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Title: The Effect of Vitamin B-6 Deficiency on Carnitine Metabolism During Fasting in Rats

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The purpose of this study was, first, to investigate whether there is a vitamin B-6 requirement for carnitine synthesis and, second, to investigate the effect of fasting on vitamin B-6 metabolism. An experimental group of 72 rats (6 per group) were fed either a vitamin B-6 deficient diet (-B6) (ad libitum, meal-fed) or a control diet (+B6) (ad libitum, pair-fed). These diets were fed for 6 weeks and then the rats were repleted with the control diet for 2 weeks. The animals were fasted for 3 days before and after repletion. Total acid soluble carnitine (TCN) and free carnitine (FCN) levels were compared in the plasma, liver, skeletal muscle, heart muscle and in the urine of rats fed +B6 diet and -B6 diets. The concentrations of pyridoxal 5'-phosphate (PLP) in the plasma, liver, skeletal muscle, and heart muscle and urinary 4-pyridoxic acid (4-PA) excretion were compared in rats fed the +B6 or -B6 diet. Similar comparisons were made in fasted and non-fasted rats. Also, plasma glucose, liver glycogen, and free fatty acid concentrations were compared.

In rats fed the -B6 vs +B6 diet, the TCN concentration was significantly ($P < 0.05$) lower in the plasma, skeletal muscle, heart muscle and urine. With fasting, the liver TCN concentration of -B6 rats was also significantly lower than that of +B6 rats. After the -B6 rats were repleted with the +B6 diet, the TCN concentrations in the plasma, liver, skeletal muscle, heart muscle, and urine returned to those of the control rats. Thus, the decrease in TCN and FCN concentrations, and the increase of these concentrations after repletion provides evidence for a vitamin B-6 requirement in the biosynthesis of carnitine.

Fasting resulted in increased concentrations of PLP in the plasma, liver, and heart muscle of rats fed a -B6 diet. The urinary 4-PA excretion of -B6 rats also increased with fasting. These changes are consistent with a redistribution of vitamin B-6 (as PLP) when there is a caloric deficit. Thus, with fasting, PLP is supplied by an endogenous source, possibly skeletal muscle glycogen phosphorylase. In -B6 vs +B6 rats, liver glycogen concentration was higher and plasma FFA concentration was lower.

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CHAPTER I

INTRODUCTION

The metabolic events of fasting allow animals to live for a period of time without caloric intake. Maintaining close to normal metabolic rate and physical activity, animals survive by undergoing a series of adaptations of their metabolism fuels. Over the past 20 years, research has suggested that vitamin B-6 may play an important role in these adaptations.

A vitamin B-6 deficiency affects two important fuel metabolism processes: carbohydrate metabolism, via glycogenolysis and gluconeogenesis, and fatty acid metabolism. There have been a number of studies of carbohydrate metabolism, or transamination in a vitamin B-6 deficient state (Angel, 1980; Helmreich & Klein, 1980; Krebs & Fischer, 1964), but few studies have examined the temporal changes in pyridoxal 5'-phosphate (PLP) in the fasting state and with a deficiency of vitamin B-6 .

A continuous supply of fuel and glucose is essential for survival during periods of fast and under normal circumstances glucose and fatty acids are the preferred fuels for energy expenditure. Cahill (1970) established that during periods of prolonged fasting about 90% of the daily energy utilization is derived from adipose tissues. Under conditions of

starvation, glycogenolysis supplied by liver glycogen is the main source of glucose (Felig, 1973).

The capacity of the liver to store glycogen is limited, and in humans, muscle glycogen is not a source of glucose which can be released into the circulation (Cahill, 1970). When Freminet (1981) subjected rats to conditions of starvation, the hepatic glycogen content of the animals was almost totally exhausted after 24 hours of food deprivation. The decrease in the muscle glycogen of the rats was progressive, while the heart glycogen immediately increased and then progressively decreased. At the same time the hepatic store of glycogen was lower in older rats than it was in the younger animals. In a subsequent parallel study (Koubi & Freminet, 1985), the hepatic glycogen content was higher in obese rats than in lean rats and after fasting hepatic glycogen was exhausted in the lean animals, while it decreased by only 56% in the obese animals. There was no difference in the glycogen content of the muscle tissue of either type of animal.

Despite the limited store of carbohydrate, a continuing supply of glucose is essential for the needs of the brain. About 115 g of glucose are metabolized by the central nervous system in a normal 24 hour period in humans (Ferrendelli, 1974). Other tissues also require glucose for anaerobic glycolysis. Erythrocytes, the bone marrow and renal medulla, and the peripheral nerves collectively metabolize about 36 g of glucose/day (Cahill et al., 1966). The production of new glucose, therefore, is an adaptation which is crucial to survival during periods of fasting. As a result, the factors that regulate fat utilization from adipose tissues and the release of precursors from muscles for gluconeogenesis assume physiological and chemical importance in the metabolic adaptation to fasting.

Metabolic adaptation during the early and gluconeogenic phase of fasting is initiated by a small decline (range of 555 to 833 μmol/L) in blood glucose resulting from continuing glucose utilization, particularly by the brain (Marliss et al., 1970). Amino acids, as well as lactate and glycerol, which are released from peripheral tissues are important factors in maintaining an adequate supply of glucose during brief periods of fasting. Amino acids, via transamination, are largely responsible for providing substrates for gluconeogenesis (Cahill et al., 1966; Exton, 1972).

In a prolonged fasting state, fat provides most of the body's energy requirements. Since all the enzymes for fatty acid oxidation are located within the mitochondrial matrix, the transport of fatty acids into the mitochondria is essential. Neither the major fatty acids, nor their respective CoA derivatives, readily penetrate the mitochondrial membrane. At this point, carnitine acts as a carrier of fatty acyl groups from the cytoplasm to the mitochondria (Bremer, 1983). While carnitine can be obtained from the diet, the body can also synthesize it. The daily carnitine requirements are unknown for all mammalian species, including humans. Cederblad and Lindstedt (1976) estimated that adult rats (160 g average body weight) consumed 113 μg of carnitine from a commercial diet and synthesized 486 μg daily. There is a total body carnitine pool of approximately 9100 μg. About 7% of the body pool was eliminated in daily urine. Thus, dietary intake was only a fraction of the amount needed to replace urinary losses, and most of the need was supplied by endogenous synthesis.

Research has suggested that vitamin B-6 is involved in fuel metabolism in three ways during fasting. First, pyridoxal 5'-phosphate (PLP), the biologically active form of vitamin B-6, acts as an integral part of glycogen phosphorylase (EC.2.4.1.1), which catalyzes the breakdown of

glycogen to glucose 1-phosphate (Krebs & Fischer, 1964). Second, PLP is a cofactor for aminotransferase, which catalyzes the transamination of amino acid for gluconeogenesis (Plebani et al., 1980). Third, PLP is a cofactor of serine transhydroxymethylase, which may be involved in the synthesis of carnitine, a carrier for fatty acid across the mitochondrial membrane.

A relationship between vitamin B-6 and carnitine synthesis has been suggested. In 1978, Hulse et al. (1978) reported that serine transhydroxymethylase, a PLP requiring enzyme, is involved in carnitine synthesis in vitro. Based on in vitro evidence, Dunn et al. (1982) suggested that there was a requirement for PLP in carnitine synthesis. In their study, when vitamin B-6 antagonist, 1-amino-D-proline, was incubated with perfused liver, there was decreased carnitine synthesis. Moreover, the activity of lysine methyltransferase, which catalyzes the initial reaction in the biosynthesis of carnitine, was reported to be reduced in the livers of vitamin B-6 deficient rats (Loo & Smith, 1986a). However, there is no information available which demonstrates there is an in vivo requirement for vitamin B-6 in carnitine synthesis.

For many years it has been assumed that vitamin B-6, a water soluble vitamin, is not stored in the body. However, Black et al. (1977; 1978) reported that muscle phosphorylase acts as a reservoir for vitamin B-6. In their study, a high intake of vitamin B-6 (10 x requirement for a rat) resulted in a steady increase in muscle phosphorylase and total muscle vitamin B-6. The amount of muscle phosphorylase decreased when a caloric deficit was introduced. However, neither the vitamin B-6 nor the PLP content of skeletal muscle was measured during a state of caloric restriction. Moreover, to date the effect of fasting on the temporal

changes of vitamin B-6 metabolism in the plasma, tissues, and urine has not been determined.

Thus, it was the objective of this study to systematically describe the changes in plasma and tissue levels of PLP and carnitine (free and esterified) with vitamin B-6 deficiency and during fasting. This thesis includes a review of the literature on vitamin B-6, metabolic adaptations to fasting, and the metabolism and the nutritional implications of carnitine, vitamin B-6 and fat metabolism. Two articles to be submitted for publication follow the review of literature.

CHAPTER II

REVIEW OF LITERATURE

The metabolic response to fasting involves a series of adaptation processes in which vitamin B-6 is directly or indirectly involved. Thus, the current state of knowledge of these adaptation processes and the role with them of vitamin B-6 is the subject of this review.

The review of the effect of vitamin B-6 deficiency on carnitine metabolism during fasting is presented in four sections. The first section on vitamin B-6 covers its history, chemistry, absorption, metabolism, storage, and functions and the effects of vitamin B-6 deficiency in the rat. The second section discusses metabolic adaptations to fasting and covers general adaptation mechanisms, gluconeogenesis, transamination, and how vitamin B-6 relates to these processes. The third section deals with the metabolism and nutritional implications of carnitine. This section covers the biosynthesis, transport, regulation, and function, as well as several factors which influence the tissue carnitine, nutritional status and various pathological and physiological conditions in the rat. The final section covers how fat metabolism may be altered by a deficiency of vitamin B-6.

Vitamin B-6

History, Chemistry, and Sources

In 1934, Gyorgy determined that a compound he named vitamin B-6 was essential in the prevention of skin lesions in rats (Gyorgy & Ekhart, 1939). Within the next few years several investigators had isolated the vitamin B-6 crystalline compound. Following this, vitamin B-6 was synthesized by Harris and Folkers (1939) and was determined to be essential to human health (Spies et al., 1939). This was confirmed when infants fed formula diets in which the vitamin B-6 had been destroyed, developed convulsions (Coursin, 1954).

Vitamin B-6 is the generic term for six forms of the vitamin. Figure 2-1 shows the chemical structure of all 6 forms and their biological interconversions. These vitamers are widely distributed in nature (Orr, 1969). Pyridoxine (PN) is the major form found in plant foods, while pyridoxal (PL) and pyridoxamine (PM) are the predominant forms found in animal products. Plant sources containing significant amounts of vitamin B-6 include nuts, beans, lentils, cereals, and some fruits such as bananas and avocados. Animal sources of vitamin B-6 are beef liver and red meat, halibut, tuna, turkey, and chicken. In the American diet, meats supply about 40%, dairy products provide 12%, cereal products and legumes provide 15%, and vegetables provide 22% of the total vitamin B-6 consumed by humans (Sauberlich, 1981).

Vitamin B-6 exists naturally as free and bound forms. In animal foods, vitamin B-6 is known to bind to protein through the formation of a

Schiff's base (Matsuo, 1957). In plant foods, vitamin B-6 is bound to glucose, forming pyridoxine-beta-glucosides (Yasumoto et al., 1977).

Digestion and Absorption

Vitamin B-6 is absorbed in the small intestine, primarily in the jejunum and secondarily in the ileum. Early reports, based on experiments with whole animals (Booth & Brain, 1962), everted sacs (Middleton, 1977), isolated loops (Middleton, 1979b) and intestinal rings (Tsuji et al., 1973), indicated that PN is absorbed by passive diffusion. Wozenski et al. (1980) found in men that equal molar amounts of PL and PN produced a quicker response in plasma and urinary vitamin B-6 parameters than PM. These investigators suggested that PM absorption is slower and/or metabolized differently than PL or PN. However, Henderson (1984) reported that in an in vitro system the absorption rate of PM is greater than PN. The hydrolysis of phosphorylated vitamers, such as the hydrolysis of PLP to PL and PMP to PM, occurs in the intestinal lumen by intestinal phosphatases (Lumeng & Li, 1975) and PL and PM are then absorbed. The alkaline phosphatase activity in the lumen is associated with the membrane (Middleton, 1982). Middleton (1979b) used a luminal perfusion of a segment of the jejunum in vivo and confirmed that some of the phosphorylated forms are slowly absorbed without hydrolysis when a high dose is given (Mehansho et al., 1979).

Many factors may influence the bioavailability of vitamin B-6. These include the ability of the body to digest food, processing and storage of food, the form of the vitamin in a food, and other constituent in the food. Studies of the bioavailability of vitamin B-6 from foods have been reported (Gregory & Kirk, 1981; Gregory & Litherland, 1986; Kabir et al.,

1983a; 1983b; Leklem et al., 1980). For certain foods that have undergone heat processing, the bioavailability can be as low as 40 to 50%. Gregory and Kirk (1978) reported that the reduction of the Schiff base formed between the PL of the PLP and the amino groups of lysine residues in the protein was the most likely cause for the loss of vitamin B-6 during thermal processing and storage. The E-pyridoxyllysine residues formed in this way accounted for about half of the PLP that was degraded in the storage of dehydrated model systems containing PLP. Further, processed foods which contained these phosphopyridoxyllysine residues had low vitamin B-6 activity in bacteria and variable activity in rats. In a subsequent report Gregory (1980) observed that bovine serum albumin-bound phosphopyridoxyllysine had about 50% of the molar vitamin B-6 activity of PLP for rat growth, feed efficiency, and maintenance of liver PLP concentration. In animals this phosphopyridoxyl protein can induce a vitamin B-6 deficiency, based on experiments in which low doses of phosphopyridoxyllysine given to rats accelerated the onset of vitamin B-6 deficiency. This phosphopyridoxyllysine may account for the convulsive seizures observed in infants who were fed nonfortified, heat-sterilized, canned infant formula (Coursin, 1954).

Various forms of dietary fiber have been shown to have little effect on the bioavailability of vitamin B-6 (Nguyen et al.,1981; Nguyen & Gregory, 1983b, Leklem et al., 1980). Several studies have indicated that the utilization of vitamin B-6 from animal derived foods often is more efficient than from plant sources (Nguyen et al., 1983a; Kabir et al., 1983a). The presence of the poorly utilized beta-glucoside of B-6 vitamers in plant tissues may account for the incomplete bioavailability of vitamin B-6 of plant-derived foods (Kabir et al., 1983a; 1983b). Differ-

ences in the apparent biological activity of various forms of vitamin B-6 may contribute to difficulty in accurately quantifying biologically available vitamin B-6. Recent studies indicated that the three non-phosphorylated B-6 vitamers exhibit different biological activity and that the magnitude of the difference depends on the criterion of B-6 nutriture being examined (Nguyen et al.,1982). Quantitative differences in the metabolism of B-6 vitamers in humans who have received a loading dose have been reported (Wozenski et al., 1980). Another potential source of inaccuracy in bio-availability studies is the influence of diet composition on the production of B complex vitamins by the intestinal microflora. Various types of dietary fiber have been shown to stimulate the intestinal production of vitamin B-6 by microorganisms in humans (Miller et al., 1980) and rats (Nguyen et al., 1981).

