino acids. Pratt concluded in his review of transport inhibition in the pathology of PKU (Pratt, 1982):

> ... Meanwhile, there is a great deal of circumstantial evidence (short of conclusive proof) linking damage and transport inhibition. It would be prudent, therefore, to assume that in PKU the supply to the brain of not just tyrosine but of any one or more of up to 11 other amino acids may be at risk (p. 81).

4. Protein requirements and intakes in PKU

Dietary protein and amino acids in the growing child and adolescent function to provide the necessary substrates to support an acceptable rate of net protein gain (Young, Steffe, Pencharz, Winterer, & Scrimshaw, 1975). The rate of total body protein turnover (synthesis and breakdown) is, however, considerably greater than the protein intake estimated to meet the needs for growth (Young, Meredith, Hoerr, Bier, & Matthews, 1985). This difference reflects an extensive reutilization of the IDAAs, which are liberated during protein breakdown, for continued protein synthesis. The process is not completely efficient, as some amino acids are lost due to oxidative catabolism. Young and Pellett (1987) have proposed that the rate of oxidation of the IDAAs is the primary determinant of their requirements. Currently, there are estimated dietary requirements for both

the IDAAs and for nitrogen, which together comprise a total dietary protein requirement for growth and maintenance in children and adolescents.

The recycling of amino acids and the rates of synthesis and breakdown of body proteins change in response to various stimuli, including alterations in the level and adequacy of protein and amino acid intakes. When intakes of total protein or of a specific IDAA are lowered, a well-documented metabolic response that occurs is a reduced rate of amino acid oxidation (Waterlow; 1968, 1986; Young et al., 1985; Munro, 1964) and a decrease in the rate of body nitrogen loss, primarily as urinary urea (Munro, 1964). Within a few days to approximately one week (Rand, Young, & Scrimshaw, 1976), a new, lower, and relatively steady-state nitrogen excretion level is attained. To maintain body nitrogen balance this obligatory urinary nitrogen loss must be compensated for by an appropriate intake of nitrogen. The level is thought to be approximately 30-40% above the obligatory nitrogen losses (Food and Agriculture Organization, World Health Organization, United Nations University [FAO/WHO/UNU], 1985).

The RDAs are the most generally accepted criteria for the adequacy of intake of protein and other nutrients in the U.S. Adult protein RDAs are based on estimated nitrogen balances of healthy subjects on protein-free diets. The estimate, 0.37 g protein/kg/day, is increased to account

for:

1. A coefficient of individual variation (30% increase to cover 97.5% of the population; 0.37 x 1.30 = 0.45 g protein/kg/day);
2. A loss of efficiency in utilization (30% increase for the difference when protein is consumed in adequate amounts; 0.45 x 1.30 = 0.59 g protein/kg/day); and
3. The net protein utilization (30% increase to cover the product of the biological value and the coefficient of digestibility; 0.59 x 1.30 = 0.77 g protein/kg/day).

Therefore, the adult allowance for mixed proteins in the U.S. diet is 0.8 g protein/kg/day (FNB, 1980). For children, protein allowances are greater, on a per kg body weight basis, based on growth rates and body nitrogen accretion data. A limitation of the estimated protein requirements of children is that an assumption is made that the efficiency of protein utilization for growth in children is comparable to that observed for maintenance in adults.

Historically, recommendations for the nutritional treatment of infants and preschool-aged children with PKU have incorporated protein intakes above the RDAs (Acosta & Wenz, 1971; 1977). Only one report (Kindt, Motzfeldt, Halvorsen, & Lie, 1983) has specifically examined protein intakes in patients with PKU aimed at or below RDA levels.

Two groups of patients were studied over their first two years of life. One group was provided a diet with amounts of protein as recommended by the FNB (RDA of protein) (FNB, 1980), while the diet of the other group supplied protein to meet the recommendations of the FAO/WHO/UNU (FAO of protein) (FAO/WHO/UNU, 1973). A protein hydrolysate low in phe (Albumaid X-P®, Scientific Hospital Supplies, Liverpool, England) provided approximately 95% of the total protein intakes of both groups. The estimated protein intakes are compared to the recommended (RDA and FAO) levels for each group in Table I.9. No gross deviations in growth were observed for either of the groups, but for two reasons the authors suspected that the FAO group received a marginal protein intake during the second year of the study: 1) Three individuals had declines in linear growth, and 2) Five of the children had hand x-rays which showed possible osteoporosis. On the basis of these findings, the authors concluded that the RDA of protein was safe for patients with PKU fed the majority of their protein as a hydrolysate. The protein intakes of the FAO group were increased, accordingly, to meet the RDA.

In spite of this report, which examined patients aged 0-2 years, nutrition protocols for patients with PKU have continued to recommend total protein intakes consistently above the 1980 RDAs (Acosta, 1989; Matalon & Matalon, 1989). Acosta (1989) suggested several reasons for these

TABLE I.9 Estimated protein intakes of two groups of treated patients with PKU compared to recommended intakes[1]

patient group and no. of subjects	year 1 (0-12 mos.)	year 2 (12-24 mos.)
	dietary protein, g/kg/day	
RDA (n=8)		
recommended intake	2.0 - 2.2[2]	1.8
estimated intake	2.4[3]	2.0
FAO (n=8)		
recommended intake	2.0 - 1.5[4]	1.3
estimated intake	1.8	1.3

[1] From Kindt et al., 1983.

[2] The 1980 RDA of protein is 2.2 g/kg/day, age 0-6 mos., and 2.0 g/kg/day, age 6-12 mos.

[3] Actual intakes were reported for 0-12 mos. and 12-24 mos. only, mean values.

[4] The 1973 FAO of protein is 2.0 g/kg/day, age 0-6 mos., and 1.5 g/kg/day, age 6-12 mos.

recommendations: 1) rapid absorption of amino acids, 2) early and high post-absorptive concentrations of plasmaamino acids, and 3) rapid catabolism of amino acids (Acosta cited Stepnick-Gropper, 1988). The previously mentioned work of Silk et al. (1979) (see Figure I.4.) supports Acosta's statements that free amino acids have a rapid absorption and produce early increases in plasma amino acid concentrations. However, these studies do not support Acosta's statements that a diet of free L-amino acids increases dietary protein requirements. On the contrary, Silk et al. (1979) suggested that caution be exercised when interpreting the clinical or nutritional significance of their data. For example, while they found that the plasma amino acid concentrations increased earlier following the peptide meal than after ingestion of the free amino acid meal, they did not find significant differences in the total areas under the curves when they examined the plasma amino acids over a three hour period. These data, they concluded, suggested that the total amount of absorbed nitrogen was similar between the two dietary forms.

There is no apparent evidence to support total protein intakes above RDAs and, in fact, the physical growth of treated patients with PKU has only been reported through age four (Holm, Kronmal, Williamson, & Roche, 1979) or six (Chang et al., 1984). In both reports, the children with PKU achieved lengths and weights that were similar to those

of unaffected age-matched children.

In Table I.10, the 1980 and 1989 protein RDAs (FNB, 1980, 1989) are compared to the recommended intakes for children and adolescents with PKU (Acosta & Wenz, 1977; Mead Johnson Nutritional Division, 1981; Acosta, 1989; Matalon & Matalon, 1989). Relative to the findings of Kindt et al. (1983) and to the total diet, there are two important points regarding the recommended protein intakes in Table I.10:

1. The study by Kindt et al. (1983) examined only young patients, ages 0-2 years, while protein intakes above RDA levels (ranging 112-120% of RDAs) are recommended for patients of nearly all ages; and
2. The nutrition protocols recommend intakes above protein RDAs from medical foods alone and do not take into account the additional protein contributed by natural foods.

Presumably, these recommendations are the result of the medical food products currently available which provide between 20-90% of their energy as protein. Medical foods were designed and initially intended for infants, when protein needs relative to energy are the highest. However, even during infancy, these products supply excess protein and amino acids (except phe) if used in the amounts that are recommended and which would be necessary to meet energy needs. Because certain L-AAs in the EMFs taste unpleasant,

TABLE I.10 Recommended protein intakes for children and adolescents with PKU compared to protein RDAs

Matalon & age Matalon	RDAs		Acosta		Mead Johnson	
years	1980	1989	1977	1989	1981	1989
	<---------- g protein/kg/day ---------->					
1<4	1.5	1.2	1.9	1.9	1.9	2.3
4<7	1.5	1.2	1.5	1.8	1.5	2.2
7<11	1.2	1.0	---	1.4	1.2	1.6
11<15						
(F)	1.0	1.0	---	1.2	---	1.0
(M)	0.8	1.0	---	---	---	---
15<19						
(F)	0.8	0.8	---	1.0	---	---
(M)	0.8	0.9	---	---	---	---
≥ 19						
(F)	0.8	0.8	---	0.9	---	---
(M)	0.8	0.8	---		---	---

(---) Dashed lines indicate no recommendations were provided.

[1] Recommended g protein was divided by the age-specific median RDA body weights in kg to compute the g protein/kg/day amounts (FNB, 1980, 1989).

[2] The nutrition protocols of Acosta & Wenz (1977), Acosta (1989), Mead Johnson (1981), and Matalon & Matalon (1989) suggest that these quantities be supplied by medical foods only; the protein intake from natural foods (5-15 g/day) would be in addition to these recommended amounts.

the wisdom of this approach, nutritionally and socially, is not clear. Based upon the aforementioned studies concerning protein requirements, shouldn't PKU be approached as a unique nutritional situation in which it would be desirable to supply protein (and especially the unpleasant-tasting L-AAs) at the lowest safe level? From a nutritional viewpoint, the ideal treatment design would provide protein in an amount that would just exceed the patients' obligatory nitrogen and amino acid losses. Nutrients to be considered with any alteration in dietary protein include energy, the individual amino acids, and vitamin B-6.

5. Impact of energy intake

Over extended periods, the processes of protein synthesis and breakdown (turnover) require dietary sources of energy. Non-protein energy intake significantly affects amino acid utilization (Calloway, 1975). The influence of energy balance on nitrogen balance extends from suboptimal up to excess levels of energy intake. As a result, any change in energy intake above or below an individual's needs is likely to influence nitrogen balance (Calloway, 1981). Balance will reach a plateau, which represents the limitations imposed by the dietary protein level. When the protein content of the diet is too low, the improvement in nitrogen balance caused by adding energy to the diet can be compromised (Calloway & Spector, 1954).

For children with PKU, adequate energy intake can be difficult to achieve because energy sources are limited to low phe foods. Current medical foods, even if consumed as prescribed to meet 120% of school-aged 1980 protein RDAs (Acosta, 1989), will provide less than 30% of the 1980 energy RDAs (computed from Acosta, 1989). Natural foods, if consumed as prescribed to meet approximately 100% of the school-aged phe requirement, will provide 5-15 g protein/day. In order to balance the energy deficit created by the medical foods, natural foods must also supply 850 to 3,000 calories/day (Acosta, 1989; Matalon & Matalon, 1989). To successfully adhere to the strict-diet, patients would need to choose natural foods which provide 150 to 600 calories for each g of protein. This is in contrast to the school-aged RDAs which recommend a diet of approximately 85 calories for each g of protein (FNB, 1980).

The protein:energy density of the allowed natural foods in the strict-diet for PKU has not been previously examined to determine if the current diet approach is practical. The results of the preliminary caregiver survey suggested that providing adequate energy from allowed natural foods is a difficult part of the diet for school-aged patients, second only to problems associated with the taste of the medical foods. Based on these data, it appears that energy intake is limited by two factors: 1) the protein (and, thus, phe) content of the natural foods, and 2) the taste of the

medical foods. Chapter II examines the protein and energy contributions of natural foods and medical foods to the total intakes of 15 treated school-aged patients.

6. Amino acid requirements and intakes in PKU

The medical foods and EMFs designed for patients with PKU vary widely in their amino acid patterns, with no apparent nutritional or clinical rationale for these variations in patterns, with the exception of the reduction in phe ([CON-AAP], 1985). These variations do not appear to be unique to modular diets for PKU; variation in the composition of amino acid solutions available in the U.S. for the total parenteral nutrition of children has been observed by Zlotkin et al. (1985), who stated:

> ... all of the amino acid solutions contain the eight known essential amino acids plus histidine, which is known to be essential in children. There is, however, quite a variation in the quantity and availability of the 'nonessential' amino acids. It has been argued by Jackson [1983] that all the 20 amino acids used in protein synthesis are essential; however, for a variety of technical reasons, not all of the nonessential amino acids are universally included in the formulations (p. 389).

Because the protein equivalent[5] in TPN solutions or EMF formulations is comprised of free L-AAs and can, therefore,

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[5] When crystalline L-amino acids supply protein, 1.2 g crystalline amino acids is the equivalent of 1.0 g intact protein due to the water of hydration lost when a protein is hydrolyzed (Acosta, 1989).

be mixed with any pattern, the differences among these products presumably reflect the various manufacturers' interpretations of a desirable L-AA profile. Nayman, Thomsen, Scriver, and Clow (1979 and cited in CON-AAP, 1985) suggested that manufacturers follow the "Montreal dictum" which suggests that products should be based on the amino acid composition of human milk. While this idea seems nutritionally sound for medical foods intended for infancy when human milk is the major protein source of normal infants, there is no nutritional basis for an extension beyond infancy. However, this has indeed occurred, as evidenced in a report by Kitigawa and co-workers (1987) in which a new improved-tasting low-phe peptide was prepared. L-amino acids were added to the product, a whey protein hydrolysate, in amounts to adjust the amino acid composition (other than phe) to the same proportions as cow's milk formulas commercially available for infant feeding.

Problems that limit the ability of manufacturers to prepare nutritionally ideal L-AA patterns in the EMFs intended for school-aged patients with PKU include:

1. Current estimates of the IDAA requirements in human adults are now being questioned for their use in providing determinations of the minimum physiological requirements (Young, Bier, & Pellett, 1989). It has been suggested that medical foods must meet the recommended IDAA

requirements (CON-AAP, 1985), proposed by various national (Williams, Harper, Hegsted, Arroyave & Holt, 1974) and international (FAO/WHO/UNU, 1985) groups. These estimates are available for healthy infants, children at age 2 years or 10 to 12 years, and adults (Table I.11). The current state of knowledge is such that there are no estimated IDAA requirements for children between 2 and 10 years, 12 years to adulthood (FNB, 1989), or for patients with PKU (other than phe) at any age.

2. The IDAA intakes (other than phe) (Acosta & Wenz, 1971; Acosta, Trahms, Wellman, & Williamson, 1983) from natural foods in patients with PKU have not been reported. Ideally, an EMF formulation would account for these intakes.
3. There are technical difficulties as described by Zlotkin et al. (1985). Tyrosine is quite insoluble and, therefore, the maximum concentration possible in EMFs may be limited.
4. The suggestion that higher intakes of certain IDAAs which compete with phe for transport might provide clinical benefit to patients has not been tested (Pratt, 1980). An ideal EMF would not only be nutritionally balanced, but might give consideration to Pratt's approach, if experimental data from actual feeding studies of patients were

Table I.11 Indispensable amino acid requirements estimated for healthy persons at various ages

Indispensable amino acid	infants[1] (3-4 mo)	children[2] (2 yr)	children[2] (10-12 yr)	adults[3]
	mg amino acid/kg/day[4]			
histidine[5]	28	19[5]	19	8-12
isoleucine	70	31	28	10
leucine	161	73	42	14
lysine	103	64	44	12
total sulphur	58	27	22	13
total aromatic	125	69	22	14
threonine	87	37	28	7
tryptophan	17	12.5	3.3	3.5
valine	93	38	25	10
Total	714	352	214	84

[1] Infant estimates are based on the amounts in human milk or infant formulas fed at levels that supported adequate growth (Fomon & Filer, 1967).

[2] Children estimates are based on an achievement of nitrogen balance which supported adequate lean tissue gain (2 yrs; Pineda, Torun, Viteria, & Arroyave, 1981) or on the upper range of intakes for positive nitrogen balance (10 yrs; Williams et al., 1974; Nakagawa, Takahashi, Suzuki, & Kobayashi, 1964).

[3] Adult estimates are based on the highest level to achieve nitrogen balance (FAO/WHO/UNU, 1985).

[4] FAO/WHO/UNU, 1985.

[5] Histidine values for children were imputed (FNB, 1989).

found to support the recommended dietary supplements. Without evidence that an increased neutral L-AA intake will raise the plasma amino acids to a desirable therapeutic range in patients with PKU, this approach remains hypothetical.

In summary, the state of our scientific knowledge level regarding an ideal IDAA intake for optimal nutrition in healthy school-aged children is still inadequate. In addition, application of recommended IDAA intakes to patients with PKU who have IDAA intakes from natural foods and medical foods or EMFs, needs to take into account the total diet, relative to nutritionally adequate or therapeutically desirable plasma amino acid concentrations.

The patterns and amounts of DAAs in EMFs intended for use in school-aged patients vary even more than the IDAAs (Table I.12). For example, Phenyl-Free® (Bristol-Meyers Co., Evansville, IN) supplies no alanine, proline, or serine but provides more aspartate, glutamate, and glycine per g protein equivalent than the other EMFs which contain at least some alanine, proline, and serine. Because there are no estimated DAA requirements for healthy persons of any age, the nutritional appropriateness of the DAA profiles in EMFs is especially difficult to evaluate. It appears, however, that the reasons for some of these decisions may be economical rather than from a nutritional, clinical, or even organoleptical basis. For example, the costs per kg of

TABLE I.12 Dispensable amino acid patterns of elemental medical foods designed for patients with PKU over age two years

dispensable amino acid	elemental medical foods		
	Phenyl-Free®[1]	PKU-2®[2]	Maxamaid X-P®[3]
	mg amino acid/g dietary protein		
arginine	34	40	93
alanine	0	46	68
aspartate-asparagine	261	113	78
glutamate-glutamine	330	239	110
glycine	163	27	74
proline	0	106	86
serine	0	60	53
% total protein as dispensable amino acids	78.8	63.1	56.2

[1] Phenyl-Free® is manufactured and distributed in the U.S. by Bristol-Meyers Co., Evansville, IN.

[2] PKU-2® is manufactured by Milupa AG - International Scientific Department, Friedrichsdorf, West Germany, and is distributed in the U.S. by Bristol-Meyers Co., Evansville, IN.

[3] Maxamaid X-P® is manufactured by Scientific Hospital Supplies, Liverpool, U.K., and is distributed in the U.S. by Ross Laboratories, Columbus, OH.

L-alanine ($70), L-proline ($98), and L-serine ($165) are considerably above the costs per kg of L-aspartate ($16), L-glutamate ($18), and L-glycine ($16) (Ajinomoto Co., 1990 price list, Torrance, CA). The omission of the DAAs L-alanine, L-proline, and L-serine in a product such as Phenyl-Free appears to be incongruent with Pratt's approach, which placed an equal importance on maintaining plasma concentrations of these L-AAs to that of glycine. Inclusion of L-aspartate and L-glutamate in this product suggests an oversight or lack of attention to an early report by Armstrong and Tyler (1955), which found that the ingestion of these compounds resulted in nausea in patients. They suggested substituting L-asparagine and L-glutamine which would be metabolized similarly and, therefore, would fulfill the nutritional needs for their respective amides.

Given the limitations in our current knowledge surrounding optimal IDAA and DAA patterns in both healthy school-aged children and in patients with PKU, what bases can be used to evaluate the current nutritional treatment for PKU and to suggest improvements?

A report by the 1985 Food and Agriculture Organization Committee on Energy and Protein Requirements (FAO/WHO/UNU, 1985), like its predecessor (FAO/WHO/UNU, 1973), supported a relationship between the amino acid composition of a dietary protein and its nutritional value. This concept of the "ideal" protein is one in which all the IDAAs are provided

in the amounts to meet the age-specific estimated requirements without any excesses. The nutritional quality of a single dietary protein is evaluated by computing the deficit of each IDAA below the amount in the "ideal" protein. This approach is the basis for the traditional amino acid scoring procedure which is used to evaluate the capacity of dietary proteins to meet IDAA needs.

To determine the IDAA requirement pattern for a dietary protein, relative to an individual, each estimated IDAA requirement (mg amino acid/g protein/day) is divided by the recommended allowance of reference protein for a given age group (g protein/kg/day) (FNB, 1989). These estimates, in mg amino acid/kg/day, are consistent with the earlier FAO/WHO/UNU report (1973). The IDAA pattern for preschool-aged children is recommended for children 1 to 6 years and the school-aged pattern is to be used for children 6 to 12 years of age (FAO/WHO/UNU, 1985). In Table I.13, these two patterns are compared to the relevant IDAA patterns: 1) EMFs intended for patients with PKU over age two years, 2) diets of healthy children aged one to three years, and in the diets of persons of all ages (U.S. Department of Agriculture, [USDA], 1987).

As a percent of the total dietary protein, current IDAA estimated requirements are: 46% (infants), 34% (preschool), 24% (school-aged), and 13% (adults). These estimates, derived largely from the results of nitrogen balance studies

TABLE I.13 Estimated indispensable amino acid requirement and intake patterns compared with elemental medical foods intended for patients with PKU over two years of age

indispensable amino acid	requirement pattern children[1]		reported composition of elemental medical foods			U.S. diet intake pattern[2]	
	1-6 yrs	6-12 yrs	Phenyl-Free	PKU-2	Maxamaid X-P	1-3 yrs	1-65 yrs
	<---------- mg amino acid/g dietary protein ---------->						
histidine	19	19	23	27	54	--	--
isoleucine	28	28	54	67	72	54	52
leucine	66	44	85	113	122	80	77
lysine	58	44	93	81	94	70	68
total sulphur	25	22	48	54	50	35	35
total aromatic	63	22	46	67	30	81	78
threonine	34	28	46	54	60	40	39
tryptophan	11	9	14	21	24	12	12
valine	35	25	62	81	78	57	54
percent total protein as indispensable amino acids	33.9	24.1	47.1	56.5	58.4	42.9	41.9

[1]FNB, 1989
[2]USDA, 1987

in limited numbers of healthy individuals, suggest that individual proteins and total dietary protein intakes with an IDAA content and pattern that effectively meet the estimated IDAA requirements of infants will exceed the estimated IDAA needs of all other age groups.

A new approach questions the adequacy of these requirements for healthy adults (Young et al., 1989). This approach proposed that the current estimates are too low, based upon kinetic experiments of several individual IDAAs. The revised estimates, in mg IDAA/g protein/day, are two to three times higher than the currently accepted IDAA estimated requirements for adults (FNB, 1989) and are more similar to the recommended patterns for the preschool-aged child (FAO/WHO/UNU, 1985) or the consumption patterns of persons of all ages (see Table I.13).

The approach by Young et al.(1989), follows that previously suggested by Millward and Rivers (1988); the minimum IDAA predictions are based on estimates of the obligatory IDAA oxidation rates. To maintain body amino acid balance, the obligatory amino acid oxidation rates must be compensated for by an appropriate dietary supply, just as when obligatory nitrogen losses are balanced by an adequate nitrogen intake (FAO/WHO/UNU, 1973). The minimum intakes of individual IDAAs required to balance these losses can be calculated, given a known efficiency of an IDAA. More direct data in humans are needed before this approach can be

used to estimate individual IDAA requirements and, ultimately, to evaluate diets to meet protein and amino acid needs. For school-aged patients with PKU, studies in this age group will be necessary. In addition, studies in patients with PKU would be useful to determine if there are differences in their efficiencies of utilization of IDAAs either as a function of PKU per se or as an adaptation to a modified protein intake.

There is a need to evaluate the appropriateness of the L-AA profiles of EMFs for patients with PKU, based on the current estimated IDAA requirements and reference intakes of healthy children. In the new approach to nutritional treatment of PKU suggested by Pratt (1980) to maintain higher plasma amino acid concentrations than the normal reference range, IDAA supplements in addition to medical foods and EMFs are proposed. These recommended L-AA supplements are presented in Table I.14, compared to the current estimated IDAA requirements for school-aged patients.

A proper evaluation of the L-AA profiles of EMFs, would also take into account the plasma amino acid concentrations of patients consuming these foods as their major protein source, the organoleptic qualities of the individual L-AAs, and the amino acid quantities contributed from both the natural foods and the EMFs. It is widely accepted that the only meaningful measure of the protein quality of a diet is

TABLE I.14 Recommended indispensable amino acid (IDAA) supplements in a new approach to the nutritional treatment of PKU compared to the estimated IDAA requirements for healthy school-aged children[1]

IDAA	recommended as supplements to PKU diet (Pratt, 1980)[2]	estimated requirements in normal diet (FNB, 1989)
histidine	100	19
isoleucine	100-200	28
leucine	50	66
lysine	---	58
met + cys	100 (as met)	25
phe + tyr	100 (as tyr)	63
threonine	---	34
tryptophan	100	11
valine	50	35

[1] Recommendations from Pratt (1980) and FNB (1989) are in mg IDAA/kg body weight.

[2] In addition to these IDAAs, Pratt suggested supplements of arginine (50 mg/kg).

(---) Dashed lines indicate IDAAs for which Pratt recommended no supplements.

one made on the total diet as consumed; specifically, on the extent to which the amino acid patterns of the various diet components complement one another (FNB, 1980). In addition, the previously reviewed work of Silk et al. (1979), Adibi and Mercer (1973), and Marrs, Addison, Burston, and Matthews (1975) support measurements of plasma free amino acid concentrations against dietary amino acid intakes in evaluations of amino acid nutriture. Chapter III examines the taste characteristics of L-AAs and the IDAA and DAA levels in the diets and plasma of 15 school-aged treated patients with PKU. The separate amino acid contributions of natural foods and medical foods are evaluated, relative to the currently accepted estimated IDAA requirements for school children (mg amino acid/kg/day) and to the DAA intakes of six age-matched healthy siblings (mg amino acid/g dietary protein/day).

C. VITAMIN B-6 METABOLISM AND FUNCTION

1. Interconversions and metabolic reactions

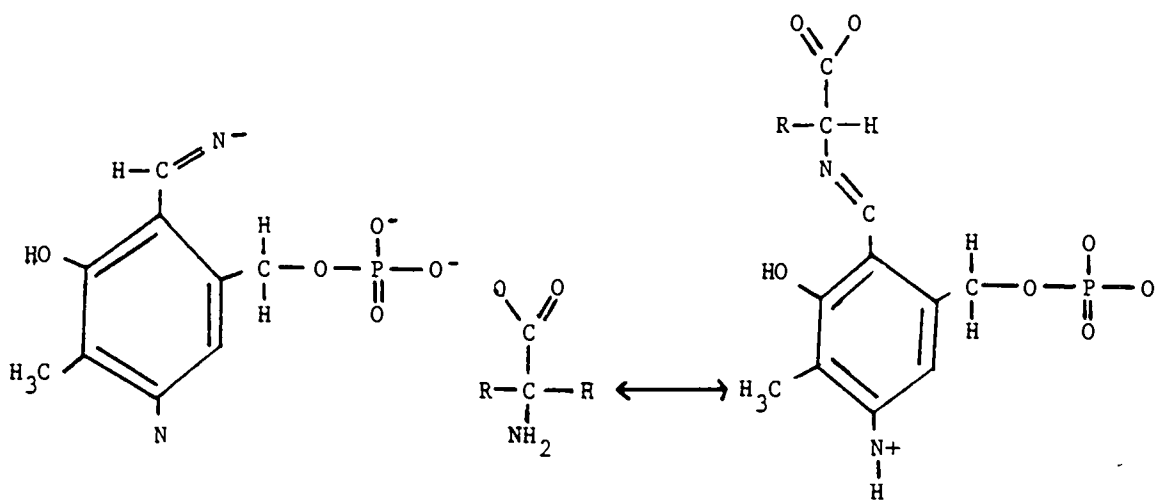
Pyridoxine (PN), pyridoxal (PL), pyridoxamine (PM) and their phosphorylated derivatives pyridoxine 5'-phosphate (PNP), pyridoxal 5'-phosphate (PLP), and pyridoxamine 5'-phosphate (PMP), are the biologically active forms of vitamin B-6. Among these vitamers, only PLP and PMP can function as coenzymes (Lui, Lumeng, Aronoff, & Li, 1985). Each of the three phosphorylated vitamers are synthesized

from PN, PL, and PM, respectively, in the human liver and erythrocytes, in the presence of the enzyme pyridoxal kinase (Lui et al., 1985).

The active coenzyme form of the vitamin, PLP, is found covalently bound to enzymes via a Schiff base with an E-amino group of lysine in the enzyme. In enzymatic reactions, the amino group of the substrate can form a Schiff base via a transamination reaction (Figure I.5.) (Leklem, 1990a). Although nearly half of the enzymatic reactions that occur as a result of a Schiff base formation are the transamination type which involve the α-carbon, there are also decarboxylation, oxidative deamination, and other reactions which involve PLP and the α-carbon, as well as reactions involving the ß- or γ- carbon. Some examples of these reactions, catalyzed by PLP, are presented in Table I.15. Those reactions that are especially relevant to vitamin B-6 status assessment or to amino acid metabolism in PKU are highlighted.

2. Interorgan exchanges

The various dietary forms of vitamin B-6 are derivatives of PL, PN, and PM. PLP is the major vitamer in animal foods in contrast to PN and PM (or their respective phosphorylated forms, PNP and PMP) which predominate in plant foods. The absorption of these forms has been studied most extensively in the rat by Middleton who found the



PLP and lysine E-amino group of enzyme

α-amino acid substrate

Schiff base

Figure I.5. Enzyme-catalyzed transamination reaction and Schiff base formation involving PLP[1]

[1] From Leklem, 1990a.

TABLE I.15 Enzyme reactions catalyzed by pyridoxal 5'-phosphate[1]

type of reaction, typical reaction, or enzyme	
transamination	alanine + α-ketoglutarate --> pyruvate + glutamate
	aspartate + α-ketoglutarate -> oxaloacetate + glutamate[2]
	phenylalanine --> phenylpyruvate[3]
racemization	D-amino acid --> L-amino acid
decarboxylation	phenylalanine --> phenylethylamine + CO_2[3]
	5-hydroxytryptophan --> 5-hydroxyphenylalanine
	3, 4-dihydroxyphenylalanine (DOPA) --> $(dophamine)_3$
oxidative deamination	histamine --> imidzole-4-acetaldehyde + NH_4
loss of the side chain	THF + serine --> glycine + N5, 10-methylene THF
involvement of the ß-carbon	
replacement	cysteine synthetase
elimination	serine and theronine dehydratase
involvement of the γ-carbon	
replacement	cystathione --> cysteine + homoserine
elimination	homocysteine desulfhydrase
cleavage	kynurenine --> anthranilic acid[2]

[1]From Leklem, 1990a.
[2]Reactions that are especially relevant to vitamin B-6 status assessment.
[3]Reactions that are especially relevant to amino acid metabolism in PKU.

absorption of PL, PN, and PM to be a non-saturable, passive process (Middleton, 1978, 1982). Absorption of the phosphorylated forms (PLP, PNP and PMP) has been shown to occur only to a limited extent in the rat intestine. While there are some interconversions in the intestinal cell of the rat (Henderson, 1985), the initially-absorbed, non-phosphorylated vitamers are the primary forms which leave the intestinal cell of the rat (Middleton, 1982; Hamm, Mehansho, & Henderson, 1979). These forms are then transferred to the circulation where uptake into the liver occurs by facilitated diffusion (Leklem, 1988).

Vitamin B-6 metabolism has been studied in the human liver. The major transformations of the vitamers: reactions, enzymes, and co-enzymes/co-factors, are shown in Figure I.6. (Merrill, Henderson, Wang, McDonald, & Millikan, 1984). The three non-phosphorylated forms are phosphorylated by pyridoxal kinase (McCormick, Gregory, & Snell, 1961); the resulting PNP and PMP are oxidized to PLP by pyridoxine (pyridoxamine) 5'-phosphate oxidase (Wada & Snell, 1961). The fate of the resulting PLP is thought to be (Merrill et al., 1986): binding to cellular proteins; release into the circulation; or de-phosphorylation in the liver to PL by pyridoxal 5'-phosphate phosphatase, which appears to be the general alkaline phosphatase (McComb, Bowers, & Posen, 1979; Whyte, Mahuren, Vrabel, & Coburn,

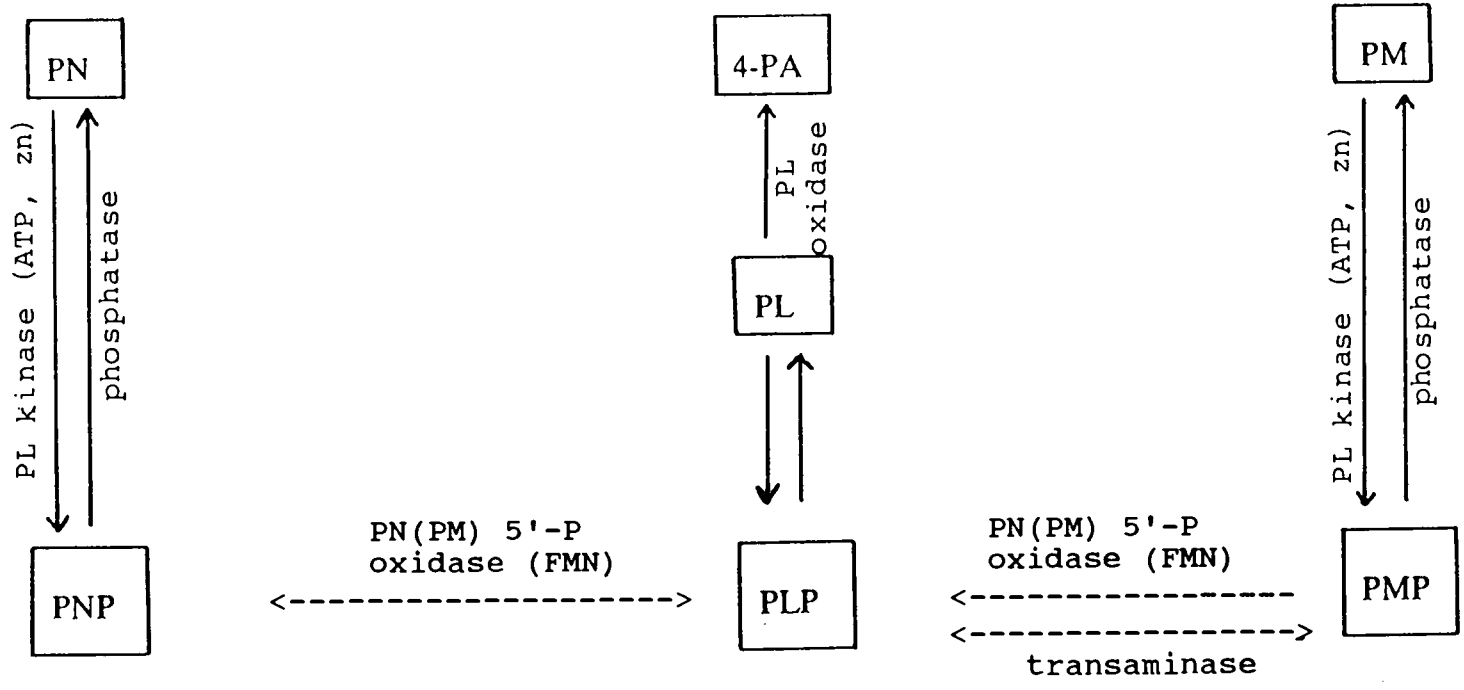


Figure I.6. Metabolism of vitamin B-6 by human liver enzymes[1]

[1] From Merrill et al., 1984.

1985). The fate of the PL thus formed is: re-phosphorylation to PLP by pyridoxal kinase; release into the circulation as PL; or oxidation to 4-pyridoxic acid (4-PA). Oxidation to 4-PA occurs as an irreversible reaction, by an aldehyde oxidase or an NAD-dependent dehydrogenase enzyme (the oxidase has been referred to as PL oxidase by Merrill and co-workers because it has not been identified conclusively in humans) (see Figure I.6., Merrill et al., 1986).

Although other tissues contribute to vitamin B-6 metabolism, the liver is thought to be the primary source of PLP synthesis (Lumeng, Brashear, & Li, 1974) and the probable site of degradation (Snell & Haskell, 1971). Measured enzyme activities in the livers of five human subjects suggested that pyridoxine (pyridoxamine) 5'-phosphate oxidase which converts PNP and PMP to PLP is the limiting enzyme in vitamin B-6 metabolism (Merrill et al., 1984). Once PLP is formed and de-phosphorylated to PL, the activity of pyridoxal oxidase appears to be sufficient to convert the PL to 4-PA (Merrill et al., 1984). In human studies, the majority of dietary vitamin B-6 has been shown to be ultimately and irreversibly converted to 4-PA (Brown, Rose, Leklem, Linkswiler, & Arnand, 1975; Wozenski, Leklem, & Miller, 1980; Shultz & Leklem, 1981). It has been suggested that this serves to prevent large amounts of the highly reactive PLP vitamer from accumulating (Leklem, 1990a).