Metabolism, Transport, and Regulation

The vitamers PL, PN and PM are the forms in which most dietary vitamin B-6 reaches the liver following absorption. All three forms are rapidly transported into organs and tissues. This transport is promoted by the metabolic trapping as the 5'-phosphate esters in all tissues that have been examined. Results from studies of perfused liver indicate that PN is rapidly taken up by passive perfusion, followed by metabolic trapping as PNP (Mehansho et al., 1980a). In perfused kidney, PN has been found to accumulate against a concentration gradient even when the PN concentration was high enough to saturate the PL kinase (Hamm et al., 1980). While this suggests active transport into kidney, it is more likely that PN accumulates in the tubules, because tubular secretion of this compound occurs when PN is present at high concentrations in the blood. Pyridoxal, which

is not excreted by tubules, is taken up by the kidney less rapidly and accumulates as result of phosphorylation (Hamm et al.,1980).

The various forms of vitamin B-6 are converted to PLP in the body. Although other tissues such as red blood cells (RBC) contribute to B-6 metabolism, the liver is thought to be responsible for forming the PLP appearing in plasma (Lumeng et al., 1974). When PN, along with PM and PL, are taken up by liver cells, they are rapidly acted upon by PL kinase and then converted to PLP by PNP oxidase (Mehansho et al., 1980a). The PL kinase has been found in most mammalian tissues, such as the liver, kidney, brain, spleen, blood cells, and small and large intestines (McCormick et al.,1961). The PL kinases purified from the liver, brain, and erythrocytes differ from each other in pH optima, metal ion requirement, and molecular weights (Karawya & Fonda, 1982). However, PNP oxidase, an enzyme which requires FMN as a cofactor, is confined to a few tissues. The liver has a very high concentration of the oxidase, but other tissues such as the lungs, heart, pancreas, and skeletal muscle have none (Pogell, 1958). Thus, it is suggested that most tissues are able to phosphorylate the B-6 vitamers, but are unable to convert the PNP and PMP vitamers to PLP. Therefore, some body tissues require the liver and red blood cells to convert PN and PM to PLP for subsequent release of these vitamers into the plasma (Mehansho & Henderson, 1980b). The PL kinase, the oxidase and the phosphotase provide a means of converting dietary PN to circulating PL, which can then serve a source of the coenzyme PLP in all tissues that contain PL kinase, whether or not they contain PNP oxidase. Lumeng & Li (1980a) found that PN is rapidly metabolized to PLP in isolated rat hepatocytes. The newly formed PLP was not freely exchangeable with endogenous PLP, but was converted to PL and

4-PA and released into the medium. In humans given physiological doses of PN, PL, or PM, PL and PN are rapidly cleared from the plasma and oxidized to 4-PA (Wozenski et al., 1980). Also, PL was the major labeled metabolite released from rat livers perfused with ^{3}H-PN (Mehansho et al., 1980a). Since PLP in plasma is bound to albumin and hence is not readily available to cells (Anderson et al., 1974), PL may be the major source of vitamin B-6 for most tissues and organs. Either free PL or PLP bound to albumin can cross the liver membrane and be transported to other tissues via the plasma. The charged phosphate group would hinder passage of PLP through the membrane (Snell & Haskell, 1971), but most tissues have membrane-bound phosphatases which can catalyze the hydrolysis of PLP to PL. This PL may then be taken up into the tissues (Lumeng & Li, 1975). Once the PL is in the cell, the cytosol kinases can convert it to PLP.

In erythrocytes, PN and PL are taken up by simple diffusion and phosphorylated by pyridoxine kinase. In human erythrocytes, the PNP oxidase forms PLP from PNP. However, the erythrocytes of rats and some other species exhibit no oxidase activity and the PNP has no known fate other than hydrolysis back to PN, which returns to the plasma and then to other tissues for conversion to PLP (Mehansho & Henderson, 1980b). Furthermore, erythrocytes cannot release the phosphorylated form from the cell (Anderson et al.,1971). Insofar as PLP is bound tightly to the hemoglobin, the red blood cells can contribute only PL to the plasma for transport to other tissues. However, the red blood cells may deliver vitamin B-6 directly to the tissues without first releasing it to the plasma. Figure 2-2 illustrates the tissue interrelationship of B-6 vitamers. The binding of PL to hemoglobin (Ink et al., 1982) and the resulting accumulation of

this vitamer in the erythrocyte provides an alternative means for PL to function as an important form in the blood.

The major metabolite of vitamin B-6 is 4-PA and is the form which is excreted in the urine. The metabolite 4-PA is formed from PL by the action of an FAD dependent aldehyde oxidase (Schwartz & Kjelgaard, 1951) or by the action of NAD dependent dehydrogenase (Stanulovic et al., 1976). While PL is acted on by dehydrogenases from many tissues, PL is a substrate for aldehyde oxidase only in the liver. The activities of the NAD dependent PL dehydrogenase were undetectable in cirrhosis patients (Merrill et al., 1986). Under normal dietary conditions urinary 4-PA is considered to be indicative of vitamin B-6 intake, not of the body stores. When physiological doses of PL, PM, and PN were given, the maximal ratio of urinary B-6 and 4-PA excretion occurred in the first three hours following the dose (Wozenski et al., 1980).

The enzymatic hydrolysis of PLP in various tissues may control the intracellular level of this coenzyme. The uptake and conversion of PN to PLP did not increase the PLP levels in the livers of rats that had been given normal vitamin B-6 intakes (Li et al., 1974). Middleton (1980) observed that low PLP levels of jejunal everted sacs from vitamin B-6 deficient rats were elevated by incubation with PN. Thus, the control of intracellular concentration of PLP would be exerted by the concentration of the PLP binding proteins in the cells, and when these proteins were saturated, the newly synthesized PLP would be hydrolyzed. Another potential control point for tissue is the activity of PNP oxidase. Because PN oxidase is subject to feedback inhibition by the product PLP, Snell and Haskell (1971) hypothesized that this control would be effective when PN or PM is the substrate. However, Li et al. (1974) found that although the

PNP oxidase affected hepatic PLP synthesis, it was not by itself sufficient to account for the high degree of regulation of PLP concentration in vivo. Merrill et al. (1978) have confirmed that the product inhibition of PNP oxidase plays a role in regulating the PLP concentration in tissues.

Storage

Unlike other water soluble vitamins, there is evidence that vitamin B-6 is stored in the body. Although the liver is rich in both PLP and PMP, the muscle, due to its mass, represents the single largest storage site of vitamin B-6 in the body (Li & Lumeng, 1981). Sixty percent or more of the vitamin B-6 present in rat muscle and 75 to 96% of that present in mouse muscle are associated with glycogen phospholyase (Krebs & Fischer, 1964). Since this enzyme comprises nearly 5% of the soluble protein of muscle, this enzyme serves as a physiological repository of vitamin B-6. Black et al. (1977; 1978) have found that in skeletal muscle approximately 90% of the PLP is bound to the glycogen phosphorylase, and muscle phosphorylase activity is lowered in vitamin B-6 deficient animals. When an excess of vitamin B-6 is given, both muscle PLP content and glycogen phosphorylase activity increase proportionately. However, these investigators found that muscle glycogen phosphorylase activity does not decrease with a vitamin B-6 deficiency unless a caloric deficit was also introduced. A decrease in the amount of glycogen phosphorylase is presumed to occur concomitantly with the release of PLP from the enzyme.

Hemoglobin-bound PLP in the red blood cells could function as a further reservoir of vitamin B-6 (Lumeng et al., 1980b). However, this may be a trap since the removal of PLP from hemoglobin is difficult,

requiring rigorous conditions in vitro for release of the vitamer for microbiological assay (Miller & Edwards, 1981).

Functions of Vitamin B-6

Vitamin B-6 is best known for its role as a coenzyme in amino acid metabolism. In most of these reactions, PLP acts as a coenzyme for aminotransferase by forming a Schiff base. Over 50 PLP-dependent aminotransferases have been discovered (Rawn, 1983). The general reaction catalyzed by these enzymes is shown in Figure 2-3. The glutamate family of amino transferases is especially important because its corresponding alpha-keto acid is a citric acid cycle intermediate, and the glutamate family of amino transferases thus provides a direct link between the citric acid cycle and amino acid metabolism. Among this family, aspartate aminotransferase (ALT; EC 2,6,1,1) and alanine aminotransferase (AST; EC 2,6,1,2) both convert amino acids to citric acid cycle intermediates. The carbon skeletons of aspartate and alanine can both be incorporated as a part of glucose by gluconeogenesis.

Most animal tissues possess alanine aminotransferase, the enzyme which catalyzes the reversible conversion of L-glutamate and pyruvate to L-alanine and alpha-ketoglutarate. The liver contains particularly large amounts of this enzyme (Rawn, 1983).

Glutamate + pyruvate $\xleftrightarrow[\text{alanine aminotransferase}]{}$ alpha-ketoglutarate + alanine

Alanine aminotransferase provides a source of pyruvate for gluconeogenesis. Pyruvate in muscle is transaminated to give alanine, and the alanine is transported to the liver and transaminated to pyruvate. The pyruvate

can then be converted to glucose. Alanine accounts for over half of the amino acid converted to glucose in the human liver (Rawn, 1983).

PLP plays an active role in glycogenolysis in the liver. As mentioned previously, PLP is a cofactor for glycogen phosphorylase (Krebs & Fisher, 1964). This enzyme catalyzes the phosphorolytic cleavage of alpha-1,4 glycosidic bonds of glycogen, starting at the nonreducing end of the polysaccharide (Helmreich & Klein, 1980). PLP binds to Lys-679 in rabbit muscle phosphorylase with the 5'-phosphate group adjacent to the substrate binding site. This suggests that the phosphate is involved in the catalysis (Sygusch et al., 1977; Titani et al., 1977).

Vitamin B-6 has long been known to be involved in lipid metabolism. As early as 1938, Halliday (1938) observed that the livers of pyridoxine deficient rats contained two to three times as much fatty acid as in the livers of normal rats. Studies in vitamin B-6 deficient rats showed there was an accumulation of lipid, consisting mainly of triglyceride and cholesterol (Okada & Iwami, 1977; Gomikawa & Okada, 1978; Suzuki & Okada, 1982a,b). Vitamin B-6 deficiency resulted in a marked decrease in the oxidation of palmitate and linoleate (Dussault & Lepage, 1979). Recently, Cunnane et al. (1984) reported that essential fatty acid turnover between triglyceride (TG) and phospholipid may be influenced by pyridoxine. Also, PLP is a cofactor of serine transhydroxymethylase (Hulse et al., 1978), which may be identical to 3-hydroxy-6-N-trimethyllysine aldolase, an enzyme involved in carnitine synthesis. Carnitine is a fatty acyl carrier from cytoplasm to mitochondria for β-oxidation of long chain fatty acids (Bremer, 1983). This is discussed in greater detail in a subsequent section.

Vitamin B-6 plays an important role in brain function. Neurotransmitters, such as dopamine, norepinephrine, serotonin, r-amino butyric acid (GABA), histamine, and taurine are synthesized in the body. L-DOPA decarboxylase, a PLP dependent enzyme, participates in the metabolic pathway leading from tyrosine to dopamine. Dopamine is an intermediate in the biosynthesis of the neurotransmitters, epinephrine and norepinephrine. L-DOPA decarboxylase also decarboxylates 5-hydroxytryptophan to produce serotonin, another neurotransmitter (Lovenberg et al., 1962). In the central nervous system, GABA, which does not penetrate the blood brain barrier, is formed primarily from glutamic acid with the aid of a PLP-dependent L-glutamic acid decarboxylase (Haber et al., 1970). GABA is catabolized to glutamate and succinic semialdehyde by GABA transaminase, a PLP-requiring enzyme (Baxter & Robert, 1958). In contrast to glutamic acid decarboxylase, PLP is bound tightly to GABA transaminase. PLP is also involved in the synthesis of taurine and histamine as a cofactor (Pasantes-Morales et al., 1976; Schayer, 1959).

Vitamin B-6 is also involved in nucleic acid synthesis and functioning of the immune system. In folate metabolism, PLP is a cofactor of serine transhydroxymethylase, which catalyzes the interconversion of tetrahydrofolate (FH_4) to N^5N^{10}-methylene-FH_4. This N^5N^{10}-methylene-FH_4 is involved in the synthesis of thymine, which is required for the formation of deoxyribonucleic acid. This is important in maintaining the integrity of the immune system, in which a vitamin B-6 deficiency can impair both humoral and cell-mediated immune responses (Robson et al., 1978).

Vitamin B-6 is involved in heme synthesis. PLP is a cofactor for aminolevulinate synthetase, which catalyzes the synthesis of aminolevulinate, an intermediate compound in the formation of porphyrin. This latter

substance is necessary for the formation of the porphyrin ring of hemoglobin (Richert & Schulman, 1959). Also, anemia was reported in pyridoxine deficient men (Raab et al., 1961).

Vitamin B-6 acts as a modulator of hormone activity by interacting at the receptor sites and decreasing the effect of glucocorticoid (Litwack, 1979) and in maintaining the integrity of the immune system where vitamin B-6 deficiency can impair both humoral and cell-mediated immune responses (Robson et al., 1978). A study (Kondo, 1985) on the activity of tryptophan oxygenase, which is known to be induced by glucocorticoid, suggested that PLP has an effect upon glucocorticoid activity since tryptophan oxygenase was higher in the livers of pyridoxine-deficient rats than in those of control rats.

Effect of Vitamin B-6 Deficiency in Rats

The dietary requirement for growth and reproduction of the rat is at least 5 mg/kg diet and approximately 7 mg/kg diet are required for maintenance of normal aminotransferase activity (National Research Council, 1978). In the absence of an adequate diet, progressive vitamin B-6 deficiency leads to anorexia, poor growth, emaciation and symmetrical scaling dermatitis on the tail, paws, face, and ears of rats (Sherman, 1954). Signs of neurological disturbance due to pyridoxine deficiency have been observed in rats (Chick et al., 1940). Anemia also occurs in pyridoxine-deficient rats (Kornberg et al., 1945).