PLP and PL are the principal B-6 vitamers found in human plasma (Lumeng & Li, 1980). In fasting adults, they account for 70-90 percent of the plasma vitamin B-6 concentration. PLP accounts for 50-75 percent of the total concentration of vitamers in human plasma (Coburn & Mahuren, 1983; Lumeng, Li, & Lui, 1985; Hollins & Henderson, 1986) and has been shown to reflect the vitamin B-6 nutritional status of human subjects (Li & Lumeng, 1981), especially as an index of the body stores of the vitamin (Lui et al., 1985). Both PLP and PL can bind via a Schiff base reaction with proteins. The binding of PLP to proteins may be the predominant factor influencing tissue levels of PLP (Li, Lumeng, & Vertch, 1974) and is hypothesized to result in metabolic trapping of PLP in cells (Li, Lumeng, & Vertch, 1974). PLP synthesized in liver cells is released and found in the circulation bound to albumin. Whether the PLP is bound to albumin prior to release from the liver or is released unbound and subsequently binds to albumin has not been determined. The binding of PLP to albumin in the circulation serves to protect it from hydrolysis and allows for the delivery of PLP to other tissues (Leklem, 1990a). Hydrolysis of PLP to PL occurs by the action of phosphatases, presumably those bound to cellular membranes, followed by uptake of PL into the cell.

In contrast, both PLP and PL in the red cell are primarily bound to hemoglobin (Mehansho & Henderson, 1980;

Ink & Henderson, 1984a). For PL, this phenomenon results in a concentration in red cells that is four to five times greater than the PL concentration in the plasma (Ink & Henderson, 1984b). While the liver is thought to be the main site of the metabolic transformations of the various B-6 vitamers (see Figure I.6.), a recent report suggests that the red cell may play a more active role in vitamin B-6 metabolism and function than has traditionally been suggested (Anderson, Perry, Clements, & Greany, 1989). This study, using IV injections of PN to a human subject, confirmed that the red cell takes up and metabolizes PN to PL <u>in vivo</u>, a finding which had been previously demonstrated <u>in vitro</u> by Anderson and co-workers (Anderson, Fulford-Jones, Child, Beard, & Bateman, 1971). In addition, these studies (Anderson et al., 1989, 1971) led the authors to speculate that the red cell may also deliver PN and PL to other sites of conversion in the tissues and could even aid in the delivery of these vitamers to the liver for ultimate conversion to PLP. When viewed in this way, the red cell may serve as an important source of vitamin B-6.

PL plays a central role in vitamin B-6 metabolism in human skeletal muscle. Due to a near or total absence of PMP (PNP) oxidase activity in this tissue (Lumeng & Li, 1980), PL is the only B-6 vitamer that can serve as a source of PLP in skeletal muscle. Based on the total skeletal muscle mass in humans, the PL and PLP content of this tissue

may be quantitatively significant, relative to their content in other tissues and fluids.

PLP also exists in a bound form in skeletal muscle. Approximately 66-69% of the vitamin B-6 content in this tissue is present as PLP (Coburn & Mahuren, 1983), bound to glycogen phosphorylase (Black, Guirard, & Snell, 1978). In rats fed a high vitamin B-6 diet, skeletal muscle B-6 content (primarily as PLP) and glycogen phosphorylase increased together over a six week study period (Black, Guirard, & Snell, 1977). A relationship between PLP and glycogen phosphorylase was later observed by these same authors (Black et al., 1978) in the skeletal muscle of rats fed a low-calorie diet. In this study, both PLP and glycogen phosphorylase content were reduced in the calorically-deprived animals. These findings led the authors to speculate that the skeletal muscle serves as a reservoir for PLP, protected in its bound form with glycogen phosphorylase and released in response to a caloric deficit (Black et al. 1977, 1978). Leklem and others (Leklem & Shultz, 1987; Manore, Leklem, & Walter, 1987) have added to these findings with their more recent studies in human subjects immediately following strenuous exercise. They observed increased PLP concentrations in plasma obtained from fasting, exercised subjects. Postulating that strenuous exercise results in a metabolic state of an acute caloric deficit and, thereby, an increased need for

gluconeogenesis, they further speculated that the increased circulating levels of PLP reflected a release of PLP from the skeletal muscle. This phenomenon was considered to be similar to the release of gluconeogenic amino acids from skeletal muscle under these conditions.

Anderson and co-workers (1989), suggested that the red cell may also transport vitamin B-6 to other sites of metabolism in tissues, and speculated that there might be an analogy in the red cell of the uptake and clearance of the PN vitamer to that of amino acids. The basis for this analogy is similar to that of the analogy which Leklem and co-workers (Leklem & Shultz, 1987; Manore et al., 1987) suggested concerning the release of PLP to that of amino acids in the skeletal muscle. The red cell and skeletal muscle have been shown to play important roles in the interorgan metabolism and transport of amino acids to and from the liver and other tissues. While it is not yet known whether an interrelationship between vitamin B-6 and transport of amino acids exists in the red cell or in the skeletal muscle, the similarities between the inter-organ metabolism of vitamin B-6 and the BCAAs are shown in Figure I.7. (modified from Hatcher, 1983; Harper et al., 1984).

3. Interrelationships with amino acids and with PKU

PLP, as a central vitamin B-6 metabolite and coenzyme, is involved in a variety of biochemical reactions (Leklem,

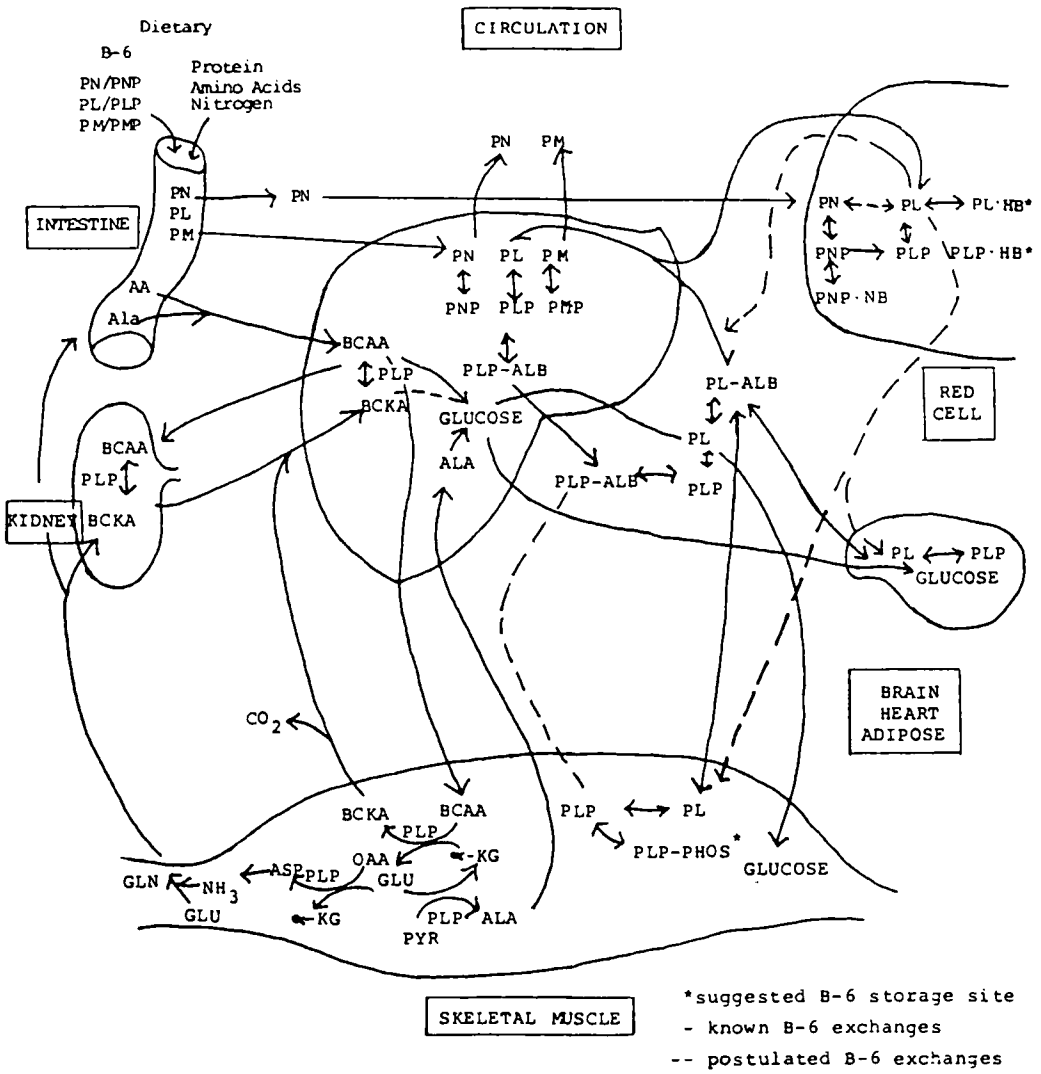


FIGURE I.7. Current understanding of the interorgan cooperativity in the metabolism of vitamin B-6 and branched-chain amino acids (BCAA)[1].

[1] Modified from Hatcher, 1983; Harper, et al., 1984.

1990a). It is primarily through reactions with amino acids and other nitrogenous compounds (Martinez-Carrion, 1986) that a majority of the metabolic processes and functional roles of vitamin B-6 have been described (Leklem, 1990a). Because of this close association with amino acid metabolism and the influence of dietary protein on vitamin B-6 requirements (Miller, Leklem, & Shultz, 1985), vitamin B-6 has been referred to as the "protein vitamin" (Sauberlich, 1985). The cellular processes and the functions/systems influenced by PLP (Leklem, 1990a) that are interrelated with amino acid metabolism are shown in Figure I.8. Production of glucose via transamination of amino acids and of neurotransmitters via the decarboxylation of several procursor aromatic amino acids are particularly significant to the present review. Both of these processes may be affected by the nutritional mileau of energy, protein, amino acids, and vitamin B-6.

a. Production of glucose

Because mammalian liver lacks the capacity to convert fatty acids to glucose, gluconeogenesis importantly serves to convert amino acids to glucose and to recycle their carbon skeletons (lactate, pyruvate, glycerol). The DAA alanine is a key amino acid in this process and accounts for more than half of the total amino acid utilization in

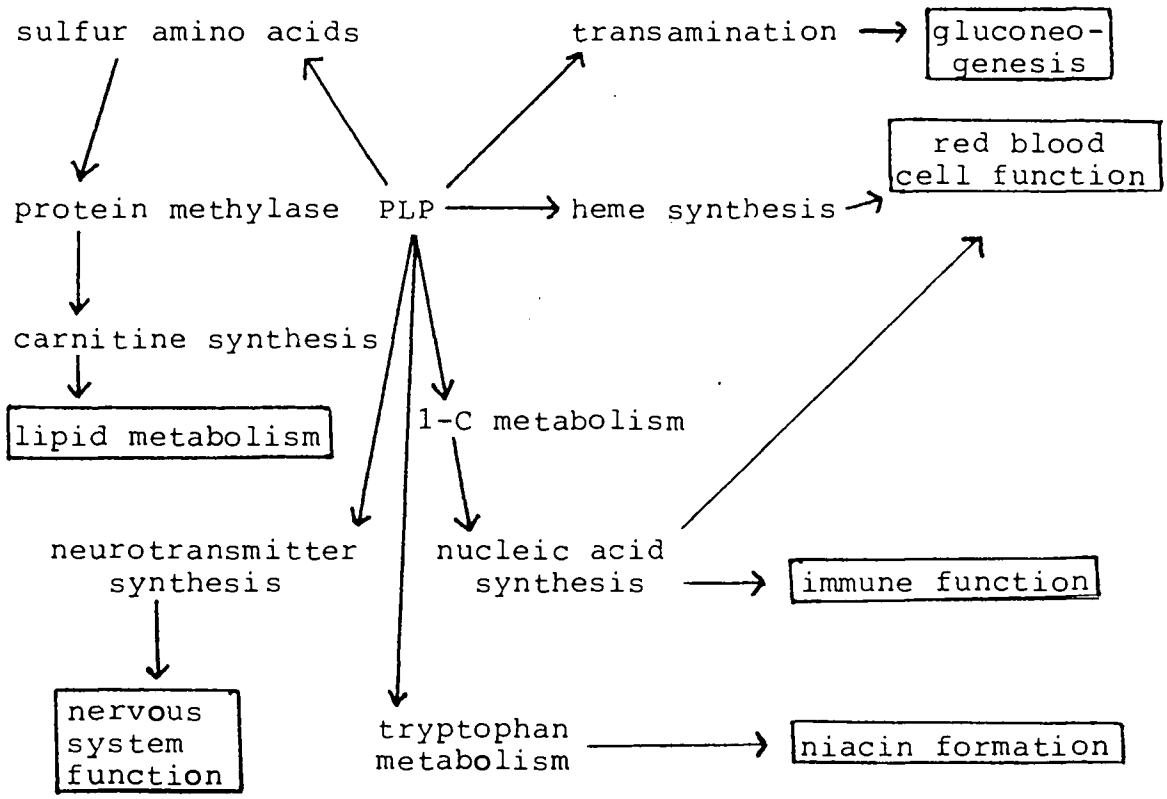


FIGURE I.8. The key roles of vitamin B-6 as pyridoxal 5'-phosphate (PLP) in amino acid metabolism.[1]

[1] Modified from Leklem, 1990a.

hepatic gluconeogenesis. Alanine, glutamine, glutamate, and glycine comprise nearly 80% of the total free amino acid nitrogen pool (Munro, 1970).

As previously described, quantitatively the most important reservoir of PLP and amino acids is the skeletal muscle. It is in this tissue that alanine is synthesized from pyruvate (resulting from the catabolism of glucose) and glutamate or aspartate (resulting from the catabolism of the BCAAs). Alanine is then transported to the liver, the primary site of gluconeogenesis (Figure I.9). Based on an understanding of the glucose-alanine cycle, Nyhan and co-workers (Kelts, Ney, Bay, Saudubray, & Nyhan, 1985; Wolff, Kelts, Algert, Prodanos, & Nyhan, 1985) hypothesized that dietary supplementation with alanine could serve to reduce BCAA catabolism in situations where BCAAs may become limiting. Such situations include genetic defects of amino acid metabolism requiring a restricted natural protein intake to decrease the accumulation of one or more amino acids (e.g. PKU).

Due to the difficulties associated with ingestion of the L-AAs in medical foods or EMFs, presumably it would be desirable to reduce the L-AA quantities in these products to the lowest safe level. If alanine is a superior DAA over others, due to its role in BCAA metabolism and glucose production, it should be given preferential consideration in

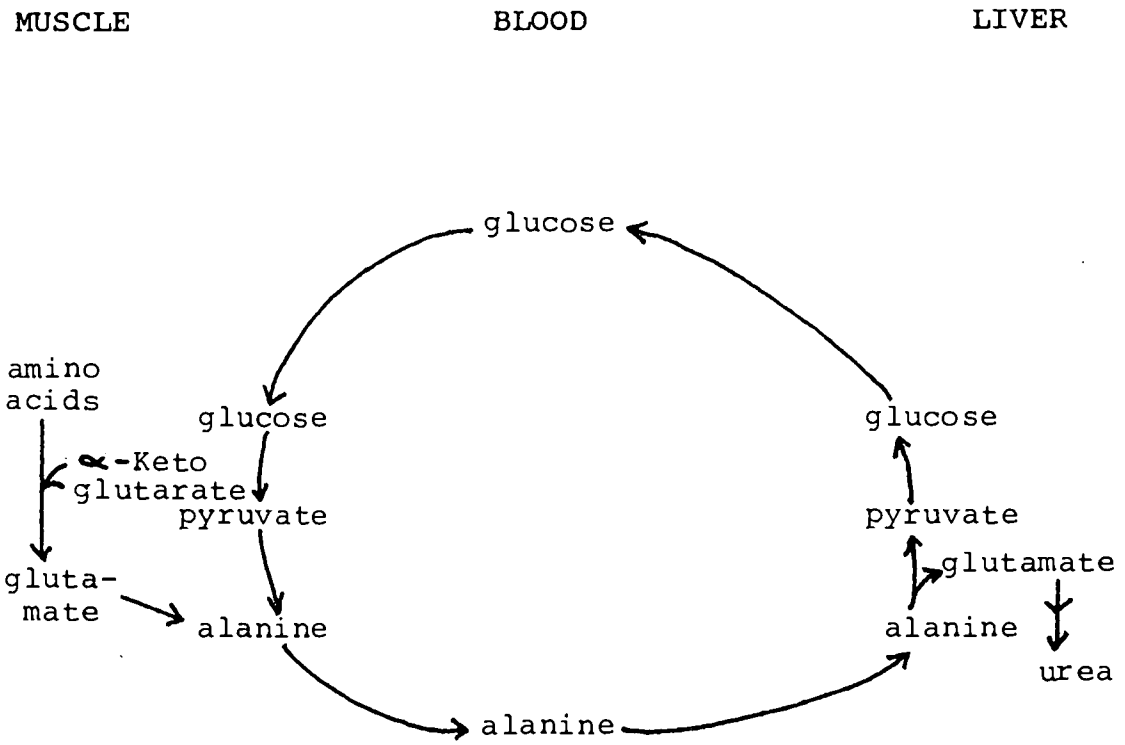


FIGURE I.9. The glucose-alanine cycle.[1]

[1] Modified from Kelts et al., 1985.

the formulation of these products. Currently there is no L-alanine in the major U.S. EMF, Phenyl-Free®, even though it is a relatively neutral, non-offensive tasting amino acid (Solms, Vuataz, & Egli, 1965).

Nyhan and co-workers studied five infants with inborn errors of amino acid metabolism requiring dietary protein restriction (Kelts et al., 1985). Various amounts of L-alanine were added to a control diet of defined energy, protein, and IDAAs which were close to the estimated minimal requirements for growth in infants. All diets were isocaloric and each diet was fed to the infants for seven days. When L-alanine was added to the control diet, weight gain increased significantly. In four of the five infants, after protein intake was lowered so that growth stopped or became minimal, alanine supplementation restored and increased growth. In several of the infants, the same growth rate was achieved with 0.75 g protein/kg with alanine, as was observed with 1.79 g protein/kg without alanine. These infants also showed graded weight gains in response to graded alanine supplements from 50-250 mg/kg. When alanine was compared to a mixture of IDAAs, glycine alone, or glutamate alone, alanine promoted greater growth.

This report suggests that alanine has a unique protein-sparing anabolic effect which permits growth at total protein intakes below those usually required for growth. The authors related the effects they observed in these

infants to the operation of the glucose-alanine cycle. In a review of these studies ("Advantages Of", 1986), two interpretations are proposed. One view is that alanine carries the amino nitrogen generated from BCAA oxidation in muscle from the muscle to the liver. Alanine formation would thus allow muscle metabolism of BCAAs without accumulations of ammonia. An alternative interpretation, suggested by Nyhan and co-workers and supported by these experiments, is that the oxidation of a considerable quantity of BCAAs may be required by muscle to generate alanine for gluconeogenesis. Secondly, exogenous alanine supplementation may spare an otherwise obligatory catabolism of the BCAAs, preserving them for protein synthesis.

What impact would this have on vitamin B-6 metabolism and status? One could speculate that the amount of PLP released from its greatest quantitative reservoir, the skeletal muscle, might be regulated at least in part by the need for PLP to be in the muscle (to degrade the BCAAs), which might further be regulated to some extent by the need for endogenous alanine synthesis. For treated patients with PKU receiving the majority of their protein as Phenyl-Free®, an EMF which supplies no L-alanine, the reduced exogenous intake of alanine might be limiting for its central metabolic role. This would place a greater need on endogenous synthesis of alanine, through BCAA catabolism, and, thereby, increased requirements for the BCAAs and for

vitamin B-6.

In a recent attempt to examine the effect of protein quality on vitamin B-6 status, two groups of rats were fed diets which contained either adequate or suboptimal vitamin B-6 and crystalline L-AA mixtures reflecting either good quality or low quality protein (the amino acid profile of the low quality protein was equivalent to that of maize) (Fisher, Willis, & Haskill, 1984). The mean values for three indices of vitamin B-6 status: urinary 4-PA, liver vitamin B-6, and plasma PLP, were lower in the rats fed the low quality protein compared to those fed the good quality protein, regardless of the vitamin B-6 intake. The authors speculated that because proteins supplying greater proportions of DAAs (low quality) are utilized less efficiently for protein synthesis, more amino acids would be degraded. This situation would favor an increased need for enzymes which degrade amino acids and, thereby, an increased need for PLP (hence, for vitamin B-6).

These studies support a closer evaluation of the vitamin B-6 status and the dietary and plasma levels of amino acids in patients with PKU receiving the majority of their dietary protein in an unnatural and potentially unbalanced form. The literature pertaining to plasma amino acid concentrations in treated patients with PKU has been reviewed; unfortunately, none of these reports quantitated amino acid intakes. Due to the natural protein restrictions

which are a part of the nutritional treatment of PKU, the natural foods consumed supply low quality (plant) protein. However, the EMFs which comprise the major protein source for patients with PKU are relatively high in IDAAs, suggesting that the total protein intake may be of higher quality (refer to Table I.13). These data suggest an increasing need for vitamin B-6 in treated patients with PKU, as dietary adherence is relaxed and lower quality protein is substituted for EMF-protein.

b. Production of neurotransmitters

The essential role of B-6 vitamers in maintaining the proper function of the central nervous system has been established (Coursin, 1964a, 1969). In studies of infants receiving a vitamin B-6 deficient diet, the extent of symptoms (i.e. convulsions and abnormal EEG tracings) appeared to be correlated with the protein content of the diet. Treatment with vitamin B-6 produced a rapid improvement in the symptoms.

Based upon these observations and similar neurological abnormalities associated with the clinical syndrome of untreated PKU, a series of animal experiments by Loo and co-workers beginning in 1964 were undertaken to determine whether an inhibition of vitamin B-6 function contributed to the pathogenesis of untreated PKU (Loo & Ritman, 1964, 1967). The basis for the experiments was the detection of a

Schiff base, formed between a metabolite of phe and vitamin B-6, in the urine of untreated patients with PKU. The Schiff base, pyridoxylidene-ß-phenylethylamine, and its precursor, the product of the decarboxylation of phe, phenylethylamine (PEA), were suggested to be neurotoxic by Loo and others (Kaufman, 1977). The possibility that the neuropathologic effects that characterize untreated PKU are caused by the accumulation of toxic metabolites of phe continues to receive attention (Michals, Lopus, & Matalon, 1988). However, in a comprehensive review of the evidence reported to date for this theory, Kaufman recently concluded that none of the phe metabolites (including those which involve B-6 vitamers) are likely to reach neurotoxic levels, either in patients with PKU or in normal humans (Kaufman, 1989). The metabolic relationships among these metabolites and vitamin B-6 are shown in Figure I.10. (modified from Kaufman, 1989).

Of greater relevance to the possible metabolic interrelationships between vitamin B-6, PKU, and central nervous system function is the synthesis of several essential neurotransmitters which require PLP and have been shown to be reduced in the plasma and in the cerebrospinal fluid (CSF) of patients with PKU. As with the plasma amino acid concentrations, there are inconsistencies in the reported depletions of these compounds depending on whether the patients were undergoing treatment at the time of the

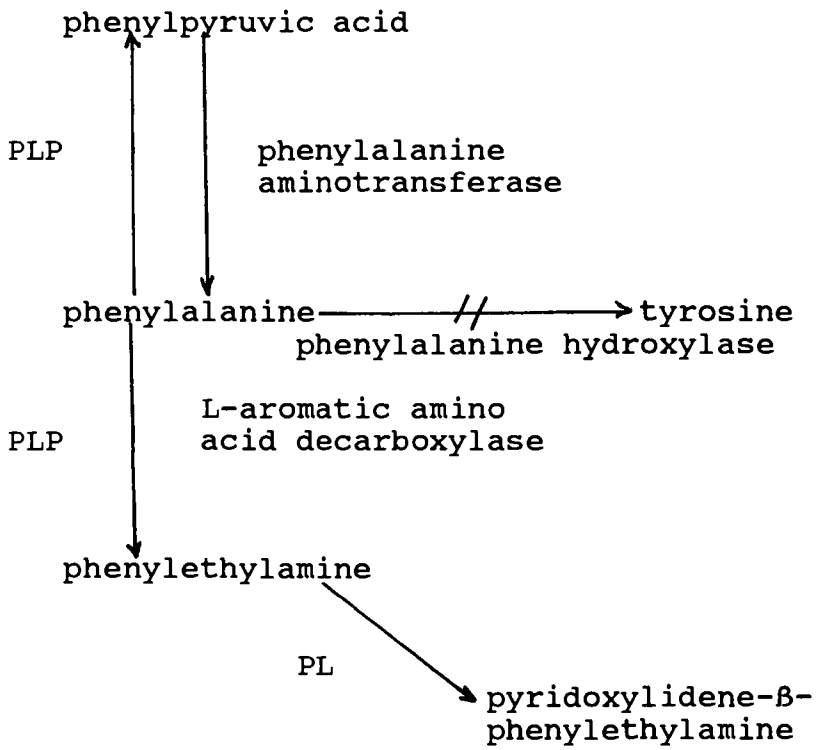


FIGURE I.10. Metabolic relationships among metabolites of phenylalanine and vitamin B-6.[1]

[1] Modified from Kaufman, 1989.

study. Two neurotransmitters have received the greatest attention in the PKU literature: 5-hydroxytryptamine (serotonin) and dopamine. Each of these compounds are synthesized from aromatic amino acid precursors (tryptophan and tyrosine, respectively) and require aromatic L-amino acid decarboxylase and PLP for their production. The synthetic pathways are illustrated in Figure I.11. The concentrations of their metabolites, 5-hydroxyindoleacetic acid (5-HIAA) and homovanillic acid (HVA), in serum, urine, and CSF have been used as indirect measures of neurotransmitter synthesis.

Decreased concentrations of serum serotonin and excretion of 5-HIAA in the urine of untreated patients with PKU compared to normal subjects were reported by Pare and co-workers in 1957. These same investigators found they could achieve a significant rise in the mean serum serotonin level and in the excretion of 5-HIAA in the urine of patients when low-phe diets were administered (Pare, Sandler, & Stacey, 1957). This led to a speculation that serotonin metabolism in the central nervous system of untreated patients with PKU might be disturbed and might be associated with the disordered behavior observed in these patients (as reviewed in McKean, 1972). Additional support for this theory was provided when Yuwiler and Louttit demonstrated decreased serotonin levels in the brain tissue of an experimental animal model for PKU (Auerbach, Waisman,

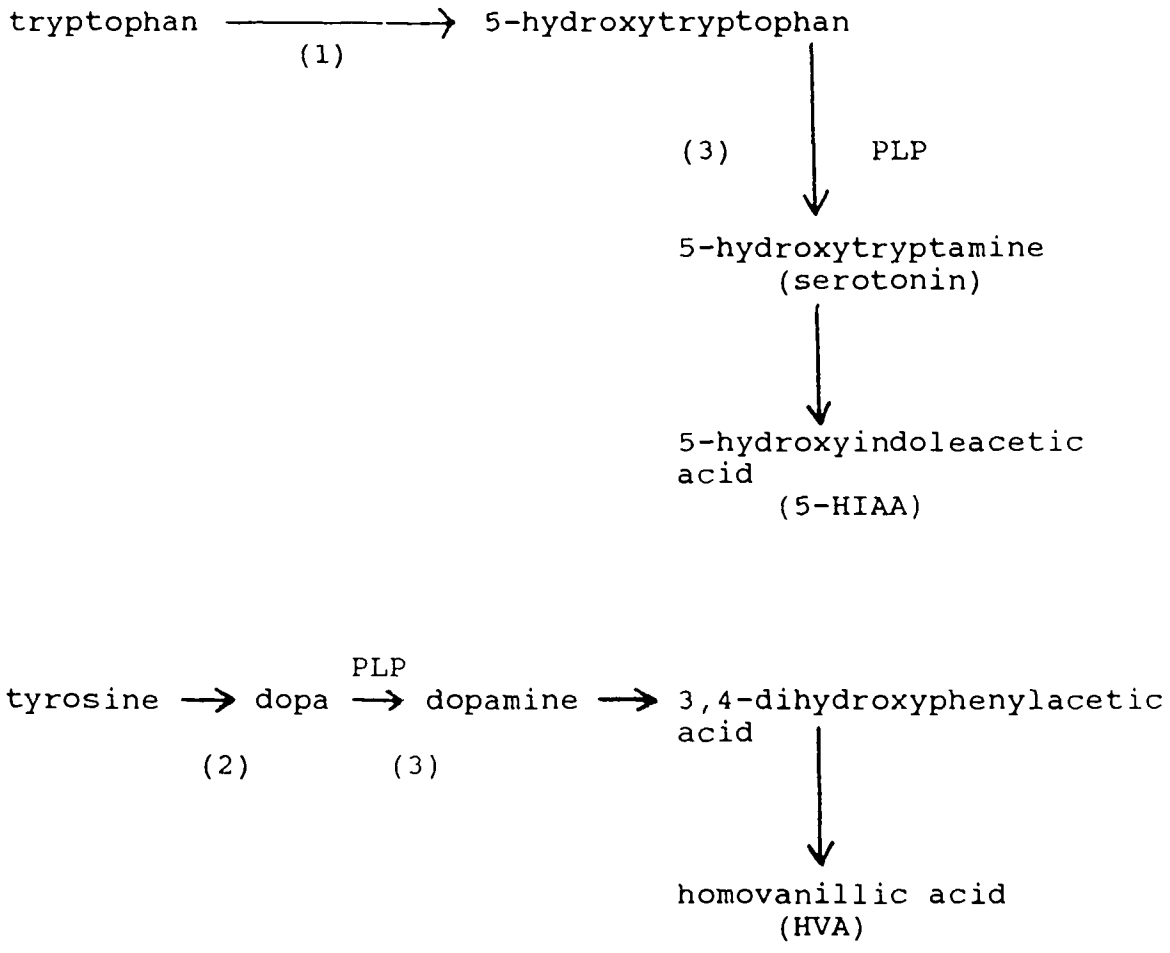


FIGURE I.11. Metabolic pathways of neurotransmitter production showing the rate-limiting enzymes: (1) tryptophan-5-hydroxylase and (2) tyrosine-3-hydroxylase and the pyridoxal 5' phosphate (PLP) - dependent enzyme: (3) L-aromatic amino acid decarboxylase.[1]

[1] Modified from Rumsby and Shepherd, 1980.

& Wyckoff, 1958) fed a high-phe diet (Yuwiler & Louttit, 1961). They also reported impairments in the behaviors and problem-solving activities of the animals during the high-phe diet (Yuwiler & Louttit, 1961).

By the early 1960s, several explanations were advancing concerning the mechanism of the brain serotonin depletion in PKU (as reviewed in Yuwiler, Geller, & Slater, 1965). These included: (1) Specific inhibition of 5-hydroxytryptophan decarboxylase (later found to be the general L-aromatic amino acid decarboxylase), supported by in vitro studies of Davidson and Sandler (1958); 2) Nonspecific inhibition of decarboxylases and other PLP-dependent enzymes; 3) Inhibition of precursor (amino acid) transport through the blood-brain barriers; and 4) Inhibition of tryptophan hydroxylase, the non-PLP-mediated enzyme for hydroxylation of tryptophan to 5-hydroxytryptophan (refer to Figure I.11.).

To test the possibility that the decreased serotonin was a result of the inhibition of PLP-dependent enzymes necessary for serotonin synthesis, the effect of vitamin B-6 supplementation on blood serotonin levels in ten untreated children with PKU was examined (Berman, Justice, & Hsia, 1970). Previous studies had shown a decrease of available PLP and depressed 5-hydroxytryptophan decarboxylase activity in a vitamin B-6 deficient state, which were corrected by vitamin B-6 administration (referred to in Berman et al.,

1970). In this study, 25-50 mg of vitamin B-6 was added to the diets of the patients for each of seven days. No significant changes in serotonin were observed, and the authors concluded that the reduced blood serotonin concentrations could not be increased by the oral administration of vitamin B-6. It should be noted that the supplementation period was relatively short (seven days), the sample size was relatively small (ten subjects), and serotonin concentrations in serum may not necessarily reflect the levels in the brain. This is the only study which has examined the effect of vitamin B-6 supplementation on the neurotransmitter deficit reported in patients with PKU.

A more recent and intriguing study was conducted by McKean (1972), using autopsy and in vivo data, to determine the effects of high phe concentrations on the cerebral metabolism of these neurotransmitters. The concentrations of phe, tryptophan, tyrosine, serotonin, and dopamine were measured in post-mortem brain tissues collected from untreated patients with PKU and control subjects. Additionally, the concentration of these compounds and of 5-HIAA and HVA were measured in vivo in the CSF of the patients, during nutritional treatment and off-treatment. From the autopsy samples, McKean found tryptophan and tyrosine concentrations in the cortical tissues of the patients which were 50-60% lower than the concentrations in

the respective tissues of the control subjects. In contrast, tryptophan and tyrosine concentrations in the CSF of the patients were increased 100% and 20%, respectively, over the concentrations of these amino acids in the CSF of the controls. This suggested to McKean that there may be an increased rate of efflux into extraneuronal fluid or a decreased rate of efflux from the CSF compartment. From the in vivo measures of 5-HIAA and HVA accumulation, McKean reported significant increases in the accumulation rates of these metabolites by lowering the blood phe concentrations, suggesting that the neurotransmitters themselves increased. Further, McKean was able to reverse the metabolic inhibition by reducing the phe concentration.

More recently, plasma and CSF concentrations of phe tryptophan, tyrosine, 5-HIAA, and HVA in four adolescent to young adult patients with PKU were measured before and after discontinuation of nutritional treatment (Lou, Guttler, Lykkelund, Bruhn, & Niederwieser, 1985). These biochemical indices, along with clinical measures of vigilance, were used to test the hypothesis that the nutritional treatment could safely be relaxed in older patients in whom (presumably) the nervous system had reached a nearly complete development. In three of the four patients studied, discontinuation of the nutritional treatment resulted in nearly a two-fold increase in plasma and CSF phe, a moderate decrease in plasma tyrosine and tryptophan,

and inconsistent changes in CSF tyrosine or tryptophan. CSF concentrations of HVA and 5-HIAA were in the low-normal range during treatment and decreased further when treatment was discontinued. The 5-HIAA levels especially became extremely low, suggesting lower levels of serotonin than dopamine. These changes in neurotransmitter metabolism were associated with clinical deteriorations in the measures of vigilance (Lou et al., 1985). The authors concluded that tyrosine and tryptophan, as judged by their CSF concentrations, were not the rate-limiting factors for dopamine and serotonin synthesis since tyrosine and tryptophan levels were not consistently lowered after the nutritional treatment was discontinued. They suggested two possible mechanisms for the reduced neurotransmitter synthesis: 1) transport inhibition across neuronal cell membranes of aromatic amino acids such as tyrosine and tryptophan by phe; or 2) competitive inhibition of brain tyrosine-3-hydroxylase and tryptophan-5-hydroxylase, the rate-limiting enzymes in the synthesis of dopamine and serotonin, respectively, by phe at the pathophysiological concentrations which characterize PKU. Experimental support for this has been demonstrated both <u>in vivo</u> and <u>in vitro</u> (Curtius et al., 1982; Herrero, Aragon, Gimenez, & Valdivieso, 1983). McKean proposed the same two-part mechanism in his 1972 report (McKean, 1972).

These potential effects on the need for vitamin B-6 in

patients with PKU have not been examined. Because the proposed inhibition precedes the PLP-dependent decarboxylation of tryptophan and tyrosine (refer to Figure I.11.), one might speculate that the need for PLP would be in part regulated by the availability of the precursor amino acids and by the extent of an inhibition of their hydroxylation. Presumably, this would be dependent on the degree of adherence to the strict nutritional treatment and the subsequent plasma concentrations of phe, tryptophan, and tyrosine, particularly relative to one another. Thus, when the nutritional treatment is strictly adhered to, characteristic of infants and preschool-aged patients, the need for PLP associated with L-aromatic amino acid decarboxylase, would be greater. This would also be at a time when protein and amino acid intakes would be greater, as a result of the acceptance of the protein-dense medical foods.

In summary, the literature in two areas of amino acid metabolism which may be altered in PKU suggests consequent effects on the need for vitamin B-6 as PLP. These metabolic factors and the previously reviewed dietary factors in the nutritional treatment of PKU and their potential effects on a need for vitamin B-6 are outlined in Table I.16. As discussed, presumably these effects would be influenced by the degree of dietary adherence. To examine the adequacy of vitamin B-6 in the current nutritional treatment, a vitamin

TABLE 1.16 Dietary and metabolic factors associated with PKU: potential effects on the need for vitamin B-6[1]

Factors	Need for vitamin B-6
dietary	
strict-adherence (increased medical foods)	
increased total protein	increase (?)
increased good quality protein	decrease (?)
inadequate calories	increase (?)
decreased alanine (limiting[2])	increase (?)
relaxed-adherence (increased natural foods)	
decreased total protein	decrease (?)
increased low quality protein	increase (?)
metabolic	
treated (increased plasma phe[3])	
increased transamination, decarboxylation of phe	increase (?)
formation of phe/B-6 metabolite[4]	increase (?)
increased plasma amino acids	increase (?)
inhibition of trp, tyr hydroxylases	decrease (?)
untreated (further increased plasma phe[5])	
all metabolic factors are increased	(?)