Disturbances of amino acid metabolism, abnormal absorption of amino acids, and abnormal tissue concentrations of amino acid have been observed in vitamin B-6 deficient animals. A considerable decrease in the rate of absorption of L-methionine, histidine, and L-tyrosine occurs in

pyridoxine-deficient rats. These rats were restored to normal by the administration of pyridoxine (Akedo, 1960). Asatoor et al. (1972) reported that the absorption rate of 18 amino acids was depressed to about half the rate in normal rats. Rats deficient in pyridoxine have a decreased concentration of tissue PLP and lower activities of 5-hydroxytryptophan decarboxylase (Buzaard, 1957), serine and homoserine dehydratases, kynureminase, alanine and aspartate aminotransferases, and muscle phosphorylase (Takami et al., 1968). Alanine aminotransferase activity in the liver is restored by incubation with PLP in vitro, but aspartate aminotransferase activity is only partially restored by the addition of PLP in vitro (Okada & Hirose, 1979). Moreover, Shibuya and Okada (1986) reported that PLP does not affect the turnover of mitochondrial aspartate aminotransferase in rat liver. Evidence of abnormal tryptophan metabolism in pyridoxine deficiency is provided by a rise in the urinary excretion of xanthurenic acid, with the amount excreted being in direct proportion to the progress of vitamin B-6 deficiency in the rat (Kornberg et al., 1945).

Evidence of abnormal amino acid metabolism with vitamin B-6 deficiency is also provided by abnormal urea metabolism. In rats fed a 70% casein diet deficient in vitamin B-6, urinary urea decreased from 80 to 50% of the total nitrogen, blood urea concentration decreased, and the urinary excretion of free ammonia decreased while the excretion of free amino acids increased (Okada & Suzuki, 1974). However, there was no difference in the total nitrogen and creatinine in the urine of pyridoxine deficient rats. Cystathionine and citrulline were excreted in large amounts by deficient rats (Okada & Suzuki, 1974).

Pyridoxine deficiency also alters carbohydrate metabolism by decreasing the activity of phosphorylase. The total phosphorylase activity, as

well as that of phosphorylase in the muscle and liver, has been found to be considerably decreased by pyridoxine deficiency in the rat (Illingworth et al., 1960; Eisenstein, 1962). A vitamin B-6 deficiency does not seem to influence the activities of hepatic glycolytic enzymes (Ribaya & Gershoff, 1977). The availability of insulin is decreased (Huber et al., 1964) and sensitivity to insulin administration increases (Sabo & Gershoff, 1971b; Makris & Gershoff, 1974) in vitamin B-6 deficient rats. Vitamin B-6 deficiency results in lowered levels of blood glucose, blood pyruvic acid, lactic acid, and liver glycogen (Beaton et al., 1954; Huber et al., 1964). Furthermore, pyridoxine deficiency severely impairs the absorption of glucose and glycine from intestine due to marked changes in chemical structure architecture of brush boarders. These changes probably explain the observed aberrations in epithelial cell function in this nutritional deficiency (Mahmood et al., 1985).

Alterations in lipid metabolism have been reported in vitamin B-6 deficient rats. Although body fat stores (Beare et al., 1953; Huber et al., 1964) and ^{14}C incorporation from labeled glucose into carcass fat (Angel & Song, 1973) are lowered in vitamin B-6 deficient rats, lipogenesis by liver slices (Audet & Lupien, 1974; Sabo et al., 1971a), adipose tissue segments (Huber et al., 1964), and adipocytes (Sabo & Gershoff, 1971b) from vitamin B-6 deficient rats were reported to be enhanced in comparison with controls fed vitamin B-6. This suggests that the lipogenic potential of vitamin B-6 deficient rat tissue is not necessarily impaired when an adequate supply of substrate is provided. However, the activities of the hexosemonophosphate shunt dehydrogenase and of malic enzyme, which generate reduced nicotinamide adenine dinucleotide phosphate (NADH) for fat synthesis, are lowered in the liver (Ribaya & Gershoff, 1977;

Radhakrishnamurty et al., 1968) and the epididymal adipose tissue (Ribaya & Gershoff, 1977; Angel & Song, 1973) of vitamin B-6 deprived rats.

Metabolic Adaptation During Fasting

Adaptation Mechanisms

The metabolic events of starvation allow animals to live for a period of time without caloric intake, while maintaining a close to normal metabolic rate and amount of physical activity. Metabolic adaptation during the early and gluconeogenic phase of starvation is initiated by a small decline (555 to 833 μmol/L) in blood glucose, which results from continuing glucose utilization, particularly by the brain (Marliss et al., 1970). Hormones such as insulin, glucagon, corticosterone, and epinephrine are known to affect many reactions that regulate the level of glucose in blood (Ruderman & Herrera, 1968). A lowered blood glucose concentration is a signal for the insulin level to fall and the glucagon level to rise. In the rat gastrocnemius muscle acute short term fasting (2 days) resulted in a 50 % depletion of muscle glycogen. The changes in the enzyme profile favored reduced muscle glycolysis with preservation of oxidative metabolism and increased fat oxidation. Prolonged food restriction presumably resulted in severe depletion of body fat stores and reduced fatty acid oxidation (Russell et al., 1984). Additionally, 72 hours starvation in the rat was characterized by a rapid loss in liver weight (50%) and proteins (50%, organ base). With the exception of the nuclei, the loss of liver protein is reflected in a loss of protein in most organelles, including mitochondria, microsomes, and cytosol. The increase in the activity of the lysosomal enzyme, cathepsin D, measured during this period is indicative of a general

increase in catabolic processes. However, the nuclear protease activity decreased during this period, suggesting an organelle compartmentation of the degradation process (Shyamala, 1984).

With food deprivation, mammals develop an efficient mechanism for nitrogen conservation, particularly for essential amino acids. The response of muscle protein metabolism to starvation is dependent on a variety of factors including the age of the animal, the size of the animal's adipose stores, and the duration of the fast. Because fat-fed obese rats were found to maintain protein synthesis and prevent the terminal increase in proteolysis twice as long as their chow-fed counterparts (Goodman et al., 1981), the availability of lipid fuels is an additional factor influencing the ability of muscle to conserve protein. With a more prolonged fast, a variety of mechanisms for nitrogen conservation occur in the body. Felig et al. (1970) reported a marked curtailment in amino acid release from the forearm muscle of men subjected to a prolonged fast. In these men an equally marked diminution of total urinary nitrogen excretion was observed. Cahill (1970) reported that the decrease in plasma urea concentration and total nitrogen excretion is consistent with the activation of a nitrogen conservation mechanism. In addition, increased free ammonia con-centrations favor gluconeogenesis from lactate by the liver, thus sparing amino acids (Grunnet & Katz, 1978).

The hypoglycemia which develops during fasting changes the humoral balance, which is reflected by increased secretion of growth hormone and glucagon, activation of the adrenergic system and pituitary adrenal axis, and decreased secretion of insulin (Tepperman, 1982). Such hormonal changes are probably responsible for the activation of lipolysis of triglyceride during prolonged fasting. Fasting has been found to blunt the

ability of insulin to promote glucose incorporation into glycogen in vitro (Kaslow & Eichner, 1984), thereby maintaining a constant blood glucose level.

The relative maintenance of plasma glucose concentration is based on the balance between the increase in liver glycogenolysis and gluconeogenesis, as well as a decrease in glucose utilization in most tissues, mainly through substitution of other substrates. Since blood glucose remains unchanged as starvation extends beyond three days, the adaptation to prolonged fasting must include a reduction in glucose utilization. In humans the brain, which normally consumes in excess of 100 to 125 g of glucose per day, meets its fuel requirements through a total glucose production rate of only 80 g/day (Owen et al., 1969). In fasting humans, the brain consumes large amounts of ketones, accounting for 50 to 60% of its total fuel requirement (Owen et al., 1969). The decrease in glucose utilization in the late phase of starvation can be accounted for by an altered brain metabolism, but in theory the decrease in glucose production could result from primary changes in hepatic gluconeogenesis, alterations in substrate release from muscle, or a combination of both mechanisms. That the liver is not the prime regulatory site of decreased gluconeogenesis was suggested by the fact that the functional extraction of alanine by the splanchnic bed is no less in prolonged fasting than in postabsorptive man, while the level of circulating alanine (Felig et al., 1969) and the output of alanine from muscle (Felig et al., 1970) were markedly reduced when compared to other amino acids.

Ketones have a dual role, serving as "substrate" as well as "signal" in the late phase of starvation. Ketones are the major energy-yielding substrate for the brain, thereby reducing the demand for glucose.

Concomitantly, they provide a signal to muscle, resulting in decreased amino acid catabolism and a reduced output of alanine. In turn, the resulting hypoalaninemia is responsible for the reduction in hepatic gluconeogenesis (Sherwin et al., 1975). Ketone bodies and fatty acids, which during starvation originate from adipose tissue, inhibit glycolysis and pyruvate oxidation in the heart and skeletal muscle (Randle et al., 1964). This extraordinary increase in plasma ketones with fasting is mainly due to increased fatty acid metabolism by the liver (Aoki et al., 1975).

Starvation is characterized by an increased availability of free fatty acids (FFA) for cellular oxidative metabolism. The stimulation of these processes is affected by a decline in plasma insulin levels consequent to food deprivation (Cahill et al., 1966; Goodman & Knobill, 1961). Palou et al. (1981) have also reported that fasting more than 24 hours is accompanied by a decrease in blood glucose and an increase in plasma FFA in the rat. An elevation of basal FFA levels, resulting in more FFA utilization in tissues (Ensinck & Williams, 1972), permits a lower glucose consumption. Strubbe et al. (1986) suggested that the decreased responsiveness of insulin secretion by the beta-cell after moderate fasting periods results in a decrease of basal insulin. In turn, this facilitates the conversion from glucose to FFA metabolism even when the first meals are missed. This effect saves the glycogen stores as long as possible as a source of glucose for fuel for the central nervous system. Although the decrease in plasma insulin may allow for increased lipolysis, excessive lipolysis is prevented by an increased antilipolytic action of insulin during fasting (Strubbe & Prins, 1986).

Gluconeogenesis

In the postabsorptive state, glycogenolysis is the main source of glucose released by the liver (Felig & Wahren, 1971). Because the capacity of the liver to store glycogen is limited, the glycogenolysis process cannot supply glucose indefinitely during prolonged fasting. Therefore, man's ability to survive after glycogen stores are depleted is in part dependent on the body's ability to derive the necessary glucose via gluconeogenesis. Gluconeogenesis from lactate, glycerol, and amino acids, which are released from peripheral tissues, is another important factor in maintaining an adequate supply of glucose during brief periods of starvation. The end glycolytic product, lactate, may be converted back to glucose in the liver and kidney (Cori cycle). However, the Cori cycle does not provide net glucose or net energy since the lactate was originally glucose-derived and the energy required for resynthesizing glucose offsets that derived from glycolysis (Exton, 1972).

In the fasting state, fat also plays an important role in the regulation of gluconeogenesis. Energy for hepatic glucose synthesis is derived from fatty acid oxidation, a plentiful energy source. Hepatic fatty acid oxidation can increase gluconeogenic flux by providing acetyl CoA and reducing equivalents (NADH). Acetyl CoA is necessary for the reaction catalyzed by pyruvate carboxylase, while NADH is required to displace the reversible reaction catalyzed by glyceraldehyde 3-phosphate dehydrogenase in the direction of gluconeogenesis (Ferre et al., 1982; Benmiloud & Frienkel, 1967). In addition to providing energy for glucose synthesis in the Cori cycle, a part of the fat molecule plays a more direct role as an

ongoing source of glucose. The glycerol skeleton of triglyceride is readily converted to glucose, yielding about 18 g of glucose each 24 hours (Cahill et al., 1966). As lipolysis increases during fasting, the released glycerol becomes a significant, if relatively minor, substrate for glucose synthesis. Administration of glycerol at a rate of 2 mmol/min resulted in a rise in renal glucose output to 0.16 mmol/min, which could be accounted for in its entirety by renal glycerol uptake (Bjorkman et al., 1980). In rats, fasting induced a four to five-fold increase in glucose synthesis from glycerol when compared to fed rats (Friedman et al., 1970).

Finally, protein derived amino acids are a major gluconeogenic substrate. Skeletal muscle represents the major protein reservoir in vertebrates. During fasting, protein synthesis in muscle is markedly reduced, while protein breakdown is accelerated (Ogata et al., 1978). The amino acids released from this net loss of tissue proteins provide more than 50% of the precursors for gluconeogenesis in the liver and the kidney (Cahill et al., 1966; Exton, 1972). Although all amino acids other than leucine have long been recognized as potentially glucogenic, the classic "glucogenic/ketogenic" classification of amino acids fails to take into account the relative availability of individual amino acids as endogenous glucose precursors. When one examines amino acid balance across muscle tissue, the major reservoir of body protein stores, a specific pattern of amino acid exchange emerges. A net release is observed for virtually all amino acids. However, the output of alanine and glutamine exceeded that of all other amino acids (Felig et al., 1970). Analysis of the composition of various proteins in muscle reveals that alanine accounts for no more than 7% of amino acid residues, yet it is responsible for 30 to 40% of the total amino acid output from muscle. According to Felig (1973), alanine is

synthesized in muscle by transamination of glucose-derived pyruvate. The alanine is then released by muscle and taken up by the liver, where its carbon skeleton is reconverted to glucose. Using an isolated rat diaphragm preparation, Odessey et al. (1974) showed that 60 to 70% of alanine coming from muscle tissue is glucose-derived. In addition, a small proportion of the carbon skeleton of alanine may be derived from the catabolism of other amino acids. With regard to the source of the amino groups for alanine synthesis in muscle, studies of isolated muscle tissue (Odessey et al., 1974; Buse et al., 1972) have suggested that the branched chain amino acids (valine, leucine and isoleucine) are preferentially catabolized in muscle and are the major source of nitrogen for alanine synthesis. Moreover, Felig et al. (1969) reported that gluconeogenesis from alanine was markedly stimulated during early fasting, but decreased during prolonged periods of fasting.

The question arises whether or not the body's adaptation to fasting involves increased efficiency of conversion of alanine into glucose, as well as an alteration in the efficiency of alanine extraction by the liver. According to Chiasson et al. (1979), fasting is associated with a gradual decrease in hepatic alanine extraction even though the fractional uptake of alanine is increased. However, gluconeogenesis from alanine is increased after 2 days and 18 days of fasting in man due to more efficient intrahepatic conversion of alanine to glucose.

Both the human liver and kidney possess the necessary enzymes for gluconeogenesis. Hepatic gluconeogenesis, which is of quantitative importance for glucose homeostasis in the postabsorptive state, is increased after two or three days of fasting (Garver et al., 1974; Chiasson et al., 1979). In contrast, renal gluconeogenesis (resulting in net renal glucose

output) was observed only after prolonged (4 to 6 weeks) starvation (Owen et al., 1969). In 60 h-fasted man, administration of glycerol (2 mmol/min) stimulated renal glucose production, while alanine infusion increased splanchnic, but did not affect renal glucose output. Thus, hepatic and renal gluconeogenesis respond differently with respect to stimulation by specific substrates (Bjorkman et al., 1980).

Transamination of Amino Acids Relation to Vitamin B-6

During starvation skeletal muscle becomes largely responsible for providing the substrates for gluconeogenesis by increasing its release of amino acids via transamination. Long term fasting induces an increase in the activity of liver aminotransferase. According to Palou et al. (1980), there was a significant increase in muscle alanine aminotransferase activity in rats following 24 hours of fasting. Furthermore, the combined peripheral tissues (muscle + adipose tissue + skin) activity showed a mean 35 % increase, and extrahepatic splanchnic tissue (intestine + stomach + kidney) activity increased by a mean 21%, while liver activity decreased by a mean 14% because of a decrease in liver size.

Various degrees of decrease in the activity of PLP dependent enzymes in the liver have been reported in pyridoxine-deficient animals (Ludwig & Kaplowitz, 1980; Plebani et al., 1980). PLP is tightly bound to alanine aminotransferase (EC 2,6,1,2). This enzyme is usually present in the form of the holoenzyme, but PLP easily dissociates from L-serine dehydratase (EC 4,2,1,13) and tyrosine aminotransferase (EC 2,6,1,5). Thus, these enzymes are largely present as the apoenzymes (Okada & Suzuki, 1974; Greenberg, 1964). The holoenzyme activity of aspartate aminotransferase decreased by 17% and 40% of the normal levels in the

liver and kidney, respectively, of rats fed 70% casein without pyridoxine for 3 weeks (Okada & Hirose, 1979). The activity in the kidney fraction of deficient rats was restored to near the control level by addition of PLP, whereas that of the liver was only partially restored.

The activity and the amount of aspartate aminotransferase may be regulated in different ways in different tissues because the enzyme activities in the liver, heart, brain, and muscle preparations of the pyridoxine deficient rats were abnormally low, whereas those in other tissues (the kidney, intestine, spleen, and lung) were similar to those of control rats (Shibuya et al., 1982). In liver and heart preparations, the antigen activities of the deficient rats were similar to those in controls, but in brain and muscle mitochondria they were abnormally low. One unit of antigen activity was defined as the amount of immuno reactive substance equivalent to the amount of antibody precipitating one unit of enzyme activity of control rat tissue. These observations suggest that in pyridoxine-deficient rats, the mitochondria of the liver and the heart contain a substance that reacts with anti-mitochondrial enzyme, but has little or no enzyme activity. Thus, Shibuya et al. (1982) suggested that PLP may regulate not only the activity, but also the amount of mitochondrial aspartate aminotransferase in muscle and brain. In addition, they reported conformational changes of the enzyme molecule in pyridoxine-deficient rat liver; i.e., a decrease in helical content and a difference in the microenvironment of tryptophan residues compared to those of the enzyme from control rat liver. This difference seems to be caused by lability of the enzyme protein, resulting from lack of PLP during or after synthesis; the coenzyme seems to be important for maintenance of the native protein structure (Kuroda et al., 1982).

According to Okada and Kondo (1982), 90% of the aspartate aminotransferase in rat liver is present as the holoenzyme. With pyridoxine deficiency, the ratio of holoenzyme activity to total activity is markedly reduced, but after pyridoxine injection the ratio was found to increase rapidly. However, the total enzyme activity remained low for a few days. The intestinal enzyme activities of pyridoxine-deficient rats were readily reconstituted in the presence of PLP in vitro, but the enzyme activities in liver and muscle in deficient rats required several days for complete recovery. Thus, Okada and Kondo (1982) suggested that active enzyme was synthesized de novo in these tissues. This was assumed to be related to an increase in the functional state of glucocorticoid hormone (Disorbo & Litwack, 1981) or thyroid hormone (Okada & Ikeda, 1984). In contrast to the inactive form of aspartate aminotransferase, inactive alanine aminotransferase is readily reconstituted with PLP in vivo or in vitro (Perry et al., 1979). Addition of PLP to liver homogenates increased liver alanine aminotransferase activity, but not aspartate aminotransferase activity, in patients with alcoholic hepatitis who had initial low concentrations of plasma PLP (Diehl et al., 1984). In rats fed excessive vitamin B-6 (500 x requirement for the rat), compared to rats fed normal amounts of vitamin B-6, no significant differences were found with regard to basal or stimulated hepatic alanine aminotransferase activities, hepatic DNA, or protein content expressed on either an organ or per gram basis (Fay & Driskell, 1981).

Russell et al. (1985) observed that the prior intake of excess vitamin B-6 does not prevent the subsequent development of vitamin B-6 deficiency when vitamin B-6 intake is minimal. This was because for two months following the removal of high levels of supplemental vitamin B-6

from the diet of rats, the rate of erythrocyte aspartate aminotransferase activity coefficient increase was different from the respective rate of increase in rats with unsupplemented diets.

Impairment of gluconeogenesis from amino acids in vitamin B-6 deficiency is a likely consequence of diminished transamination prior to oxidative deamination. The activities of other enzymes involved in gluconeogenesis, such as glutamate dehydrogenase, pyruvate kinase, pyruvate carboxylase, and phosphoenolpyruvate carboxykinase were not influenced by vitamin B-6 deficiency (Angel, 1980). Although gluconeogenesis from amino acids is impaired as a result of diminished transamination, the serum glucose concentration remains normal in pyridoxine-deficient rats. Furthermore, a low concentration of liver glucose and a marked decrease in liver glycogen was apparent in pyridoxine deficient rats. Suzuki and Okada (1982a) suggested that glucose which was newly synthesized by either glycogenolysis or gluconeogenesis is preferentially released into the blood to maintain normal glucose levels. In contrast to the serum glucose level, liver glucokinase activity in pyridoxine-deficient rats was lower than in control rats. Serum immunoreactive insulin was also lower in pyridoxine-deficient rats than in controls. Thus, a lower level of physiologically active insulin decreases the utilization of glucose in liver cells and, conversely, glucagon and other hyperglycemic hormones may promote secretion of glucose from liver cells of pyridoxine-deficient rats (Suzuki & Okada, 1982b).

Strenuous exercise such as running, can be viewed as acute starvation (Lemon & Nagle, 1981). Leklem and Shultz (1983) demonstrated an increase in circulating PLP directly after a strenuous exercise in adolescent males and hypothesized that the significant changes in plasma PLP

concentration found in their study were related to an increased need for cofactor for gluconeogenesis. Dreon and Bulterfield (1986) reported that physical activity was associated with lower 4-PA excretion, reflecting less conversion of the vitamin to 4-PA in active men (running 5 miles/day) than that portion in sedentary men during a period of similar dietary intake. Physical activity is known to increase the rate of gluconeogenesis, which in turn increases the requirement for additional transaminase in the liver and muscle (Bemzi et al., 1975). Moreover, physical activity increases the rate of glycogenolysis, which in turn increases the need for glycogen phosphorylase in muscle tissue (Taylor et al., 1972). Because PLP is a cofactor of these enzymes, the enzyme pools of the active individual could require a greater proportion of the dietary pyridoxine than required by the sedentary individual. Thus, as has been suggested by Dreon and Bulterfield (1986), exercise may promote storage of the vitamin B-6 that is necessary for subsequent redistribution requirements.

Metabolism and Nutritional Implications of Carnitine

Introduction

Toward the beginning of this century carnitine (3-hydroxy-4-N-trimethylammoniobutanoate) was found to be an important compound in meat (muscle tissue) extracts. The chemical structure was determined in 1927 by Tomita and Sendju (Friedman & Fraenkel, 1972). In 1952, Carter et al. (1952) created new interest in carnitine when they established that it is a growth factor for the meal worm Tenebrio molitor and thus named the compound vitamin B_T ("T" for Tenebrio).

The first clue of the metabolic role of carnitine came from Bhattacharya et al. (1955). They observed the carnitine analogue gamma-butyrobetaine in Tenebrio larvae when they were starved. Six years later, Fritz (1961) showed that carnitine stimulates fatty acid oxidation in liver homogenates, particularly those having chain lengths greater than C_8. Carnitine is most likely present in all animal species, in many microorganisms, and in some high plants (Fraenkel, 1953; Mitchell,1978a; Panter & Mudd, 1969). Carnitine has vitamin-like properties for a few organisms which do not synthesize it and has a vital biological role. The marked variation in the abundance of carnitine in different organisms and in different tissues within an organism has led to speculation that probably it has more than one biological function (Fraenkel, 1980).

The amount of carnitine in different species and the concentration in different tissues varies over a wide range. The highest concentrations reported have been found in horseshoe crab muscle (Fraenkel, 1980) and in rat epididymal fluid, a fluid in which carnitine can reach a concentration of 60 mmol/L (Brooks et al., 1974). In mammalian tissues the concentration usually varies between 0.1 and a few millimoles/kg (with the highest concentrations in the heart and skeletal muscle) and there are relatively great interspecies variations. For example, the carnitine concentration is about 1 mmol/L in rat skeletal muscle (Brooks & McIntosh, 1975), about 3 mmol/L in human muscle (Cederblad et al., 1974), and up to 15 mmol/L in ruminant muscle (Snoswell & Henderson,1980). When carnitine is found in plants, the concentration is only a few micromoles/kg (Panter & Mudd, 1969).

The requirement for carnitine in higher animals must be met either by the diet, particularly animal protein foods, or from de novo synthesis.

Hence, any consideration of carnitine as a nutrient must take into account the relative contribution of these two sources. Mitchell (1978a) has pointed out that information concerning the carnitine content of foods is scarce and in many ways unsatisfactory. In general, carnitine is low in plant foods and high in animal foods. For example, the edible portion of beef tenderloin and beef shoulder were reported to contain 59.80 and 67.40 mg, respectively, of carnitine per 100 g. In contrast, no carnitine was detected in a 100 g portion of vegetable protein mixture (soyameal, 50 g; rice, 30 g; pinto beans 20 g). Moreover, it is precisely those foodstuffs which are deficient in carnitine that are also limiting in its amino acid precursors, lysine and methionine. Such considerations have led a number of investigators to consider the effect on carnitine deficiency of the consumption of cereal grain diets in animals and in man. Human carnitine deficiency occurs both as a classical deficiency and in a number of other commonly occurring disease entities (Rudman et al., 1977; 1980; Bohmer et al., 1978).

Biosynthesis of Carnitine

While carnitine can be obtained from the diet, the body synthesizes carnitine. Broquist and Borum (1982) have suggested that two essential amino acids (lysine and methionine), three vitamins (ascorbate, niacin, and vitamin B-6), and a metal ion (reduced iron) are required for the biosynthesis of carnitine (see Figure 2-4).

The first clues to the mechanism of carnitine biosynthesis were obtained in 1961, when it was shown that the methyl groups of carnitine come from methionine (Bremer, 1961; Wolf & Berger, 1961). Gamma-butyrobetaine was then converted to carnitine (Bremer, 1962; Lindstedt &

Lindstedt, 1965). These results, however, did not provide information on the origin of the carnitine four carbon chain. The origin of this chain was established 10 years later when several investigators showed that labeled lysine is converted to carnitine in N. crassa (Horne & Broquist, 1973) and in the rat (Tanphaichitr & Broquist, 1973), with 6-N-trimethyllysine (TML) as an intermediate. Figure 2-4 shows the suggested pathway of carnitine biosynthesis. In N. crassa, free lysine is methylated, with S-adenosylmethionine serving as the methyl donor (Borum & Broquist, 1977b). In mammals, there is no evidence for methylation of free lysine to TML, as is observed in N. crassa. The protein methylase III, detected by Paik and Kim (1970) in rat liver, suggests that TML may arise from the hydrolysis of methylated proteins. Labadie et al. (1976) have provided direct evidence for this hypothesis of the carnitine biosynthesis in the rat.

S-adenosyl homocysteine is a potent inhibitor of rat liver protein methylase III in vitro (Ueland, 1982). This enzyme appears to be regulated by modulation of the intracellular molar ratio of S-adenosylmethionine to S-adenosyl homocysteine (SAM: SAH ratio). Trimethyllysine is then hydroxylated in a complex manner to yield 3 -hydroxy-6-N-trimethyllysine. This hydroxy-TML is cleaved to 4-N-trimethylaminobutyraldehyde (gamma-butyrobetaine aldehyde) and glycine. In 1978, Hulse et al. (1978) reported that serine transhydroxymethylase, a PLP requiring enzyme, has the capacity to cleave 3-hydroxy-6-N-trimethyllysine in vitro and in turn, this enzyme has been implicated in the scheme of carnitine biosynthesis. The aldehyde is oxidized by an apparently specific NAD-requiring dehydrogenase to gamma-butyrobetaine (Hulse & Henderson, 1979). The complex oxygenase system required for the hydroxylation of gamma-butyrobetaine to form carnitine has been well described in both rat liver (Lindstedt, &

Lindstedt, 1970a) and a microbial system (Lindstedt et al., 1970). The dioxygenase is present in the soluble portion of the cell and requires alpha-ketoglutarate, molecular oxygen, and ascorbic acid. Alpha-ketoglutarate and oxygen are required as substrates and react with gamma-butyrobetaine, likely forming a peroxide intermediate which decomposes, forming carnitine, succinate, and CO_2 (Lindstedt & Lindstedt, 1970).

Most animal tissues contain the enzymes necessary to convert trimethyllysine to butyrobetaine. However, butyrobetaine hydroxylase (gamma-butyrobetaine 2-oxoglutarate dioxygenase EC 1.14.11.1), which catalyzes the conversion of butyrobetaine to carnitine, is present only in a few tissues and shows species variation in tissue distribution. Butyrobetaine hydroxylase is found in the liver of all species studied. In the rat, this hydroxylase is also found to a small extent in the testis, but is absent from all other tissues (Cox & Hoppel, 1974). In humans the enzyme is present in the liver, kidney, and brain (Rebouche & Engel, 1980a). In the liver, conversion of trimethyllysine to butyrobetaine seems to take place in both parenchymal and nonparenchymal cells, whereas the hydroxylation of butyrobetaine to carnitine occurs only in parenchymal cells (Dunn & Englard, 1981). Thus, the liver of the rat and the liver and kidney of the human are the major sites of carnitine production.