[1] Question marks illustrate the current state of knowledge.
[2] Assuming the medical food Phenyl-Free® that contains no L-alanine.
[3] Phenylalanine is increased relative to the normal state.
[4] Pyridoxylidene-ß-phenylethylamine is formed from phenylethylamine and pyridoxal.
[5] Phenylalanine is increased to a greater extent than in the treated state. The metabolic effects of PKU are increased.

B-6 status assessment of patients who differed in their degree of dietary adherence was undertaken (Chapter IV). Because of the dietary factors and the interrelationships between PKU per se, amino acid and vitamin B-6 metabolism, the design, measurement, and analysis of a good status evaluation required careful planning. A review of the various approaches, their applications and limitations, preface the vitamin B-6 status report of the sample of school-aged children who were examined in this research.

D. VITAMIN B-6 STATUS

1. Approaches, indices, and methods

The assessment of B-6 status is central to an understanding of vitamin B-6 nutrition in an individual human subject (Leklem, 1990a). Numerous approaches, indices, and methods have been developed and utilized to assess the vitamin B-6 status of human subjects. The most commonly used indices are presented in Table I.17, grouped by their three currently accepted categories: 1) direct, 2) indirect, and 3) diet intake methods (Leklem, 1990a).

Direct indices of human vitamin B-6 status have traditionally measured one or more of the B-6 vitamers (e.g. PL, PLP) in the plasma or erythrocytes of subjects, or the urinary excretion of the major vitamin B-6 metabolite, 4-PA. This is in contrast to a direct assessment of the vitamin B-6 status of animals, in which the various vitamers are

TABLE I.17 Indices for assessing vitamin B-6 status in humans[1]

indices	blood	urine
1. **direct**		
plasma pyridoxal-5'-phosphate	x	
plasma pyridoxal	x	
plasma total vitamin B-6	x	
erythrocyte pyridoxal-5'-phosphate	x	
4-pyridoxic acid		x
total vitamin B-6		x
2. **indirect**		
erythrocyte (or serum) alanine aminotransferase	x	
erythrocyte (or serum) aspartate aminotransferase	x	
tryptophan load test (2 g)		x
methionine load test (3 g)		x
3. **diet intake**		
vitamin B-6		
vitamin B-6:protein ratio		
pyridoxine β-glucoside		

[1] Modified from Leklem, 1990a.

measured in the actual tissues of interest (liver, brain, muscle). This difference is a limitation in human studies of vitamin B-6 status and requires that certain assumptions be made about the association between the quantities of the vitamers or their metabolites in biological fluids and in the tissues, even with the direct indices.

Over the past 15 years, plasma PLP has been the most frequently used direct measure of vitamin B-6 status (Leklem, 1990b). Recognition of the factors that may influence plasma PLP concentration is a necessary antecedent to its use and interpretation as a status indicator. The factors that have been found to influence plasma PLP are summarized in Table I.18 (Leklem, 1990b). Dietary vitamin B-6 and protein are known to have the most significant (and opposite) effects. An increase in vitamin B-6 intake increases the plasma PLP concentration, while an increased protein intake decreases the concentration of PLP in plasma (Miller et al., 1985). Because of these factors and of the possible effects of various medications on plasma PLP, the historically accepted usefulness of plasma PLP as the single best indicator of vitamin B-6 status has been questioned more recently (Leklem & Reynolds, 1988; Leklem, 1990b).

Based on studies in rats in which the tissue (muscle) PLP level was significantly correlated with the plasma PLP concentration (Lumeng, Ryan, & Li, 1978), plasma PLP has been considered to be a direct measure of the active

TABLE I.18 Factors affecting plasma pyridoxal 5'-phosphate (PLP) concentrations[1]

factors		plasma PLP
<u>diet</u>		
vitamin B-6	(increase)	increase
protein	(increase)	decrease
glucose	(increase)	decrease (acute effect)
<u>physiological</u>		
plasma volume	(increase)	decrease
physical activity	(increase)	increase (acute effect)
age	(increase)	decrease
uptake into nonhepatic tissues	(decrease)	increase

[1] Modified from Leklem, 1990b.

coenzyme form of the vitamin which is available to the tissues (Leklem, 1990b). However, circulating PLP must first be de-phosphorylated to PL in the plasma, prior to tissue uptake, and then re-phosphorylated to PLP for coenzyme activity in the tissues. In situations whereby the phosphorylation/de-phosphorylation mechanism is altered, plasma PLP concentration may be less valid as a direct measure of vitamin B-6 status. One such condition is hypophosphatasia, an inborn error of metabolism which is characterized by a deficient plasma activity of the tissue-nonspecific (including liver, bone, kidney) isoenzyme of alkaline phosphatase (AP) (TNSALP). Whyte et al. (1985) reported increased plasma PLP concentrations in hypophosphatasia, supporting the dephosphorylation of PLP by TNSALP. Further studies by Whyte et al. (1988) on post-mortem autopsy samples in patients with the lethal perinatal form of the disease provided more information about the metabolism of vitamin B-6, particularly the relationship between tissue levels of PLP and plasma concentrations of PLP, PL and TNSALP. Three patients with perinatal hypophosphatasia were found to have markedly elevated plasma PLP levels (25-fold greater than the levels of normal control newborns) and undetectable plasma PL levels. However, 4-PA excretion in the urine and total vitamin B-6, PLP, and PL concentrations in the tissues were unremarkable. Prior to death from the metabolic defect, none of the

patients exhibited symptoms of vitamin B-6 deficiency or excess, further suggesting to Whyte et al. that TNSALP acts as an ectoenzyme to regulate extracellular but not intracellular levels of PLP. A variety of evidence has indicated that, in the circulation, soluble ALP contributes little to PLP hydrolysis (Lumeng, Schenker, Li, Brashear, & Compton, 1984; Whyte et al. 1984). TNSALP activity on cell surfaces would appear, therefore, to dephosphorylate PLP to PL extracellularly (suggested by Whyte et al., 1988). These data are consistent with a reduced dephosphorylation of PLP to PL with extreme TNSALP deficiency. Whether circulating ALP activity reflects ALP activity on cell surfaces remains unknown. Additionally, there is a need to examine the PLP concentrations in clinical situations in which ALP activity is reduced, but to less extreme levels than what characterizes patients with hypophosphatasia. Kant and co-workers (1988) have reported a significant inverse correlation between plasma PLP and serum alkaline phosphatase levels in healthy adults.

Other direct measures of vitamin B-6 status are plasma PL and total vitamin B-6. Plasma total vitamin B-6 measures both the phosphorylated and nonphosphorylated forms of the vitamin (Kant, Moser-Veillon, & Reynolds, 1988). Plasma PLP has been found to contribute from 52% (Coburn & Mahuren, 1983) to 66% (Lumeng et al., 1985) of the total B-6 vitamers in human plasma (fasting). PL was reported (in these same

studies) to contribute 21% and 27%, respectively. Because PL is the primary vitamer that crosses all membranes under post-prandial conditions, direct measurement of the PL vitamer in addition to PLP in plasma is recommended in a vitamin B-6 status assessment (Leklem, 1990b). Alternatively, PL is sometimes estimated (by difference) when total vitamin B-6 and PLP are measured in plasma, since PLP and PL comprise the majority of the B-6 vitamers in plasma (Leklem, 1990a). However, this may not be appropriate in situations in which the dephosphorylation of PLP to PL is altered (as previously discussed, e.g. hypophosphatasia).

Urinary 4-PA and total urinary vitamin B-6 are additional direct measures of vitamin B-6 status. Produced in the liver, 4-PA is the major metabolic product of vitamin B-6 (Leklem, 1990a) and, as observed with plasma PLP, there is an inverse relationship between protein intake and excretion of 4-PA. Miller and co-workers (1985) reported in eight healthy adults fed a constant intake of vitamin B-6 and three different levels of protein, significantly different urinary 4-PA concentrations at each level of dietary protein. All subjects except one excreted the lowest amount of urinary 4-PA in response to the highest protein diet; all of them excreted the highest amount of 4-PA in response to the lowest protein diet. The excretion of urinary total vitamin B-6, in contrast, decreased only

slightly as the level of protein intake was increased.

An interpretation of urinary 4-PA and/or total vitamin B-6 excretion as indices of vitamin B-6 status should be done with three considerations. First, since 4-PA is produced by oxidation of PL, PN and PM must first be converted to PLP and then to PL before they can contribute to urinary 4-PA. Thus, the turnover of PLP via conversion to PL is the main control point which determines the subsequent amount of 4-PA (Leklem, 1990b). Second, urinary 4-PA excretion changes rapidly in response to a change in vitamin B-6 intake and, thus, is considered to be a short-term indicator of vitamin B-6 (Brown et al., 1975). Urinary vitamin B-6 is not considered to be a good measure of vitamin B-6 status (Leklem, 1990b), representing 8-10% of the daily vitamin B-6 intake (Leklem, 1988). However, if dietary vitamin B-6 is measured and information is obtained on how representative the measured intake is of an individual's usual diet, 4-PA excretion is considered to be a useful status indicator (Leklem, 1990b). Third, urinary measures of B-6 vitamers (e.g. 4-PA) should be interpreted along with some assessment of protein intake. Plasma PLP and total vitamin B-6 concentrations in plasma and urinary 4-PA excretion have been shown to be inversely related to protein intake. This suggests that as the level of dietary protein is increased, more vitamin B-6 is retained, presumably in the liver, to supply PLP for the PLP-dependent

enzymes involved in amino acid degradation (Miller et al., 1985). As a result, less vitamin B-6 is available for conversion to 4-PA and PLP (Lumeng et al., 1974).

Indirect methods of vitamin B-6 status include the measures of the activities of PLP-dependent enzymes before and after stimulation with PLP, and the excretion of one or more products of PLP-dependent amino acid metabolic pathways before and after a loading dose of the amino acid substrate (refer to Table I.17). The advantage of these measures is their functional nature; they are sensitive to situations in which PLP-dependent enzymes are inhibited which may or may not be accompanied by changes in plasma (or even tissue) levels of PLP. A relative weakness of these more functional indices, however, is their indirect nature in that they do not directly measure any of the B-6 vitamers. Therefore, they are ideally used in conjunction with one or more direct indices. Plasma PLP and urinary 4-PA excretion, along with the excretion of tryptophan metabolites following a tryptophan load test, is the combination of measures that has been cited most extensively in human adult vitamin B-6 status reports.

Historically, the tryptophan load has been the most widely used indirect index of vitamin B-6 status (Leklem, 1990b). The tryptophan metabolic pathway involves several PLP-dependent enzymes. The liver is the primary site of tryptophan metabolism and the majority of plasma PLP

synthesis occurs in the liver. The steps in the tryptophan metabolic pathway that require PLP are shown in Figure I.12. In a vitamin B-6 deficiency state, urinary xanthurenic acid (XA) excretion is increased in humans following a loading dose of L-tryptophan (Coursin, 1964a, 1964b). At first this observation seems paradoxical as XA is formed from 3-hydroxykynurenine by the kynurenine aminotransferase (KAT) which is itself PLP-dependent (Figure I.12.). Thus, one would expect XA excretion to decrease rather than increase in a vitamin B-6 deficiency. However, the other PLP-dependent enzymes involved in tryptophan metabolism occur in cytoplasm and are readily depleted of their coenzyme. The KAT, located in the mitochondria, is protected from such depletion. The steps leading to kynurenic acid (KA), anthranilic acid, and 3-hydroxyanthranilic acid are therefore inhibited in the vitamin B-6 deficient state. This leaves more 3-hydroxykynurenine available as substrate for the mitochondrial KAT, resulting in the increased formation and excretion of XA.

Abnormal metabolite excretion patterns following a tryptophan load test alone can only lead to speculative conclusions about vitamin B-6 status. There are other factors besides vitamin B-6 adequacy that can affect the urinary excretion of tryptophan metabolites. For example, Tada and Bessman (1960) reported a decreased urinary excretion of both KA and XA in the urine of untreated

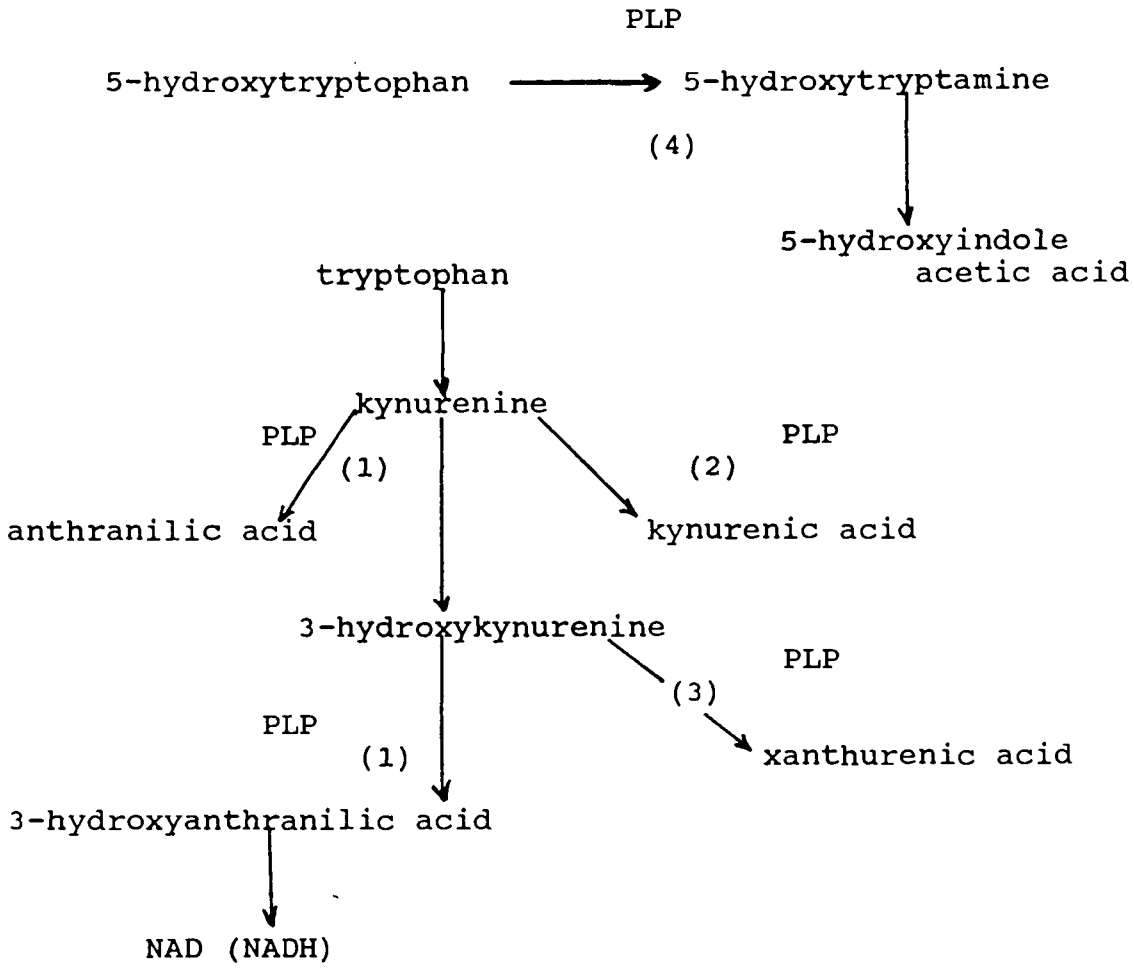


FIGURE I.12 Principal pathways of tryptophan metabolism showing the steps that require pyridoxal 5′-phosphate (PLP) as a coenzyme: (1) cytoplasmic kynureninase; (2) cytoplasmic kynurenine aminotransferase (KAT); (3) mitochondrial kynurenine aminotransferase; and (4) aromatic L-amino acid decarboxylase.[1]

[1]Modified from Rumsby & Shepherd, 1980.

patients with PKU following an L-tryptophan load test. Subsequent to this report, Heeley (1965) examined tryptophan metabolite excretion patterns in 12 patients with PKU during nutritional treatment, compared to the excretion patterns following discontinuation of the nutritional treatment and to a sample of normal children. Compared with the controls, Heeley found a significant increase in the quantities of 3-hydroxykynurenine (HK) to that of 3-hydroxyanthranilic acid (HA) excreted in the urine of the untreated patients with PKU. The increased HK:HA ratio was accompanied by a significant mean increase in the ratio of XA:HA excreted, suggesting a reduced activity of the kynureninase enzyme necessary for HA production. These abnormal patterns were not significantly changed during nutritional treatment with a low-phe diet. To determine whether the abnormalities were related to an inadequate supply of PLP, Heeley repeated the tryptophan load test in three patients (off the low-phe diet) and in two control children following a period of PN supplementation. The increased HK/HA and XA/HA ratios of the patients fell to normal; in the control children there were no changes in the urinary metabolite excretion patterns.

This study design is an example of an appropriate approach to evaluating an indirect vitamin B-6 status assessment index such as the L-tryptophan load test. By providing vitamin B-6 as a supplement to the diets of the

patients, readministering the test, and finding normal metabolite excretion patterns, Heeley's work suggested that some untreated patients with PKU have a decreased availability of PLP for the kynureninase step.

A second important point this study illustrates is the weakness of a single index of vitamin B-6 status. The results in the treated patients suggest that there are perhaps factors other than vitamin B-6 that reduce the ability of treated patients to adequately perform the kynureninase step. When Heeley repeated the L-tryptophan load test in two patients, during a low-phe diet, he found that the values for HK/HA remained abnormal. Heeley concluded that the nutritional treatment itself (i.e. low-phe diet) lacks a factor that would enable PN to be utilized. In the earlier report of Tada and Bessman (1960), phe and/or its metabolites were suggested as possible inhibitors of the tryptophan pyrrolase system, resulting in a reduced excretion of some of the kynurenine pathway metabolites following an L-tryptophan load test. Therefore, the differences in the excretion of tryptophan metabolites may be related to differences in phe and/or its metabolites and their effects on enzymes in the tryptophan pathway, rather than vitamin B-6 status. Unfortunately, there have been no other reports that have examined the tryptophan metabolite excretion patterns of patients with PKU, untreated or treated, with or without PN supplementation.

Brown (1988) has suggested that tryptophan metabolism is adversely affected by some factors, which may be independent of vitamin B-6 status (e.g. cytokines stimulate peripheral indole oxygenase). Leklem (1990b) maintains that under conditions in which the factors known to influence tryptophan metabolism are not present (e.g. hormonal receptor activities in breast cancer), urinary excretion of XA acid remains a valid indicator of vitamin B-6 status.

The measurement of erythrocyte alanine aminotransferase (EALT, EGPT) and aspartic acid aminotransferase (EAST, EGOT) activities or stimulation are commonly used indirect indices of vitamin B-6 status (Leklem, 1990b). Specifically, the basal activities of these transaminases are measured initially and subsequent to excess PLP. A stimulation index (the ratio of stimulated to unstimulated enzyme activity) and a percent stimulation (the stimulated activity minus the unstimulated activity times 100) are calculated and reported. One concern with the utility of these measures as status indices is the rate of change in specific enzyme activities with PLP stimulation (Leklem, 1990b). Deficiency studies conducted for four weeks in women have suggested that EALT activity is more responsive to changes in the intake of vitamin B-6 (Brown et al., 1975) than EAST activity. Longer-term studies (over 6 to 8 week periods), in which vitamin B-6 intake is varied and EALT and/or EAST activity is measured, are necessary to address this concern.

In the interim, erythrocyte transaminase activities are considered to be useful long-term measures of vitamin B-6 status due to the relatively long life span of the erythrocyte (Leklem, 1990b).

Dietary measures used to assess vitamin B-6 status have routinely included the total amount of vitamin B-6 in the diet, calculated from food composition tables or analyzed in the actual foods consumed by the subjects. There are inherent difficulties associated with accurately obtaining and analyzing dietary intake data, which should be accounted for and discussed in an evaluation of the overall assessment data, and which include: 1) the methodological problems in collecting intake data from free-living subjects, 2) the variability in the completeness of data in the nutrient databases which are used to calculate the vitamin B-6 content of foods consumed by the subjects, and 3) the commonly used food composition tables and computerized nutrient databases do not yet include bioavailability data. Therefore, short of a controlled metabolic ward experiment, the conclusions regarding the available vitamin B-6 to experimental subjects will remain less than complete. Because such rigid dietary conditions are time-consuming and labor-intensive, few experiments today are conducted under these conditions. Particularly in studies of free-living subjects and in field studies, such rigor is sacrificed in order to study subjects under more natural circumstances and

at a reduced cost.

Equally important to these methodological problems is an evaluation of vitamin B-6 intake relative to dietary protein. This has been an often overlooked, but critical, part of an adequate evaluation of a dietary assessment of vitamin B-6 status. The Food and Nutrition Board has discussed this relationship in the last two editions of the RDAs (FNB, 1980, 1989) and, accordingly, the vitamin B-6 RDA is expressed per gram of dietary protein in these texts. However, the widely referred to "table values", which are published in the back of each text, provide an absolute amount of vitamin B-6 in mg/day for the various age-sex category RDAs. The 1980 and 1989 RDA table values for protein and vitamin B-6 intakes are compared to the average intakes of healthy U.S. children and adolescents in Table I.19. The 1989 vitamin B-6 table values were based on the average protein intakes which ranged between 172-300% of their age- and sex-respective RDA values (USDA, 1984). The 1980 and 1989 RDA texts cite the vitamin B-6 RDA for children and adolescents as a 0.020 vitamin B-6 (mg): protein (g) ratio. The 1985 Nationwide Food Consumption Survey found that school-aged children consumed vitamin B-6 and protein in amounts which reflected a 0.023 ratio (USDA, 1987). Other studies have reported average vitamin B-6 intakes of 0.019-0.021 (mg/g protein) for children ranging in age between 1-10 years, and intakes of 0.017 (mg/g

TABLE I.19. Recommended Dietary Allowance (RDA), table values for protein and vitamin B-6 compared to average protein and vitamin B-6 intakes of healthy U.S. children and adolescents

age range		RDA table[1] 1980	RDA table[1] 1989	average intake[2]	RDA table[1] 1980	RDA table[1] 1989	average intake[3]
(yrs)		<--- protein (g/d) --->			<- vitamin B-6 (mg/d) ->		
1-3		23	16	48	0.9	1.0	1.1-1.2
4-6		30	24	59	1.3	1.1	1.1-1.2
7-10		34	28	66	1.6	1.4	1.1-1.2
11-14	(M)	45	45	80	1.8	1.7	---
	(F)	46	46	81	1.8	1.4	1.2
15-18	(M)	56	59	101	2.0	2.0	---
	(F)	46	44	79	2.0	1.5	1.2

(---) Dashed lines indicate no data is available.

[1] From FNB 1980, 1989.

[2] From USDA, 1984.

[3] From Anderson, 1986; Driskell & Moak, 1986; Fries, Chrisley, & Driskell, 1981; Lewis & Nunn, 1977.

protein for girls 11-18 years of age (Anderson, 1986; Driskell & Moak, 1986; Fries, Chrisley, & Driskell, 1981; Lewis & Nunn, 1977).

A potential problem when using the vitamin B-6 RDAs as normal reference values is the assumption made in the table values regarding protein intakes. In a vitamin B-6 status assessment which includes dietary data of children and adolescents receiving protein intakes significantly different from the normal reference protein intakes, it would be critical to take these differences into account. One way to do this would be to use the 0.020 mg vitamin B-6 per gram protein RDA, rather than to refer to the RDA table values which may make invalid assumptions concerning protein intakes for certain subgroups of the U.S. population (e.g. school-aged children with PKU consuming relaxed diets).

There are two points of caution which preface this discussion of the published status reports concerning school-aged children and patients with PKU. First, tissue levels of the various B-6 vitamers would be most desirable (Leklem and Reynolds 1981). However, it is generally not possible to obtain samples of human tissues for analyses. Therefore, an assessment of the dietary conditions that preceded the biochemical data collections is essential in order to adequately evaluate the biochemical results. Plasma PLP and urine 4-PA concentrations correlate with absolute vitamin B-6 intakes under controlled conditions in

adult subjects (Shultz and Leklem 1981) and are inversely related to protein intake (Miller et al., 1985). The consensus of a workshop concerning human vitamin B-6 status (Leklem & Reynolds, 1981) was that an adequate evaluation should include dietary intakes of both vitamin B-6 and protein, along with at least two (direct or indirect) biochemical indices. Second, a vitamin B-6 status assessment needs to be evaluated relative to the effect PKU may have on tissues which are actively involved in the metabolism of vitamin B-6 (e.g. the liver) or on compounds for which there are metabolic interrelationships with vitamin B-6 (e.g. amino acids, phe metabolites, aminotransferase and decarboxylase enzymes) (Leklem and Reynolds, 1981). In summary, a vitamin B-6 status assessment of patients with PKU represents a unique clinical opportunity to support the premise of Leklem and Reynolds that the choice of the approach, indices, and methods to be used should consider the particular situation under study (Leklem and Reynolds, 1981).

2. Reports in healthy children and adolescents and in PKU

Four reports have examined the vitamin B-6 status of healthy children and adolescents (Anderson, 1986; Driskell & Moak, 1986; Fries, Chrisley, & Driskell, 1981; Lewis & Nunn, 1977). To date there is only one report in the literature which has examined the vitamin B-6 status of patients with

PKU (Anderson, 1986).

Each of these reports included dietary data as a part of the status assessment; only two of the four reports included two or more of the recommended biochemical indices (Anderson, 1986; Driskell & Moak, 1986). The indices included in each of these reports, relative to the recommended status indices (Leklem & Reynolds, 1981), are compared in Table I.20. The specific methods used and the applications and limitations of these data are discussed below.

Each of these studies examined free-living children or adolescents. Challenges associated with studying free-living subjects, particularly children, include measuring food intakes, withdrawing blood in the fasting state, obtaining complete 24-h urine collections, and locating subjects who are not receiving vitamin B-6-containing nutritional supplements at the time of the investigation. These potential methodological problems were addressed differently by each of the authors of the four reports. The dietary data and the methods are compared for these four reports in Table I.21. A direct comparison of the dietary data between reports is difficult due to the differences in ages of the subjects studied and the methods used to measure the intakes. Another confounding variable is whether the subjects were receiving vitamin B-6-containing nutritional supplements at the time of the study. In Anderson's report,

TABLE I.20. Indices of vitamin B-6 status included in four reports which examined healthy children and adolescents.[1]

recommended indices[2]	reports Anderson 1986	Driskell 1986	Fries 1981	Lewis 1977
<u>direct</u>				
plasma PLP	x	x	x	
plasma PL				
plasma total B-6				
urine 4-PA	x			x
urine total B-6				
<u>indirect</u>				
EALT/EAST[3]		x		
trp load test				
met load test				
<u>diet</u>				
vitamin B-6	x	x	x	x
protein	x	x	x	x

[1] Reports are listed by first author; references are Anderson, 1986; Driskell & Moak, 1986; Fries, Chrisley, & Driskell, 1981; Lewis & Nunn, 1977.

[2] Recommendations of Leklem & Reynolds, 1981.

[3] Erythrocyte alanine/aspartate aminotransferases-stimulation with added PLP.

TABLE I.21 Dietary intakes and methods used in four reports which examined the vitamin B-6 status of healthy children and adolescents

report[1]	ages (y)	subjects no.	methods	protein (g) <- intake/d, mean ± SD (range)[3] ->	vitamin B-6 (mg) <- intake/d, mean ± SD (range)[3] ->	B-6:protein[2] ratio
Anderson	3-11	5	3-d records or 24-h recalls	61.6±8.4 (50-63)	1.15±0.12 (1.0-1.3)	0.019
Driskell[4]	12-16	186	2 24-h records	67.4	1.25	0.018
Fries[5]	3-4	18	2-d records + 1-24-h recall	56.2	1.10	0.021
Lewis	2-9	22	3-d records	55.2±19.4 (36-75)	1.10±0.47 (0.5-2.4)	0.019

[1] Reports are listed by first author; references are Anderson, 1986; Driskell & Moak, 1986; Fries, Chrisley, & Driskell, 1981; Lewis & Nunn, 1977.

[2] Mean vitamin B-6:protein ratios were computed based on mean protein and B-6 intakes.

[3] Intake data are presented as mean values ± standard deviation (SD) for those reports which provided individual data.

[4] Driskell studied 186 subjects; the mean protein intake is based on the total sample. Mean vitamin B-6 intake is based on the 162 subjects who were not receiving vitamin B-6-containing supplements.

[5] Fries studied 35 subjects; the mean protein and vitamin B-6 intakes are based on the 18 subjects who were not receiving vitamin B-6-containing supplements.

information concerning the intakes of vitamin supplements was collected, but was not reported (Anderson, 1986). Driskell & Moak (1986) included some subjects in their study who were consuming vitamin B-6-containing supplements at the time of the study; however, the vitamin B-6 contribution from the supplements was not included in the reported dietary intakes. Fries, Chrisley, & Driskell (1981) accounted for the vitamin B-6-containing supplements in the 17 of their 35 subjects who were receiving them at the time of the data collection. The study by Lewis & Nunn (1977) is the only one of these four reports in which an attempt was made to control for the possibility of vitamin B-6-containing supplements. The authors stated in this report that none of their subjects had received vitamin supplements for at least two weeks before the data collection.

In spite of these methodological problems and differences in the dietary data collections, the average intakes in Table I.21 fall within the range of usual intakes presented in Table I.19. The mean vitamin B-6:protein ratios were close to the 0.020 RDA standard (FNB, 1980, 1989).

The mean values for the biochemical indices examined in three of these four reports are presented in Table I.22. The data of Anderson (1986) are not included in the table because the methods used for vitamin B-6 analyses were not those currently recommended (Reynolds & Leklem, 1981) and,

TABLE I.22 Mean values for biochemical indices[1] of vitamin B-6 status using accepted methods in three reports which examined healthy children and adolescents

report[2]	plasma PLP (nmol/L)	urine 4-PA (% excretion)	EALT (% stimulation)
Driskell	45.2	---	15.5
Fries	70.0	---	---
Lewis	---	57	---

(---) Dashed lines indicate this index was not measured in the report.

[1] Pyridoxal 5'-phosphate (PLP) in fasting plasma, 4-pyridoxic acid (4-PA) excretion/24-h compared to vitamin B-6 intake/24-h, erythrocyte alanine aminotransferase (EALT) activity with added PLP compared to without PLP added to the assay.

[2] Reports are listed by first author; references are Diskell & Moak, 1986; Fries, Chrisley, & Driskell, 1981; Lewis & Nunn, 1977.

therefore, preclude a direct comparison. Specifically, blood for plasma PLP was collected in the non-fasting state and the direct/indirect radioenzymatic assay of Camp, Chipponi, & Faraj (1983) was used, rather than the recommended method of Chabner and Livingston (1970) on fasting samples. Urine was collected randomly, rather than as 24-h volumes which is the generally accepted method for quantitation of urine 4-PA excretion.

Using the individual data from each of these reports, compared to the available suggested criteria for inadequate vitamin B-6 status of children and adolescents, the number and percentage of the subjects with inadequate status is presented in Table I.23. Based on these data, a relationship between the dietary and biochemical indices, using the currently available criteria for inadequate status, is not clear. For example, Driskell & Moak (1986) reported 49-58% of their subjects met the dietary criteria for inadequate vitamin B-6 status. In contrast, only 23-26% of their subjects met the biochemical criteria for inadequate status. The authors reported that no significant correlations were found between individual estimated vitamin B-6 intakes and either plasma PLP values or EALT activities.

These findings are inconsistent with those of Shultz and Leklem (1981) and others who have reported significant correlations between biochemical and dietary measures of vitamin B-6 status. However, these studies were done using

TABLE I.23 Number and percentage of subjects with inadequate vitamin B-6 status using acceptable methods in three reports which examined healthy children and adolescents

report[1]	<-------dietary[2]------->		<----------biochemical[3]---------->		
	B-6 intake < 2/3 RDA	B-6 protein < 0.020	plasma PLP < 34 nmol/L	urine 4-PA < 30%	EALT activity ≥ 16%
	no. %	no. %	no. %	no. %	no. %
Driskell[4] (n=186)	--- 49	--- 58	--- 26	not examined	--- 23
Fries[5] (n=18)	5 17	8 23	3 17	<-----not examined----->	
Lewis[6]	2 9	13 59	not examined	2 23	not examined

[1] Reports are listed by first author; references are Driskell & Moak, 1986; Fries, Chrisley, & Driskell, 1981; Lewis & Nunn, 1977.

[2] Criteria use for inadequate vitamin B-6 status as follows: absolute vitamin B-6 intake <2/3 1980 RDA (FNB, 1980); vitamin B-6: protein ratio < 0.02 (FNB, 1980; 1989).

[3] Criteria used for inadequate vitamin B-6 status as follows: plasma PLP < 34 nmol/L (Rose, 1976); urine 4-PA excretion < 30% of vitamin B-6 intake (Lewis & Nunn, 1977); coenzyme stimulation of EALT activity ≥ 16% (Kirksey, Keaton, Abernathy, & Greger, 1978).

[4] Driskell did not report the number of subjects who met the criteria (percentages only).

[5] The assumption was made that the three subjects reported by Fries with PLP values < 34 nmol/L were in the group of 18 unsupplemented subjects used to compute the mean diet B-6 and plasma PLP values to Tables 1.20 and 1.21

[6] Dietary data were compared to 1974 RDAs.

adult subjects under controlled conditions. Replication of these correlations in children may be possible in adequately controlled studies during which the children examined are taken off of nutritional supplements and dietary data are collected with more care. None of these studies reported in children used estimated energy intakes or other measures to validate the completeness of the dietary data, nor discussed the reliability of the 2-3 days examined and used to compute mean intakes.

The methodological problems of these reports and their inconsistent and rather difficult to evaluate results underscores the need for additional vitamin B-6 status assessments of healthy children and adolescents. For this reason, the approach recommended by Leklem and Reynolds (1981), which utilizes a triad of accepted indices (one dietary and two biochemical) and the accepted methods for vitamin B-6 analysis (Reynolds & Leklem, 1981), were combined in a vitamin B-6 status evaluation of six healthy school-aged subjects (reported in Chapter IV). Although the subjects were free-living, as a result of careful planning, design and administration, as well as a trusting rappoire with the subjects and their families, the collection and analysis of the data met the recommended standards. These data also served to supply normal reference values, collected and analyzed under the same conditions, for a

vitamin B-6 status evaluation of children with PKU which was the focus of this study.

The single report that examined the vitamin B-6 status of patients with PKU is, unfortunately, the study by Anderson (1986) in which the methods used for data collection and analysis were not the currently recommended methods. However, the study results are worth reviewing because: 1) They are the only dietary and biochemical measures of vitamin B-6 status in patients with PKU in the literature; and 2) The study did include a control group whose data were subjected to the same methods. Therefore, while it would be inappropriate to compare the biochemical values against other reports which used different methods, the patients' values can be compared against the control subjects' values for significant differences.

Anderson examined the vitamin B-6 status of 12 patients with PKU and five control subjects using the dietary measures of absolute vitamin B-6 and protein intakes and the biochemical measures of plasma PLP and urinary 4-PA excretion. The results of the study are presented in Table I.24 except for the 4-PA data which were reported as ug/g creatinine (rather than as a percent excretion). Anderson found significantly lower protein intakes in the patients with PKU ($p \leq 0.01$) and significantly greater non-fasting PLP values in the plasma of the patients with PKU ($p \leq$

TABLE I.24 Vitamin B-6 status report of patients with PKU compared to control subjects[1]

	age	dietary measures			biochemical indices
	(y)	B-6[2] mg/d	protein g/d	B-6: protein ratio	plasma PLP[3] nmol/L
control subjects (n=5)	6.2	1.15 (±3.3)	61.6* (±0.12)	0.019 (±8.4)	52.9# (±15.8)
patients with PKU (n=12)	3.5	9 (±3.7)	34.5* (±0.53)	0.028 (±14.5)	185# (±121.6)

---

1 Values are given as mean ± 1 SD (Anderson, 1986).

2 Values do not include vitamin B-6 containing supplements.

3 Values are from non-fasting samples, analyzed by the method of Camp et al. (1984).

* Significantly different at $p \leq 0.01$.

Significantly different at $p \leq 0.05$.

0.05). Whether these differences in the plasma PLP values reflect the greater vitamin B-6 intakes of the patients relative to their protein intakes or some other differences associated with PKU per se are not clear from this study. Anderson concluded that status was adequate in patients with PKU, based upon the relatively high plasma PLP values.