The synthesis of carnitine in the rat has been calculated to be 2 $mol/100g \cdot d^{-1}$ (Cederblad & Lindstedt, 1976). Under normal conditions, very little carnitine is metabolized. Carnitine is excreted intact in the urine. In some physiological states such as cold stress, starvation, diabetes, and pregnancy, decarboxylation of carnitine to 5-methylcholine has been reported, but this observation needs confirmation (Khairallah & Wolf, 1967).

Transport of Carnitine Into Tissues

Since tissues such as cardiac muscle and skeletal muscle depend upon fatty acid oxidation as a major source of energy, but cannot synthesize carnitine, transport of carnitine from its site of synthesis to its site of action is important to cellular energy metabolism. Brooks and McIntosh (1975) have shown that the turnover times for carnitine in the kidney, liver, heart, skeletal muscle, and brain of the rat are 0.4, 1.3, 21, 105, and 220 hours, respectively. In rat plasma, carnitine concentration is controlled by sex hormones. While androgens raise the concentration, estrogens tend to lower it. Thus, adult males tend to have a higher concentration than adult females (Borum, 1980). Serum carnitine is mostly present primarily as free carnitine and short chain acylcarnitine (Pace et al., 1978).

L-carnitine has a greater affinity for binding to the transport system than D-carnitine (Molstad et al., 1977). Increasing the concentration of L-carnitine from 2 to 100 μmol/l in the growth medium of the human heart cell line increased the rate of uptake by 50%. This effect of L-carnitine could be inhibited by cycloheximide, indicating dependence on protein synthesis (Molstad et al., 1978). A cardiac carnitine binding protein has been isolated from rat hearts that may serve as the carrier for the transport of carnitine across the heart cell membrane (Cantrell & Borum, 1982).

At a low concentration, carnitine was absorbed by the small intestine of the rat by a stereospecific sodium dependent active process. At higher carnitine concentrations, such as those obtained after a large thera-

peutic dose, the small intestine absorbed carnitine by passive diffusion (Shaw et al., 1982). With carnitine concentrations normally present in the plasma, the uptake of carnitine by eipdidymal cells from normal rats was 10 times greater than that of cells from castrated rats. Uptake by epididymal cells from normal rats was a saturable process, whereas the uptake by cells from castrated rats was a nonsaturable process (James et al., 1981). Despite the existence of an active transport mechanism, carnitine disappearance from the intestinal lumen and its appearance in the blood was remarkably slow (James et al., 1981). Although the structure of carnitine closely resembles that of known substrates for the imino acid intestinal transport mechanism, Gudjonsson et al. (1985) reported evidence for a partially saturable absorption which appeared to be separate from the imino transport system. Because no inhibitory effects of L-proline and L-alanine on carnitine absorption were evident, these authors suggested that although esterification appears to have little to do with intestinal transport of carnitine, the enzyme responsible for the formation of acetylcarnitine may influence the slow mucosal release of carnitine by binding the substrate.

Regulation of Tissue Carnitine Concentration

The concentration of carnitine in tissues at one particular time is the summation of several metabolic processes. Normally tissue carnitine concentration remains constant, but tissue concentration and the relative distribution of free and esterified carnitine in animals have been shown to be influenced by diet, nutritional status, and various pathological, physiological conditions.

Nutritional status. Carnitine deficiency may arise because of a dietary deficiency of either of the precursor amino acids (lysine or methionine) or any of the cofactors (iron or ascorbic acid) required by the enzymes of the lysine-carnitine pathway. A diet limited in lysine has been shown to result in reduced carnitine concentrations in plasma, heart, skeletal, and cardiac muscle, and the epididymis, presumably owing to impaired carnitine biosynthesis (Borum & Broquist, 1977a). However, Lennon et al. (1986) reported that there was no significant relationship between either protein or carnitine intake and skeletal muscle carnitine concentrations, while there was a significant relationship of both dietary carnitine ($r = 0.50$) and protein ($r = 0.48$) intake with blood plasma total acid soluble carnitine concentrations in humans. Glucagon and insulin act as major regulators of intracellular protein catabolism that takes place within hepatic lysosomes. One important metabolic fate of these digestive products is the conversion of released trimethyllysine to carnitine (Aronson, 1980). Rebouche et al. (1986) suggested that the availability of E-N-trimethyllysine limits the rate of carnitine biosynthesis in growing rats.

Study of the in vivo effect of ascorbic acid deprivation was found to result in the severe depression of carnitine in guinea pigs (Nelson et al., 1981). Scorbutic young male guinea pigs had 50% less carnitine concentration in heart and skeletal muscles than control animals, but they were equal in liver, kidney, and plasma. The conversion of ^{14}C-trimethyllysine to trimethylaminobutyrate in the kidney was 8 to 10 times greater in control animals compared to the scorbutic animals. On the other hand, the hydroxylation of trimethylaminobutyrate, which occurs only in the liver, was not affected by ascorbic acid deficiency. Thus, it would appear that ascorbic acid deficiency affects the hydroxylation of trimethyllysine to a

greater extent than the hydroxylation of trimethylaminobutyrate (Nelson et al., 1981). These results differed from those of Thoma and Henderson (1984), who observed a decrease in liver carnitine concentration, but did not find a comparable decrease in the heart muscle. Both groups of investigators agreed that ascorbate deficiency caused a reduction in carnitine pool sizes, except for that in the kidney where the carnitine concentration was unchanged. When guinea pigs were fed a diet low in ascorbic acid, muscle carnitine concentrations were reduced even before the emergence of the symptoms regarded as characteristic of hypovitaminosis (Hughes et al., 1980). Guinea pigs supplemented with dietary carnitine survived longer on an ascorbate-free regime than unsupplemented animals (Jones & Hughes, 1982). This "sparing" effect of carnitine supports a role for ascorbic acid in the conversion of lysine and methionine to carnitine. Moreover, the reduction in synthesis of carnitine was reversed by the injection of ascorbate along with substrate in the guinea pig liver (Thoma & Henderson, 1984).

The role of iron in the biosynthesis of carnitine was the subject of another in vivo nutrient study. According to Bartholmey and Sherman (1985), severe iron deficiency in the suckling rat alters carnitine metabolism, leading to lowered carnitine concentration and greater triacylglycerol concentration in the liver on day 16 of lactation. In iron deficient pups the concentration of carnitine in the liver and heart was lower than the levels in the control pups. Carnitine biosynthesis may have been impaired in the former. In vitro studies have shown that ferrous iron is required in the synthesis of carnitine from TML (Hulse et al., 1978; Lindstedt, 1967).

PLP, a biologically active form of vitamin B-6, was suggested as one of the cofactors for carnitine biosynthesis (Hulse et al., 1978). In

this study, serine transhydroxymethylase, a PLP requiring enzyme, was able to cleave 3-hydroxy-6-N-TML to glycine and 4-N-trimethylaminobutyraldehyde in a liver homogenate system. This study has since been the basis for enzyme implication in the scheme of carnitine biosynthesis. Dunn et al. (1982) provided evidence that PLP is essential for the biosynthesis of carnitine by using a vitamin B-6 antagonist in isolated perfused rat liver systems. In the presence of 1-amino-D-proline, a vitamin B-6 antagonist, the total production of gamma-butyrobetaine, carnitine, and acetylcarnitine from protein-bound trimethyllysine was depressed by as much as 60 to 80% in the perfused rat liver. The decreased synthesis of carnitine was accompanied by the accumulation of an intermediate in the carnitine biosynthetic pathway (3-hydroxy 6-N-trimethyllysine). The effects of 1-amino-D-proline were almost completely reversed by inclusion of pyridoxine in the perfusing medium (Dunn et al., 1982). Furthermore, Loo and Smith (1986a) reported that in rats fed a pyridoxine deficient diet for 7 weeks, the total acid soluble carnitine concentration in skeletal and muscle heart was 29% and 25% less, respectively, than the values of pair fed or ad libitum fed rats. In addition, lysine methyltransferase activity, which catalyzes the initial reaction for the biosynthesis of carnitine, was reduced in the liver tissue during pyridoxine deficiency. It was also shown that a pyridoxine-deficient diet can increase the level of S-adenosylhomocystein (Eloranta et al., 1976). Protein methylase III, i.e, lysine methyltransferase, activity appeared to be regulated by modulation of the intracellular molar ratio of SAM SAH. Thus, the reduced molar ratio of SAM:SAH in pyridoxine deficient rats decreased the methylation of the ϵ-amino group of lysine residues present in histone by protein methyltranferase III and may have reduced the supply

of the initial substrate for the biosynthesis of carnitine (Loo and Smith, 1986b).

Although serine transhydroxymethylase, a PLP requiring enzyme, has the capacity to cleave 3-hydroxy-6-N-TML and D-proline, a vitamin B-6 antagonist, decreased synthesis of carnitine in vitro (Hulse et al., 1978; Dunn et al., 1982), no study of the effect of a vitamin B-6 deficiency on the in vivo synthesis of carnitine has been conducted. Moreover, no study of systematic changes of variable derivatives of carnitine in tissues and plasma has been done in conjunction with a vitamin B-6 deficient state.

Pathological conditions. Liver and kidney dysfunction could also impair carnitine synthesis. Apart from defective synthesis, other factors, such as increased metabolic losses due to catabolism, impaired kidney tubular resorption, defective transport machinery for transporting carnitine from tissues where it is synthesized (liver and kidney) to the tissues where it is maximally utilized (muscle and heart), and defective uptake by the tissues, may also lead to carnitine deficiency symptoms.

Rudman et al. (1977; 1980) reported low levels of plasma carnitine in 30% of human patients suffering from liver cirrhosis. To differentiate between the roles of exogenous (dietary) and endogenous (biosynthetic) factors in the causation of hypocarnitinaemia in cirrhotics, an excess supply of carnitine precursors was given to the patients and controls. In the patients, plasma carnitine levels failed to normalize, whereas the controls maintained their levels. From these results the investigators concluded that the hepatocellular disease blocks the pathway for biosynthesis of carnitine.

The kidney can influence carnitine levels in two ways: synthesis and excretion. Several patients with renal insufficiency have been

reported to have higher levels of plasma carnitine compared to controls. However, patients undergoing haemodialysis developed hypocarnitinaemia, as was suggested by low levels of plasma and muscle carnitine (Chu & Lincoln, 1977; Bohmer et al., 1978). In renal Fanconi syndrome, failure to reabsorb free and acyl carnitine resulted in a secondary plasma and muscle free carnitine deficiency (Bernardini et al., 1985). However, according to Pan and Wang (1985), kidney dysfunction may affect the plasma carnitine level, but not the total skeletal muscle carnitine concentration. This indicates that the availability of carnitine is not the sole factor controlling muscle carnitine level. Rat liver is the major site for carnitine synthesis, but the process is mainly dependent on the kidney to supply the precursor, gamma-butyrobetaine, for carnitine synthesis. In bilateral nephrectomized rats the incorporation of labeled trimethyllysine into carnitine in the liver is decreased 8-fold (Carter & Fraenkel, 1979). This led the authors to suggest that the low level of plasma carnitine observed in uremic rats was probably related to a greater reduction in carnitine synthesis despite the increase in carnitine retention. In 1973, Engel and Angelini (1973) reported a case of muscle dystrophy associated with lipid accumulation and impaired ability to oxidize long chain fatty acids. The carnitine concentration of the muscle was very low in these patients. In vitro addition of carnitine to the muscle restored the fatty acid oxidizing capacity.

Fatty acid oxidation is a major source of energy for cardiac tissue and any disturbance of fatty acid oxidation leads to severe metabolic impairment of cardiac tissue (Vary et al., 1981). In vitro studies showed that diphtheria toxin inhibits carnitine transport into an established cell line from the human heart by inhibiting the synthesis of carriers (Molstad &

Bohmer, 1981). According to Suzuki et al. (1982), the beneficial effect of L-carnitine on the ischaemic heart may be due to restoration of energy metabolism of ischaemic myocardium and prevention of ventricular arrhythmias.

Diabetic rats have decreased carnitine concentrations in the heart (Stearns, 1983; Vary & Neely, 1982; Brooks et al.,1985), the diaphragm (Stearns, 1983) and serum (Vary & Neely,1982; Brooks et al., 1985) when there is an elevated concentration of carnitine in the liver (Brooks et al., 1985; McGarry et al., 1975) and increased urinary excretion (Brooks et al.,1985). Brooks et al. (1985) concluded that changes in tissue carnitine seen with diabetes result from the redistribution and regulation of carnitine, which is due to an as yet undefined process.

Physiological conditions. Animals of all ages synthesize gamma-butyrobetaine, but the activity of gamma-butyrobetaine hydroxylase is age-dependent. The ability of the rat liver to synthesize carnitine from gamma-butyrobetaine increased from low levels in the fetus to adult values on the eighth day after birth (Hahn, 1981). In three month-old human infants, the gamma-butyrobetaine hydroxylase activity was 12% of normal adult men. By 2.5 years, the activity was 30% of adult men (Rebouche & Engel, 1980a). Newborn infants fed a diet devoid of carnitine rapidly showed a fall in plasma total carnitine levels (Penn et al., 1981), whereas in the adult such a fall was observed only after a prolonged total parenteral nutrition (Worthly et al. ,1983; Bowyer et al., 1986; Hahn et al., 1982). This suggests that carnitine synthesis in the newborn is less efficient than in the adult. According to Hahn and Novak (1985), the addition of L-carnitine to soybean-based formulas decreases plasma triglyceride and free fatty acids levels in premature infants who have lower carnitine

levels at birth than full-term babies. Daily urinary carnitine excretion increases with age from 3.5 to 18 years in normal children and adolescents (Eanes & Biniek, 1980). Adult men excreted more urinary carnitine than adult women (Maebashi et al., 1976), but adult male rats excreted less carnitine than adult female rats (Carter & Stratman, 1982).

Maebashi et al. (1976) reported that carnitine excretion varied widely in women during the menstrual cycle, reaching a maximum at the time of ovulation. In rats (Seccombe et al., 1978) and men (Frohlich et al., 1978), starvation led to a rise in the blood levels of acylcarnitine and ketones, reflecting an increased rate of fatty acid oxidation. At the same time, hepatic carnitine acetyltransferase activity was also increased. The plasma ratios of acid-soluble acylcarnitine to free carnitine have been directly correlated with the plasma ketone levels of metabolites in starved rats and humans (Brass & Hoppel, 1978; Hoppel, 1980). According to Brass and Hoppel (1978), plasma total carnitine concentration in rats decreased during the first 24 hours of starvation and then increased, reaching levels higher than controls due to an increase in acid soluble acylcarnitine. However, Kerner & Bieber (1983) reported that acylcarnitine concentration decreased with fasting, mainly due to the decrease of acetylcarnitine levels. They also showed that the use of anesthetics may induce significant quantitative changes in specific acylcarnitine levels, presumably reflecting changes in specific acylCoA levels.