Anderson reported that 74% of the mean protein intake of the patients was supplied by medical foods and 57% of the mean vitamin B-6 intake was supplied by medical foods. Ten of the 12 patients were preschool-aged. To provide a more complete assessment, broader in scope and using currently accepted methods, the study described in Chapter IV was undertaken.

CHAPTER II

CONTRIBUTION OF NATURAL FOODS AND MEDICAL FOODS TO PROTEIN AND ENERGY INTAKES IN PHENYLKETONURIA

A. ABSTRACT

The contribution of medical and natural foods to the total diets of 15 patients with phenylketonuria (PKU), aged 7-17 years, was computer-analyzed from four-day dietary records. The medical food most widely used, Phenyl-Free®, contributed 5 g protein and 0 mg phenylalanine (phe/100 calories. Eight children were considered to be adhering to a strict low-phe diet of which natural foods provided 0.9 g protein and 43 mg phe/100 calories. In contrast, the diets of six non-PKU control siblings, aged 6-14 years, provided 3.3 g protein and 153 mg phe/100 calories. In spite of this degree of natural protein restrictions, blood phe levels of patients consuming a strict diet were above the acceptable treatment range. Medical foods provided 74% of the protein requirements of these patients compared to current recommendations that they contribute approximately 120% of the Recommended Dietary Allowances (RDAs). These data suggest that an increase in the energy content of medical foods might improve satiety from these products and contribute more to overall energy needs. This would better balance the energy from natural food intakes and, thereby, improve the long-term dietary control of PKU. However, until palatable products are available, it is unclear whether development of any medical foods with a greater energy: protein density would improve the dietary adherence of school-aged patients.

B. INTRODUCTION

Patients with phenylketonuria (PKU) cannot tolerate adequate protein (phe). Therefore, the effective nutritional treatment of PKU requires the use of chemically-defined medical foods in which the protein is specially modified to reduce this amino acid ([CON-AAP], 1985). Natural foods are prescribed along with medical foods, in the quantities necessary to supply the essential dietary requirement for phe (Acosta, 1989). During infancy, when the phe requirement is the highest relative to total protein (Holt, Gyorgy, Pratt, Snyderman, & Wallace, 1960), medical foods serve to dilute the phe content of breast milk or a standard infant formula and to provide approximately 100% of the protein requirement and 50% of the energy needs of the newborn infant with PKU (calculated from Acosta). The mixture is acceptable for the patient and the family when formula is the only source of nutrition. As the children grow older, natural foods low in protein are prescribed along with medical goods (Hunt et al., 1985) and the diet is usually well-accepted through the preschool years (Michals et al., 2988). At school-aged, strict dietary adherence becomes more difficult to manage (Michals et al., 1988). Due to the adverse effects of the loss of strict dietary adherence and the consequent rises in blood phe levels (Holtzman, Kronmal, Van Doorninck, Azen, & Koch, 1986), the National PKU Collaborative Study (NPKUCS) strongly suggests

that PKU treatment clinics try to maintain all patients on a strict diet through school age (Michals et al., 1988).

There are several contributing factors to the decline in dietary adherence at school age. Some of the L-amino acids which comprise the modified protein in the elemental medical foods (EMFs) have a sulphurous, bitter, unpleasant taste (Schiffman & Gagnon, 1981) which complicates adherence to the EMF prescription (Acosta, 1989). At the same time, the requirement for phe and protein relative to body weight declines, and by seven years of age approximately 10 g of natural food protein can meet the estimated requirement for phe of most children (Acosta, 1989). In order to achieve this severe natural protein restriction, food choices are limited to fruits, vegetables, specially-made starch-based flours, baking mixes and their products (e.g. breads, pastas), and pure fats and sugars (Acosta, 1989). The protein and individual amino acid values of these foods is often underestimated in the calculations of the total protein intake required by a medical food product. As a result, most EMFs are designed to provide almost all of the protein intake and have between 20-90% of their energy content as amino acids. This leads to protein intakes in excess of the current Recommended Dietary Allowances (RDAs) (FNB, 1980) when the products are used according to recommendations. While the EMFs are designed and prescribed to contribute more than 100% of the RDAs for protein, most

EMFs will provide less than 30% of the energy needs of school-aged patients (calculated from Acosta, 1989).

The conceptual framework for this study was based on the following observations: 1) Natural foods contribute to protein and individual amino acid intakes and, therefore, should be accounted for in the dietary prescriptions of patients with PKU; 2) Due to the low energy: protein ratio of EMFs, patients ingest unnecessarily large quantities of amino acids relative to energy from these products; 3) The poor organoleptic qualities of several of the free amino acids in EMFs renders them progressively less acceptable as children grow and their special senses mature; and 4) This combination leads to diminishing intakes of EMFs and increasing intakes of natural foods to meet both satiety and social needs. The result is a reduced ability to adhere to the natural protein restriction which is necessary to maintain biochemical control of PKU.

This study was designed to evaluate the effectiveness of current medical foods to meet the protein and energy needs of school-aged patients with PKU. It is for these patients that limited dietary information exists and medical food acceptance and dietary adherence is a problem. Although each of the 15 patients who participated in this study had been encouraged to maintain strict diet adherence, the dietary records of only eight of these subjects reflected medical food and natural phe-restricted food

intakes which we considered indicative of strict adherence to the diet. The four-day dietary records collected from these eight patients were the primary focus of this study. They were computer-analyzed, with and without the contributions from medical foods, which allowed us to describe the protein and energy content of the natural foods and to evaluate the abilities of the medical foods, in the amounts consumed, to provide a total dietary balance of protein and energy.

C. METHODS

1. Subjects

One distinct birth cohort was selected to represent the entire school-aged population of children and adolescents with PKU[6], managed by The Oregon Health Sciences University Metabolic Clinic. The sampling frame consisted of all patients living in Oregon, 7-17 years of age, who had maintained contact with the clinic within the previous two years. Involvement of entire families was encouraged to increase awareness of the importance and accuracy of the dietary recording. Additionally, sibling data served as control values. Sixteen of the 18 eligible families

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[6] The classification of PKU was based on the newborn pretreatment blood phe levels determined by the Guthrie assay on whole blood (Guthrie & Susi, 1963). All patients in the study had pretreatment blood phe levels $\geq$ 12 mg/dl by the Guthrie assay ($\geq$ 720 umol/L).

participated in the study; 16 patients and six siblings completed the study. One of the 16 patients reported a mean energy intake at 39% of estimated caloric requirements, in spite of a body weight at the 95th percentile of the National Center for Health Statistics standards (Hamill et al., 1979). We considered the diet records for this subject to be a non-valid measure of actual intake and, therefore, chose not to include them in the final data analyses.

2. Procedure

The clinic dietitian (AP) made home visits to review the dietary recording procedures on an individual household basis. Each family member who agreed to be involved in dietary recording was asked to estimate, in writing, portion sizes of eight lifelike food models. This exercise provided the dietitian with baseline data of each family's abilities to describe foods. Families were then shown how to record quantities of food intake on forms specially designed for this study. A descriptive language to quantitate foods was agreed upon with each family, using their own household measuring tools and the commercial food models. Subjects were encouraged to make diet record entries immediately after eating and parents were asked to supervise when possible. Plastic wallets were given to children to carry diet records in, as suggested by Jackson et al. (1986), to maximize accuracy and reliability. A 4-day recording period

(Friday, Saturday, Sunday, Monday) was selected to allow for the possibility that intakes of school children might be consistently different on weekdays compared to weekend days. A fasting venous blood sample was collected from each subject into a heparinized Vacutainer tube®. Samples were obtained two days after the dietary recording period and kept on ice until they were centrifuged. Plasma was removed and phe was quantitated using an automated Beckman Amino Acid Analyzer®[7]

3. Description of the data base

Current nutrition protocols (Acosta, 1989) recommend the Amino Acid Analyzer®[8] software for computerized nutrient analyses of dietary intakes of patients with inborn errors of amino acid metabolism. At the time of this project, Amino Acid Analyzer was limited to complete data on 12 amino acids in 750 foods. Because of these limitations, an existing computerized nutrient data base, Nutrition and Diet Services®[9] was expanded to provide a profile for each of 18 amino acids in 1800 foods. The revised USDA Agricultural Handbook No. 8 series (U.S. Department of

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[7] Beckman Instruments, 111 California Avenue, Palo Alto, CA 94303.

[8] Nutrition Management Systems, Inc., 1916 Wahalaw Court, Tallahassee, FL 32301.

[9] Nutrition and Diet Services, Inc., 727 S.E. Rimrock Lane, Portland, OR 97267.

Agriculture [USDA], 1976-1987) was used as the primary data source of energy, protein, and amino acids in foods. The composition of brand name foods was obtained from manufacturers and distributors of specialty food products used by patients[10]. When amino acid data were not available from these sources, additional references (Paul & Southgate, 1978; Food and Agriculture Organization [FAO], 1970; Orr & Watt, 1968) were used to impute values, resulting in no missing values for the energy, protein and amino acid content of all foods reported by the sample.

4. Statistical analysis of nutrient intakes

Computer-generated printouts of daily energy, protein, and amino acid intakes were entered into a UNIX HCX/UX main-frame computer and descriptive statistics were determined using the Statistical Package for the Social Sciences (Statistical Package for the Social Sciences [$SPSS_X$], 1986). There were no consistent differences between weekday vs weekend intakes and, therefore, mean (± SD) daily energy, protein, and indispensable amino acid (IDAA) intakes for natural foods, medical foods, and in the total diets of the patients, were computed across the 4-day recording period for each individual. Mean intakes of energy, protein, and

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[10] Dietary Specialties, PO Box 227, Rochester, NY 14601; Ener-G-Foods, Inc., 6901 Fox Avenue South, PO Box 24724, Seattle, WA 98124; Kingsmill Food Co., Ltd., 1399 Kennedy Road, Unit 17, Scarborough, Ontario, M1P 2L6; Med-Diet, Inc., 1409 Fairfield Road South, Minnetonka, MN 55343.

IDAAs were compared to the 1980 RDAs (FNB, 1980). At the time of the study, the 1980 RDAs were the generally accepted nutritional standards in the U.S. Because energy and protein RDAs are based on median reference weights, we individualized them to account for differences in the subjects' actual body weights. We compared IDAA intakes to the school-aged standards (FNB, 1980; [FAO/WHO/UNU], 1985). Cystine and methionine intakes were considered together, relative to the total sulphur-containing amino acid requirement. Tyrosine and phe intakes, generally considered together relative to the standard total aromatic amino acid requirement (22 mg/kg) (FNB, 1980; [FAO/WHO/UNU], 1985), were examined separately since the metabolic block in PKU is in the conversion of phe to tyrosine. Tyrosine intakes were also compared to the estimated tyrosine requirement (125 mg/kg) for school-aged patients with PKU to achieve adequate plasma tyrosine levels (Acosta, 1989).

D. RESULTS

The physical characteristics, 4-day mean intakes of phe, and plasma phe values of each subject are presented in Table II.1. Based upon the mean intakes of phe and natural protein, the degree of natural food restrictions, and the use of medical foods, the individuals with PKU were divided into three groups: 1) strict-diet, 2) relaxed-diet, and 3) off-diet. This method of classification is a modification

TABLE II.1 Physical characteristics and phenylalanine in diets and plasma of 15 patients with PKU and 6 control subjects

					phenylalanine		
subject group[1] and no.	**age**	**sex**	**height**	**weight**	**diet**		**plasma**
strict-diet	**(y)**		**percentile**		**mg**	**mg/kg**	**umol/L**
1	10.8	male	30[2]	40	496[3] ± 116	16	520[4]
2	8.2	male	75	75	627 ± 513	22	942
3	8.4	female	10	40	321 ± 126	13	1136
4	11.0	female	80	80	796 ± 355	19	1146
5	11.2	male	50	50	828 ± 412	23	517
6	10.4	male	40	50	695 ± 369	19	938
7	11.1	male	60	55	675 ± 48	18	950
8	7.8	female	30	50	550 ± 214	22	648
group mean	**9.9**		**47**	**55**	**627 ± 269**	**19**	**854 ± 252**
relaxed-diet	**(y)**		**percentile**		**mg**	**mg/kg**	**umol/L**
9	16.7	female	50	60	1835 ± 574	31	1364
10	14.9	female	50	50	1755 ± 1259	33	1268
11	16.7	male	95	80	2310 ± 925	38	1026
group mean	**16.1**		**65**	**60**	**1967 ± 868**	**34**	**1219 ± 174**

TABLE II.1 Physical characteristics and phenylalanine in diets and plasma of 15 patients with PKU and 6 control subjects (continued)

					phenylalanine		
subject group[1] and no.	age	sex	height	weight	diet	plasma	
off-diet	(y)		percentile		mg	mg/kg	umol/L
12	7.4	male	30	40	1575 ± 676	68	437
13	10.5	female	>95	>95	2845 ± 1428	55	1064
14	7.5	male	40	35	1349 ± 122	59	515
15	7.6	male	65	65	2845 ± 581	98	814
group mean	**8.2**		**58**	**59**	**2154 ± 696**	**70**	**708 ± 228**
control	(y)		percentile		mg	mg/kg	umol/L
16	6.5	male	25	1060	2601 ± 245	141	67
17	11.7	male	70	80	3959 ± 1113	78	74
18	5.5	female	40	35	2719 ± 598	156	72
19	11.4	female	35	50	3022 ± 496	79	48
20	13.9	male	95	80	4321 ± 1851	73	88
21	7.95	male	80	80	2626 ± 1125	91	69
group mean	**9.5**		**58**	**56**	**3208 ± 905**	**103**	**70 ± 13**

TABLE II.1 Physical characteristics and phenylalanine in diets and plasma of 15 patients with PKU and 6 control subjects (continued)

[1] PKU patients are grouped by dietary characteristics, as a modification of the 1990 National Survey methods, regardless of plasma phenylalanine values (Schuett, 1990).

[2] Heights and weights are compared to the National Center for Health Statistics growth percentiles (Hamill et al., 1979).

[3] Mean ± standard deviation computer-analyzed from 4-day dietary records. The recommended phenylalanine intakes for patients with PKU 7<11 years of age = 7-15 mg/kg; for 11<19 years of age = 5.5-13 mg/kg (Matalon & Matalon, 1989)

[4] A fasting venous blood sample was collected two days after the dietary recording period. Plasma phenylalanine was quantitated by an automated amino acid analyzer. The normal range (mean ±2 standard deviations) of 136 fasting healthy 6-18 (y) children = 42-74 umol/L (Armstrong & Stave, 1973). The acceptable treatment range for patients with PKU = 120-605 umol/L (Schuett, 1990).

protein, the degree of natural food restrictions, and the use of medical foods, the individuals with PKU were divided into three groups: 1) strict-diet, 2) relaxed-diet, and 3) off-diet. This method of classification is a modification of the method chosen by the most recent national survey (Schuett, 1990) to describe patients across treatment programs at various ages. The non-affected siblings comprised a fourth (control) group.

The strict-diet group (n=8) included those patients who reported intakes that were considered to be significantly restricted in phe (13-23 mg/kg body weight); whether or not their intakes were in the recommended treatment range (5.5-15 mg/kg) (Matalon & Matalon, 1989), or their plasma phe levels (517-1146 umol/L) were in the acceptable treatment range (120-605 umol/L) (Schuett, 1990). Each of these patients consumed medical foods daily and none of them included protein-dense foods (meat, fish, poultry, legumes, egg, dairy) in their diets. Three patients were considered to be consuming a relaxed-diet, with phe intakes which ranged from 31-38 mg/kg. Only one patient in this group regularly consumed medical foods at the time of the study. Again, none of these patients included protein-dense foods in their diets; phe intakes were increased due to a more liberal use of starchy foods. The remaining four patients were considered to be off-diet, although one subject in this group continued to consume medical foods daily. Each of

these patients included restricted amounts of protein-dense foods in their daily intakes.

The mean ages of the subjects were similar across the groups (8.2-9.9 years), including the control group, with the exception of the three patients in the relaxed-diet group who were older (16.1 years). Heights and weights of all subjects were within the normal growth percentile range (5th-95th%) established by the National Center for Health Statistics (Hamill et al., 1979), except for one patient in the off-diet group whose height and weight were above the 95th percentile.

The mean phe intakes of each of the patient groups were above the levels recommended for their mean ages. Consequently, the mean plasma phe values for each of the groups were above the acceptable treatment range. The mean phe intake of the off-diet group was greater than the other two patient groups, yet the mean plasma phe level for this group was lower. These patients presumably had greater residual PAH enzyme activities, greater individual physiological requirements for phe, or both.

Mean protein and energy intakes of the 15 patients with PKU and the six control subjects are presented in Table II.2. Intakes from natural and medical foods are shown separately for the ten patients who used medical foods (subjects 1-8, 9 and 12). Mean protein intakes from natural foods were adequate and exceeded protein RDAs (FNB, 1980)

TABLE II.2 Protein and energy intakes of 15 patients with PKU and 6 control subjects

subject group and no.	protein				energy			
	natural foods	medical foods	total	%RDA	natural foods	medical foods	total	%RDA
strict-diet	<----------(g)---------->				<----------(kcal)---------->			
1	11[1]±2	36±0	47±2	142[2]	2401±161	632±155	3034±185	153
2	12±4	36±0	49±4	145	1288±222	720±0	2008±222	81
3	9±3	33±6	42±8	138	1160±204	628±55	1788±259	82
4	21±9	29±0	50±9	117	1815±118	119±2	1935±117	96
5	18±8	24±0	42±8	118	1479±271	480±0	1935±117	96
6	14±5	16±11	31±11	83	1318±314	540±360	1858±426	97
7	15±2	15±10	30±12	82	2125±275	300±200	2424±376	110
8	12±4	14±1	27±49	92	1217±340	277±12	1419±339	70
group mean	14±4	25±9	40±9	115	1601±462	462±210	2063±274	98
relaxed-diet								
9	40±6	4±6	45±12	96	1872±338	75±114	1947±371	87

[1] Mean ± standard deviation.

[2] Mean intakes are compared to the 1980 Recommended Dietary Allowances (FNB, 1980), individualized to account for differences in the subjects' actual body weights (see text).

TABLE II.2 Continued

subject group and no.	protein				energy			
	natural foods	medical foods	total	%RDA	natural foods	medical foods	total	%RDA
rrelaxed-diet	<----------(g)---------->				<----------(kcal)---------->			
10	42±28	--	42±28	112	2187±690	--	2187±690	110
11	53±20	--	53±20	109	2586±337	--	2586±337	102
group mean	42±6	--	42±6	106	2240±323	--	2240±323	110
off-diet								
12	33±12	20±0	53±12	190	1482±173	400±0	1882±173	94
13	63±31	--	63±31	122	2854±1315	--	2854±1315	114
14	34±5	--	34±5	125	1782±428	--	1782±428	90
15	56±14	--	56±14	183	2177±468	--	2177±468	100
group mean	52±12	--	52±12	155	2173±484	--	2173±484	100

TABLE II.2 Continued

subject group and no.	protein				energy			
	natural foods	medical foods	total	%RDA	natural foods	medical foods	total	%RDA
control	<----------(g)---------->				<----------(kcal)---------->			
16	57±7	--	57±7	206	1512±251	--	1512±251	97
17	91±28	--	91±28	180	2017±624	--	2017±624	67
18	58±12	--	58±12	224	1883±338	--	1883±338	127
19	64±13	--	64±13	168	1746±350	--	1746±350	94
20	91±38	--	91±38	153	2618±857	--	2618±857	74
21	54±21	--	54±21	158	2443±562	--	2036±421	98
group mean	69±17	--	69±17	182	2036±421	--	2036±421	91

for each group except for the eight patients consuming a strict-diet (mean protein intake 39% RDAs). When the protein contributions of the medical foods were added to the natural food intakes, the mean total protein intake for this group increased to an adequate level (115% RDAs). Total energy intakes for all groups were close to 100% energy RDAs, supporting a careful and complete dietary intake recording and analysis.

Medical foods were necessary to supply adequate amounts of IDAAs for the strict-diet group (Figure II.1.). However, the IDAA levels provided in the current medical foods, even when these products were consumed in the quantities our patients in the strict-diet group achieved, which were below the recommended amounts (Acosta, 1989), were more than adequate and increased the total IDAA intakes to 147-288% RDAs (FNB, 1980; [FAO/WHO/UNU], 1985). Medical foods increased the tyrosine intakes to 245% of the aromatic amino acid RDA, but only reached 50% of the tyrosine recommended for patients with PKU to achieve adequate plasma tyrosine levels (Acosta, 1989).

The natural foods consumed by the eight patients in the strict-diet group supplied mean intakes of 0.9 g protein, 3 mg phe, and 28 mg tyrosine/100 kcal. Based upon these data, we tried to balance a sample diet prescription (Table II.3) for an 11 year old male patient, according to the currently accepted Ross Metabolic Formula System Nutrition Support

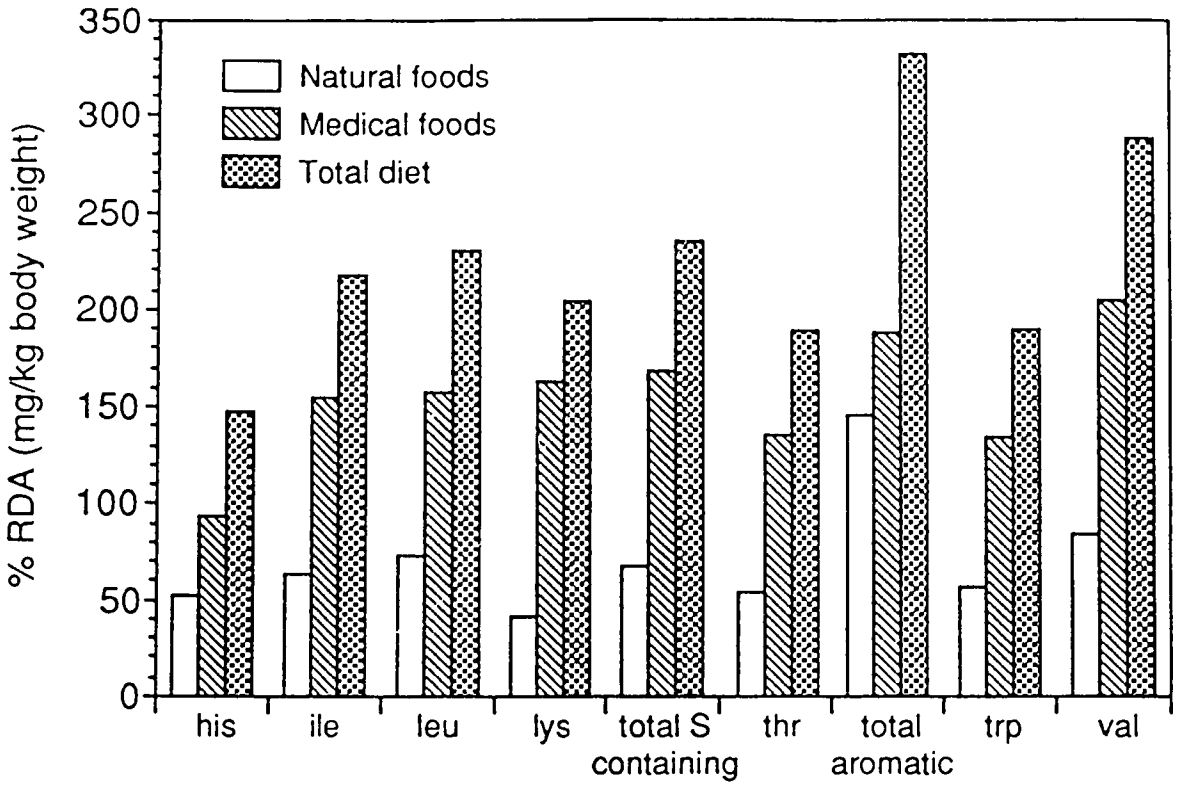

FIGURE II.1. Percent of estimated indispensable amino acid requirements met by natural foods, medical foods, and the total diets of 8 patients with PKU consuming a strict-diet.

TABLE II.3 Sample diet prescription for an 11 year-old male patient with PKU

1. **establish prescription**	recommended[1] daily intake
phenylalanine (mg)	450
protein (g)	34
energy (kcal)	2400
tyrosine (mg)	3500

2. **fill phenylalanine prescription from natural foods**

	natural food content[2]		
	/100 kcal	/1 g protein	/450 mg phe
phenylalanine (mg)	39	45	450
protein (g)	0.9	--	10
energy (kcal)	--	114	1140
tyrosine (mg)	28	31	310

3. **determine tyrosine, protein and energy needed from medical foods**

	recommended daily intake		theoretical intake from natural foods		remaining prescription
phenylalanine (mg)	450	-	450	=	0
protein (g)	34	-	10	=	24
energy (kcal)	2400	-	1140	=	1260
tyrosine (mg)	3500	-	310	=	3190

4. **fill remaining prescription from medical foods**

	quantities medical foods necessary to fill remaining prescription					
	Phenyl-Free®[3]		Maxamaid X-P®[4]		PKU-2®[5] PKU-3®	
	Tbsp	g	Tbsp	g	Tbsp	g
protein (24 g)	12	120	8	80	4	40
energy (1260 kcal)	31	310	46	460	45	450
tyrosine (3190 mg)	34	340	8	80	9	90

[1] Recommended phenylalanine, tyrosine, protein and energy intakes from 1989 Ross Metabolic Formula System Nutrition Support Protocols for school-aged patients (Acosta, 1989).
[2] Based on mean natural food intakes computed from 4-day dietary records of eight patients consuming a strict-diet in this study.
[3] Phenyl-Free® is manufactured and distributed by Mead Johnson Nutritional Division, Bristol-Meyers Company, Evansville, IN. 1 Tbsp. is 9.8 g.
[4] Maxamaid X-P® is manufactured by Scientific Hospital Supplies Ltd., United Kingdom and is distributed in the US by Ross Laboratories, Columbus, OH. 1 Tbsp is approximately 10 g.
[5] PKU-2® (for children) and PKU-3® (for adolescents) are manufactured by Milupa A.G. International Scientific Department, Friedrichsdorf, West Germany and are distributed in the US by Bristol-Meyers Company, Evansville, IN. 1 Tbsp. is approximately 10 g.

Protocols (Acosta, 1989). After natural foods are prescribed to meet the essential requirement for phe and their quantities of phe, protein, calories, and tyrosine are accounted for, the remaining protein, energy, and tyrosine prescription must be filled by medical foods. The sample patient needs a medical food which will provide 24 g protein (1.9 g/100 kcal) and 1260 calories in order to balance the diet. Current medical foods provide 5-23 g protein/100 kcal. Distributors of these products recognize the low energy content relative to protein and suggest the addition of 1 cup of sugar (Bristol-Meyers Co., 1989), 1/2 cup of oil (calculated from Acosta, 1989), or other nitrogen-free energy sources (free-foods) such as fruit juices and candies (Acosta, 1989). Our patients used the specialty low-phe food products extensively; these foods enabled patients in the strict-diet group to achieve mean natural food intakes which supplied less than 1 g protein/100 kcal, compared with our control group mean intakes of 3.3 g protein/100 kcal. However, patients were unable to incorporate the suggested amounts of sugar or oil into their daily diets to meet remaining energy needs without additional phe. The sample patient needs a medical food which will provide 3190 mg tyrosine (130 mg/g protein-equivalent); current products contain 10-90 mg tyrosine/g protein-equivalent.

E. DISCUSSION

The primary objective of this study was to examine the practicability of the current nutrition protocols established for the dietary management of school-aged patients with PKU (Acosta, 1989). The natural food intakes reported by eight patients consuming a strict-diet which included medical foods, provided the basis for an evaluation of the protein and energy content of medical foods, when considered in the context of the total diet. These data show that the current medical foods supply inadequate levels of energy and tyrosine to fill the remaining diet prescription for school-aged patients, if the necessary natural food restrictions to maintain acceptable plasma phe values are strictly adhered to (Matalon & Matalon, 1989).

We also found that the level of protein equivalent in the current medical foods is higher than is necessary to balance the diet. Some of the free L-amino acids which provide the protein equivalent in EMFs have a bitter, unpleasant taste (e.g. L-methionine, L-glutamic and L-aspartic acids) (Schiffman & Gagnon, 1981), which makes strict dietary adherence to the medical food prescription more difficult (Acosta, 1989). Mean methionine/cystine intakes were 238% of the sulphur-containing amino acid RDAs for our strict-diet group, when the intakes from both natural foods and medical foods were taken into account. The significance of these findings is reflected in the

unacceptable plasma phe levels of our patients. Nearly all individual plasma phe values were above the acceptable treatment range of ≤ 605 μmol/L (Michals et al., 1988). The mean plasma phe level of the strict-diet group (mean age 10 years) was 854 μmol/L (14 mg/dL), which is consistent with the mean whole blood phe level of 13 mg/dL for the 44 patients consuming a strict-diet (mean age 8 years) reported by the NPKUCS (Koch, Friedman, Williamson & Azen, 1982).

Plasma phe levels in patients with PKU that are above the acceptable treatment range are attributed to excess phe from natural foods or to inadequate total energy intake (Acosta, 1989). In this study, the mean phe intakes of each group of patients, even for the eight patients in the strict-diet group, were above the recommended intakes (Matalon & Matalon, 1989), while the mean energy intakes for each of the groups met the energy RDAs. These data indicate that the unacceptable biochemical control of PKU achieved by our patients was due to excessive dietary phe rather than insufficient calories. If the medical foods are not accepted in amounts which provide adequate energy and, thus, satiety for the patients, energy must be obtained from other sources. Natural foods provided 39 mg phe/100 kcal for the eight patients in the strict-diet group, in contrast to 153 mg phe/100 kcal for the control group. However, even this amount of phe was too high. Natural foods were the major energy source (78% RDAs) for these patients; medical foods

contributed only 22% of energy RDAs. These findings support our hypothesis that, given the current medical foods, excess natural foods are consumed to meet energy needs, at the expense of exceeding the phe requirements, and with consequent rises in the plasma phe levels.

1. Limitations

Not unexpected for a study of patients with a rare disorder, the number of subjects was small and the reported intakes of natural foods and medical foods among the patients showed considerable heterogeneity. To meet the study's primary objective, this report focused on the protein and energy intakes of eight of the 15 patients with PKU, those who most strictly adhered to the low-phe diet and consumed medical foods daily. Six of these patients used Phenyl-Free®, which limits the extension of our results to the other medical foods currently available for older patients. However, the data in Table II.3 demonstrate the inabilities of Maxamaid X-P®, PKU-2®, and PKU-3® to balance the remaining energy, protein, and tyrosine needs of an 11 year old male, once the phe requirement is filled from natural foods, and the energy, protein, and tyrosine intakes from the natural foods are accounted for. Historically, Phenyl-Free® has been the EMF most frequently used by school-aged patients with PKU in the U.S. (Schuett et al., 1985). This study is a unique and necessary evaluation of

this product which focused on the protein and energy contribution of Phenyl-Free®, relative to the natural food intakes of school-aged patients considered to be adhering to a strict low-phe diet. The lack of other studies to evaluate Phenyl-Free® may be a result of the proven efficacy of this EMF and of its predecessor, Lofenalac®, the primary medical food for infants in the U.S., in preventing mental retardation.

The contribution of medical food protein (73% RDAs) was conservative in our group of patients on a strict-diet, and lower than the amount recommended by the current nutrition protocols to supply at least 90% protein RDAs (calculated from Acosta, 1989). However, the average amount of Phenyl-Free® the six patients in our strict-diet group were able to consume daily, 12 Tbsp (120 g), was the result of extensive dietary counseling in our Metabolic Clinic over the patients' lifetimes and is consistent with observations by Hogan et al. (1986), who reported a maximum daily Phenyl-Free® intake of 13 Tbsp. (130 g) in a group of school-aged patients.

2. Implications

More than 4,000 patients with PKU are now followed in 105 U.S. treatment centers (Schuett, 1990); approximately 30% of the population is within the age range of patients in this study, and current policies are generally to advocate

continued strict diet adherence at least through adolescence (Fishler et al., 1987). In spite of recommendations, data from the most recent U.S. survey of these centers suggests that most patients are no longer adhering to a strict diet by age ten (Schuett, 1990). Our experience is that currently available EMFs provide unacceptable tasting phe-free protein and unsatisfactory amounts of phe-free energy to encourage school-aged patients to continue strict-diet adherence. Because EMF protein is synthesized from free L-amino acids and additional energy is added from fats and carbohydrates, the amino acid pattern and the protein:energy density should be amenable to change at the point of manufacturing.

A separate paper discusses results of L-amino acid taste-testing experiments from our laboratory and the total dietary amino acid intakes, relative to plasma amino acid levels in this same sample of children. It is our expectation that these organoleptic, nutritional, and biochemical assessments will provide sufficient data to propose a new EMF formulation and an improved dietary protocol for school-aged children and adolescents with PKU. We suggest that metabolic nutritionists begin to question the designs of current medical foods and their use according to current nutrition protocols and continue to evaluate their nutritional and social impact on strict adherence to the

therapeutic diet which is now recommended indefinitely for patients with PKU.

CHAPTER III: AN ALTERNATIVE APPROACH TO THE NUTRITIONAL TREATMENT OF SCHOOL-AGED PATIENTS WITH PHENYLKETONURIA BASED ON TASTES, INTAKES, AND PLASMA LEVELS OF AMINO ACIDS

A. ABSTRACT

We have examined the tastes of L-amino acids and elemental medical foods (EMFs) and quantitated the amino acid levels in the diets and plasma of 15 school-aged patients with phenylketonuria (PKU) and six healthy controls. L-methionine, L-aspartate, L-glutamate, and each of the EMFs taste-tested were rated the lease pleasant. Indispensable amino acid intakes of patients exceeded estimated requirements by 147-288% and dispensable amino acids ranged from 39-227% of control intakes. Mean intakes of each of the least pleasant L-amino acids exceeded these standards. Plasma tyrosine and leucine values of patients were consistently low and glycine levels were high. These data suggests that the amino acid profiles of current EMFs provide unduly unpalatable products and contribute to dietary and plasma amino acid surpluses and reductions. This research supports a need for a new approach to improve both the organoleptic and nutritional qualities of EMFs intended for school-aged patients with PKU.

B. INTRODUCTION

Chemically-defined medical foods ([CON-AAP], 1987) are the only phenylalanine-depleted sources of dietary protein available to patients with phenylketonuria (PKU). When recommended quantities of medical foods and natural foods low in protein are strictly adhered to, the irreversible mental and neurological damage associated with untreated or

poorly managed PKU is prevented and these children can reach their full developmental potential (Chang et al., 1984; Dobson et al., 1977).

The efficacy of the nutritional treatment for PKU declines sharply at school age (Michals et al., 1985). Strict diet adherence is rarely achieved beyond the preschool years. Declines in intellectual and academic performance and in behavior have been reported in school-aged patients (Smith et al., 1978; Matthews et al., 1986). There is evidence to suggest these impairments are reversible if strict dietary control is regained (Krause et al., 1985). For these reasons, PKU treatment programs in the U.S. currently advocate maintenance of the strict diet through the school years; however, the ability of the patients and their families to achieve this recommendation is uniformly poor, according to a 1988 national survey of treatment programs (Schuett, 1990).

The current approach to the nutritional treatment of PKU is to prescribe natural foods to meet the essential requirement for phenylalanine (phe) and to provide some variety (Acosta, 1989). As much as 90% of the protein intake is expected to come from medical foods. Natural foods are not considered to contribute significantly to the total recommended protein intake, although the prescribed amounts do supply 13-38% of the protein Recommended Dietary Allowances (RDAs) (calculated from Acosta, 1989; Matalon &

Matalon, 1989; FNB, 1980). Because medical foods themselves are prescribed in quantities which range from 105-135% of the 1980 protein RDAs, the total recommended protein intake is approximately 120-175% of the protein RDAs when prescribed medical and natural foods are accounted for (calculated from Acosta, 1989; Matalon & Matalon, 1989; FNB, 1980). The current approach thus provides an unnecessarily high intake of protein from medical foods and, because certain L-amino acids (L-AAs) have unpleasant organoleptic qualities, results in unduly unpalatable products. As the children grow older and become increasingly sensitive to taste discrimination (Acosta, 1989) and to the differences in their diets compared to others ([CON-AAP], 1976), ingestion of modular or elemental medical foods (EMFs) in the recommended quantities becomes more difficult (Acosta, 1989).

An alternative approach would be to take into account the protein and amino acids in the natural foods prescribed and to use these data to design an EMF to complement the natural food intakes. The levels of amino acids could then be reduced in the EMF; this change would significantly improve the taste of an EMF and could improve adherence to the total diet. The traditional approach views the EMF as providing nearly all of the essential nutrients, with the natural foods as palatable supplements of relatively little nutritional value. Our approach reverses this perspective

so that the EMF and its constituent L-AAs are a supplement to the natural foods. This allows the development of a more nutritionally balanced EMF with improved palatability.

To explore the feasibility of such an approach, the purpose of this study was to examine: 1) taste qualities of L-AAs, relative to EMFs; and 2) protein and amino acid intakes of school-aged patients, with and without contributions from EMFs, relative to estimated requirements and to control intakes. Plasma amino acid levels and physical growth were also examined, as indices of dietary protein adequacy. Reported energy intakes and urinary urea nitrogen excretions were used to validate the recorded and analyzed intakes of protein and amino acids. These data were used to evaluate the organoleptic and nutritional properties of EMF-protein and to provide experimental evidence to support a new approach.

C. MATERIALS AND METHODS

1. Taste comparison of L-AAs and EMFs

A study was designed to identify specific L-AAs which contribute to the undesirable organoleptic properties of current EMFs. Nine indispensable L-AAs (Ajinomoto Co, Los Angeles, CA) in isolation: L-his, L-ile, L-leu, L-lys, L-met, L-thr, L-trp, L-val, and L-tyr (conditionally indispensable in PKU) (Laidlaw & Kopple, 1987); the same L-AAs in combination (IDAA mixture); nine dispensable L-AAs

(Ajinomoto Co) in isolation: L-ala, L-asn, L-asp, L-citr, L-glu, L-gln, L-gly, L-pro, and L-ser; and three EMFs: Phenyl-Free® (Mead Johnson Nutritional Division, Bristol-Meyers Co, Evansville, IN), PKU-2® (Mead Johnson), and Maxamaid X-P® (Ross Laboratories, Columbus, OH), were tasted as 0.5% (wt/vol) aqueous solutions (0.25 g crystalline L-AAs were diluted to 50 mL using tap water), which permitted an evaluation of all stimuli at the same wt/vol ratio, as suggested by Solms et al. (1965).

Twenty-four normal volunteers and one patient with PKU and his mother served as experimental subjects. Solutions were presented in 30 mL plastic cups at room temperature. Subjects were asked to describe in writing which of the four primary tastes they experienced (salty, sweet, sour, bitter) (Schiffman & Erickson, 1971). Additional adjectives were encouraged to describe more complex taste qualities. Subjects were also asked to rate each solution on a semantic differential scale by making a mark along a 12 cm line numbered 1 (pleasant) to 10 (unpleasant).

Evaluations were collected and scored. For each solution, two numbers were assigned, one based on the description of taste and one based on the pleasant to unpleasant rating scale, using the following criteria: 1) Solutions described as sweet, flat, neutral or by other adjectives not suggestive of undesirable taste qualities were assigned a 0; a 1 was assigned to those solutions

described as salty, sour, bitter, or by other adjectives suggestive of more obtrusive taste qualities; and 2) Solutions rated 1-5 (pleasant-neutral) were assigned a 0; a 1 was assigned to those rated 6-10 (unpleasant).

2. Subjects for dietary and biochemical studies

Families of all patients with PKU living in Oregon, who were 7-17 y at the time of the data collection and had maintained contact with our clinic within the previous 2 y, were invited to participate in the study. The classification of PKU was based on the newborn pretreatment blood phe levels determined by the Guthrie assay on whole blood (Guthrie & Susi, 1963). All patients contacted had pretreatment blood phe levels $\geq$ 12 mg/dL by the Guthrie assay (720 umol/L) and had been advised by the clinic to maintain the strict diet which includes EMFs at school age. Involvement of entire families was emphasized, to increase their awareness of the importance of the accuracy of the study procedures (diet records and urine collections). Additionally, sibling data served as control values. Sixteen of 18 eligible families participated in the study; 16 patients and six siblings recorded their dietary intakes with parental supervision. One of the 16 patients reported a mean energy intake at 39% of estimated caloric requirements, in spite of a weight measurement at the 95th percentile of the National Center for Health Statistics

standards (Hamill et al., 1979). We considered the recorded intake of this subject to be a non-valid measure of actual intake and, therefore, excluded the data from this report. The purpose and requirements of the study were fully explained to each subject and to all families. Children over age 7 y gave assent to project participation and parents or caregivers of all subjects gave informed consent. The study protocol was approved in advance by the Committee on Human Research of the Oregon Health Sciences University and the Oregon State University Committee for Protection of Human Subjects.

3. Dietary measurements and analyses

Home visits were made by the clinic dietitian (AP) to review dietary recording procedures on an individual basis. Each family member who agreed to be involved in dietary recording was asked to estimate, in writing, portion sizes of eight lifelike food models. This exercise provided the dietitian with baseline data of each family's abilities to describe foods. Families were then shown how to record intakes on forms which were specially designed for this project. A descriptive language to quantify foods was agreed upon with each family, using their own household measuring tools and the commercial food models. Subjects were encouraged to make diet record entries immediately after eating occasions and parents were asked to supervise

when possible. Plastic wallets were given to children to carry diet records in, as suggested by Jackson et al. (1986), to maximize accuracy and reliability. A 4-d recording period (Fri, Sat, Sun, Mon) was selected to allow for the possibility that intakes of school children might be consistently different on weekdays compared to weekend days and to coincide with two 24-h urines which were collected over the weekend.

To meet our needs, an existing computerized nutrient data base, Nutrition and Diet Services® (927 S.E. Rimrock Lane, Portland, OR), was expanded to provide a profile for 18 amino acids in 1800 foods. The revised Agricultural Handbook No. 8 series ([USDA], 1976-1987) was the primary data source of the energy, protein, and amino acid content of foods. The composition of brand name foods was obtained from manufacturers and distributors of specialty food products used by patients. When complete amino acid data were not available from these sources, other references (Paul & Southgate, 1978; [FAO], 1970; Orr & Watt, 1968) were used to impute values, resulting in no missing values for the energy, protein, and amino acid content of all foods reported by the sample.

4. Plasma and urinary measurements and analyses

A fasting venous blood sample was collected from each subject into a heparinized Vacutainer tube®. Samples were

obtained two days after the dietary recording period, and kept on ice until they were centrifuged, at which time plasma was removed and stored at -20 °C for 14-30 months. Samples were thawed, deproteinized using 3% sulfosalicylic acid containing glucosaminic acid and beta-alanine as internal standards, and centrifuged prior to amino acid analysis. A Beckman Model 6300 Amino Acid Analyzer® (Beckman Instruments, Palo Alto, CA) was utilized for the analyses. Cystine, methionine, asparagine, glutamine, aspartate, glutamate, arginine, and tryptophan were not quantitated because an extended storage time (Perry & Hansen, 1969), a storage temperature above -68° C (Perry & Hansen, 1969), and a delay in deproteinization (Perry & Hansen, 1969; Stein & Moore, 1954) are factors which have been reported to affect the values for these amino acids.

Urine was collected into plastic bottles containing 15 mL toluene and 40 mL 2N HCL as preservatives for separate studies of vitamin B-6 and phe metabolites, respectively. All bottles were transferred to home freezers at the end of each 24-h collection until transport to the laboratory 3-4 days later. Aliquots of each 24-h volume were frozen at -20 °C until analysis. Samples were analyzed for creatinine and urea nitrogen with a Technicon Autoanalyzer® (Technicon Corp., Tarrytown, NY).

Urinary creatinine was measured to assess the completeness of the collections using an automated

modification of the Jaffé reaction (Pino, Bennotti, & Gordyna, 1965). In addition, each family was interviewed regarding the collections. These procedures allowed us to determine with confidence two 24-h urinary urea nitrogen values (Sat, Sun) for each subject, with several exceptions. In particular, two patients with PKU reported missing one collection and, therefore, we used a single 24-h urinary urea nitrogen excretion value for each of these subjects.

Urinary urea nitrogen was used as an indirect objective measure to validate the recorded food intakes as suggested by Huse, Nelson, Briones, and Hodgson (1974). We encountered difficulties in the interpretation of the urinary urea nitrogen excretion for those subjects whose protein intake fluctuated over the 4-d recording period. To account for these effects, we calculated the percent of the protein nitrogen intake excreted as urinary urea nitrogen on Sat, using the 24-h protein nitrogen intake averaged over Fri and Sat and, similarly, the percent of the protein nitrogen intake excreted as urinary urea nitrogen on Sun, using the 24-h protein nitrogen intake averaged over Sat and Sun. The urinary urea nitrogen excretion value reported, % dietary nitrogen, is the mean of these two percent excretion values which were computed for each subject.

5. Calculations and statistical analyses

Nutrient intakes were entered into a UNIX HCX/UX mainframe computer and descriptive statistics were determined using the Statistical Package for the Social Sciences software ($SPSS_x$, 1986). There were no consistent trends between weekday vs weekend intakes and, therefore, mean daily intakes of energy, protein, and amino acids were computed across the 4-d recording period for each individual. The values were compared to the 1980 RDAs (FNB, 1980) to determine the energy, protein, and indispensable amino acid (IDAA) adequacy of the diets. At the time of the data analyses, the 1989 RDAs (FNB, 1989) were not available. Because energy and protein RDAs are based on median reference weights, we individualized them to account for differences in the subjects' actual body weights. We compared IDAA intakes to the estimated requirements for healthy school-aged children (FNB, 1980). Cystine and methionine intakes were considered together, relative to the total sulphur-containing amino acid requirement. Tyrosine and phe intakes, generally considered together relative to the total aromatic amino acid requirement, were examined separately since the metabolic block in PKU is in the conversion of phe to tyrosine. Tyrosine intakes were also compared to the estimated requirement (125 mg/kg) for school-aged children with PKU to maintain adequate plasma tyrosine values (Acosta, 1989), which is approximately five

times above the standard for aromatic amino acids (22 mg/kg) (FNB, 1980). Because there are no standards for the dispensable amino acids (DAAs), intakes of the PKU patient groups were compared to the control group on a per g dietary protein basis (rather than per kg body weight) to account for the differences in mean protein intakes between the patients and their siblings.

D. RESULTS

1. Taste comparisons of L-AAs and EMFs

Twenty-four of the 26 evaluations were completed and scored. The possible range of scores was from 0 (a solution assigned two 0's on each of the 24 evaluations) to 48 (a solution assigned two 1's on each of the 24 evaluations). The actual scores ranged from 0 to 36 (Figure III.1.). The three EMFs, the IDAA mixture, and three of the 18 L-AAs (L-glu, L-met, L-asp) received the highest scores (20-36) and, therefore, were rated the least pleasant. The patient with PKU and his mother described the tastes of L-glu and L-met as being similar to Phenyl-Free® and they gave all three EMF solutions a highly unpleasant taste rating.

2. Dietary, physical characteristics, and phe status of subjects

Based upon the mean intakes of phe and natural protein, the degree of natural food restrictions, and the use of

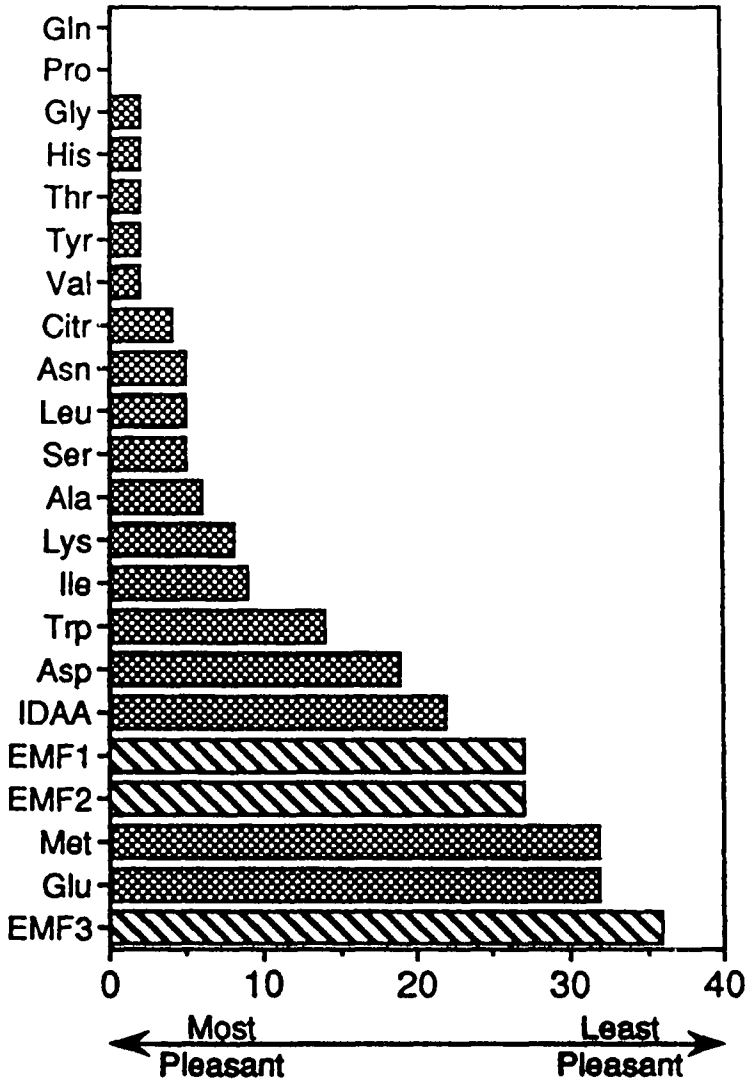

FIGURE III.1. Relative palatabilities of 0.5% (wt/vol) aqueous solutions of free L-amino acids, a mixture of indispensable L-amino acids (IDAA), and three elemental medical foods (EMFs)[1].

[1] EMF 1 (PKU-2®) is manufactured by Milupa A.G. International Scientific Department, Friedrichsdorf, West Germany and is distributed in the U.S. by Brisol-Meyers Company, Evansville, IN; EMF,

[2] (Phenyl-Free®) is manufactured and distributed by Bristol-Meyers Company; EMF

[3](Maxamaid X-P®) is manufactured by Scientific Hospital Supplies, Ltd., United Kingdom and is distributed in the U.S. by Ross Laboratories.

medical foods, the patients with PKU were divided into three groups (Table III.1): strict-diet, relaxed-diet, and off-diet. This method is consistent with the approach chosen by the most recent national survey (Schuett, 1990) to classify patients across treatment programs based on their degree of dietary adherence. The non-affected siblings comprised a fourth (control) group.

The strict-diet group (n=8) included those patients who reported intakes that were considered to be significantly restricted in phe (13-23 mg phe/kg body weight), whether or not their plasma phe levels (517-1146 umol/L) were within the acceptable treatment range (120-605 umol/L) (Schuett, 1990). Each of these subjects consumed medical foods daily and none of them consumed protein-dense foods. Three patients were considered to be consuming a relaxed-diet, with phe intakes which ranged from 31-38 mg/kg body weight. Only one patient in this group regularly consumed medical foods at the time of the study. Again, none of these patients consumed protein-dense foods; phe intakes were increased due to a more liberal intake of starchy foods. The remaining four patients were considered to be off-diet, although one subject in this group continued to consume medical foods daily. Each of these patients consumed restricted amounts of protein-dense foods.

TABLE III.1 Dietary, physical characteristics, and phenylalanine (phe) status of subjects[1]

	strict-diet (n=8)	relaxed-diet (n=3)	off-diet (n=4)	control (n=6)
dietary characteristics				
medical food intake	daily (n=8)	daily (n=1)	daily (n=1)	none (n=6)
natural food intake[2]				
protein-dense	none	none	restricted	daily
starches	restricted	daily	daily	daily
protein-poor	daily	daily	daily	daily
physical characteristics				
age (y)	9.9(8.2-11.0)	15.3(14.5-16.4)	8.3(7.4-10.5)	9.5(5.5-13.9)
sex	3F 5M	2F 1M	1F 3M	2F 4M
height (%)[3]	47 (10-90)	65 (50-95)	58 (35->95)	58 (25-95)
weight (%)	55 (40-80)	60 (50-80)	59 (35->95)	56 (10-80)
phe status[4]				
dietary phe (mg/d)	627 (321-828)	1967(1175-2310)	2154(1349-2845)	3208(2601-4321)
plasma phe (umol/L)	854 (517-1446)	1219(1026-1364)	708(437-1064)	70 (48-88)

[1] Values are means, with ranges in parentheses.
[2] Protein-dense foods include meat, poultry, fish, eggs, legumes, dairy; starches include grains, cereals, starchy vegetables; protein-poor foods include fruits, non-starchy vegetables, fats, sugars, and specially-made baked goods, pastas, and gelatins, reduced in protein.
[3] Heights and weights are compared to the National Center for Health Statistics growth percentile standards (Hamill et al., 1979).
[4] Recommended phe intakes for patients with PKU 7<11 y = 196-420 mg/d; for 11<19 y = 248-858 mg/d (calculated from Matalon & Matalon, 1989; FNB, 1980). Acceptable treatment range of plasma phe for patients with PKU = 120-605 umol/L (Schuett, 1990); reference mean and range (x±2 SD) for healthy children 6-18 y = 42-74 umol/L (Armstrong & Stave, 1973).

The mean ages of three of the four groups were similar; each of the patients in the relaxed-diet group were in their teens. Heights and weights of all subjects were within the normal growth percentile ranges (5th-95th%) established by the National Center for Health Statistics (Hamill et al., 1979), except for one patient in the off-diet group whose height and weight were above the 95th percentile.

The mean phe intakes of each of the patient groups were above the recommended levels. Consequently, the mean plasma phe values for each of the groups were above the acceptable treatment range. The mean phe intake of the off-diet group was greater than the other two patient groups, yet the mean plasma phe level for this group was lower. These patients presumably had greater residual PAH enzyme activities, individual physiological requirements for phe, or both.

3. Protein and amino acid intakes

The mean protein intakes for each group were adequate at 106-182% of the individualized protein RDAs (FNB, 1980) (Figure III.2.). Medical foods contributed to 73% of individualized protein RDAs for the patients who consumed a strict-diet. Total mean energy intakes for each group were adequate at 93-100% of individualized energy RDAs. Medical foods contributed to only 22% of individualized energy RDAs for the patients who consumed a strict-diet.

The mean IDAA intakes of each group were adequate

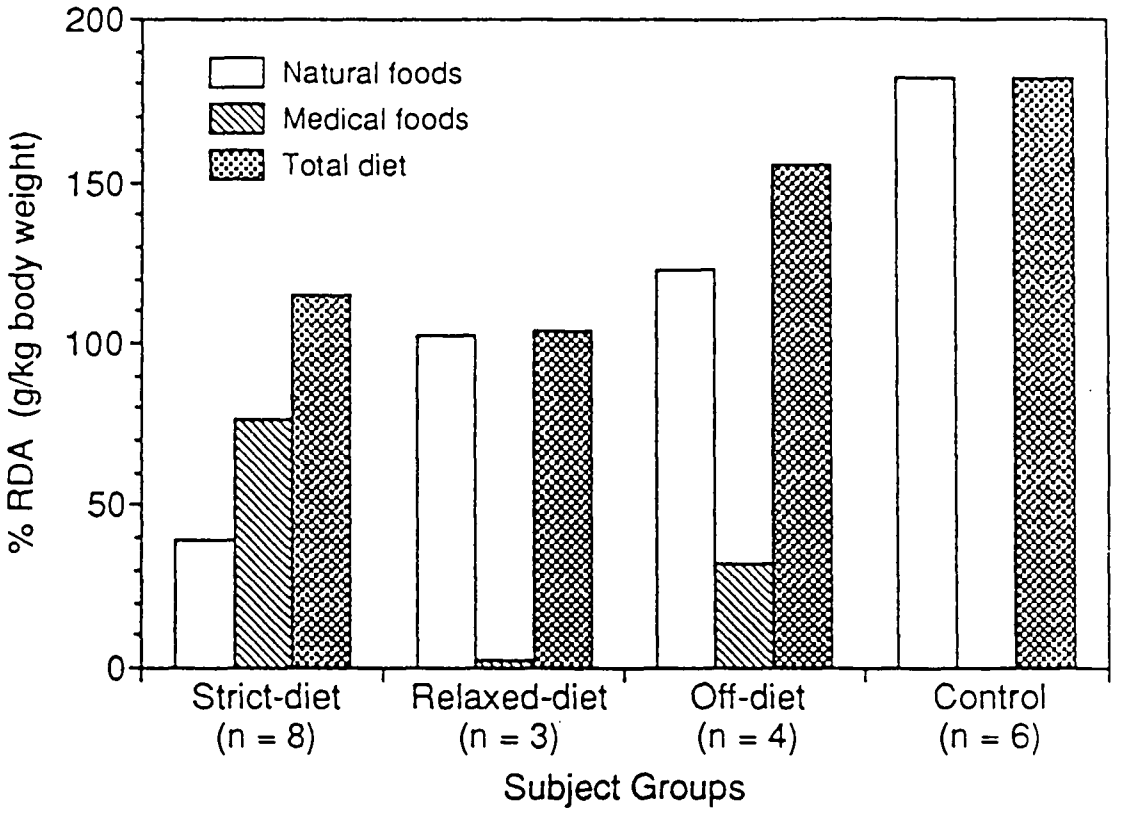

FIGURE III.2. Mean protein intakes from natural foods, medical foods, and in the total diets of each subject group compared with the 1980 Recommended Dietary Allowance (RDA).

(above the mg/kg standards) (FNB, 1989). The separate contributions from natural foods and medical foods are presented in Figure III.3. for the strict-diet group. Natural foods provided 41-145% of the IDAA standards, with lysine being the limiting IDAA. With the inclusion of the medical foods, IDAA intakes increased to 147-332% of the standards. For the patients in the strict-diet group, the mean DAA intakes from natural foods were within 85-112% of the control group intakes (except for proline which was 78% of controls) (Figure III.4.). With the inclusion of the medical foods, DAA intakes consistently moved further away from the control pattern, resulting in total intakes for the strict-diet group which ranged from 39-222% of controls. For the relaxed-diet or off-diet PKU patient groups, in which medical foods were used in lower quantities, the mean total DAA intakes were closer to the control group pattern.

4. Plasma amino acid and urinary urea nitrogen analyses

The mean fasting plasma amino acids of each group are presented, relative to one another and to the standards established for plasma amino acids in 136 healthy fasting children, 6-18 y (Armstong & Stave, 1973) in Figure III.5A. (IDAAs) and Figure III.5B. (DAAs). Our mean control values for each IDAA fell within $\pm$ 1 SD of these reference mean values. For each of the PKU patient groups, mean IDAA values consistently fell below these reference means (-1 to

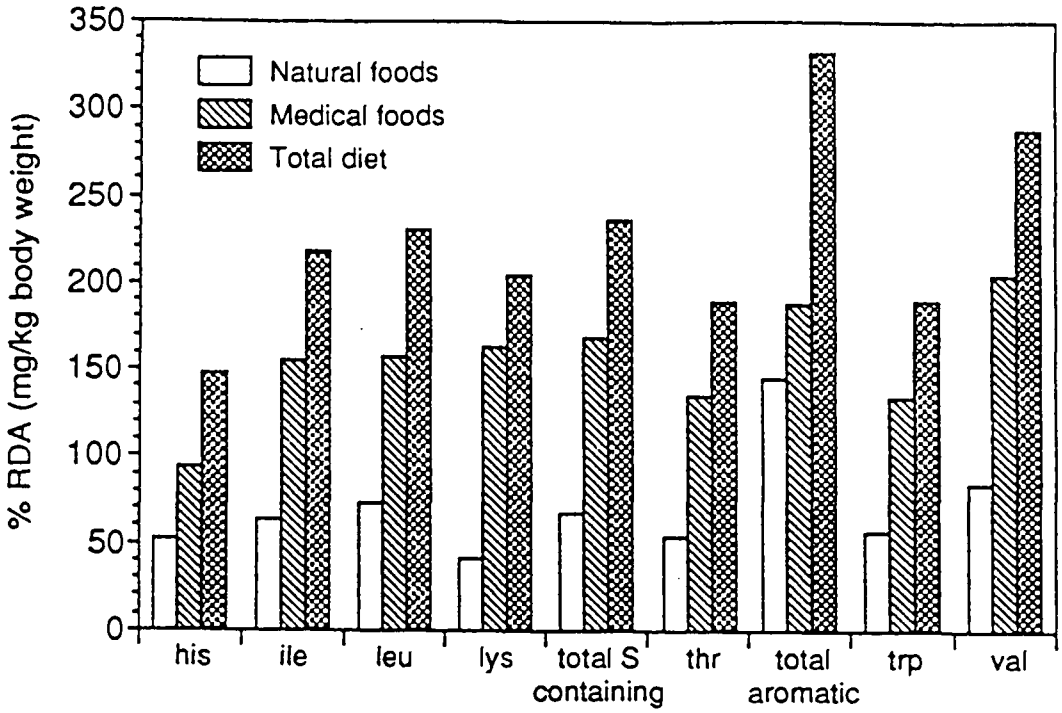

FIGURE III.3. Mean indispensable amino acid intakes from natural foods, medical foods, and in the total diets of eight patients with PKU consuming a strict-diet compared with the 1980 Recommended Dietary Allowance (RDA).

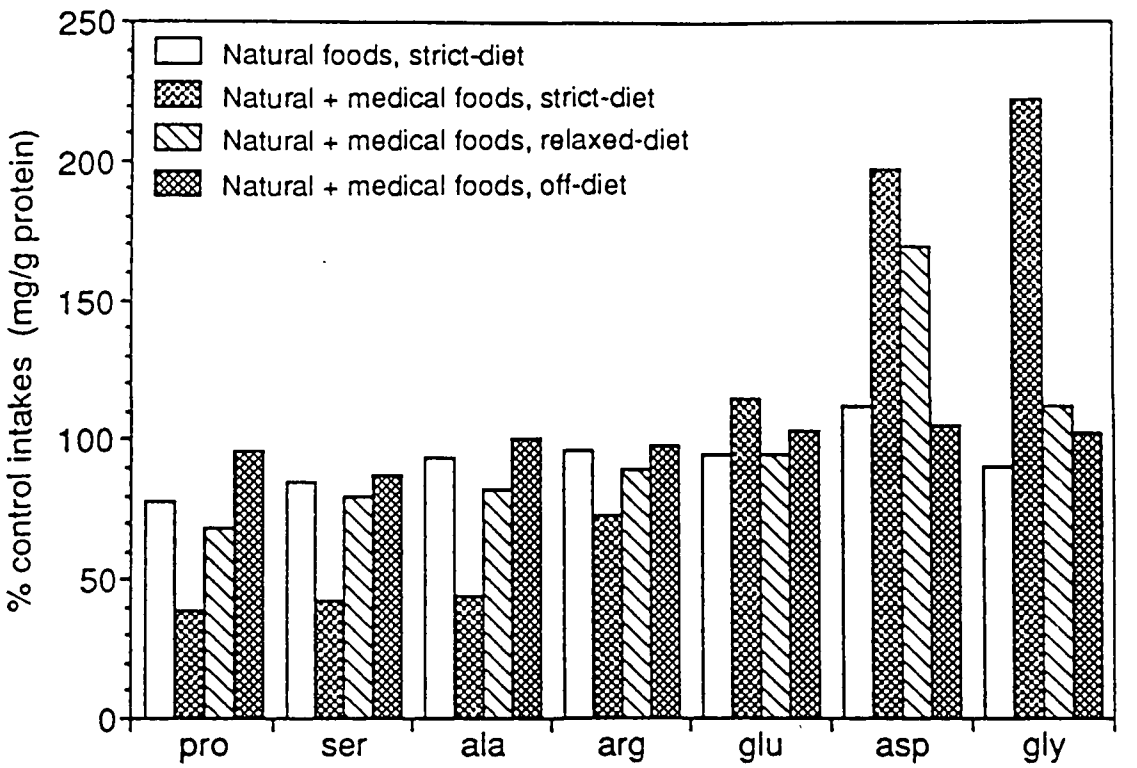

FIGURE III.4. Mean dispensable amino acid intakes from natural foods, medical foods, and in the total diets of each patient group compared with the mean intakes of the control group.

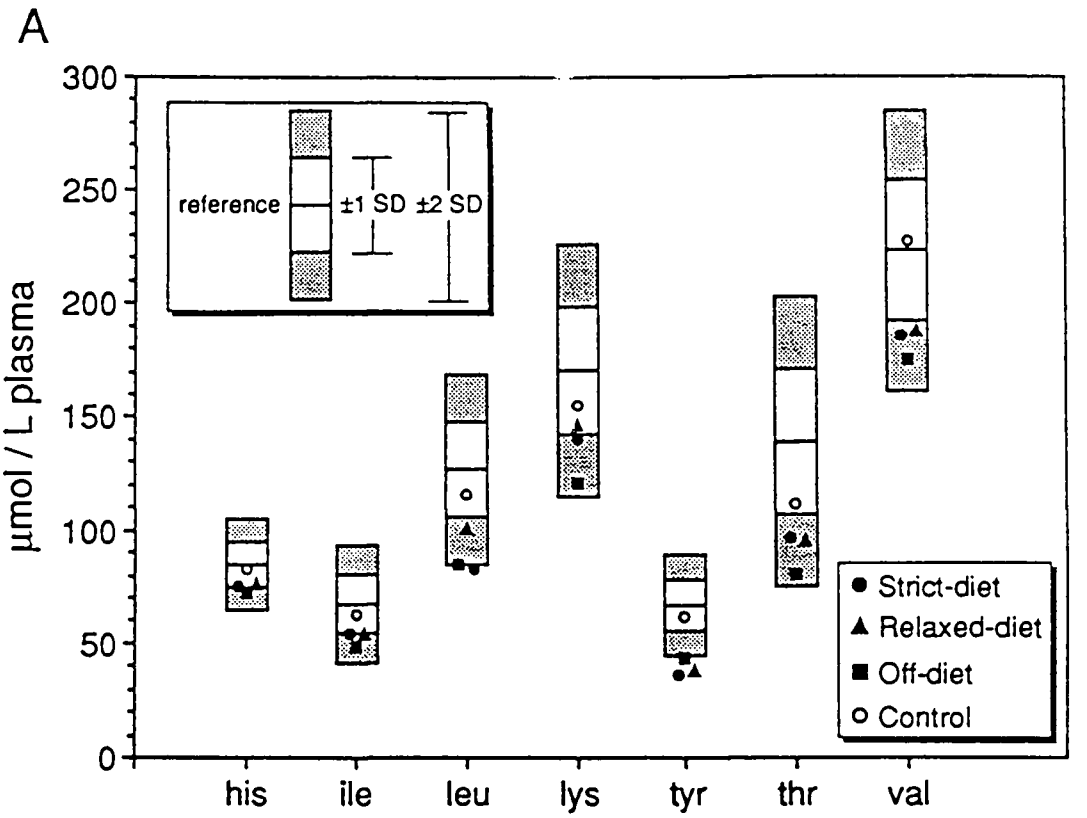

FIGURE III.5A. Mean fasting plasma indispensable amino acid values of each subject group compared to reference mean values[1].

[1] Reference values from Armstrong and Stave, 1973.

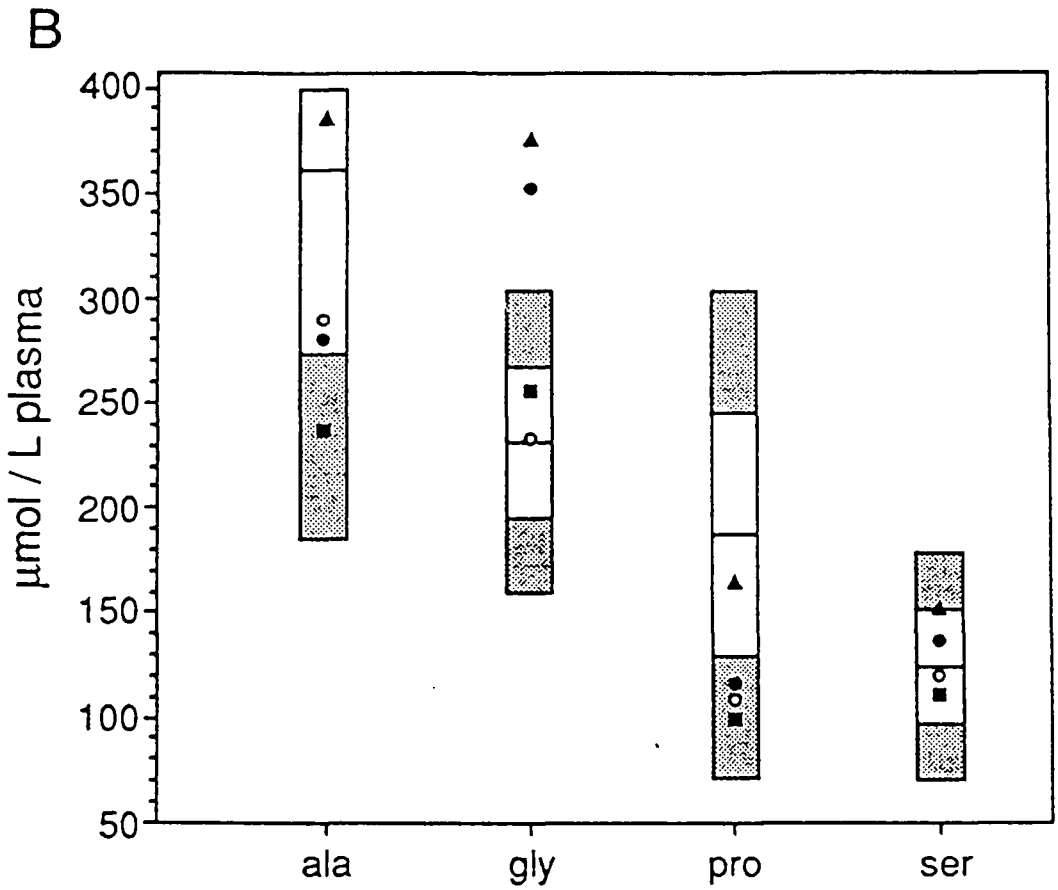

FIGURE III.5B. Mean fasting plasma dispensable amino acid values for each subject group compared to reference mean values.[1]

[1] Reference values from Armstrong and Stave, 1973.

-2 SD). The mean leucine value of the strict-diet group was less than 2 SD below the reference mean and the mean tyrosine values for all PKU patient groups were less than 2 SD below the reference mean (Figure III.5A.). Of the four DAAs we measured, values for each group were within ±2 SD of the normal reference mean except for glycine which was more than 2 SD above the reference mean for the strict-diet and relaxed-diet groups (Figure III.5B.). The mean urinary urea nitrogen excretions of each group are compared to their respective mean protein nitrogen intakes for each subject group in Table III.2. The amounts of urinary urea nitrogen excreted by the control subjects were consistently greater than patients, reflecting higher protein nitrogen intakes. The percent protein nitrogen excreted was similar between the groups.

E. DISCUSSION

PKU is the best known inborn error of amino acid metabolism for which the response to nutritional treatment has been studied extensively (reviewed in [CON-AAP], 1976). The treatment is based on medical foods or EMFs which provide modified protein, as hydrolysates or free L-AAs, severely limited in their content of phe. There are at least 35 published reports on the clinical evaluation of these products (summarized in [CON-AAP], 1985). The present study is the first to examine the impact of these products

TABLE IV.2 Energy, protein, and vitamin B-6 intakes of subjects[1]

group	protien N intake[2]		urinary urea N[3]		urinary urea N (% protein N[4]
	<----------g N/24-h---------->				
	day 1	day 2	day 1	day 2	
strict-diet	6.33 ± 1.69	6.07 ± 1.81	3.55 ± 1.24	3.66 ± 1.37	61 ± 18
relaxed-diet	7.55 ± 1.66	7.79 ± 1.94	3.77 ± 1.94	7.53 ± 4.97	63 ± 16
off-diet	7.10 ± 1.54	7.32 ± 1.55	3.71 ± 1.40	3.96 ± 1.15	51 ± 9
control	11.79 ± 5.14	11.54 ± 3.46	6.62 ± 2.57	7.32 ± 2.44	61 ± 10

[1]Values are mean ± SD.

[2]24-h protein N intake averaged over Fri, Sat (day 1) and over Sat, Sun (day 2).

[3]24-h urine urea N on Sat (day 1) and Sun (day 2).

[4]Mean value: % protein N intake (day 1) excreted as urine urea N (day 1) and % protein N intake (day 2) excreted as urine urea N (day 2).

on the total protein intakes and which addresses both protein quantity and quality. In addition, we examined the taste of the elements (the L-AAs) which constitute the protein equivalent of the EMFs currently available for school-aged patients with PKU.