The source of circulating acylcarnitines is not known. That various origins are possible has been demonstrated in lean and obese humans (Hoppel, 1980) and lean and obese rats (Brady et al., 1986). In the liver, the incomplete products of beta-oxidation in the mitochondria could be transported out of the mitochondria as acylcarnitines or, alternatively,

acylcarnitines could be formed by hepatic peroxisomes, which also contain enzymes for beta-oxidation and carnitine acyltransferases (Lazarous & De-Duve, 1976; Markwell et al., 1973). The possibility exists that the acyl-carnitines are produced in muscle from the increased oxidation of fatty acid and/or branched-chain amino acids (Bieber et al., 1982). Total carnitine in skeletal and heart muscle increased in Spargue-Dawley rats (Brass & Hoppel, 1978), but not in Zucker rats (Brady et al., 1986) as starvation progressed. In the Zucker rats it may be that heart and muscle cellular constituents, such as fat and glycogen, are not utilized to the same extent and/or water content per gram does not decrease rapidly. Liver free carnitine and total carnitine concentration increased with starvation and the increase was suggested as a reflection of the loss of other cellular constituents, rather than an increase in total hepatic carnitine (Brady et al., 1986; Brass & Hoppel, 1978). Furthermore, Davis and Hoppel (1986) reported that carnitine biosynthesis is limited by the availability of trimethyllysine, which in starved rats is limited by the rate of protein turnover.

With starvation, urinary excretion of total carnitine has been found to increase, mainly due to an increase in acylcarnitine (Suzuki et al., 1983). The urinary excretion of acetylcarnitine correlated with blood beta-hydroxy-butyrate concentration in human diabetic and fasting ketosis (Hoppel & Genuth, 1982). When protein is degraded, trimethylysine is released, becoming available for conversion to carnitine during fasting (LaBadie et al., 1976). Therefore, it is suggested that during fasting and periods of metabolic stress, increased protein degradation may provide extra substrate for carnitine biosynthesis. However, an exogenous supply of

carnitine has not shown any influence on protein turnover during periods of fasting (Hibbert et al., 1986).

Functions of Carnitine

Major function. Since all the enzymes for beta-oxidation are located within the mitochondrial matrix, the transport of fatty acid into the mitochondria is essential. Neither the major fatty acids nor their respective CoA derivatives readily penetrate the mitochondrial membrane. The fatty acylCoA reacts with carnitine to form fatty acylcarnitine. This reaction is catalyzed by carnitine palmitoyltransferase, which is located in the outer portion of the inner mitochondrial membrane. These carnitine derivatives are reconverted to intramitochondrial fatty acylCoA derivatives in the inner mitochondrial membrane. The fatty acylCoA molecule then enters the beta-oxidation sequence, whereas the carnitine molecule is freed for another shuttle (Figure 2-5). Thus, carnitine acts as a carrier of fatty acyl groups from the cytoplasm to mitochondria (Bremer, 1983). Because of this essential role of carnitine for the transport of long chain fatty acid (LCFA) into the mitochondrial matrix (Bremer, 1962; Fritz & Yuiki, 1963), tissues must contain an adequate concentration of carnitine, or beta-oxidation of LCFA will be impaired and cellular energy metabolism will be impaired. A lack of carnitine or an absence of carnitine transferase reveals a well-defined clinical picture, characterized mainly by intolerance to LCFA, such as lipid storage myopathy (Mitchell, 1978b).

However, peroxisomal fatty acid oxidation does not require the participation of carnitine. Apparently the peroxisomal membrane contains a direct carrier or a permease for acylCoA (Theirriault & Mehlman, 1965; McGarry & Foster, 1980). Glucagon and fasting do not activate

peroxisomal fatty acid beta-oxidation in rat liver (Gudjonsson et al., 1985), while glucagon (McGarry et al., 1975) increases liver carnitine in rats.

A lipid-lowering effect of carnitine therapy has been found in patients with type IV hyperlipoproteinemia (Maebashi et al., 1978), haemodialysis (Lacour et al., 1980), chronic uremic patients (Gaurniri et al., 1980), and alcohol induced fatty liver (Sachan et al., 1984). Furthermore, Abdel-Aziz et al. (1984) reported that carnitine caused a significant reduction in the level of serum total lipids, triglyceride, cholesterol, and nonesterified fatty acids, with a significant increase in the serum level of phospholipids in diabetic patients. L-carnitine treatment of diabetic rats significantly reduced the concentration of serum glucose, free fatty acids, and ketones, prevented a decrease in myocardial total carnitine content, and improved the recovery of cardiac output (Paulson et al., 1984). Hulsmann et al.(1982) showed that excess carnitine (5 mM L-carnitine) inhibits acylglycerol accumulation in heart during in vitro perfusion with a lipid emulsion. The mechanism of this phenomenon is probably the inhibition of lipid accumulation in the heart by acylcarnitine, rather than stimulation of fatty acid oxidation by excess carnitine.

Other functions. In addition to its key metabolic function, as the transfer medium of LCFA from cytoplasm into the mitochondria, carnitine may have other effects on metabolism. It is generally accepted that carnitine plays an important role in thermogenesis in brown adipose tissue. The body pool of carnitine was eight times greater in cold acclimated rats than in rats maintained at 25°C (Theirriault & Mehlman, 1965).

Carnitine also plays an important role in ketogenesis. Treatment of fed rats with anti-insulin serum or glucagon, a 24 hour fast, or the presence of alloxan diabetes enhanced long chain fatty acid oxidation and

ketogenesis in perfused rat liver and increased liver carnitine concentration two to four-fold (McGarry & Foster, 1980).

Carnitine serves a facilitative, if not an obligatory, role in branched chain amino acids catabolism (Bieber et al., 1982). L-carnitine increased the oxidation of branched chain 2-oxoacids derived from leucine and valine (VanHinsbergh et al., 1980). The use of carnitine therapy in isovaleric acidemia, a defect in leucine metabolism, appears to be as effective as glycine therapy in the removal of isovaleryl CoA and is more effective in reducing plasma isovaleric acid (Roe et al., 1984a).

Bieber et al. (1982) suggested that carnitine and short and medium chain carnitine acyltransferases in peroxisomes are involved in the shuttling of acetyl and medium chain acyl residues out of peroxisomes. These acyl residues are the products of the chain shortening processes in the peroxisome.

The rate of gluconeogenesis from propionate in rat kidney cortex slices was stimulated up to three and one-half times by D, L-carnitine, and by bicarbonate (Weidemann & Krebs, 1969). Addition of carnitine during the incubation of slices of rabbit liver with alanine-U ^{14}C increased the radioactivity of the glucose that was recovered in the suspending medium and tissue glycogen. Carnitine also promoted the incorporation of alanine carbon into glucose by the slices of kidney cortex from fasted rabbits and the labeling of glucose by liver slices incubated in the presence of NaH $^{14}CO_3$ (Benmiloud & Frienkel, 1967). In propionic acidemia, L-carnitine facilitates excretion of toxic acylCoA as propionylcarnitine, while restoring the acylCoA to free CoA ratio in the mitochondria (Roe et al., 1984a). Carnitine may influence cholesterol metabolism. It has been

reported that cholesterol feeding is associated with increased levels of plasma carnitine (Gillies & Bell, 1976; 1979; Bell et al., 1983).

Vitamin B-6 and Fat Metabolism

Vitamin B-6 and Fatty Liver Formation

Pyridoxine has long been known to be involved in lipid metabolism. As early as 1938, Halliday (1938) observed that the livers of pyridoxine deficient rats were significantly heavier than those of normal rats and contained two to three times as much fatty acid. The administration of choline to these deficient animals reduced the amount of liver fat slightly, but even large doses of choline (220 mg in 11 days) failed to lower the fat concentration to a normal value.

Although the vitamin B-6 deficient rat is characterized by markedly diminished fat stores, epididymal adipocytes (Beaton et al., 1954) and liver tissue (Sabo et al., 1971a) from vitamin B-6 deficient rats showed greatly enhanced lipid formation when incubated with labeled glu-cose. Enhanced lipid formation has also been noted in epididymal fat pads from vitamin B-6 deficient rats incubated with both glucose and insulin (Huber et al., 1964). Audet and Lupien (1974) have also reported that liver slices from vitamin B-6 deficient rats showed greater incorporation of labeled acetate into fatty acids, triglycerides, and cholesterol compared to liver slices from ad libitum-fed and pair-fed controls. All these data indicate that the adipose tissue and liver of pyridoxine deficient rats have a greater than normal capacity to synthesize fat when provided conjointly with adequate substrate and insulin. It has been shown that rats given a 70% casein diet deficient in pyridoxine showed significant accumulation of liver lipid

consisting mainly of TG and cholesterol (Okada & Iwami, 1977; Gomikawa & Okada, 1978; Suzuki & Okada, 1982b).

The exact mechanisms involved in liver lipid accumulation in pyridoxine deficiency are unknown. Accumulation of liver triglyceride could be due to increased triglyceride synthesis in the liver, increased uptake of triglyceride from the circulation, decreased triglyceride hydrolysis in the liver, decreased fatty acid oxidation in the liver, or decreased secretion of triglyceride from the liver into blood. Pyridoxine-deficient rats have shown a decreased concentration of phospholipid and cholesterol in the serum, reduced triglyceride synthesis, and a low level of liver lipid (Suzuki & Okada, 1982a; 1982b). These findings suggest that the accumulation of triglyceride in the liver is due to decreased release of lipoprotein from the liver.

The level of free fatty acid (FFA) in the serum was observed by investigators to be lower in pyridoxine deficient rats than in pair-fed controls (Audet & Lupien, 1974; Suzuki & Okada, 1982b). Audet and Lupien (1974) suggested that pyridoxine deficient animals were unable to release as much FFA from the adipose tissue as were control animals, presumably because the fat content of the pyridoxine deficient animals was lower. Moreover, the incorporation of labeled linoleate into liver lipid and the count of beta-lipoprotein as a percentage of total liver lipid were significantly lower in pyridoxine deficient rats than in control rats. The concentration of protein in chylomicrons and VLDL were lower than those of pair-fed controls. Thus, Suzuki and Okada (1984) suggested that the excretion of VLDL from liver into blood was impaired or catabolism of VLDL was increased in pyridoxine deficient rats and dietary lipid seemed

the most probable source of TG accumulated in the liver of pyridoxine deficient rats.

In rats fed a 70% protein diet without pyridoxine, numerous lipid droplets appeared in secondary lysosomes near the cell membrane and crystals of lipid were formed (Abe & Kishino, 1982). The investigators suggested that the fatty liver induced resulted from impaired lysosomal degradation of lipid. In addition, they postulated that one of the causes of accumulation of lipid in hepatocytes, including that of triglyceride and cholesteryl ester, might be associated with a relative deficiency of intralysosomal digestion in pyridoxine deficiency.

Vitamin B-6 and Fat Synthesis and Fat Oxidation

The increased lipogenesis observed in the pyridoxine deficient rats may be accompanied by changes in enzymes critical to glucose metabolism. According to Ribaya & Gershoff (1971), vitamin B-6 deficiency did not significantly affect the glycolytic enzyme activity in the liver, kidney, and adipose tissue or the phosphate shunt dehydrogenase activity measured in adipose tissue and kidney. Liver glucose 6-phosphate dehydrogenase and adipose tissue and liver malic enzyme activity were significantly lowered in vitamin B-6 deficient rats, compared to ad libitum and pair-fed controls. Adipose tissue and liver ATP citrate lyase activities were also decreased with vitamin B-6 deficiency. Thus, Ribaya and Gershoff (1977) suggested that the increased glucose utilization by the adipose tissue and liver of vitamin B-6 deficient rats is not directly related to changes in these enzymes.

In studies done in vitro on the fatty acid metabolism in vitamin B-6 deficient rats (Dussault & Lepage, 1979), there was a marked decrease in

the oxidation of palmitate and linoleate, but not in the oxidation of arachidonate. However, the mitochondrial oxidative process was not altered by the vitamin B-6 deficiency. Thus, the lower level of fatty acid oxidation may be due to lower availability of fatty acids rather than a reduction of oxidative capacity of the mitochondria.

Vitamin B-6 and Essential Fatty Acid Metabolism

In 1936, Birch and Gyorgy reported that essential fatty acids (EFA) had a sparing effect on the dermatitis caused by pyridoxine deficiency in the rat. Later, Swell et al. (1961) reported that linoleic acid (18;2n6) accumulation in liver of pyridoxine deficient rats may be caused by decreased 18;2n6 metabolism to arachidonic acid(20;4n6). Dussault and Lepage (1975) maintained that pyridoxine deficiency does not affect 18;2n6 metabolism, but rather influences the rate of 18;2n6 or 20;4n6 oxidation and may also decrease 20;4n6 incorporation into tissue total phospholipids. More recently, Cunnane et al. (1984) reported on the effect of pyridoxine on EFA metabolism. In pyridoxine deficient rats, phospholipid levels of linoleic and gamma-linolenic acid were increased, but arachidonic acid was decreased, compared to the controls, in the plasma, liver, thymus, and skin. In the liver, triglyceride from pyridoxine deficient rats, all the EFA (n3 and n6) were increased. Thus, the investigators suggested that both linoleic desaturation and gamma-linolenic acid elongation may be impaired in pyridoxine deficient rats, and EFA turnover between triglyceride and phospholipid may be influenced by pyridoxine.

Vitamin B-6 and Cholesterol Metabolism

Early studies by Greenberg (1964) reported that vitamin B-6 deficient rhesus monkeys developed widespread atherosclerotic lesions in their vessels. Cholesterol metabolism was also affected because the feeding of a vitamin B-6 deficient diet to the monkeys caused a degree of hypercholesterolemia than in the control animals.

Vitamin B-6 deficiencies in vitro (Lupien et al., 1969) and in vivo (Hinse & Lupien, 1971) were also reported to enhance the incorporation of labeled acetate into hepatic cholesterol in rats although the levels of cholesterol in serum and liver remain practically unaffected. Furthermore, Gomikawa & Okada (1978) observed the incorporation of labeled linoleic acid into cholesterol in the liver of rats fed a 70% casein pyridoxine deficient diet. Later, they reported that 3-hydroxy-3-methylglutaryl CoA (HMG-CoA) reductase, which catalyzes the rate-limiting reaction of hepatic cholesterologenesis, was about twice as high in pyridoxine deficient rats compared to control rats (Gomikawa & Okada, 1980). This increased enzyme activity in pyridoxine deficient rats was due to increased enzyme synthesis or increased specific activity induced by some unknown mechanism , but not to a change in the state of phosphorylation of the HMG-CoA reductase.