We are aware of only one previous report which has suggested that the nutritional quality of the protein source in medical foods needs improvement. Over a decade ago, it was proposed that products for infants with inborn errors of metabolism could be improved by bringing their amino acid composition closer to that of human milk (Nayman et al., 1979). There are no reports which have evaluated the amino acid composition of the EMFs intended for older patients, relative to the amino acid composition of the natural foods consumed by these patients, to the estimated amino acid requirements, or to the plasma amino acids. This may be a reflection of the current approach to the nutritional treatment. With the exception of phe and tyrosine (Elsas & Acosta, 1988), the current goals of nutritional support for patients with PKU do not include maintenance of dietary or plasma amino acid levels within desirable ranges. The computerized nutrient data base which has been designed particularly for metabolic nutritionists to evaluate intakes of patients with inborn errors of metabolism (Aminoacid Analyzer®, Tallahassee, Florida) does not have complete data for all of the amino acids. However, valid and reliable

data on the amino acid composition of foods are available ([USDA], 1976-1987). Our specific efforts to update an existing nutrient data base for both the IDAAs and the DAAs from the research and industry literature allowed us to quantitate complete amino acid intakes. None of the foods that contained more than 0.1 g protein, in the amounts recorded by these subjects, had missing amino acid data in our analyses.

While the idea of examining the protein and individual amino acid intakes of patients with and without medical foods is unique, the need for a better understanding of these products and their impact upon protein nutriture is well-documented. The Committee on Nutrition of the American Academy of Pediatrics [CON-AAP] produced four reports between 1967 and 1987 which addressed the nutritional treatment of inborn errors of metabolism ([CON-AAP], 1987, 1976, 1985, 1967). The first two ([CON-AAP], 1967, 1976), recognized several problems with the dietary treatment: 1) The total protein and individual amino acid needs of patients consuming amino acid-modified diets were not defined; and 2) Although nutritional treatment was known to yield dramatic clinical improvement of PKU and other inherited disorders, there was a gap between the theory and practice of treatment. The CON-AAP recommended that this gap be narrowed through better use of food technology and improved application of nutritional information ([CON-AAP],

1976). The two more recent reports ([CON-AAP], 1987, 1985) were produced in response to such issues as diet continuation for PKU and the safety and efficacy of medical foods. Variations found among products in both IDAAs and DAAs led to a recognition that: 1) with current knowledge, the biological significance of the differences in amino acid composition cannot be determined; and 2) totally synthetic products (EMFs) can be prepared to any desired L-AA pattern. However, the reports did not recommend that studies be undertaken to determine the biologic significance of the amino acid composition of products or to produce a desirable L-AA mix. They concluded that the current products are safe, provide sufficient nutrition for growth ([CON-AAP], 1985), and that they are efficacious, based on their abilities to prevent mental retardation.

The literature and the results of the present study do not support these conclusions with respect to the safety nor to the efficacy of current medical foods intended for school-aged patients. Two-thirds of the children in the largest clinical trial in the U.S., the National Collaborative Study of Children Treated for PKU (NPKUCS), were unable to maintain blood phe levels within the acceptable treatment range of 120-605 umol/L (2-10 mg/dL) by 8 years of age (Michals et al., 1988). Consistent with this report, the group of patients in our study who adhered most strictly to the recommended diet (strict-diet) had a mean

plasma phe value of 854 umol/L (14 mg/dL) at a mean age of 10 years. Normal growth of children with PKU has been reported through 6 years of age (Chang et al., 1984). Our patients who consumed a strict-diet tended to have lower mean heights and weights, compared to the other patients and to the controls, but still within the normal range. Sutherland, Berry and Umbarger (1970) reported periods of inconstant growth and elevated blood phe levels in patients with PKU which they suggested were related to an imbalance between IDAAs in the medical foods and phe in the natural foods.

The most recent CON-AAP report ([CON-AAP], 1987) was the first to recognize that natural foods can contribute to the nutrition of patients. This report suggested that medical foods should meet nutrient requirements when used with natural foods. However, this concept was contradicted later in the same report by a statement that the nutrient content of the medical foods themselves must at least meet the age-specific RDAs. This approach theoretically assures clinicians that nutrient requirements, including protein and IDAAs, are met. However, in practice it is contingent upon the patients' strict adherence to the medical food prescription which is jeopardized by the taste of the modified protein and is currently not being achieved by older patients (Hogan et al., 1985).

Using the currently accepted approach results in total protein intakes of 20-75% above protein RDAs if prescriptions are strictly adhered to (calculated from Acosta, 1989; Matalon & Matalon, 1989; & FNB, 1980). Because the current EMF-proteins we examined were found to be unpleasant in taste and unbalanced in their amino acid profiles, we question this approach which Matalon and Matalon (1989) and Acosta (1989) currently advocate. They suggest that protein intakes need to be above RDAs to allow for any problems with utilization of the modified protein. Specifically, Acosta suggests that when L-amino acids (in EMFs) supply the protein equivalent, protein requirements may be higher than RDAs due to rapid absorption and early and high concentrations of plasma amino acids. Although neither Matalon and Matalon (1989) nor Acosta (1989) cite evidence to support this theory, Silk et al. (1979) demonstrated this phenomenon in healthy humans fed 50 g protein as peptides compared to free amino acids. However, when plasma amino acid concentrations were examined over a three hour period, there were no significant differences in the total areas under the curves. Silk et al. concluded that caution be used when interpreting the clinical or nutritional significance of early plasma amino acid concentrations and that their data suggested that the total amount of absorbed nitrogen was similar between peptides and free amino acids.

Only one report has specifically examined protein intakes in patients with PKU aimed at RDA levels. Kindt et al. (1983) examined the growth outcomes and hand x-rays of 16 patients followed from infancy to age 24 months who received 95% of their protein as a low-phe hydrolysate. The total protein intakes were provided in amounts to meet the 1980 protein RDAs (FNB, 1980) or to meet the 1973 Food and Agriculture Organization (FAO) recommended amounts ([FAO/WHO/UNU], 1973). Three of the eight patients in the FAO group had linear growth declines during the second year of the study and five patients had hand x-rays which showed possible osteoporosis. There was no evidence of growth declines or osteoporotic bone changes in any of the patients in the RDA group. The authors concluded that the RDA protein level was safe for patients with PKU who consume the majority of their protein from medical foods. The intakes of the FAO group were increased, accordingly, to meet the protein RDAs. Although this study examined infants and young children who consumed protein hydrolysates, it is one of the few studies which has examined an alternative approach to treatment. These data provide long-term evidence to support the safety of lowered (RDA level) protein intakes in patients with PKU who consume modified protein sources.

Plasma amino acids have also been used as one measure of the safety and efficacy of nutritional treatment. There

is a well-documented characteristic reduction of plasma amino acids in untreated patients with PKU (Christensen, 1953; Efron et al., 1969). Christensen has proposed that this is a result of the phe accumulation in the plasma which inhibits the transport of some amino acids out of the tissues and into the plasma (Christensen, 1987). With the use of medical foods or EMFs and a reduction in plasma phe, the other amino acids have been shown to be increased in the plasma of treated patients (Berry et al., 1976; Held et al., 1983; Link & Wachtel, 1984). Compared to values for 20 amino acids obtained in 18 normal children, 11 amino acids in the plasma of patients fed a casein hydrolysate (Lofenalac®, Bristol-Meyers Co, Evansville, IN) and six amino acids in the plasma of those receiving a protein hydrolysate from beef serum (Albumaid X-P®, Scientific Hospital Supplies, Ltd, Liverpool UK) were increased to levels more than two SD above the normal mean values. The sum of the mean concentrations was approximately 40% higher with Albumaid X-P® and 75% higher with Lofenalac®, compared to the sum of the normal mean values. Link and Wachtel (1984) examined the plasma amino acid concentrations of patients receiving a new L-amino acid mixture (PKU-2®, Milupa Corp, Friedrichsdorf, Germany) as one measure of safety and efficacy. In these patients, the sum of the mean plasma amino acid concentrations was approximately 24% above the respective sum for the normal children. These reports

didn't discuss the plasma amino acid findings relative to protein and amino acid intakes nor to general protein nutriture.

Pratt (1980) proposed an alternative approach to nutritional treatment based on inhibition of transport of certain bulky dipolar amino acids by phe. His approach suggests that plasma levels of 13 amino acids be maintained two to three times above the normal reference ranges. To achieve these concentrations, Pratt recommends amino acid supplements of 50-100 mg/kg for eight amino acids, regardless of the intakes from natural or medical foods. A similar approach advocated by Berry, Brunner, Hunt, and White (1990) recommends supplements of the three branched chain amino acids only. One of the objectives of the study by Berry et al. (1990) was to determine whether the supplements could be consumed over a long period. More than 25% of the subjects were dropped from the study before the end of six months as a result of noncompliance with the nutritional treatment.

The long-term safety and efficacy of one or more amino acids increased above the normal reference range in plasma and the effect of amino acid supplements from medical foods, EMFs, or in addition to these sources, on overall protein and amino acid metabolism and nutrition has not been evaluated. The data from the present study demonstrated a trend in the DAA intake patterns of patients away from the

nutritional standards we selected (mg/g dietary protein compared to control intakes) when the intakes of medical foods were added to natural foods. Six of the eight patients in the strict-diet group consumed the EMF Phenyl-Free® (Bristol-Meyers Co), which contains no L-alanine, L-serine, or L-proline and supplies disproportionately high quantities of L-glycine. The significance of disproportionate amounts of dietary amino acids was reviewed by Harper, Benevenga, and Wohlhueter, 1970. An amino acid imbalance traditionally refers to disproportional dietary surpluses of IDAAs, especially when added to low-protein diets. It has been suggested that DAA surpluses may also create an amino acid imbalance (Abernathy & Miller, 1965). This idea was supported by Harper et al. (1970) who further suggested that this occurs when the disproportion is great enough to cause an adverse effect, regardless of the dispensability of the surplus amino acids. In rats, depressed growth has been shown to occur with DAA surpluses (Harper et al., 1970; Fisher, Griminger, Leveille, & Shapiro, 1960; Savage & Harper, 1964); usually much larger amounts are necessary than with surpluses of IDAAs. Effects depend on the physiological and nutritional state of the animal and on the composition of the diet. In healthy animals fed diets low in protein, adverse effects on growth have been observed with individual dietary IDAAs fed at levels 20% above requirements (Harper et al., 1970). There

is evidence to suggest that if the quantity of a surplus amino acid is increased in direct proportion to the increase in dietary protein (as occurs with medical foods or EMFs added to natural foods), the additional protein will not ameliorate the adverse effects (Daniel & Waisman, 1969; Harper, Becker, & Stucki, 1966).

1. Limitations

Our purpose was not to determine the physiological or biochemical effects of the disproportionate amino acid intakes we observed. We did not measure indices of protein status other than growth outcomes and fasting plasma amino acids. The lower mean weight and height percentiles observed in the patients in the strict-diet group were previously mentioned. Some of our patients had plasma amino acid patterns similar to aminograms of infants fed diets in which protein was below RDAs (Snyderman, Holt, Norton, Roitman & Phansalker, 1968), with a depression of valine, isoleucine, leucine, lysine, and tyrosine, and an elevation in glycine. Reductions in plasma valine, isoleucine, and leucine concentrations were also reported in infants fed L-AAs as their source of protein (Snyderman et al., 1968). Mean leucine and tyrosine levels were at least 2 SD below their reference means for each of our PKU patient groups and glycine levels were more than 2 SD above the reference mean for the strict-diet and relaxed-diet groups. Whether these

deviations were the result of disproportions in amino acid intakes, inadequate total protein, or PKU per se cannot be determined from these data. However, because all natural protein is severely restricted for patients with PKU, medical food and EMF-protein are the only available sources of nitrogen and amino acids for these patients. Because of the levels of certain L-AAs in the EMFs intended for school-aged patients, our data show that higher dosages of current products may not be beneficial and, in their present forms, would not likely be accepted by our patients.

We recognize that these results are not generalizable to school-aged patients who are able to achieve lower natural protein and amino acid intakes. However, we are not aware of a report which describes successful adherence to the strict diet in school-aged patients. Therefore, we believe our eight patients who adhered the closest to a strict diet are representative of the general target population of school-aged patients and that the reported protein and amino acid intakes were complete, based on the mean energy intakes which were 98% of the individualized energy RDAs, and the urinary urea nitrogen and creatinine data.

We also recognize that our results cannot be extended to other EMFs which are available for school-aged patients but were not used by our patients (PKU-2®, Maxamaid X-P®). However, both of these EMFs contain even higher levels of L-

glutamate than Phenyl-Free® (per g protein) and the total amount of the three least pleasant-tasting L-AAs is nearly identical in these products. As expected, the overall tastes of these EMFs were comparably unpleasant to Phenyl-Free® in our taste-tests (Figure III.1.).

2. Applications

Shortland and co-workers have defined the protein requirement for children as the protein intake needed to maintain optimal growth and plasma amino acid concentrations (Shortland, Smith, Francis, Ersser & Wolff, 1985). The goals of nutritional support for children and adolescents with PKU are to maintain adequate growth and plasma phe and tyrosine concentrations within their desirable treatment ranges. Based upon this research, we expand the aims of nutritional treatment for children and adolescents with PKU to include: 1) to meet the total energy, protein and IDAA standards established for healthy non-PKU children; 2) to provide DAAs at levels consistent with patterns provided by the diets of healthy non-PKU children; 3) to maintain adequate growth and fasting plasma amino acid values within the reference ranges established for healthy children (except for phe); and 4) to supply an acceptable source of EMF protein which would encourage patients to maintain plasma phe values in the acceptable treatment range. The data in the present study support the national experience

that the current approach to the nutritional treatment, in its design and prescription does not meet these aims. We propose a new approach to the design and use of an EMF for school-aged patients with PKU. Specifically, we recommend bringing the L-AA content, when combined with allowed quantities of natural foods, closer to that of normal mixed diets and with consideration of the nutritional and organoleptic qualities of the individual L-AAs. Until improvements are made in the palatability of EMFs, it may not be possible for school-aged patients to strictly adhere to the nutritional treatment which is necessary in order to maintain adequate biochemical control of PKU. We recommend that these data serve as the basis for further research and product development.

CHAPTER IV

VITAMIN B-6 STATUS OF TREATED SCHOOL-AGED PATIENTS WITH PHENYLKETONURIA

A. ABSTRACT

Fifteen school-aged patients with phenylketonuria (PKU) were divided into three groups, on the basis of their adherence to the nutritional treatment. Six non-affected age-matched siblings served as controls. Diet intakes were recorded for 4 days and computer-analyzed for protein and vitamin B-6 intakes; urine samples were collected for a 24-h period for 2 days and analyzed for 4-pyridoxic acid (4-PA) excretion; and a fasting plasma sample was analyzed for pyridoxal 5'-phosphate (PLP), total vitamin B-6 (TB6), and alkaline phosphatase (AP). Significant differences ($p \leq 0.05$) between the control group and two patient groups (overall and relaxed-diet) were observed for dietary protein (below control), dietary vitamin B-6:protein (above control), plasma PLP (above control), plasma PLP:TB6 (above control), and plasma AP (below control). These findings, along with urine 4-PA excretions which were below control values, suggest a reduced turnover of PLP in treated patients with PKU at school-age, especially when dietary adherence is relaxed.

B. INTRODUCTION

Vitamin B-6, in the form of the coenzyme pyridoxal 5'phosphate (PLP), plays a key role in the metabolism of amino acids. Phenylketonuria (PKU) is a rare inborn error of amino acid metabolism which results in rapid and irreversible mental retardation and neurologic damage if not

treated in the newborn period (Bickel et al., 1953). The reduced hydroxylation of phenylalanine (phe) in PKU places a greater reliance on the secondary metabolic pathways for phe degradation, transamination and decarboxylation, which require PLP. Acceptable biochemical control of PKU can only be achieved through a severe dietary restriction of natural protein, below the level necessary to support normal growth and development (FNB, 1989; Acosta, 1989). The need for vitamin B-6 is reduced when dietary protein intake is low (Miller et al., 1985). The nutritional treatment of PKU is based on chemically-defined medical foods, intended to supply patients with a concentrated source of low or no-phe protein, as well as vitamins and minerals to meet or to exceed requirements, and to supply some energy (Acosta, 1989). The medical foods used by school-aged patients contain up to 0.04 mg of vitamin B-6 per g of protein; levels which exceed the 1980 and the 1989 Recommended Dietary Allowances (RDAs) of 0.02 mg/g protein for infants and children (FNB, 1980, 1989).

PKU and its treatment represent a unique clinical condition in which there are metabolic and nutritional factors which might affect vitamin B-6 status. At school age, when adherence to the nutritional treatment becomes less homogeneous, these factors would be more variable within patients. There is only one other published vitamin B-6 status report which examined patients with PKU

(Anderson, 1986). In the report, status was found to be adequate, based upon plasma PLP values of the patients which were significantly higher than a control group. In contrast to the present report, the subjects were younger (preschool-aged) and presumably, adhering to the strict diet and achieving acceptable biochemical control; urine 4-PA values were based on random, rather than 24-h, samples; and PLP values were measured in a non-fasting state and analyzed by the method of Camp et al. (1983), which does not determine the total amount present in plasma. Thus, the present study was designed to expand this research, specifically to assess vitamin B-6 status by multiple accepted methods in a free-living sample of school-aged patients with PKU and their non-affected age-matched siblings. These subjects were chosen since it is possible that vitamin B-6 status might be different in patients with varying degrees of adherence to the therapeutic diet compared to normals, and also possible that status would be different in school-aged patients with plasma phe levels above the acceptable treatment range compared to younger patients previously studied.

C. METHODS

1. Subjects

Families of all PKU patients living in Oregon, who were 7-17 y at the time of the data collection and had maintained clinic contact within the previous 2 y were invited to join

the study. Sixteen of 18 eligible families agreed to participate; both of the two families who chose not to join had caregivers who were unable to commit the necessary supervision for the diet recording and urine collections. Home visits were made to review procedures on an individual basis. Non-PKU siblings were invited to fully participate along with the PKU patients and caregivers were asked to supervise the diet recording and urine collections. We encouraged the involvement of entire family units to increase awareness of the importance of the accuracy and desired detail in data collection. Sixteen children with PKU and six siblings (controls) completed the study. One of the 16 patients with PKU reported a mean energy intake at 39% of estimated caloric requirements (FNB, 1980), in spite of a weight measurement at the 95th percentile of the National Center for Health Statistics standards (Hamill et al., 1979). We considered the reported intake of this subject to be a non-valid measure of actual intake and, therefore, complete data for 15 PKU patients and six controls were analyzed and are included in this report.

This data collection was part of a larger study which examined additional dietary components, biochemical indices, and clinical parameters of the sample. The total project required a 4-d diet record, two 24-h urine collections, a fasting blood sample, and 5-h psychometric testing. With 89% of the accessible population included in the final data

set, we considered the sampling to be successful and representative of our target population of school-aged PKU patients receiving dietary advice and treatment. The purpose and requirements of the study were fully explained to each subject and to all families. With the exception of one family, all agreed to discontinue vitamin and mineral supplements at least 3 weeks prior to the study. This family included two subjects (one patient off-diet and his sibling). Each of these children habitually consumed a multivitamin supplement containing 1.06 mg vitamin B-6 which was discontinued 3 days prior to the data collection. The supplemental vitamin B-6 was, therefore, not included in the dietary intakes computed for these two subjects.

Children over age 7 y gave consent to participation and parents or caregivers of all subjects gave informed consent. The study protocol was approved in advance by the Committee on Human Research of the Oregon Health Sciences University and the Oregon State University Committee for the Protection of Human Subjects.

2. Dietary measurements and analyses

At the home visit, each family participant was asked to estimate, in writing, portion sizes of eight lifelike food models. This exercise provided baseline data of each family's ability to describe foods. Families were then shown how to record foods on forms specially designed for

the project. Subjects were encouraged to make diet record entries immediately following eating occasions and parents were asked to supervise when possible. Plastic wallets in which to carry diet record forms were given to subjects, as suggested by Jackson et al. (1986), to maximize validity and reliability. A 4-d recording period (Fri, Sat, Sun, Mon) was selected to allow for the possibility that intakes of school children might be consistently different on weekdays compared to weekend days and to coincide with the two 24-h urine collections (Sat, Sun) for the measurement of 4-PA excretion.

Records were returned two days after the recording period, reviewed with each family and checked for any ambiguous data. A computerized nutrient data base, Nutrition and Diet Services® (Portland, Oregon), was used to analyze the 84 diet records collected. The revised Agricultural Handbook No. 8 series ([USDA], 1976-1987) was the primary data source of energy, protein, and vitamin B-6 content of foods. Special efforts were made to locate protein, amino acid, and vitamin B-6 data on the low-protein foods which were unique to the sample. The composition of brand name foods was obtained from manufacturers and distributors of specialty food products used by patients. As a result of these methods, there were no missing values for the energy, protein, phe, and vitamin B-6 content of the foods consumed by the subjects.

The mean intakes were compared to the 1980 RDAs (FBN, 1980) to determine adequacy of the diets. At the time of the data analyses, the 1989 RDAs (FNB, 1989) were not available. In order to more accurately evaluate individual intakes of energy and protein against the 1980 standards, we individualized the 1980 RDAs to the subjects' actual body weights, rather than use the age- and sex-specific median body weights which constitute the energy and protein RDAs (FNB, 1980). Vitamin B-6 intakes were compared to the 0.02 mg/g protein standard, rather than the total mg given in the RDA table which assumes an average protein intake (approximately two times the protein RDAs). This assumption would have been inappropriately high for the PKU patients.

3. Plasma measurements and analyses

A fasting venous blood sample was obtained from each subject two days after the 4-d dietary recording period. Blood was kept on ice until centrifugation for 15 min. at 4° C and the plasma was stored at -20° C until analysis. Samples were thawed and re-centrifuged prior to determination of PLP, total vitamin B-6 and alkaline phosphatase activity. PLP and total vitamin B-6 assays were performed in subdued light. Plasma PLP was measured enzymatically with tyrosine decarboxylase apoenzyme by the method of Chabner and Livingston (1970). Recoveries for PLP samples averaged 94±16%. Plasma total vitamin B-6 was

determined by a microbiological assay with Saccharomyces uvarum (ATCC No. 9080) as the assay organism (Miller & Edwards, 1981). The colorimetric method for alkaline phosphatase activity was used (Roy, 1970). The samples of individual patients and their respective siblings were assayed in the same run when possible; all samples were assayed in duplicate.

4. Urine measurements and analyses

Urine was collected into plastic bottles containing 15 mL toluene and 40 mL 2N HCL as preservatives for 4-PA measurement and for a separate study of phe metabolites, respectively. All bottles were transferred to home freezers at the end of each 24-h collection until transport to the laboratory 3-4 d later. Aliquots for each 24-h volume were frozen at -20° C until analysis.

Samples were thawed and centrifuged prior to urinary 4-PA determination by high-pressure liquid chromatography (Gregory & Kirk, 1979). An internal standard solution containing 0.1 ug/mL pyridoxamine and an external standard solution containing 20 ug/mL 4-PA were freshly prepared. Samples were injected onto an Econosil C-18, 10 u column, length of 25 cm and internal diameter of 4.6 mm (Alltech Assoc., Inc., Deerfield, IL) and water-jacketed at 25° C. The mobile phase consisted of 0.034 M potassium phosphate buffer (pH 2.2), 3.5% acetonitrile, and 5% methanol in

redistilled water (v:v:v) filtered through a 0.45 u filter and degassed by vacuum. 4-PA was detected by fluorescence (excitation 320 nm, emission 425 nm). All urine samples were titrated to a pH $\leq$ 2.0 and we used a peak area measurement because the peak height mode resulted in excessive (> 5%) inter-assay variation when there were differences in the adjusted pH of the samples.

Completeness of the 24-h urine collections was assessed by measuring creatinine using an automated modification (Technicon Autoanalyzer, Technicon Corp., Tarrytown, NY) of the Jaffé reaction (Pino et al., 1965). In addition, each family was interviewed regarding the collections. These procedures allowed us to determine with confidence two 24-h 4-PA excretion values (Sat, Sun) for each subject, with several exceptions. In particular, two patients with PKU reported missing one collection and, therefore, we used a single 24-h 4-PA excretion value for each of these subjects.

Lui et al. (1985) have described difficulties in the interpretation of the urinary 4-PA excretion on days when the vitamin B-6 intake differs. We calculated the percent of the vitamin B-6 intake excreted as 4-PA on Sat, using the 24-h vitamin B-6 intake averaged over Fri and Sat and, similarly, the percent of the vitamin B-6 intake excreted as 4-PA on Sun, using the 24-h vitamin B-6 intake averaged over Sat and Sun. The 4-PA excretion value reported, % 4-PA, is

the mean of these two values which were computed for each individual.

5. Calculations and statistical analyses

All data (diet, plasma, and urine variables) were entered into a UNIX HCX/UX mainframe computer and summarized using the Statistical Package for the Social Sciences ($SPSS_X$, 1986). There were no consistent differences in the mean daily intakes of the subjects across the 4-d recording period and, therefore, a 4-d mean value for energy, protein, phe, and vitamin B-6 was used to describe the intake of each subject. Individual patients were then classified, according to adherence patterns to the therapeutic diet, into three groups: strict-diet, relaxed-diet, and off-diet. This is consistent with the method chosen by the authors of a national survey (Schuett, Gurda & Yandow, 1983) to describe patients across treatment programs and at various ages. In the present study, the six siblings comprised a fourth group (control).

A one-way analysis of variance was used to compare group means on 10 selected variables and, thus, to answer two questions concerning vitamin B-6 status: 1) Were there significant differences between an "average" group of school-aged patients with PKU and a group of healthy school-aged children; and 2) Were there significant differences within subgroups of patients with PKU, who differed in their

adherence to the nutritional treatment? To account for the problem of multiplicity associated with multiple comparisons of means, the Bonferroni method (Guenther, 1964) was applied to the results of the ANOVA which tested for differences within subgroups of patients. The F ratio was, therefore, adjusted to account for the chance of error per experiment (10 tests), instead of per test. The Bonferroni adjustment was chosen because it is deliberately conservative; (Godfrey, 1985). In a practical sense, the p value we were willing to accept for differences within groups, based upon this adjustment, was $p \leq 0.005$.

The ANOVA assumes equality of variances in the different groups. Because subgroup cell sizes were as small as three (relaxed-diet group) and there were large standard deviations relative to some of the mean values, Cochran's test for homogeneity of variances was used to test for significance differences in group variances. For those variables which showed significant differences among group means after applying these conservative criteria, Scheffe's test of multiple comparisons was used to identify the subgroup means which differed. To answer the question regarding differences between an "average" group of school-aged patients and healthy children, the contrast procedure within $SPSS_X$ was used. Paired comparisons were made contrasting the combined patient group means, in which each subgroup mean was given equal weight, against the control

group means for each variable. For these contrasts, a $p \leq 0.05$ was considered to be statistically significant.

D. RESULTS

1. Subject characteristics

Physical and dietary characteristics of the four groups of subjects are described in Table IV.1. The strict-diet group (n=8) included those patients who reported mean intakes that were considered to be significantly restricted in phe (13-23 mg/kg body weight), whether or not their mean plasma phe values (854 $\pm$ 254 umol/L) were within the acceptable treatment range (120-605 umol/L) (Schuett, 1990). None of these subjects reported consuming protein-dense foods (e.g., meat, fish, poultry, legumes, eggs, dairy products) and they restricted their intakes of starches (e.g., grains, cereals, starchy vegetables). Three subjects were considered to be consuming a relaxed-diet, with phe intakes that ranged from 31-38 mg/kg body weight. Again, none of these subjects reported consuming protein-dense foods; phe intakes were increased because starchy foods were used more liberally. The remaining four subjects with PKU,considered off-diet, consumed some protein-dense foods with no restrictions on starches. The mean ages were

TABLE IV.1 Physical, dietary characteristics, and phenylalanine status of subjects[1]

group	n	age	sex		<--height-->		<---weight-->		<--phenylalanine-->	
		y	M	F	cm	%	kg	%	plasma umol/L	diet mg/d
control	6	9.5±3.3	4	2	138	58[2](25-95)	32	56(10-80)	70± 13[3]	3208±905
PKU patients										
strict-diet	8	9.9±1.4	5	3	137	47(10-80)	33	55(40-80)	854±252[4]	627±269[5]
relaxed-diet	3	16.1±1.0	1	2	168	65(50-95)	58	60(50-80)	1219±174	1967±868
off-diet	4	8.2±1.5	3	1	130	58(30->95)	33	59(35->95)	708±288	2154±696

[1] Mean ± SD (or with ranges given in parentheses).

[2] Growth percentiles based on the National Center for Health Statistics standards (Hamill et al., 1979).

[3] Reference mean and range ($\bar{x}$ ±2 SD) for plasma phenylalanine values of fasting, healthy children, 6-18 y = 42-74 umol/L (Armstrong & Stave, 1973).

[4] Acceptable treatment range of plasma phenylalanine for patients with PKU = 120-605 umol/L (Schuett, 1990).

[5] Recommended phenylalanine intakes for patients with PKU 7<11 y = 196-420 mg/d; 11<19 y = 248-858 mg/d (calculated from Acosta, 1989 and Matalon & Matalon, 1990).

considered off-diet, consumed some protein-dense foods with no restrictions on starches. The mean ages were similar across the groups (8.2-9.9 y), including the control group, with the exception of the three patients in the relaxed-diet group, who were older (16.1 y). The mean phe intake of the off-diet group was greater, compared to the other PKU patient groups, yet plasma phe values of three of the four off-diet patients were among the lowest of the sample. Greater residual PAH enzyme activities and/or greater individual physiological requirements for phe may have accounted for these differences. Height and weight percentiles were within the normal range of the National Center for Health Statistics standards (Hamill et al., 1979) for all subjects with the exception of one patient off-diet whose height and weight measurements were above the 95th percentile.

2. Nutrient intakes

Mean energy, protein, and vitamin B-6 intakes are compared to RDAs in Table IV.2. Reported mean energy intakes ranged from 93-100% of the individualized 1980 RDAs, suggesting that individualizing values was appropriate and that dietary records were carefully kept and analyzed. Mean protein intakes for each of the PKU patient groups were adequate and ranged from 106-155% of the individualized 1980 RDAs. The mean protein intakes for the overall PKU patient

TABLE III.2 Urinary urea nitrogen (N) excretion of subjects[1]

group	n	<----energy---->			<----protein---->			<----vitamin B-6----->		
		kcal/d	kcal/kg	%RDA	g/d	g/kg	%RDA	mg/d	mg/g protein	%RDA
control	6	2198±498	67±28	93[2]	69.2±17	2.2±0.8	182[2]	1.00±0.30	0.018±0	90[3]
PKU patients[4]	15				45.9±6*			1.97±0.42	0.044± 0.001	
strict-diet	8	2062±470	64±13	98	39.6±9*	1.2±0.3	115	1.86±0.62	0.046±0*	230
relaxed-diet	3	2381±414	39±6	100	46.7±6	0.8±0.0	106	2.44±1.32	0.051± 0.022*	255
off-diet	4	2149±512	75±14	100	51.5±12	1.8±0.5	155	1.62±0.32	0.034± 0.012	170

[1]Mean ± SD

[2]The energy and protein RDAs were individualized as described in the text.

[3]The vitamin B-6 RDA of 0.020 mg vitamin B-6 per g protein was used as described in the text

[4]Mean ± SD based on equally-weighted patient subgroup means.

*Significantly different from the control group ($p \leq 0.05$)

group and the strict-diet group were significantly less than the mean protein intake of the control group ($p \leq 0.05$). The mean vitamin B-6 intakes (mg/d) consistently showed an inverse pattern to the mean protein intakes (g/d) across all groups. The mean vitamin B-6:protein ratios for the overall PKU patient group, and the strict-diet and relaxed-diet groups were significantly greater than the ratio of the control group ($p \leq 0.05$) which was 90% of the RDA ratio of 0.02.

3. Plasma measurements

Mean values for fasting PLP, total vitamin B-6, and alkaline phosphatase activity in the plasma are presented in Table IV.3. The mean plasma PLP concentrations and the mean alkaline phosphatase activities of the overall PKU patient group and the relaxed-diet subgroup were significantly different from the respective control group means ($p \leq 0.05$) and were inversely related. The differences between the PLP:total B-6 ratio in the plasma of patients and controls reached statistical significance for the overall patient group only ($p \leq 0.05$).

4. Urine 4-PA measurements

The mean urinary 4-PA excretions are presented in Table IV.4, umol/24-h, and as a percent of vitamin B-6 intake. In

TABLE IV.3 Pyridoxal 5′-phosphate (PLP) values, total vitamin B-6 (TB6) concentration, and alkaline phosphatase (AP) activities of subjects[1]

group	n	PLP <--------nmol/L--------->	TB6	PLP:TB6 ratio	AP U/L
control	6	35.1±16	53.5±21	0.66±0.16	81.7±6
PKU patients[2]	15	94.4±35*	105.3±23	0.93±0.18	63.2±25*
strict-diet	8	77.2±38	96.9±42	0.78±0.14	78.7±16
relaxed-diet	3	134.8±85*	131.1±104	1.13±0.36	34.5±18*
off-diet	4	71.0±27	88.0±75	0.88±0.09	76.6±20

[1]Mean ± SD.

[2]Mean ± SD based on equally-weighted patient subgroup means.

*Significantly different than the control group ($p \leq 0.05$)

TABLE IV.4 Urinary 4-pyridoxic acid (4-PA) excretion of subjects[1]

group	n	B-6 intake <----------umol/24-h---------->	4-PA <----------umol/24-h---------->	4-PA excretion %[2]
control	6	7.8[3]±2.1	3.4±2.1	44.3±27
PKU patients[4]	15	11.4±2.0	4.1±1.5	35.4±8
strict-diet	8	10.7±3.0	5.5±4.1	45.0±25
relaxed-diet	3	13.6±8.4	4.3±1.5	31.7±7

[1]Mean ± SD

[2]4-PA excretion as a percent of vitamin B-6 intake (see Methods section):

$$\left[\left(\frac{\text{urine umol 4-PA Sat}}{\text{mean diet umol B-6 Fri, Sat}}\right) \cdot \left(\frac{\text{urine umol 4-PA Sun}}{\text{mean dietumol B-6 Sat, Sun}}\right) \div 2 \times 100\right]$$

[3]Vitamin B-6 intake expressed as umol/24-h (1 umol = 0.169 mg as pyridoxine).

[4]Mean ± SD based on equally-weighted patient subgroup means.

spite of greater mean vitamin B-6 intakes and lower mean protein intakes, both the relaxed-diet and off-diet groups had mean 4-PA excretions, as a percent of intake, which were below the control group mean excretion. Eight of 15 patients with PKU excreted 4-PA below the criterion for inadequate vitamin B-6 status in children (Lewis & Nunn, 1977).

The numbers and percentages of subjects meeting the suggested dietary or biochemical criteria for inadequate vitamin B-6 status are summarized in Table IV.5. Only one patient with PKU consumed a diet which was less than two-thirds of the RDA for vitamin B-6 and none of the patients had fasting plasma PLP values below 34 nmol/L. However, over one-half of the 15 PKU patients had urinary 4-PA excretions below 30% of their respective vitamin B-6 intakes.

E. DISCUSSION

The primary objective of this study was to assess the vitamin B-6 status of school-aged patients with PKU to determine if there were differences between school-aged patients and healthy children, and if there were differences within patients, grouped on the basis of their adherence to the nutritional treatment for PKU. There are only four other reports which provide reference values for school-aged subjects for the currently recommended vitamin B-6 status

TABLE IV.5 Number and percentage of subjects with inadequate vitamin B-6 status

		<-------diet indices[1]------->				<-biochemical indices[2]->			
		absolute vitamin B-6 intake < 2/3 RDA		vitamin B-6: protein ratio < 0.020		plasma PLP <34 nmol		urinary 4-PA excretion < 30%	
group	n	(n)	(%)	(n)	(%)	(n)	(%)	(n)	(%)
control	6	2	33	5	83	3	50	2	33
PKU patients	15	1	7	0	0	0	0	8	53
strict-diet	8	1	12	0	0	0	0	3	38
relaxed-diet	3	0	0	0	0	0	0	2	66
off-diet	4	0	0	0	0	0	0	3	75

[1]Diet criteria based on 1980 RDAs (FNB, 1980).

[2]Plasma PLP criterion suggested by Rose et al. (1976); urinary 4-PA excretion criterion suggested by Lewis and Nunn (1977).

indices. Two of these reports (Driskell & Moak, 1986; Lewis & Nunn, 1977) measured two or more indices, as is currently suggested for an adequate assessment of vitamin B-6 status in humans (Leklem & Reynolds, 1981). The present study is the first to describe a recommended triad of diet, plasma, and urine measurements of the vitamin in a free-living school-aged sample. There is only one other report of the vitamin B-6 status of treated patients with PKU (Anderson, 1986). The sample was younger (mean age 3.5 y with 9 of 12 patients less than two years of age). Presumably, these patients were still strictly adhering to the nutritional treatment.