In pyridoxine deficient rats, the rate of bile secretion was found to be normal, but the levels of bile acids, cholesterol, and phospholipid in bile were increased (Iwami & Okada, 1982). Thus, increased cholesterologenesis in pyridoxine deficiency might result in the increased secretion of bile acids. Moreover, increased cholesterol catabolism in pyridoxine deficient rats was shown by the shorter half-life of labeled cholesterol injected into

rats (Iwami & Okada, 1982). Further study will be required to determine whether increased cholesterologenesis or increased cholesterol catabolism is the primary cause of the disturbance of cholesterol metabolism in pyridoxine deficiency.

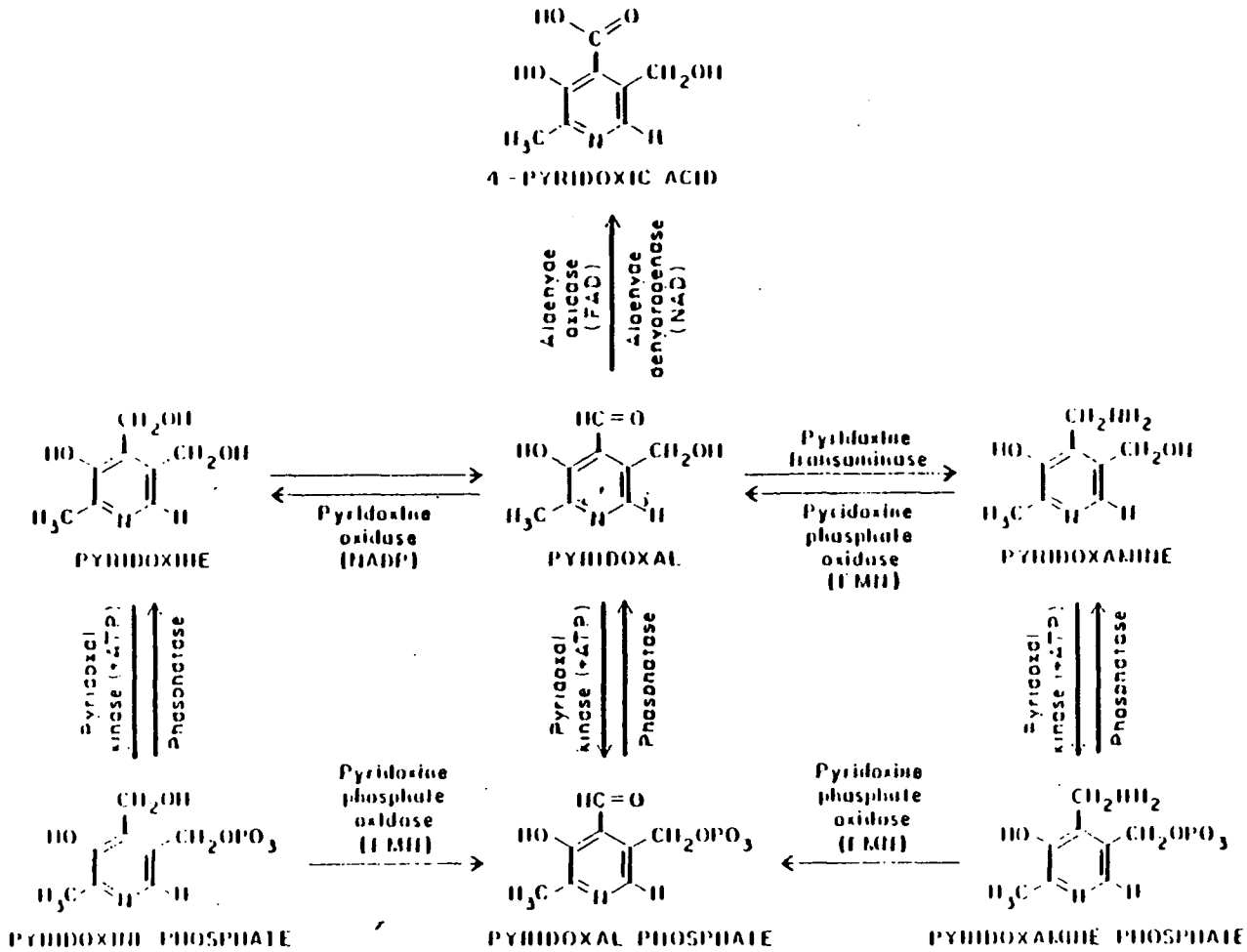

Figure 2-1. Interconversions and structures of B-6 vitamers (Tryfiates & Morris, 1983).

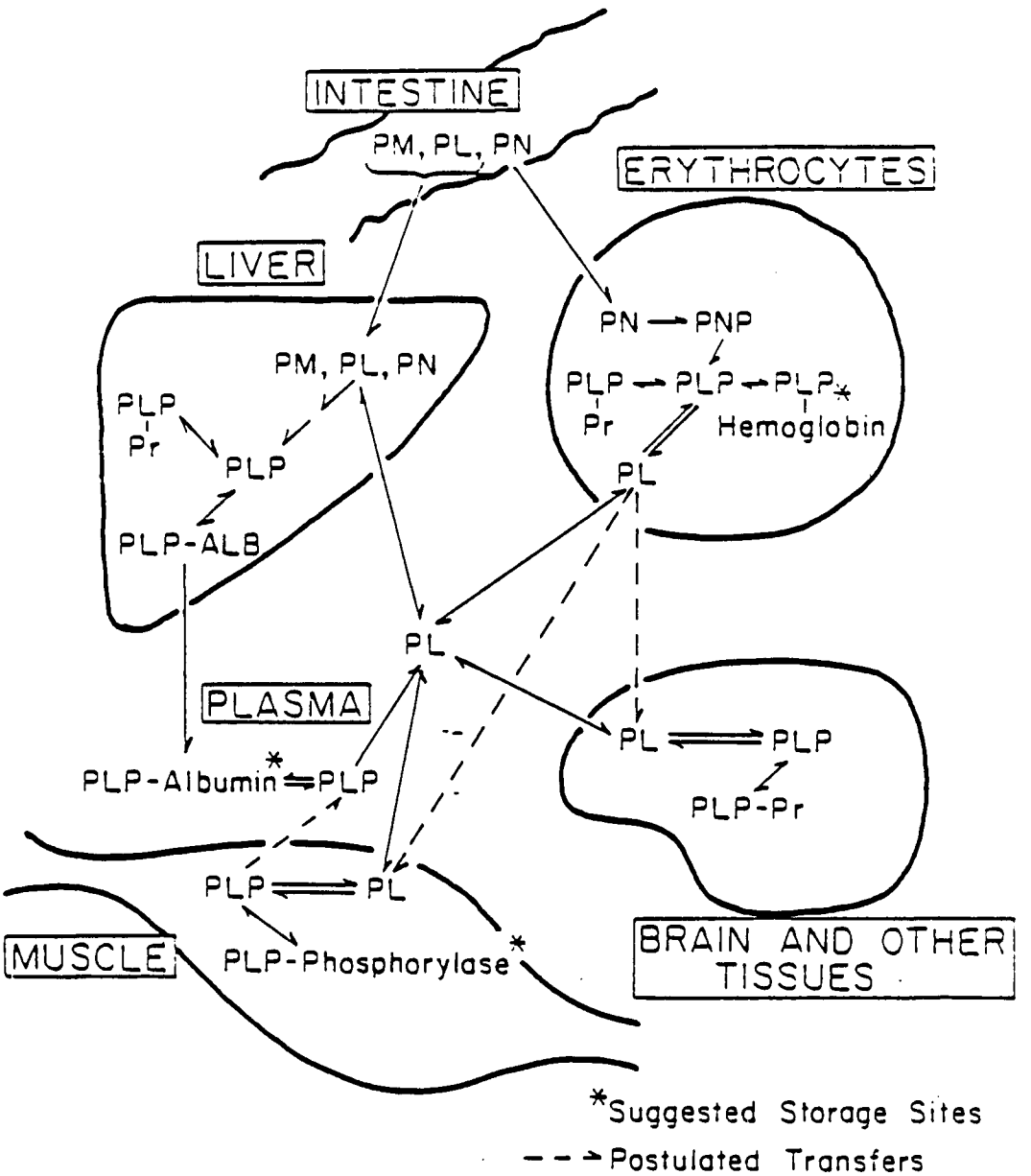

Figure 2-2. Tissue interrelationships of pyridoxal and pyridoxal 5'-phosphate (Hatcher et al., 1982).

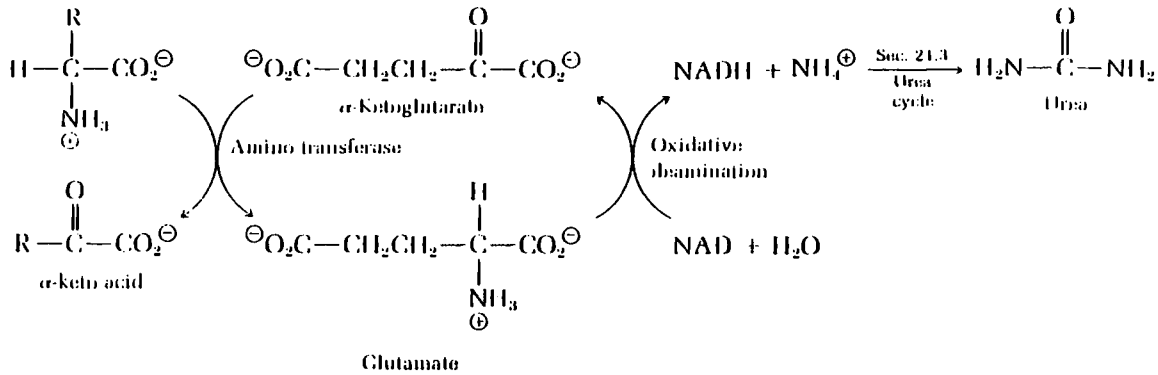

Figure 2-3. Deamination of amino acids (Rawn, 1983).

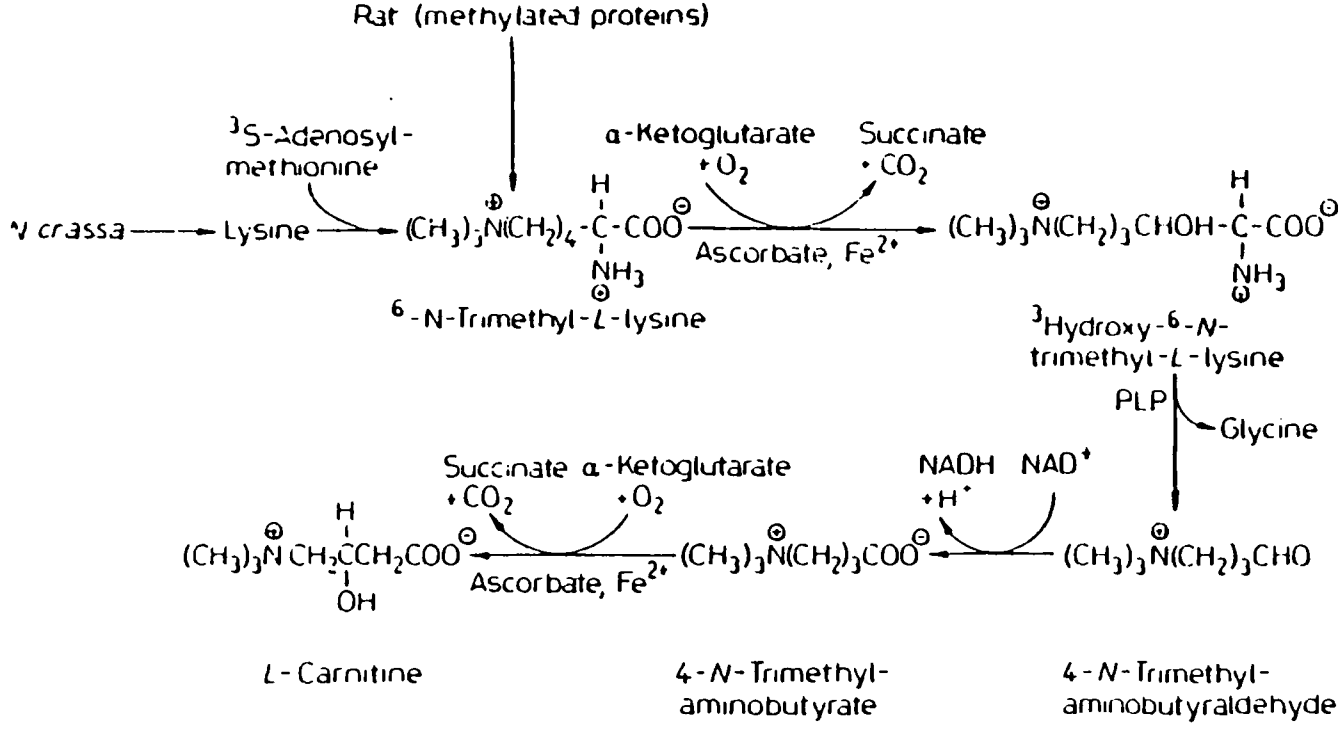

Figure 2-4. Pathway for carnitine biosynthesis (Bamji, 1984).

CHAPTER III

IN VIVO EVIDENCE FOR A VITAMIN B-6 REQUIREMENT IN CARNITINE SYNTHESIS

Abstract

The purpose of this study was to determine if there is a requirement for vitamin B-6 (B6) in carnitine synthesis. Rats (6 per group) were fed a B6 deficient (-B6) (0.04 mg pyridoxine [PN]/kg) diet (ad libitum, meal-fed) or a control (+B6) (5.7 mg PN/kg) diet (ad libitum, pair-fed). These diets were fed 6 weeks and then the rats were repleted with the +B6 diet for 2 weeks and fasted for 3 days before and after repletion. Total acid soluble carnitine (TCN) and free carnitine (FCN) levels were compared in the plasma, liver, skeletal muscle, heart muscle, and urine of rats fed +B6 or -B6 diets. In -B6 rats vs +B6 rats, TCN levels were significantly lower ($p < 0.05$) in the plasma, skeletal muscle, heart muscle, and urine, but not in the liver. However, with fasting, liver TCN concentration of -B6 rats was significantly lower than that of +B6 rats. After -B6 rats were repleted with the +B6 diet, the TCN level in the plasma, liver, skeletal muscle, heart muscle, and urine returned to the levels of control rats. Thus, the decrease in TCN and FCN levels and the increase of these levels after repletion provides evidence for a B6 requirement in the biosynthesis of carnitine.