1. Vitamin B-6 and protein intakes

The mean protein intake of the control group (182% RDA) was consistent with the estimated average protein intake for U.S. children 9-11 y, which is 193% of the 1980 RDA (Young & Pellett, 1987). The patients with PKU consumed diets that were increased in vitamin B-6 and reduced in protein, relative to those of the control siblings and for other treated patients with PKU as was also found by Anderson (1986). The strict diet for patients with PKU restricts natural dietary protein intakes to 5-15 g/d at school age (Acosta, 1989). Each of the eight patients consuming a strict diet regularly consumed phe-deficient medical foods to meet total protein needs. These products supplied an

average of 1.3 mg of vitamin B-6 (68% of the total mean vitamin B-6 intake for the strict-diet group) as PN•HCl, a form of the vitamin with a high bioavailability. Only one patient in each of the other two patient groups (relaxed- and off-diet) reported the use of medical foods and both consumed more of their total protein from natural foods, compared to the eight patients consuming a strict-diet. The natural foods that contributed the greatest amounts of vitamin B-6 to the PKU diet were fortified breakfast cereals (which provide vitamin B-6 as PN•HCL) and bananas, a unique plant food which provides 98% of its B-6 in a free form which has a high bioavailability (Kabir, Leklem, & Miller, 1983). These data suggest a high quantity and quality (bioavailability) of vitamin B-6 in the foods which comprise the intake of patients with PKU.

2. Plasma PLP values

The patients with PKU in the present study had high mean fasting plasma PLP values relative to their non-affected siblings, consistent with the report by Anderson (1986) which examined younger patients. Fasting plasma PLP is considered to be a sensitive and reliable indicator of the vitamin B-6 status of healthy adults (Li & Lumeng, 1981). There are two reports of PLP values in school-aged subjects. Reinken (1972) studied 26 children, aged 4-13 y, and reported a mean plasma PLP value of 35.2 nmol/L.

Unfortunately, the dietary conditions (e.g. whether the subjects were fasting) were not provided. Driskell and Moak (1986) studied slightly older subjects (12, 14 and 16 y females) and reported mean fasting plasma PLP values for the different age groups which ranged from 42.1-48.1 nmol/L. Rose et al. (1976) suggested a plasma PLP value below 34 nmol/L as one criterion of inadequate vitamin B-6 status.

3. Plasma total vitamin B-6 values, alkaline phosphatase activity and urine 4-PA excretion

We also observed high plasma PLP:total vitamin B-6 ratios in PKU patients compared to the control group values. The control group had the lowest mean plasma PLP:total B-6 ratio (0.66), a value within the range generally observed for adults in our laboratory (0.60-0.80). Compared to the control group, the ratios for each of the PKU patient subgroups were higher (0.78-1.13); 10 of the 15 patients had ratios which were more than 0.80. The mean ratio for the patients consuming a relaxed diet (above 1.00) was due to one individual with a 1.55 ratio. Multiple assays were performed for both PLP and total vitamin B-6 for this patient which replicated these results. The plasma PLP:total B-6 ratios of the other two patients in the relaxed-diet group were between 0.90 and 1.00.

In the two groups with the most extreme mean plasma PLP values, we observed an inverse relationship between these values and their respective mean alkaline phosphatase

activities. The control group had the lowest mean plasma PLP value and the highest mean alkaline phosphatase activity, while the relaxed-diet group had the highest mean plasma PLP value and the lowest mean alkaline phosphatase activity.

In spite of adequate status as suggested by the vitamin B-6 intakes and the plasma PLP concentrations, urinary 4-PA excretion was lower in most of the patients compared to controls. There is only one published report of 4-PA excretion in children (Lewis & Nunn, 1977). In this study of six school-aged subjects (6-9 y), the mean 4-PA excretion was 4.01 $\pm$ 22 umol/24-h, compared to a mean vitamin B-6 intake of 7.96 $\pm$ 33 umol/24-h (57% excretion). The control group in the present study had values consistent with this report. In spite of a mean intake which was nearly twice that of the control group, the relaxed-diet group had a mean 4-PA excretion which was approximately 30% of intake. Lewis and Nunn (1977) suggested a 4-PA excretion below 1.78 umol/24-h or below 30% of intake as criteria of inadequate vitamin B-6 status in children. By either of these criteria, one-third of the control group had inadequate vitamin B-6 status, which is consistent with the report by Lewis and Nunn for healthy subjects within this age range. Presumably because the vitamin B-6 intakes were greater for the PKU patient groups, fewer patients met the 1.78 umol/24-h criterion (three of 15 subjects) than when we examined

excretion as a percent of intake. Eight of 15 subjects excreted 4-PA below the 30% of vitamin B-6 intake criterion. Using the most conservative approach, we applied the percent intake criterion to the subjects of this study.

The present study demonstrates that high levels of plasma PLP are common in school-aged patients with PKU, most of whom are not in acceptable biochemical control. The question arises as to why the circulating PLP levels are high. We have discussed the potential impact of the combination of dietary factors. Alternatively, a reduced turnover of PLP could lead to high plasma PLP levels. The low plasma alkaline phosphatase activities and urine 4-PA excretions, particularly in those patients consuming a relaxed-diet, who had the highest absolute plasma PLP values and plasma PLP:total vitamin B-6 ratios, support a reduced PLP hydrolysis via alkaline phosphatase. Patients in the relaxed-diet group also had the highest plasma phe levels and phe is known to inhibit alkaline phosphatase activity <u>in vitro</u> (Whyte et al., 1985). Circulating PLP concentrations are markedly increased (214-3839 nmol/L) in patients with hypophosphatasia, an inborn error in which activity of the tissue nonspecific isoenzyme of alkaline phosphatase is deficient (Whyte et al., 1985).

The question then arises whether there are clinical implications of the high circulating PLP concentrations. The levels in patients with hypophosphatasia are much higher

than the levels we observed in our patients with PKU. In hypophosphatasia, vitamin B-6 nutrition does not appear to be affected, as evidenced by the lack of clinical correlations with vitamin B-6 deficiency or toxicity states and a normal urinary 4-PA excretion (Whyte et al., 1985). In contrast, there are some similar neurological characteristics in untreated or poorly managed PKU and in vitamin B-6 deficiency or toxicity states (e.g. abnormal EEGs, seizures, convulsions). In addition, we observed lower than anticipated quantities of 4-PA excreted in the urine of some of the patients, particularly those who were no longer consuming a strict diet. These observations lend further support to speculate that vitamin B-6 metabolism may be altered in patients who are not adhering strictly to the nutritional treatment. Blood samples are currently being collected in younger patients who are still maintaining acceptable biochemical control of PKU. Future studies would ideally include one or more functional tests of vitamin B-6 status (e.g., urinary excretion of tryptophan metabolites or aminotransferase activities in erythrocytes). Measures of these along with other forms of the vitamin (e.g., pyridoxal) in the plasma could help to understand vitamin B-6 metabolism in PKU, to further evaluate status, and to ultimately determine the appropriate level of vitamin B-6 in the medical foods which are used in the nutritional treatment of PKU.

SUMMARY

PKU, in its treated state, provides a unique metabolic and nutritional situation for a study of energy, protein, and amino acid intakes and for vitamin B-6 status. The treatment for PKU is a therapeutic diet, consisting of plant-based foods which are relatively low in energy and high in vitamin B-6 compared to protein and amino acids. The nutritional treatment, when strictly adhered to, reduces plasma phenylalanine (phe) and its metabolites in plasma, increases other amino acids in plasma, and supplies high-quality protein as L-amino acids and vitamin B-6 as PN•HCl. At school-age, strict adherence to the treatment is less consistent across patients and, therefore, the consequent nutritional and biochemical factors that affect plasma amino acids and vitamin B-6 metabolism and status become less homogeneous within treated patients.

The purpose of this study was to assess the vitamin B-6 status, energy and protein intakes, and amino acids in the diets and plasma of school-aged patients with phenyl (PKU). Fifteen patients with PKU, 7-17 years of age, and six non-affected healthy siblings, 6-14 years of age, served as subjects. A four-day dietary record, two 24-h urine collections, and a fasting blood sample were obtained from each subject. Descriptive statistics were employed to summarize the data and to characterize subjects on the following variables: intakes of energy, protein, amino

acids, and vitamin B-6; plasma concentrations of amino acids, pyridoxal 5'-phosphate (PLP), total vitamin B-6, and alkaline phosphatase (AP) activities; and urinary excretions of creatinine, urea nitrogen, and 4-pyridoxic acid (4-PA). Patients were categorized into three groups, based on adherence to the nutritional treatment: strict-diet, relaxed-diet, and off-diet. Inferential statistics were used to answer the research questions: 1) Were there significant differences in vitamin B-6 status between an overall group of patients and controls; and 2) Were there significant differences in vitamin B-6 status within the subgroups of patients?

Mean energy and protein intakes of the patients met the Recommended Dietary Allowances (RDAs). However, natural low-protein foods contributed more to phe intakes than recommended and the medical foods were used in less than recommended dosages. The patients consuming a strict-diet had dispensable amino acid intake patterns which deviated from the control group intake patterns. Mean plasma amino acid concentrations of each of the patient subgroups were below the reference range for healthy children (mean $\pm$ 1 SD) for some of the indispensable amino acids (leucine, tyrosine, threonine, and valine), in spite of intakes which exceeded estimated requirements for healthy children. The mean plasma glycine values for each of the patient subgroups were above the reference range for glycine. All patients

had phenyl values which exceeded acceptable treatment levels. These data suggested that the current approach to treatment at school age needs improvement.

The overall patient group and a subgroup of patients on a relaxed-diet had significantly lower protein intakes and significantly higher dietary vitamin B-6:protein ratios than the control group ($p \leq 0.05$). Mean plasma PLP concentrations and plasma PLP:total vitamin B-6 ratios were significantly higher for these patient groups and plasma AP activities were lower compared to controls. In spite of these findings, the mean percent of vitamin B-6 intake excreted as 4-PA was lower for the overall PKU patient group; over 50% of the patients excreted quantities which met the criterion for inadequate vitamin B-6 status. These findings suggest that treated patients with PKU, at school age, may have an altered metabolism of vitamin B-6. The lowered excretion of the primary end-product of vitamin B-6 metabolism, 4-PA, in spite of a dietary intake pattern which should favor a lowered need to retain the vitamin (high in vitamin B-6 and low in protein), and increased concentrations of PLP in plasma, suggest a problem with conversion of PLP to 4-PA. The data support further research aimed at explaining these findings and determining their functional significance.

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APPENDIX

Appendix Table 1. Energy intakes of subjects from natural foods

subject group and no.	day 1	day 2	day 3	day 4	mean ± SD
	kcal/day				
strict-diet					
1. BS3	2153	2472	2505	2476	2402 ± 166
2. JY6	1615	1160	1234	1143	1288 ± 222
3. MS19	1029	1094	1464	1055	1160 ± 204
4. AW4	1645	1918	1846	1854	1314 ± 287
5. RR5	1144	1549	1429	1797	1480 ± 271
6. TM21	1471	853	1415	1534	1318 ± 314
7. MB4	2143	2026	1838	2491	2124 ± 275
8. MS15	1434	1560	1049	826	1217 ± 340
mean ± SD	1579 ± 410	1579 ± 541	1597 ± 456	1647 ± 627	1538 ± 463
relaxed-diet					
9. CH17	1901	1394	2174	2020	1872 ± 338
10. WH18	1504	3121	2230	1894	2187 ± 690
11. KH22	2512	2816	2140	2879	2856 ± 337
mean ± SD	1972 ± 508	2444 ± 922	2181 ± 45	2263 ± 533	2305 ± 502
off-diet					
12. SM14	1224	1551	1599	1552	1482 ± 173
13. MH12	4813	2045	2325	2226	2854 ± 1315
14. SL23	1493	1583	2322	1329	1682 ± 440
15. BC10	2386	1777	1807	2739	2177 ± 468
mean ± SD	2480 ± 1636	1739 ± 227	2013 ± 368	1962 ± 643	2049 ± 611
combined PKU					
mean ± SD	1898 ± 922	1810 ± 612	1825 ± 447	1854 ± 624	1827 ± 578
control					
16. SY7	1476	1785	1599	1187	1512 ± 251
17. PJY8	1841	2942	1671	1615	2010 ± 627
18. KC11	1856	1871	1489	2316	1883 ± 338
19. SS20	1815	1986	1233	1949	1746 ± 350
20. ZB5	3317	3341	2206	1607	2618 ± 857
21. MH13	2645	2215	1801	3109	2442 ± 562
mean ± SD	2158 ± 686	2357 ± 637	1666 ± 326	1964 ± 676	2198 ± 498

Appendix Table 2. Energy intakes of subjects from medical foods

subject group and no.	day 1	day 2	day 3	day 4	mean ± SD
	kcal/day				
strict-diet					
1. BS3	720	401	687	720	632 ± 155
2. JY6	720	720	720	720	720 ± 0
3. MS19	600	600	710	600	628 ± 55
4. AW4	121	118	118	118	119 ± 2
5. RR5	480	480	480	480	480 ± 0
6. TM21	720	720	0	720	540 ± 360
7. MB4	400	0	400	400	300 ± 200
8. MS15	291	267	266	284	277 ± 12
mean ± SD	506 ± 224	413 ± 269	423 ± 278	505 ± 226	462 ± 210
relaxed-diet					
9. CH17	240	0	0	60	75 ± 114
10. WH18	0	0	0	0	0
11. KH22	0	0	0	0	0
mean ± SD	80 ± 138	0	0	20 ± 35	25 ± 43
off-diet					
12. SM14	400	400	400	400	400 ± 0
13. MH12	0	0	0	0	0
14. SL23	0	0	0	0	0
15. BC10	0	0	0	0	0
mean ± SD	100 ± 200	100 ± 200	100 ± 200	100 ± 200	100 ± 200
combined PKU					
mean ± SD	313 ± 287	210 ± 280	252 ± 290	300 ± 294	278 ± 270
control					
16. SY7	0	0	0	0	0
17. PJY8	0	0	0	0	0
18. KC11	0	0	0	0	0
19. SS20	0	0	0	0	0
20. ZB5	0	0	0	0	0
21. MH13	0	0	0	0	0
mean ± SD	0	0	0	0	0

Appendix Table 3. Total energy intakes of subjects					
subject group and no.	day 1	day 2	day 3	day 4	mean ± SD
	kcal/day				
strict-diet					
1. BS3	2873	2873	3192	3196	3034±185
2. JY6	2335	1880	1954	1863	2008±222
3. MS19	1629	1694	2174	1655	1788±259
4. AW4	1766	2036	1964	1972	1934±117
5. RR5	1624	2029	1909	2277	1960±271
6. TM21	2191	1573	1415	2254	1858±426
7. MB4	2543	2026	2238	2891	2424±376
8. MS15	1725	1827	1315	1110	1494±338
mean ± SD	2085±472	1992±394	2020±578	2152±666	2062±470
relaxed-diet					
9. CH17	2141	1394	2174	2080	2100±435
10. WH18	1504	3121	2230	1894	2187±690
11. KH22	2512	2816	2140	2874	2856±337
mean ± SD	2052±510	2081±999	2181±45	2283±520	2381±414
off-diet					
12. SM14	1624	1951	1999	1952	1882±173
13. MH12	4818	2045	2325	2226	2854±1315
14. SL23	1493	1583	2322	1329	1682±440
15. BC10	2386	1777	1807	2739	2177±512
mean ± SD	2310±1313	1839±204	2113±255	2062±587	2149±512
combined PKU					
mean ± SD	2211±844	1839±204	2113±255	2062±587	2149±512
control					
16. SY7	1476	1785	1599	1187	1512±251
17. PJY8	1841	2942	1671	1615	2010±627
18. KC11	1856	1871	1489	2316	1883±338
19. SS20	1815	1986	1233	1949	1746±350
20. ZB5	3317	3341	2206	1607	2618±857
21. MH13	2645	2215	1801	3109	2442±562
mean ± SD	2158±686	2357±637	1666±326	1964±676	2198±498

Appendix Table 4. Protein intakes of subjects from natural foods					
subject group and no.	day 1	day 2	day 3	day 4	mean ± SD
	g/day				
strict-diet					
1. BS3	10	8	13	12	11±2
2. JY6	19	9	10	14	13±4
3. M519	5	9	11	11	9±3
4. AW4	31	24	17	11	20±9
5. RR5	14	14	15	30	18±8
6. TM21	18	6	16	16	14±5
7. MB4	16	13	16	16	15±2
8. MS15	15	17	11	7	12±4
mean ± SD	16±8	12±6	14±3	15±7	14±4
relaxed-diet					
9. CH17	27	32	55	51	41±14
10. WH18	19	82	29	36	42±28
11. KH22	45	66	28	72	53±20
mean ± SD	30±13	60±26	37±15	53±18	45±7
off-diet					
12. SM14	23	29	29	50	33±12
13. MH12	91	20	78	63	63±31
14. SL23	35	28	36	39	34±5
15. BC10	47	43	74	59	56±14
mean ± SD	58±30	30±12	63±23	54±13	46±15
combined PKU					
mean ± SD	28±21	27±22	29±22	32±22	29±18
control					
16. SY7	53	67	56	51	57±7
17. RJY8	83	131	68	71	88±29
18. KC11	43	67	69	55	58±12
19. SS20	58	56	84	60	64±13
20. ZB5	130	112	76	45	91±38
21. MH13	54	30	52	82	54±21
mean ± SD	70±32	77±37	68±12	61±14	69±16

Appendix Table 5. Protein intakes of subjects from medical foods

subject group and no.	day 1	day 2	day 3	day 4	mean ± SD
	g/day				
strict-diet					
1. BS3	36	36	36	36	36±0
2. JY6	36	36	36	36	36±0
3. M519	30	30	43	30	33±6
4. AW4	29	29	29	28	29±0
5. RR5	24	24	24	24	24±0
6. TM21	22	23	0	20	16±11
7. MB4	20	0	20	20	15±10
8. MS15	14	14	13	15	14±1
mean ± SD	26±8	24±12	25±14	26±8	25±9
relaxed-diet					
9. CH17	12	0	0	4	4±6
10. WH18	0	0	0	0	0
11. KH22	0	0	0	0	0
mean ± SD	4±7	0	0	1.3±2	1.3±2
off-diet					
12. SM14	20	20	20	20	20±0
13. MH12	0	0	0	0	0
14. SL23	0	0	0	0	0
15. BC10	0	0	0	0	0
mean ± SD	5±10	5±10	5±10	5±10	5±10
combined PKU					
mean ± SD	22±11	18±15	24±13	23±10	23±11
control					
16. SY7	0	0	0	0	0
17. RJY8	0	0	0	0	0
18. KC11	0	0	0	0	0
19. SS20	0	0	0	0	0
20. ZB5	0	0	0	0	0
21. MH13	0	0	0	0	0
mean ± SD	0	0	0	0	0

Appendix Table 6. Total protein intakes of subjects					
subject group and no.	day 1	day 2	day 3	day 4	mean ± SD
	g/day				
strict-diet					
1. BS3	46	44	49	48	47±2
2. JY6	55	45	46	50	49±4
3. M519	35	39	54	41	42±8
4. AW4	60	53	46	39	50±9
5. RR5	38	38	39	54	42±8
6. TM21	40	29	16	39	31±11
7. MB4	36	13	36	36	30±12
8. MS15	29	31	24	22	26±4
mean ± SD	42±11	36±12	39±13	41±10	36±10
relaxed-diet					
9. CH17	39	32	55	55	45±12
10. WH18	19	82	29	36	42±28
11. KH22	45	66	28	53	53±20
mean ± SD	34±14	60±26	37±15	50±7	47±6
off-diet					
12. SM14	43	49	49	70	53±12
13. MH12	91	20	78	63	63±31
14. SL23	35	28	36	39	34±5
15. BC10	47	43	74	59	56±14
mean ± SD	53±18	35±13	59±20	58±13	52±12
combined PKU					
mean ± SD	44±16	41±17	44±17	47±12	44±10
control					
16. SY7	53	67	56	51	57±7
17. RJY8	83	131	68	71	88±29
18. KC11	43	67	69	55	58±12
19. SS20	58	56	84	60	64±13
20. ZB5	130	112	76	45	91±38
21. MH13	54	30	52	82	54±21
mean ± SD	70±32	77±37	68±12	61±14	69±16

Appendix Table 7. Carbohydrate intakes of subjects from natural foods					
subject group and no.	day 1	day 2	day 3	day 4	mean ± SD
	g/day				
strict-diet					
1. BS3	243	356	288	271	290±48
2. JY6	308	269	263	257	274±23
3. MS19	167	190	161	162	170±14
4. AW4	231	322	337	252	286±52
5. RR5	208	252	170	283	228±50
6. TM21	244	161	264	202	218±46
7. MB4	357	310	257	411	334±66
8. MS15	237	268	171	173	212±48
mean ± SD	**249±59**	**266±66**	**239±64**	**251±79**	**252±53**
relaxed-diet					
9. CH17	360	278	369	333	335±41
10. WH18	315	492	395	283	371±93
11. KH22	386	464	337	471	414±64
mean ± SD	**354±36**	**411±116**	**367±29**	**362±97**	**373±40**
off-diet					
12. SM14	261	246	231	237	244±13
13. MH12	777	376	305	326	446±223
14. SL23	265	286	357	224	283±56
15. BC10	429	296	259	331	329±73
mean ± SD	**433±242**	**301±54**	**288±55**	**280±57**	**326±88**
combined PKU					
mean ± SD	**319±146**	**304±90**	**278±74**	**281±84**	**296±77**
control					
16. SY7	187	246	272	206	228±38
17. PJY8	262	364	242	223	273±63
18. KC11	334	313	211	315	293±56
19. SS20	226	276	107	239	212±73
20. ZB5	451	441	252	229	343±119
21. MH13	486	430	273	449	410±94
mean ± SD	**324±122**	**345±80**	**226±63**	**277±92**	**293±74**

Appendix Table 8. Carbohydrate intakes of subjects from medical foods

subject group and no.	day 1	day 2	day 3	day 4	mean ± SD
	g/day				
strict-diet					
1. BS3	117	117	117	117	117±0
2. JY6	117	117	117	117	117±0
3. MS19	97	97	99	102	92±28
4. AW4	1	1	1	1	1±0
5. RR5	78	77	171	78	95±33
6. TM21	91	92	0	91	89±37
7. MB4	65	0	65	64	48±32
8. MS15	47	43	43	46	45±2
mean ± SD	77±39	68±48	77±60	77±40	76±40
relaxed-diet					
9. CH17	39	0	0	10	12±18
10. WH18	0	0	0	0	0
11. KH22	0	0	0	0	0
mean ± SD	13±22	0	0	3±6	4±6
off-diet					
12. SM14	65	65	65	65	65±0
13. MH12	0	0	0	0	0
14. SL23	0	0	0	0	0
15. BC10	0	0	0	0	0
mean ± SD	16±32	16±32	16±32	16±32	16±32
combined PKU					
mean ± SD	48±46	41±48	45±57	43±47	45±41
control					
16. SY7	0	0	0	0	0
17. PJY8	0	0	0	0	0
18. KC11	0	0	0	0	0
19. SS20	0	0	0	0	0
20. ZB5	0	0	0	0	0
21. MH13	0	0	0	0	0
mean ± SD	0	0	0	0	0

Appendix Table 9. Total carbohydrate intakes of subjects

subject group and no.	day 1	day 2	day 3	day 4	mean ± SD
	g/day				
strict-diet					
1. BS3	360	473	405	388	406 ± 48
2. JY6	425	386	380	374	391 ± 23
3. MS19	264	287	260	264	269 ± 12
4. AW4	232	323	338	253	286 ± 52
5. RR5	286	329	341	361	329 ± 32
6. TM21	335	253	264	293	286 ± 80
7. MB4	422	310	322	475	382 ± 80
8. MS15	284	311	214	219	257 ± 48
mean ± SD	326 ± 72	334 ± 68	316 ± 65	328 ± 86	326 ± 60
relaxed-diet					
9. CH17	399	278	369	343	347 ± 52
10. WH18	315	492	395	283	371 ± 93
11. KH22	386	464	337	471	414 ± 64
mean ± SD	367 ± 45	411 ± 116	367 ± 29	366 ± 96	377 ± 34
off-diet					
12. SM14	326	311	296	302	309 ± 13
13. MH12	777	376	305	326	446 ± 227
14. SL23	265	286	357	224	283 ± 56
15. BC10	429	296	259	331	329 ± 73
mean ± SD	449 ± 229	317 ± 40	304 ± 40	296 ± 49	342 ± 72
combined PKU					
mean ± SD	367 ± 130	345 ± 76	323 ± 56	327 ± 78	340 ± 59
control					
16. SY7	187	246	272	206	228 ± 38
17. PJY8	262	364	242	223	273 ± 63
18. KC11	334	313	211	315	293 ± 56
19. SS20	226	276	107	239	212 ± 73
20. ZB5	451	441	252	229	343 ± 119
21. MH13	486	430	273	449	410 ± 94
mean ± SD	324 ± 122	345 ± 80	226 ± 63	277 ± 92	293 ± 74

Appendix Table 10. Indispensable amino acid intakes of subjects from natural foods compared to estimated school-aged requirements											
subject group and no.	his[1] (19)	ile (28)	leu (44)	lys (44)	met (-)	total s - containing (22)	phe (-)	total aromatic (22)	thr (28)	trp (9)	val (25)
	mg/kg body weight										
strict-diet											
1. BS3	8 ± 2[2]	15 ± 3	31 ± 10	17 ± 4	6 ± 1	11 ± 2[3]	16 ± 3	27 ± 6[4]	14 ± 3	4 ± 0	18 ± 3
2. JY6	8 ± 3	14 ± 6	26 ± 10	14 ± 6	8 ± 4	14 ± 6	16 ± 6	27 ± 10	13 ± 5	4 ± 2	19 ± 8
3. MS19	8 ± 3	19 ± 6	26 ± 10	18 ± 6	5 ± 2	11 ± 4	16 ± 5	27 ± 9	14 ± 5	4 ± 1	17 ± 6
4. AW4	11 ± 6	23 ± 12	37 ± 20	24 ± 15	8 ± 4	16 ± 7	24 ± 11	40 ± 19	19 ± 10	6 ± 4	24 ± 12
5. RR5	12 ± 6	24 ± 10	37 ± 17	17 ± 6	8 ± 4	19 ± 9	23 ± 11	39 ± 18	17 ± 7	6 ± 3	24 ± 11
6. TM21	8 ± 3	14 ± 6	27 ± 11	12 ± 6	9 ± 7	16 ± 10	16 ± 7	26 ± 11	12 ± 6	4 ± 2	17 ± 7
7. M84	11 ± 2	15 ± 2	30 ± 4	18 ± 3	10 ± 2	17 ± 2	20 ± 2	33 ± 3	15 ± 3	7 ± 2	22 ± 2
8. MS15	12 ± 5	21 ± 8	39 ± 17	22 ± 9	9 ± 4	18 ± 9	22 ± 9	39 ± 16	19 ± 6	6 ± 2	25 ± 10
mean ± SD	10 ± 2	18 ± 4	32 ± 5	18 ± 4	8 ± 2	15 ± 3	19 ± 4	32 ± 6	15 ± 3	5 ± 1	21 ± 4
relaxed-diet											
9. CH17	18 ± 6	31 ± 10	53 ± 19	40 ± 15	14 ± 5	24 ± 8	32 ± 10	55 ± 18	27 ± 9	9 ± 2	36 ± 11
10. WH18	19 ± 12	33 ± 22	57 ± 42	43 ± 26	14 ± 12	25 ± 9	34 ± 24	59 ± 41	29 ± 19	10 ± 6	41 ± 28
11. KH22	22 ± 11	39 ± 16	61 ± 27	53 ± 25	17 ± 9	30 ± 15	39 ± 15	69 ± 27	34 ± 13	12 ± 5	48 ± 18
mean ± SD	20 ± 2	34 ± 4	57 ± 4	45 ± 7	15 ± 2	26 ± 3	35 ± 4	61 ± 7	30 ± 3	10 ± 2	42 ± 6
off-diet											
12. SM14	33 ± 16	62 ± 26	109 ± 47	68 ± 36	26 ± 12	49 ± 23	66 ± 27	114 ± 48	50 ± 18	17 ± 6	74 ± 28
13. MH12	33 ± 16	58 ± 29	101 ± 48	74 ± 38	27 ± 13	44 ± 19	58 ± 27	103 ± 50	48 ± 23	15 ± 7	68 ± 33
14. SL23	39 ± 4	68 ± 6	114 ± 9	80 ± 12	28 ± 4	53 ± 5	65 ± 5	115 ± 10	56 ± 5	18 ± 2	75 ± 6
15. 8C10	60 ± 18	107 ± 34	179 ± 52	144 ± 62	52 ± 18	86 ± 23	101 ± 25	182 ± 52	88 ± 28	27 ± 7	123 ± 36
mean ± SD	41 ± 13	74 ± 22	126 ± 36	92 ± 35	33 ± 12	58 ± 19	72 ± 19	128 ± 36	60 ± 19	19 ± 5	85 ± 26

Appendix Table 10. (continued)

subject group and no.	his[1] (19)	ile (28)	leu (44)	lys (44)	met (-)	total s - containing (22)	phe (-)	total aromatic (22)	thr (28)	trp (9)	val (25)
	mg/kg body weight										
combined PKU											
mean ± SD	20±15	36±27	62±44	43±36	16±12	29±21	36±25	64±46	30±22	10±7	42±31
control											
16. SY7	83±12	158±19	260±22	206±34	73±10	115±18	141±13	263±22	122±16	37±4	172±14
17. PJY8	47±17	88±29	144±42	115±52	40±16	65±24	78±22	146±41	68±25	21±6	98±27
18. KC11	93±23	166±41	279±68	231±71	82±22	131±28	156±34	283±67	138±34	42±10	190±45
19. SS20	52±10	86±22	144±27	133±31	43±11	65±18	79±13	142±31	76±15	23±3	98±18
20. ZB5	43±18	74±30	130±58	95±40	34±15	56±23	73±31	130±55	62±26	23±10	86±39
21. MH13	50±23	91±42	154±75	109±56	40±20	66±30	91±39	163±72	72±33	24±10	106±49
mean ± SD	61±21	110±40	185±66	148±56	52±20	83±32	103±36	188±67	90±32	28±9	125±44

[1] Requirement pattern was calculated from amino acid requirements divided by the recommended allowance of reference protein. Because protein allowance (in g/kg) is 0.99 for children 10-12 years of age, amino acid requirements are equivalent, whether expressed as mg/kg body weight or mg/g dietary protein (WHO, 1985; FNB, 1989). Dashed lines indicate no standard available.

[2] Mean ± standard deviation computed from 4-day dietary records.

[3] Includes methionine + cystine.

[4] Includes phenylalanaine + tyrosine.

Appendix Table 11. Total indispenseble amino acid intakes of subjects compered to estimeted school-aged requirements											
subject group and no.	his[1] (19)	ile (28)	leu (44)	lys (44)	met (-)	total s - conteining (22)	phe (-)	total aromatic (22)	thr (28)	trp (9)	val (25)
	mg/kg body weight										
strict-diet											
1. 8S3	33 ± 2[2]	74 ± 3	122 ± 10	117 ± 4	40 ± 1	64 ± 2[3]	16 ± 3	77 ± 6[4]	64 ± 3	19 ± 0	85 ± 3
2. JY6	37 ± 3	82 ± 6	132 ± 10	130 ± 6	47 ± 4	74 ± 6	16 ± 6	85 ± 10	71 ± 5	22 ± 2	97 ± 8
3. MS19	35 ± 3	82 ± 6	124 ± 9	125 ± 6	42 ± 2	67 ± 4	16 ± 4	81 ± 9	68 ± 5	20 ± 1	89 ± 6
4. AW4	29 ± 6	69 ± 12	113 ± 21	78 ± 16	26 ± 4	52 ± 7	24 ± 11	100 ± 20	55 ± 10	20 ± 4	78 ± 13
5. RR5	27 ± 6	56 ± 9	93 ± 17	79 ± 6	29 ± 4	51 ± 9	23 ± 11	70 ± 18	48 ± 7	15 ± 3	66 ± 11
6. TM21	17 ± 6	40 ± 16	72 ± 29	64 ± 29	22 ± 10	29 ± 11	18 ± 7	55 ± 20	38 ± 14	10 ± 4	56 ± 23
7. MB4	21 ± 8	36 ± 17	64 ± 24	55 ± 27	22 ± 9	36 ± 14	20 ± 2	52 ± 12	34 ± 15	13 ± 2	48 ± 18
8. MS15	25 ± 5	51 ± 8	86 ± 17	74 ± 8	26 ± 4	45 ± 9	22 ± 9	64 ± 16	44 ± 6	13 ± 2	60 ± 10
meen ± SD	28 ± 7	61 ± 18	101 ± 25	90 ± 29	32 ± 10	52 ± 15	19 ± 3	73 ± 16	53 ± 14	17 ± 4	72 ± 18
relexed-diet											
9. CH17	20 ± 6	37 ± 7	62 ± 18	50 ± 7	17 ± 5	29 ± 9	31 ± 14	59 ± 20	32 ± 5	10 ± 2	44 ± 9
10. WH18	19 ± 12	33 ± 22	57 ± 42	43 ± 26	14 ± 12	25 ± 19	34 ± 24	59 ± 41	29 ± 19	10 ± 6	41 ± 28
11. KH22	22 ± 11	39 ± 16	61 ± 27	53 ± 25	17 ± 9	30 ± 15	39 ± 15	69 ± 27	34 ± 13	12 ± 5	48 ± 18
mean ± SD	20 ± 2	36 ± 3	60 ± 3	49 ± 5	16 ± 2	28 ± 3	35 ± 4	62 ± 6	32 ± 2	11 ± 1	44 ± 4
off-diet											
12. SM14	53 ± 16	109 ± 26	181 ± 47	147 ± 36	52 ± 12	91 ± 23	66 ± 27	154 ± 48	90 ± 18	29 ± 6	127 ± 28
13. MH12	33 ± 16	58 ± 29	101 ± 48	74 ± 38	27 ± 13	44 ± 19	58 ± 27	103 ± 50	48 ± 23	15 ± 7	68 ± 33
14. SL23	39 ± 4	68 ± 6	114 ± 9	80 ± 12	28 ± 4	53 ± 5	65 ± 5	115 ± 10	56 ± 5	18 ± 2	75 ± 6
15. 8C10	60 ± 18	107 ± 34	179 ± 52	144 ± 62	52 ± 18	86 ± 23	101 ± 25	182 ± 52	88 ± 28	27 ± 7	123 ± 36
mean ± SD	46 ± 12	86 ± 26	144 ± 42	111 ± 40	40 ± 14	68 ± 23	72 ± 19	138 ± 36	70 ± 22	22 ± 3	98 ± 31

Appendix Table 11. (continued)

subject group and no.	his[1] (19)	ile (28)	leu (44)	lys (44)	met (-)	total s - containing (22)	phe (-)	total aromatic (22)	thr (28)	trp (9)	val (25)
	mg/kg body weight										
combined PKU											
mean ± SD	**35 ± 16**	**63 ± 25**	**104 ± 40**	**88 ± 35**	**31 ± 13**	**52 ± 21**	**37 ± 25**	**88 ± 38**	**53 ± 20**	**17 ± 6**	**74 ± 27**
control											
16. SY7	83 ± 12	157 ± 19	260 ± 22	206 ± 34	73 ± 10	115 ± 18	141 ± 13	263 ± 22	122 ± 16	37 ± 4	172 ± 14
17. PJY8	47 ± 17	88 ± 29	144 ± 42	115 ± 52	40 ± 16	65 ± 24	78 ± 22	146 ± 41	68 ± 25	21 ± 6	98 ± 27
18. KC11	93 ± 23	166 ± 41	279 ± 68	231 ± 71	87 ± 22	131 ± 28	156 ± 34	283 ± 67	138 ± 34	42 ± 10	190 ± 45
19. SS20	52 ± 0	86 ± 22	144 ± 27	133 ± 31	43 ± 11	65 ± 18	79 ± 13	142 ± 31	76 ± 15	23 ± 3	98 ± 18
20. ZB5	43 ± 18	74 ± 30	130 ± 58	95 ± 40	34 ± 15	56 ± 23	73 ± 31	130 ± 55	62 ± 26	23 ± 10	86 ± 39
21. MH13	50 ± 23	91 ± 42	154 ± 75	109 ± 56	40 ± 20	66 ± 30	91 ± 39	163 ± 72	72 ± 33	24 ± 10	106 ± 49
mean ± SD	**61 ± 21**	**110 ± 40**	**185 ± 66**	**148 ± 56**	**52 ± 20**	**83 ± 32**	**103 ± 36**	**188 ± 67**	**90 ± 32**	**28 ± 9**	**125 ± 44**

[1] Requirement pattern is calculated from amino acid requirements, divided by the recommended allowance of reference protein. Because protein allowance (in g/kg) is 0.99 for children 10-12 years of age, amino acid requirements are equivalent, whether expressed as mg/kg body weight, or mg/g dietary protein (WHO, 1985; FNB, 1989)

[2] Mean ± standard deviation computed from 4-day dietary records.

[3] Includes methionine and cystine.

[4] Includes phenylalanine and tyrosine.

Appendix Table 12. Indispensable amino acid intakes of subjects from natural foods compared to the U.S. diet											
subject group and no.	his[1] (-)	ile (52)	leu (77)	lys (68)	met (-)	total s - containing (35)	phe (-)	total aromatic (78)	thr (39)	trp (12)	val (54)
	mg/g dietary protein										
strict-diet											
1. BS3	24±1[2]	47±1	92±12	52±2	18±1	35±2[3]	49±0	83±3[4]	42±1	13±2	55±2
2. JY6	18±6	31±7	58±21	31±8	18±5	30±8	36±8	61±16	28±7	9±2	41±11
3. MS19	23±4	52±5	74±6	49±4	15±2	29±4	45±3	76±6	40±2	12±1	48±5
4. AW4	22±3	48±6	73±10	46±11	16±3	33±2	48±3	81±6	38±5	12±2	48±5
5. RR5	22±2	49±20	73±10	34±3	17±2	36±3	44±4	76±8	33±1	12±0	48±3
6. TM21	20±3	38±7	71±8	33±10	22±13	40±17	41±8	70±10	32±7	10±2	46±7
7. MB4	27±2	35±3	72±10	43±4	23±3	41±6	48±7	81±12	36±4	18±8	55±5
8. MS15	23±3	41±8	76±14	44±2	18±3	35±8	44±5	76±12	37±3	11±0	50±4
mean ± SD	22±3	43±8	74±9	42±8	18±3	35±4	44±4	75±7	36±4	12±2	49±5
relaxed-diet											
9. CH17	25±2	45±1	75±5	56±4	19±3	34±5	46±3	78±4	39±1	13±1	52±4
10. WH18	25±2	42±3	71±5	56±8	18±3	31±4	43±1	76±2	38±4	13±1	53±1
11. KH22	24±3	44±4	69±6	59±6	18±4	33±6	45±3	79±4	39±1	14±1	55±2
mean ± SD	25±0	44±2	72±3	57±2	18±1	33±2	44±2	78±2	39±1	13±1	53±2
off-diet											
12. SM14	23±3	44±2	76±8	46±8	18±2	34±3	46±2	80±5	36±0	12±1	52±2
13. MH12	27±1	48±1	83±3	60±6	22±1	38±5	48±2	84±2	39±2	13±2	56±2
14. SL23	26±3	46±3	76±7	53±4	19±2	35±4	44±4	77±7	38±2	12±1	50±4
15. BC10	27±2	48±3	81±6	64±12	24±2	39±1	46±2	82±4	40±3	12±1	55±3
mean ± SD	26±2	46±2	79±4	56±8	21±3	36±2	46±2	81±3	38±2	12±0	53±3

Appendix Table 12. (continued)

subject group and no.	his[1] (-)	ile (52)	leu (77)	lys (68)	met (-)	total s - containing (35)	phe (-)	total aromatic (78)	thr (39)	trp (12)	val (54)
	mg/g dietary protein										
combined PKU											
mean ± SD	**24±3**	**44±3**	**75±7**	**55±16**	27±16	35±4	45±3	77±6	37±4	12±2	51±4
control											
16. SY7	27±1	51±2	85±4	66±3	24±0	37±1	46±2	86±4	40±1	12±1	56±4
17. PJY8	27±1	50±2	83±4	64±7	23±1	37±1	45±2	84±4	38±2	12±1	57±4
18. KC11	28±2	49±4	82±6	68±11	24±2	39±2	46±4	84±7	41±3	12±1	56±4
19. SS20	31±3	51±8	86±7	79±4	26±3	39±6	47±4	84±10	45±4	14±2	58±4
20. ZB5	28±2	48±4	84±8	61±6	22±2	36±3	47±3	84±6	40±4	15±6	55±5
21. MH13	26±2	47±5	80±11	56±7	20±3	34±4	48±5	86±9	38±3	13±1	55±6
mean ± SD	**28±2**	**49±2**	83±2	**66±8**	**23±2**	**37±2**	**47±1**	**85±1**	**40±3**	**13±1**	**56±1**

[1] Reference values are from the 1977-1978 USDA Nationwide Food Consumption Survey (USDA, 1984), all ages included. Dashed lines indicate no reference value available.

[2] Mean ± standard deviation computed from 4-day dietary records.

[3] Includes methionine + cystine.

[4] Includes phenylalanine + tyrosine.

Appendix Table 13. Total indispensable amino acid intakes of subjects compared to the U.S. diet											
subject group and no.	his[1] (-)	ile (52)	leu (77)	lys (68)	met (-)	total s - containing (35)	phe (-)	total aromatic (78)	thr (39)	trp (12)	val (54)
	mg/g dietary protein										
strict-diet											
1. BS3	23 ± 0[2]	52 ± 0	86 ± 3	83 ± 1	28 ± 0	45 ± 1[3]	11 ± 2	54 ± 1[4]	45 ± 1	14 ± 0	60 ± 1
2. JY6	22 ± 1	48 ± 2	77 ± 5	76 ± 4	28 ± 1	43 ± 2	9 ± 2	50 ± 3	43 ± 4	14 ± 1	56 ± 3
3. MS19	22 ± 3	50 ± 8	76 ± 11	77 ± 11	26 ± 4	41 ± 6	10 ± 3	50 ± 7	40 ± 6	12 ± 2	55 ± 9
4. AW4	25 ± 1	59 ± 3	96 ± 5	67 ± 3	22 ± 2	45 ± 3	20 ± 6	86 ± 3	49 ± 4	18 ± 1	67 ± 2
5. RR5	22 ± 1	47 ± 1	79 ± 4	68 ± 6	25 ± 2	43 ± 1	19 ± 5	59 ± 4	41 ± 2	13 ± 0	56 ± 2
6. TM21	21 ± 1	48 ± 4	85 ± 8	74 ± 20	26 ± 5	35 ± 2	24 ± 12	66 ± 14	48 ± 8	13 ± 3	66 ± 10
7. MB4	25 ± 1	42 ± 7	78 ± 4	64 ± 15	26 ± 3	45 ± 2	29 ± 19	68 ± 18	61 ± 38	19 ± 10	58 ± 2
8. MS15	23 ± 2	48 ± 3	80 ± 8	70 ± 3	25 ± 2	42 ± 4	20 ± 6	60 ± 8	42 ± 1	13 ± 0	56 ± 3
mean ± SD	23 ± 2	49 ± 5	82 ± 7	72 ± 6	26 ± 2	42 ± 3	18 ± 7	62 ± 12	46 ± 7	14 ± 3	59 ± 4
relaxed-diet											
9. CH17	24 ± 2	47 ± 3	76 ± 4	63 ± 6	22 ± 1	36 ± 2	37 ± 8	73 ± 8	40 ± 3	13 ± 1	55 ± 1
10. WH18	25 ± 2	42 ± 3	71 ± 5	56 ± 8	18 ± 3	31 ± 4	43 ± 1	76 ± 2	38 ± 4	13 ± 1	53 ± 1
11. KH22	24 ± 3	44 ± 4	69 ± 6	59 ± 6	18 ± 4	33 ± 6	45 ± 3	79 ± 4	39 ± 1	14 ± 1	55 ± 2
mean ± SD	24 ± 0	44 ± 2	72 ± 4	59 ± 4	19 ± 2	33 ± 2	42 ± 4	76 ± 3	39 ± 1	13 ± 0	54 ± 1
off-diet											
12. SM14	23 ± 2	48 ± 1	79 ± 5	65 ± 4	23 ± 1	40 ± 1	28 ± 5	67 ± 5	39 ± 2	12 ± 1	56 ± 1
13. MH12	27 ± 1	48 ± 1	83 ± 3	60 ± 6	22 ± 1	38 ± 5	48 ± 2	84 ± 2	39 ± 2	13 ± 2	56 ± 2
14. SL23	26 ± 3	46 ± 3	76 ± 7	53 ± 4	19 ± 2	35 ± 4	44 ± 4	77 ± 7	38 ± 2	12 ± 1	50 ± 4
15. BC10	27 ± 2	48 ± 3	81 ± 6	64 ± 12	24 ± 2	39 ± 1	39 ± 1	82 ± 4	40 ± 3	12 ± 1	55 ± 3
mean ± SD	26 ± 2	48 ± 1	80 ± 3	60 ± 5	22 ± 2	38 ± 2	40 ± 9	78 ± 8	39 ± 1	12 ± 0	54 ± 3

Appendix Table 13. (continued)

subject group and no.	his[1] (-)	ile (52)	leu (77)	lys (68)	met (-)	total s - containing (35)	phe (-)	total aromatic (78)	thr (39)	trp (12)	val (54)
	mg/g dietary protein										
combined PKU											
mean ± SD	**24±2**	**48±4**	**79±6**	**67±8**	**23±3**	**39±4**	**28±14**	**69±12**	**43±6**	**14±2**	**57±4**
control											
16. SY7	27±1	51±2	85±4	66±3	24±0	37±1	46±2	86±4	40±1	12±1	56±4
17. PJY8	27±1	50±2	83±4	64±7	23±1	37±1	45±2	84±4	38±2	12±1	57±4
18. KC11	28±2	49±4	82±6	68±11	24±2	39±2	46±4	84±7	41±3	12±1	56±4
19. SS20	31±3	51±8	86±7	79±4	26±3	39±6	47±4	84±10	45±4	14±2	58±4
20. ZB5	28±2	48±4	84±8	61±6	22±2	36±3	47±3	84±6	40±4	15±6	55±5
21. MH13	26±2	47±5	80±11	56±7	20±3	34±4	48±5	86±9	38±3	13±1	55±6
mean ± SD	**28±2**	**49±2**	**83±2**	**66±8**	**23±2**	**37±2**	**47±1**	**85±1**	**40±3**	**13±1**	**56±1**

[1] Reference values are from the 1977-1978 USDA Nationwide Food Consumption Survey (USDA, 1984), all ages included. Dashed lines indicate no reference value available.
[2] Mean ± standard deviation computed from 4-day dietary records.
[3] Includes methionine + cystine.
[4] Includes phenylalanine + tyrosine.

Appendix Table 14. Dispensable amino acid intekes of subjects from neturel foods compared to controls

subject group and no.	erg (52)[1]	asp (83)	ela (45)	glu (221)	gly (41)	pro (90)	ser (55)
	mg/g dietery protein						
strict-diet							
1. BS3	55±5[2]	113±24	50±7	222±13	40±2	71±7	52±5
2. JY6	48±9	81±16	39±16	145±47	29±10	50±13	35±11
3. MS19	49±8	112±26	41±4	203±37	37±5	62±15	48±9
4. AW4	49±14	91±8	36±8	204±60	39±14	66±10	46±6
5. RR5	44±7	81±6	39±8	243±40	33±5	87±12	49±7
6. TM21	44±10	71±11	40±5	220±82	32±5	77±34	44±11
7. MB4	62±8	106±15	47±7	218±38	44±7	73±17	53±6
8. MS15	49±8	90±22	48±7	210±22	42±8	72±10	51±6
meen ± SD	**50±6**	**93±16**	**42±5**	**208±28**	**37±5**	**70±11**	**47±6**
relaxed-diet							
9. CH17	47±5	110±38	41±4	219±24	40±6	76±14	40±12
10. WH18	44±4	108±55	38±2	184±22	33±5	64±15	46±2
11. KH22	50±4	172±42	40±5	202±35	38±4	56±16	46±5
meen ± SD	**47±3**	**130±36**	**39±2**	**202±18**	**37±4**	**65±10**	**47±2**
off-diet							
12. SM14	----[3]	----	----	----	----	----	----
13. MH12	49±8	89±12	53±26	236±29	40±9	94±15	47±23
14. SL23	53±14	88±9	70±30	234±37	46±17	81±15	48±7
15. BC10	52±10	83±7	103±39	214±30	39±9	83±17	50±3
meen ± SD	**51±2**	**87±3**	**75±3**	**228±12**	**42±4**	**86±7**	**48±2**
combined PKU							
mean ± SD	**50±5**	**94±35**	**48±18**	**211±24**	**38±5**	**72±12**	**47±5**
control							
16. SY7	48±8	78±6	43±6	220±13	35±8	87±11	53±2
17. PJY8	46±10	75±13	41±9	225±23	38±8	112±39	66±17
18. KC11	55±9	88±9	48±8	212±32	43±7	80±16	50±1
19. SS20	62±9	90±14	57±8	214±47	55±11	80±24	54±6
20. ZB5	50±7	78±4	43±3	227±12	38±5	87±9	51±4
21. MH13	50±10	87±13	38±2	229±21	38±4	96±12	57±8
mean ± SD	**52±6**	**83±6**	**45±7**	**221±7**	**41±7**	**90±12**	**55±6**

[1] Control pattern was calculated as the mean intake from 4-day dietary records of the six sibling (control) subjects.

[2] Mean ± standard deviation computed from 4-day dietary records.

[3] Dashed lines indicate missing data for subject 12; group mean ± SD for off-diet group based on remaining three subjects.

Appendix Table 15. Total dispensable amino acid intakes of subjects compared to controls

subject group and no.	arg (52)[1]	asp (83)	ala (45)	glu (221)	gly (41)	pro (90)	ser (55)
	mg/g dietary protein						
strict-diet							
1. 8S3	39 ± 1[2]	230 ± 11	12 ± 3	309 ± 7	136 ± 4	16 ± 4	12 ± 2
2. JY6	38 ± 2	217 ± 10	10 ± 4	286 ± 15	130 ± 9	13 ± 5	9 ± 4
3. MS19	36 ± 5	217 ± 36	9 ± 3	289 ± 36	128 ± 20	14 ± 6	11 ± 4
4. AW4	44 ± 7	104 ± 2	42 ± 3	223 ± 21	31 ± 5	89 ± 6	54 ± 3
5. RR5	38 ± 3	188 ± 15	16 ± 4	298 ± 14	110 ± 12	37 ± 13	21 ± 6
6. TM21	21 ± 18	37 ± 27	21 ± 16	113 ± 66	71 ± 11	39 ± 18	22 ± 13
7. MB4	46 ± 12	133 ± 75	28 ± 16	218 ± 104	17 ± 50	42 ± 22	31 ± 15
8. MS15	41 ± 5	180 ± 23	22 ± 7	275 ± 19	108 ± 12	33 ± 10	23 ± 4
mean ± SD	38 ± 8	163 ± 67	20 ± 11	251 ± 65	91 ± 46	35 ± 25	23 ± 15
relaxed-diet							
9. CH17	44 ± 0	139 ± 75	33 ± 8	236 ± 1	61 ± 24	62 ± 30	40 ± 12
10. WH18	44 ± 4	108 ± 55	37 ± 2	184 ± 22	33 ± 5	64 ± 15	46 ± 2
11. KH22	50 ± 4	172 ± 42	40 ± 5	202 ± 35	38 ± 4	56 ± 16	46 ± 2
mean ± SD	46 ± 4	140 ± 32	37 ± 4	207 ± 26	46 ± 8	61 ± 4	44 ± 4
off-diet							
12. SM14	----[3]	----	----	----	----	----	----
13. MH12	49 ± 8	89 ± 12	44 ± 6	236 ± 29	40 ± 9	94 ± 15	47 ± 23
14. SL23	53 ± 14	88 ± 9	46 ± 14	234 ± 37	46 ± 17	81 ± 15	48 ± 7
15. 8C10	52 ± 10	83 ± 7	46 ± 9	214 ± 30	39 ± 9	83 ± 17	50 ± 3
mean ± SD	51 ± 2	87 ± 3	45 ± 1	228 ± 12	42 ± 4	86 ± 7	48 ± 1
combined PKU							
mean ± SD	42 ± 8	142 ± 60	29 ± 14	237 ± 52	70 ± 43	52 ± 28	33 ± 16
control							
16. SY7	48 ± 8	78 ± 6	43 ± 6	220 ± 13	35 ± 8	87 ± 11	53 ± 2
17. PJY8	46 ± 10	75 ± 13	41 ± 9	225 ± 23	38 ± 8	112 ± 39	66 ± 17
18. KC11	55 ± 9	88 ± 9	48 ± 8	212 ± 32	43 ± 7	80 ± 16	50 ± 1
19. SS20	62 ± 7	90 ± 14	57 ± 8	214 ± 47	55 ± 11	80 ± 24	54 ± 6
20. Z85	50 ± 7	78 ± 4	43 ± 3	227 ± 12	38 ± 5	87 ± 9	51 ± 4
21. MH13	50 ± 10	87 ± 13	38 ± 2	229 ± 21	38 ± 4	96 ± 12	57 ± 8
mean ± SD	52 ± 6	83 ± 6	45 ± 7	221 ± 7	41 ± 7	90 ± 12	55 ± 6

[1] Control pattern was calculated as the mean intake from 4-day dietary records of the six sibling (control) subjects.

[2] Mean ± standard deviation computed from 4-day dietary.

[3] Dashed lines indicate missing data for subject 12; group mean ± SD for off-diet group based on remaining three subjects.

Appendix Table 16. Fasting plasma indispensable amino acid values of subjects									
subject group and no.	his (65-105)[1]	leu (85-169)	lys (114-226)	ile (41-93)	met (17-37)	phe (42-74)	tyr (45-89)	thr (75-203)	val (161-285)
	umol/L								
strict-diet									
1. BS3	72.3	96.2	158.2	53.4	20.4	520.4	31.8	127.2	187.9
2. JY6	65.1	93.4	119.9	53.0	22.0	942.2	28.5	87.1	201.2
3. MS19	69.6	82.0	113.9	49.4	18.9	1136.3	26.6	67.5	168.4
4. AW4	88.2	93.2	182.2	49.6	23.7	1446.0	43.2	79.9	189.8
5. RR5	75.1	78.0	127.2	43.9	18.6	517.4	42.7	96.2	155.8
6. TM21	72.2	81.0	99.3	53.0	20.2	937.5	33.6	104.5	150.0
7. MB4	78.4	110.4	144.1	67.5	24.6	950.0	34.4	101.2	210.5
8. MS15	81.8	107.9	170.5	60.3	24.5	684.4	43.7	105.5	219.2
mean ± SD	75.3±7	92.8±12	139.4±29	53.6±7	21.6±2	891.8±315	35.6±7	96.1±18	185.4±25
relaxed-diet									
9. CH17	75.0	92.9	145.5	48.4	24.1	1364.0	38.3	104.8	174.6
10. WH18	82.3	108.9	143.9	57.6	23.9	1268.0	38.0	89.1	216.4
11. KH22	76.1	93.0	148.9	56.8	32.2	1025.8	37.0	91.0	171.6
mean ± SD	77.8±4	98.3±9	146.1±2	54.3±5	26.7±5	1225.5±177	37.8±1	95.0±8	187.5±25
off-diet									
12. SM14	72.7	92.1	138.7	49.8	17.5	437.0	49.3	102.8	173.8
13. MH12	85.1	101.0	136.4	59.1	18.1	1064.2	57.6	88.4	209.1
14. SL23	62.4	72.8	97.8	42.6	17.9	515.0	29.0	71.7	161.6
15. 8C10	69.2	82.1	127.6	44.6	23.1	813.5	43.2	80.9	153.3
mean ± SD	72.4±10	87.0±12	125.1±19	49.0±7	19.2±3	707.4±288	44.8±12	86.0±13	174.4±24

Appendix Table 17. Fasting plasma dispensable amino acid values of subjects													
subject group and no.	ala (185-537)[1]	arg (49-129)	asn (34-62)	asp ((0-14)	asn + asp (-)	gln 464-728)	glu (23-250)	gln + glu (-)	gly (160-304)	orn (21-77)	pro (71-303)	ser (70-178)	tau (0-240)
	umol/L												
strict-diet													
1. BS3	261.9	99.7	27.7	26.7	54.4	333.4	137.2	470.6	369.6	33.9	99.5	118.3	37.8
2. JY6	233.3	73.2	35.7	20.9	56.6	262.1	131.5	393.6	303.9	27.0	82.5	124.3	32.5
3. MS19	288.1	85.6	33.9	18.7	52.6	429.0	83.8	512.8	458.7	52.3	125.0	152.6	52.4
4. AW4	236.6	72.7	54.1	14.0	68.1	386.6	82.6	469.2	245.0	36.5	139.5	102.1	54.3
5. RR5	236.1	72.0	30.8	27.9	58.7	328.8	173.6	502.4	429.0	36.2	80.0	135.6	37.6
6. TM21	256.0	74.8	34.8	21.9	56.7	474.9	82.6	557.5	364.1	28.0	153.1	145.6	40.7
7. MB4	480.0	77.8	34.2	25.0	59.2	376.4	168.5	544.9	311.6	38.7	140.1	143.1	45.3
8. MS15	251.3	90.1	36.0	17.8	53.8	461.3	80.0	541.3	339.0	40.7	106.1	166.0	47.3
mean ± SD	280.4 ±83	80.7 ±10	35.9 ±8	21.6 ±5	57.5 ±5	389.1 ±75	119.6 ±42	449.0 ±54	352.6 ±69	36.7 ±8	115.7 ±28	136.0 ±20	43.5 ±8
relaxed-diet													
9. CH17	337.9	68.5	43.5	22.8	66.3	402.1	77.1	479.2	397.4	37.9	159.8	146.6	45.8
10. WH18	497.7	66.3	50.3	18.2	68.5	472.3	67.5	539.8	334.7	32.4	190.7	129.3	45.0
11. KH22	319.6	76.4	49.0	13.2	62.2	528.4	50.4	578.8	395.0	45.2	141.8	123.0	61.2
mean ± SD	385.1 ±98	70.4 ±5	47.6 ±4	18.1 ±5	65.7 ±3	467.6 ±63	65.0 ±14	532.6 ±50	375.7 ±36	38.5 ±6	164.1 ±25	138.0 ±12	50.7 ±9
off-diet													
12. SM14	206.0	73.0	31.7	19.5	51.2	311.7	135.2	446.9	315.9	33.7	88.2	124.0	43.9
13. MH12	291.8	75.5	16.7	23.1	39.8	345.0	164.4	509.4	210.5	35.2	101.4	117.0	61.4
14. SL23	208.3	49.1	30.1	16.6	46.7	299.1	121.9	421.0	241.4	22.5	104.4	104.4	79.8

Appendix Table 17. (continued)

subject group and no.	ala (185-537)[1]	arg (49-129)	asn (34-62)	asp ((0-14)	asn + asp (-)	gln 464-728)	glu (23-250)	gln + glu (-)	gly (160-304)	orn (21-77)	pro (71-303)	ser (70-178)	tau (0-240)
	umol/L												
combined PKU													
mean ± SD	287.6 ±90	75.1 ±12	35.8 ±10	20.2 ±4	56.1 ±8	383.2 ±76	106.6 ±40	492.7 ±55	316.9 ±104	35.3 ±7	120.3 ±33	129.6 ±18	48.4 ±12
control													
16. SY7	231.2	84.9	27.9	23.3	51.2	346.2	129.0	475.2	175.6	31.4	90.9	109.2	35.3
17. PJY8	262.6	100.2	29.8	23.7	53.5	328.7	140.0	468.7	176.3	34.5	103.3	92.7	35.6
18. KC11	308.8	87.9	26.6	22.2	48.8	322.4	146.4	468.8	265.0	48.1	110.4	132.5	82.9
19. SS20	395.5	112.4	41.7	25.7	67.4	529.2	93.6	622.8	347.2	21.4	51.3	144.9	51.3
20. ZB5	388.5	105.1	36.8	26.7	63.5	397.2	118.7	519.9	225.3	42.2	178.9	136.4	37.6
21. MH13	260.1	84.4	20.9	22.9	43.8	373.1	165.9	539.0	210.9	33.1	114.2	105.1	56.0
mean ± SD	307.7 ±70	95.8 ±12	30.6 ±7	24.1 ±2	54.7 ±9	382.8 ±77	132.3 ±25	515.7 ±60	233.4 ±65	35.1 ±9	108.2 ±41	120.1 ±21	49.8 ±18

[1] Range reference values based upon mean values ± 2 standard deviations of 116 fasting children, 6-18 yrs (Armstrong, 1973).

Appendix Table 18. Fasting plasma pyridoxal 5'-phosphate (PLP), total vitamin B-6 (TB6), PLP: TB6 ratio, and alkaline phosphatase (AP) values of subjects

subject group and no.	PLP	TB6	PLP : TB6	AP
	nmol/L		ratio	U/L
strict-diet				
1. BS3	62.64	96.05	0.65	61.5
2. JY6	126.20	149.61	0.84	86.4
3. MS19	66.91	77.68	0.86	78.9
4. AW4	147.07	170.02	0.86	90.9
5. RR5	67.37	100.24	0.67	57.9
6. TM21	55.80	55.77	0.91	70.5
7. MB4	56.80	61.04	0.93	71.4
8. MS15	34.92	64.75	0.54	106.0
mean ± SD	77.2±38	96.9±42	0.78±0.1	78.7±16
relaxed-diet				
9. CH17	110.90	71.50	1.55	25.9
10. WH18	229.30	250.90	0.91	25.2
11. KH22	64.60	70.80	0.92	62.3
mean ± SD	134.9±85	131.08±104	1.13±0.4	34.6±18
off-diet				
12. SM14	109.00	115.40	0.94	55.2
13. MH12	59.48	62.50	0.95	68.3
14. SL23	46.85	54.16	0.86	81.7
15. BC10	68.54	90.66	0.76	101.0
mean ± SD	71.0±27	87.99±75	0.88±0.1	76.6±20
combined PKU				
mean ± SD	87.1±50	99.40±54	0.88±0.2	69.5±23
control				
16. SY7	19.75	53.56	0.37	78.6
17. PJY8	34.56	50.93	0.68	78.4
18. KC11	63.35	90.50	0.70	78.2
19. SS20	28.98	34.35	0.84	85.2
20. ZB5	20.60	31.53	0.65	91.9
21. MH13	43.61	60.26	0.72	78.1
mean ± SD	35.1±16	57.3±21	0.66±0.2	81.7±6

Appendix Table 19. Vitamin B-6 intakes of subjects from natural foods

subject group and no.	day 1	day 2	day 3	day 4	mean ± SD
	mg/day				
strict-diet					
1. BS3	0.36	0.69	0.81	0.73	0.65
2. JY6	1.75	1.23	1.22	1.01	1.30
3. M519	0.37	0.27	0.28	0.72	0.54
4. AW4	0.55	1.68	0.96	0.50	0.92
5. RR5	0.72	0.47	0.66	1.77	0.90
6. TM21	0.48	0.86	1.18	0.91	0.94
7. MB4	0.51	0.17	1.48	0.38	0.85
8. MS15	0.85	0.47	0.71	0.34	0.59
mean ± SD	0.70±0.5	0.73±0.5	0.91±0.4	0.79±0.5	0.84±0.2
relaxed-diet					
9. CH17	1.67	0.85	1.93	0.54	1.25
10. WH18	1.90	1.89	3.02	1.04	1.96
11. KH22	3.50	3.53	3.38	5.31	3.93
mean ± SD	2.30±1.0	2.09±1.4	2.78±0.8	2.30±2.6	2.38±1.4
off-diet					
12. SM14	1.42	1.07	1.20	1.00	1.17
13. MH12	2.71	0.36	1.60	1.34	1.50
14. SL23	1.76	0.90	1.08	1.79	1.38
15. BC10	1.09	0.82	1.90	1.55	1.34
mean ± SD	1.74±0.7	0.79±0.3	1.44±0.4	1.42±0.3	1.35±0.1
combined PKU					
mean ± SD	1.31±0.9	1.02±0.8	1.43±0.8	1.26±1.2	1.28±0.8
control					
16. SY7	0.68	0.77	1.26	0.70	0.85
17. RJY8	1.92	2.23	1.13	1.53	1.70
18. KC11	1.14	1.24	1.92	1.79	1.52
19. SS20	0.55	0.67	1.23	1.35	0.94
20. ZB5	1.34	1.30	1.30	1.22	1.29
21. MH13	1.94	0.63	1.16	1.22	1.23
mean ± SD	1.26±0.6	1.14±0.6	1.33±0.3	1.30±0.4	1.26±0.3

Appendix Table 20. Vitamin B-6 intakes of subjects from medical foods					
subject group and no.	day 1	day 2	day 3	day 4	mean ± SD
	mg/day				
strict-diet					
1. BS3	1.62	1.62	1.62	1.62	1.62
2. JY6	1.62	1.62	1.62	1.62	1.62
3. M519	1.35	1.35	1.35	1.35	1.35
4. AW4	1.34	1.34	1.34	1.34	1.34
5. RR5	1.08	1.08	1.08	1.08	1.08
6. TM21	0.43	0.43	0	0.43	0.32
7. MB4	0.90	0	0.90	0.90	0.68
8. MS15	0.66	0.60	0.60	0.64	0.62
mean ± SD	1.12±0.4	1.00±0.6	1.06±0.6	1.12±0.4	1.08±0.5
relaxed-diet					
9. CH17	0.54	0	0	0.13	0.17
10. WH18	0	0	0	0	-
11. KH22	0	0	0	0	-
mean ± SD	-	-	-	-	-
off-diet					
12. SM14	0.90	0.90	0.90	0.90	0.90
13. MH12	0	0	0	0	-
14. SL23	0	0	0	0	-
15. BC10	0	0	0	0	-
mean ± SD	-	-	-	-	-
combined PKU					
mean ± SD	-	-	-	-	-
control					
16. SY7	0	0	0	0	-
17. RJY8	0	0	0	0	-
18. KC11	0	0	0	0	-
19. SS20	0	0	0	0	-
20. ZB5	0	0	0	0	-
21. MH13	0	0	0	0	-
mean ± SD	-	-	-	-	-

Appendix Table 21. Total vitamin B-6 intakes of subjects

subject group and no.	day 1	day 2	day 3	day 4	mean ± SD
	mg/day				
strict-diet					
1. BS3	1.98	2.31	2.43	2.35	2.22 ± 0.45
2. JY6	3.37	2.85	2.84	2.63	2.92 ± 0.74
3. M519	1.72	1.62	1.58	2.08	1.75 ± 0.05
4. AW4	1.93	3.02	2.31	1.84	2.28 ± 1.71
5. RR5	1.80	1.55	1.74	2.85	1.98 ± 1.30
6. TM21	0.91	1.30	1.15	1.34	1.18 ± 0.43
7. MB4	1.41	0.17	2.38	1.28	1.31 ± 1.10
8. MS15	1.51	0.07	1.31	0.98	1.22 ± 0.53
mean ± SD	1.83 ± 0.7	1.74 ± 1.0	1.97 ± 0.7	1.92 ± 0.7	1.86 ± 0.6
relaxed-diet					
9. CH17	2.21	0.85	1.93	0.67	1.42 ± 1.54
10.WH18	1.90	1.89	3.02	1.04	1.96 ± 1.98
11.KH22	3.50	3.53	3.38	5.31	3.93 ± 1.93
mean ± SD	2.54 ± 0.8	1.69 ± 1.0	2.78 ± 0.8	2.34 ± 2.6	2.44 ± 1.3
off-diet					
12. SM14	2.32	1.97	2.10	1.90	2.08 ± 0.18
13. MH12	2.71	0.36	1.60	1.34	1.50 ± 1.50
14. SL23	1.76	0.90	1.08	1.79	1.38 ± 0.46
15. BC10	1.09	0.82	1.90	1.55	1.34 ± 0.48
mean ± SD	1.97 ± 0.7	1.1 ± 0.7	1.67 ± 0.4	1.64 ± 0.3	1.58 ± 0.3
combined PKU					
mean ± SD	2.01 ± 0.7	1.61 ± 0.9	2.05 ± 0.7	1.93 ± 1.1	1.90 ± 0.7
control					
16. SY7	0.68	0.77	1.26	0.70	0.85 ± 0.58
17. RJY8	1.92	2.23	1.13	1.53	1.70 ± 0.79
18. KC11	1.14	1.24	1.92	1.79	1.52 ± 0.78
19. SS20	0.55	0.67	1.23	1.35	0.94 ± 0.80
20. ZB5	1.34	1.30	1.30	1.22	1.29 ± 0.12
21. MH13	1.94	0.63	1.16	1.22	1.23 ± 1.31
mean ± SD	1.26 ± 0.6	1.14 ± 0.6	1.33 ± 0.3	1.30 ± 0.4	1.26 ± 0.3

Appendix Table 22. Urinary 4-pyridoxic acid values of subjects[1]					
subject group and no.	day 1	day 2	day 3	day 4	mean ± SD
	umol/24-h				
strict-diet					
1. BS3		9.87	11.09		10.5
2. JY6		9.63	11.59		10.6
3. M519		1.39	1.96		1.7
4. AW4		9.78	8.28		9.0
5. RR5		7.28	6.26		6.8
6. TM21		1.73	3.04		2.4
7. MB4		1.10	1.24		1.2
8. MS15		2.25	1.28		2.0
mean ± SD		5.38±4.1	5.59±4.3		5.5±4.1
relaxed-diet					
9. CH17		1.91	4.83		3.4
10. WH18		2.08	4.80		3.4
11. KH22		3.40	8.67		6.0
mean ± SD		2.19±1.2	6.10±2.2		4.3±1.5
off-diet					
12. SM14		3.69	4.39		3.7
13. MH12		2.80	4.80		2.5
14. SL23		2.34	1.86		2.1
15. BC10		2.29	0.86		1.8
mean ± SD		2.78±0.6	2.31±1.5		2.5±0.8
combined PKU					
mean ± SD		4.10±3.3	4.81±3.6		4.5±3.3
control					
16. SY7		3.12	6.30		4.7
17. RJY8		1.95	1.18		1.6
18. KC11		2.35	1.16		1.8
19. SS20		6.13	8.05		7.0
20. ZB5		2.60	2.17		2.4
21. MH13		2.42	2.68		2.6
mean ± SD		3.10±1.5	3.59±2.9		3.4±2.1

[1] Urine was collected for days 2, 3 only.

Appendix Table 23. Urinary creatinine values of subjects[1]

subject group and no.	day 1	day 2	day 3	day 4	mean ± SD
	g/24-h				
strict-diet					
1. BS3		-	0.96		0.96
2. JY6		0.59	0.66		0.62
3. M519		0.41	0.48		0.44
4. AW4		0.98	-		0.98
5. RR5		-	1.05		1.05
6. TM21		0.66	0.68		0.67
7. MB4		0.67	-		0.67
8. MS15		0.40	0.43		0.42
mean ± SD		0.62±0.21	0.71±0.25		0.73±0.24
relaxed-diet					
9. CH17		1.19	1.08		2.14
10. WH18		0.82	1.14		0.98
11. KH22		0.98	1.84		1.41
mean ± SD		0.99±0.18	1.35±0.42		1.18±0.22
off-diet					
12. SM14		0.43	0.31		0.37
13. MH12		0.75	0.75		0.75
14. SL23		0.40	0.43		0.42
15. BC10		0.42	0.48		0.45
mean ± SD		0.50±0.17	0.49±0.18		0.50±0.17
combined PKU					
mean ± SD		0.67±0.26	0.79±0.42		0.75±0.32
control					
16. SY7		0.36	0.38		0.37
17. RJY8		1.04	0.94		0.99
18. KC11		0.34	0.43		0.38
19. SS20		0.79	0.81		0.80
20. ZB5		0.89	1.43		1.16
21. MH13		0.51	0.45		0.48
mean ± SD		0.66±0.29	0.74±0.41		0.64±0.30

[1] Urine was collected for days 2, 3 only.

Appendix Table 24. Urinary urea nitrogen values of subjects[1]

subject group and no.	day 1	day 2	day 3	day 4	mean ± SD
	mg/24-h				
strict-diet					
1. BS3		4.627	4.762		4.717
2. JY6		5.188	4.970		5.079
3. M519		-	2.410		2.410
4. AW4		2.958	2.172		2.565
5. RR5		4.635	5.874		5.254
6. TM21		2.738	3.512		3.125
7. MB4		1.989	2.942		2.466
8. MS15		2.677	2.668		2.672
mean ± SD		3.544 ± 1.24	3.664 ± 1.37		
relaxed-diet					
9. CH17		5.193	4.011		4.602
10. WH18		3.187	5.359		4.273
11. KH22		5.927	-		5.927
mean ± SD		4.769 ± 1.42	4.685 ± 0.95		
off-diet					
12. SM14		5.633	4.125		4.879
13. MH12		3.700	4.939		4.320
14. SL23		2.313	2.313		2.313
15. BC10		3.184	4.468		3.826
mean ± SD		3.708 ± 1.40	3.961 ± 1.15		
combined PKU					
mean ± SD		3.854 ± 1.31	3.895 ± 1.23		3.895 ± 1.21
control					
16. SY7		6.822	5.971		6.396
17. RJY8		10.890	10.750		10.820
18. KC11		4.155	5.368		4.762
19. SS20		7.334	7.153		7.244
20. ZB5		6.785	9.832		8.308
21. MH13		3.700	4.838		4.269
mean ± SD		6.614 ± 2.58	7.319 ± 2.44		6.966 ± 2.42

[1] Urine was collected for days 2, 3 only.