Key words: carnitine, vitamin B-6 deficiency, pyridoxal 5'-phosphate, fasting.

Introduction

Over the past 20 years, research has suggested that vitamin B-6 is involved in lipid metabolism. Although the body fat stores and ^{14}C incorporation from labeled glucose into carcass fat (Beare et al., 1953; Angel & Song, 1973) are lower in vitamin B-6 deficient rats, liver slices (Audet & Lupien, 1974; Sabo et al., 1971a) and adipocytes (Sabo & Gershoff, 1971b) from vitamin B-6 deficient, as compared to vitamin B-6 sufficient rats, have been shown to have enhanced lipid accumulation when incubated with glucose. Furthermore, the concentration of free fatty acids in serum was found to be lower in vitamin B-6 deficient rats than in pair-fed controls (Audet & Lupien, 1974). Although several mechanisms (Abe & Kishino, 1982; Kishino & Abe, 1982; Ribaya & Gershoff, 1977; Suzuki & Okada, 1984) have been proposed to explain the abnormal fat metabolism of vitamin B-6 deficient animals, the exact mechanism(s) is not known. One of the possible mechanisms may involve the synthesis of carnitine.

Carnitine plays a major part in the oxidation of long chain fatty acids by transferring free fatty acids across the mitochondrial membrane (Bremer, 1962; Fritz & Yuiki, 1963). A relationship between vitamin B-6 and carnitine synthesis has been suggested. In 1978, Hulse et al. reported that serine transhydroxymethylase, a pyridoxal 5'-phosphate (PLP) requiring enzyme, has the capacity to cleave 3-hydroxy-6-N-trimethyllysine and in turn, this enzyme has been implicated in the scheme of carnitine biosynthesis. Based on in vitro evidence, Dunn et al. (1982) suggested that there was a requirement for PLP, the biological active form of vitamin B-6, in

carnitine synthesis. In their study, when the vitamin B-6 antagonist, 1-amino-D-proline, was added to a perfused liver system there was decreased carnitine synthesis. Moreover, the activity of lysine methyltransferase, which catalyzes the initial reaction in the biosynthesis of carnitine, was reported to be reduced in the livers of vitamin B-6 deficient rats (Loo & Smith, 1986a). A pyridoxine deficiency affects this enzyme activity via altering the hepatic molar ratio of S-adenosylmethionine to S-adenosylhomocysteine (SAM: SAH ratio) (Loo & Smith, 1986b). However, there is no information available which has demonstrated there is an in vivo requirement for vitamin B-6 in carnitine synthesis. To date, no study of the effect of vitamin B-6 deficiency on the systematic changes in the concentrations of carnitine derivatives in tissues has been done.

The objectives of this study were: (a) to determine if vitamin B-6 is required in the biosynthesis of carnitine in vivo; and (b) to determine the effect of vitamin B-6 deficiency on the changes of plasma, tissue concentrations, and urinary excretion of carnitine and its derivatives during the stress of fasting. The hypothesis of this study was that with a vitamin B-6 deficiency there would be a decreased tissue concentration of the coenzyme pyridoxal 5'-phosphate (PLP) and thus, decreased carnitine synthesis. This decrease in carnitine synthesis would then result in lower concentrations of carnitine and carnitine derivatives in plasma and tissues and decreased urinary excretion.

Materials and Methods

Animals and Diets

A total of 72 male weanling Wistar rats[1] were divided into 4 groups: group 1 (ad libitum control, 6 rats), Group 2 (pair-fed controls, 30 rats), group 3 (vitamin B-6 deficient, ad libitum, 30 rats), and group 4 (vitamin B-6 deficient, meal-fed, 6 rats). Animals were housed individually in wire bottomed stainless steel cages exposed to controlled lighting (12 hrs on, 12 hrs off). Animals received a vitamin-free, casein-based semisynthetic diet[2] which met AIN-76 recommendations, with the exception of vitamin B-6. The diet contained by weight 20% protein, 5% fat, and 65% carbohydrates. Based on analysis, the control (+B6) diet contained 5.7 mg pyridoxine (PN)/kg diet. The deficient (-B6) diet contained no added vitamin B-6, and analysis of this diet prior to the start of the study indicated that it contained 0.04 mg PN/kg diet.

The length of the study was 10 weeks. The control diet was fed ad libitum for 1 week to adapt the animals to the diet and feeding schedule and to facilitate a similar metabolic status. Training for the meal-fed animals (group 4) began during the adaptation period. Cohn and Joseph (1960) reported that "meal-feeding," an inherent consequence of pair-

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[1]From Charles River-Kingstar Laboratory, Wilmington, MA.

[2]Supplied by U.S. Biochem, Cleveland, OH. Provided per kg diet: casein, vitamin-free, 200 g; D.L.-methionine, 3 g; cornstarch, 150 g; sucrose, 511.1 g; fiber-celufil, 50 g; corn oil, 50 g; choline bitartrate, 2 g; $CaHPO_4$, 17.5 g; NaCl, 2.5 g; potassium citrate$\cdot H_2O$, 7.7 g; H_2SO_4, 1.82 g; magnesium carbonate (43-48% Mg), 123 mg; ferric carbonate (16-17% Fe), 210 mg; zinc carbonate (70% ZnO), 56 mg; cupric carbonate (53-55% Cu), 10.5 mg; KIO_3, 0.35 mg; $Na_2S_2O_3 \cdot 5H_2O$, 0.35 mg; $CrK(SO_4)_2 \cdot 12H_2O$, 19.25 mg; thiamin HCl, 6 mg; riboflavin, 6 mg; nicotinic acid, 30 mg; D-calcium panthothenate, 16 mg; folic acid, 2 mg; D-biotin, 0.2 mg; cyanocobalamin, 10 mg; retinyl palmitate, 8 mg; dl-d-Tocopheryl acetate, 200 mg; cholecalciferol, 25 mg; menaquinone, 50 mg.

feeding, elicits adaptations related to energy utilization by the rats, the outcome of which is increased fat synthesis and storage. Thus, the present study was conducted by imposing a similar feeding pattern on both the +B6 and -B6 groups. The meal-fed animals had access to food (vitamin B-6 deficient diets) for two hours a day, from 8:00 to 10:00 a.m. At the end of the adaptation period, the rats were fed either the +B6 or the -B6 diet.

After feeding the respective diets for 6 weeks, 6 animals from the pair-fed control and 6 animals from the vitamin B-6 deficient group were fasted for 1 and 3 days, respectively. From the 6th to the 8th week, 12 animals from each of the groups were repleted with the +B6 diet. The diet was pair-fed against the intake of the prior vitamin B-6 deficient animals. At the end of this period, 6 animals from each group were fasted for 3 days. Water was provided during all fasts.

At each of the respective time points (six weeks, two week repletion, and during the respective one and three day fast periods), animals were anesthetized in a CO_2 chamber and then decapitated. Immediately following decapitation, blood was collected in heparinized tubes and centrifuged to separate the plasma. Plasma was stored at -40°C. The liver, heart, and skeletal muscle (gastrocnemius) were rapidly removed, frozen in liquid nitrogen, and stored at -40°C until analyzed. Twenty-four hour urine samples were collected over acetic acid prior to and during fasting. Urine was stored at -40°C until analyzed. The completeness of urine collections was checked by determination of creatinine.

Analysis

The vitamin B-6 content of the diet was determined microbiologically, using *Saccharomyces uvarum* (ATCC No. 9080) as the assay organism (Association of Official Analytical Chemists, 1980). Urinary creatinine was measured by an automated procedure (Pino et al., 1965). Pyridoxal 5'-phosphate was measured by a modified radiometric-enzyme method (Chabner & Livingston, 1970). A control sample was analyzed with each assay. The coefficient of variation within an assay was 4.2%, n = 6, and between assays was 8.5%, n = 21. Tissue samples were homogenized in cold sodium phosphate buffer (pH 7.4, 80 mM). Aliquots of the tissue homogenates and plasma were added to perchloric acid (1M) and allowed to sit for one hour to release PLP from protein. This mixture was then centrifuged (18000 x g, 4°C, 15 min) and the supernatant removed. After neutralization with potassium acetate, an aliquot of the supernatant was subsequently assayed for PLP. Carnitine was assayed by a modification of the radiometric method of Pace et al. (1978). A control sample was analyzed with each assay. The coefficient of variation for FCN within an assay was 7.8%, n = 9, and between assays was 10.4%, n = 36. Duplicate samples of each tissue were homogenized in 3% perchloric acid, centrifuged (18000 x g, 4°C, 15 min), and the supernatant was used for the subsequent assay of carnitine derivatives. Free carnitine (FCN) was measured directly from the neutralized perchloric acid extract or plasma. Total acid-soluble carnitine (TCN) was obtained by alkaline hydrolysis with 0.2 ml of 1M Tris base and 0.1 ml of 0.4 N KOH at 37°C for 1 hour of a 0.1 ml aliquot of the neutralized extract or plasma. This sample was then neutralized and the TCN measured.

Statistical Analysis

All data were first evaluated by analysis of variance. For those F values which were significant, the least significant difference test was performed (Heintz, 1986). A p value < 0.05 was considered to be statistically significant.

Results

At various points during the study the vitamin B-6 status of the rats was evaluated using body weight as an indirect, long-term measure and tissue and plasma PLP concentration as a direct measure. The effect of the feeding patterns and vitamin B-6 deficiency on body weight is shown in Figure 3-1. At week 6 the mean body weight of the -B6 ad libitum-fed group was significantly lower than those of the +B6 ad libitum or +B6 pair-fed control groups. Also, the mean body weight of the -B6 meal-fed groups was significantly lower than that of the -B6 ad libitum group. However, the ratio of body weight change to food intake was similar (see Table 3-1). After the -B6 rats were repleted with a +B6 diet, the body weights of the the +B6 and -B6 rats were essentially identical. There was an 11 to 12% body weight loss after the 3 day fasts in both groups at the 6th and 8th weeks. The concentration of PLP in the plasma, liver, and skeletal muscles of -B6 rats (ad libitum, meal-fed) was significantly lower than that of both groups of +B6 fed rats (see Table 3-2). Thus, the data from Figure 3-1 and Table 3-2 indicate that by the 6th week the animals were severely B-6 deficient. After the vitamin B-6 deficient rats were repleted with the +B6 diet, the PLP concentrations in plasma, liver, and heart muscle returned to concentrations comparable to those of rats

fed the +B6 diet for 8 weeks. The PLP concentration in skeletal muscle increased but was significantly lower than the PLP concentration in rats fed the +B6 diet for 8 weeks.

Table 3-3 shows the effect of vitamin B-6 deficiency on the mean concentrations of TCN and FCN in plasma and tissues and the mean urinary excretion of TCN and FCN. Compared to +B6 pair-fed control rats, the mean TCN concentrations for the -B6 rats were 20%, 35%, and 34% lower in plasma, skeletal muscle, and heart muscle, respectively, and the mean urinary TCN excretion was 30% lower. Although the mean liver TCN concentration of the -B6 rats was 21% lower than that of the +B6 rats, this difference was not significant because of the large standard deviation of both groups. There were no significant differences in plasma, liver, skeletal muscle, or heart muscle mean TCN concentrations and urinary TCN excretion between +B6 ad libitum and the +B6 pair-fed rats. Similarly, there were no significant differences between the mean TCN levels for the two -B6 fed groups. Furthermore, there was no significant difference between +B6 rats and -B6 rats for the percentage of TCN as FCN in the liver, skeletal muscle, heart muscle, and urine. However, for plasma there was a lower percentage of TCN as FCN in -B6 rats, compared to the +B6 pair-fed control rats, which indicates that more of the carnitine in the plasma of the -B6 rats was esterified.

There was a significant correlation ($r = 0.641$) between plasma PLP and plasma TCN concentration, as well as a significant correlation ($r = 0.576$) between skeletal muscle PLP and skeletal muscle TCN concentrations. Moreover, there was a significant correlation between plasma TCN and skeletal muscle TCN ($r = 0.369$, $n = 24$), as well as between plasma TCN and heart TCN ($r = 0.512$, $n = 24$).

Figure 3-2 shows the effect of vitamin B-6 repletion of deficient rats on plasma and tissue concentrations and urinary excretions of TCN and FCN. After repletion, the mean concentrations of TCN and FCN in plasma, liver, skeletal muscle, and heart muscle and the level of TCN and FCN in the urine returned to levels observed in rats fed the +B6 diet for 8 weeks.

The effect of fasting on plasma and tissue concentrations and urinary excretion of TCN and FCN in +B6 rats and -B6 rats is shown in Figure 3-3. With fasting, there was a significant increase of TCN levels in plasma, liver, and urine for both groups. However, for both groups there was no significant increase of TCN concentration in heart muscle with fasting. Although there was a significant increase of TCN concentration in the skeletal muscles of the -B6 rats, the increase for the +B6 rats was not significant. The patterns of TCN and FCN change were basically similar in both groups. More important, while the difference of liver TCN concentrations between +B6 rats and -B6 rats was not significant before fasting, with fasting the difference was significant. Similarly, the mean concentrations of TCN reflected in the plasma, skeletal muscle, and heart muscle were significantly lowered. With fasting there was an increase in the mean urinary excretion of both TCN and FCN in the +B6 and the -B6 rats. Fasting was also associated with an increased renal clearance of TCN and FCN in both groups. The acylated forms were excreted more efficiently than FCN in both groups, and this increased clearance was even more dramatic in -B6 rats (Table 3-4).

In the repleted group (Figure 3-2) there was no further significant increase of TCN concentration in the plasma, liver, skeletal muscle, or heart muscle or in urinary excretion with a three-day fast. However,

with this treatment there was a decrease in the percentage of TCN as FCN in the plasma (20 to 26%), skeletal muscle (5 to 14%), heart muscle (12 to 18%), and urine (9 to 10%). This decrease of FCN levels indicates that with fasting more FCN was esterified, although the TCN level per se was not increased. For the fast conditioning there was a significant correlation between the PLP concentration and TCN concentration for plasma ($r = 0.405$) and skeletal muscle ($r = 0.574$). While the correlations of the respective concentrations between liver PLP and liver TCN was not significant before fasting, with fasting the correlation between liver PLP concentration and liver TCN concentration was significant ($r = 0.696$).

Discussion

This study provides the first in vivo evidence for a vitamin B-6 requirement in carnitine biosynthesis. The data presented in Table 3-3 are consistent with the hypothesis that carnitine levels in plasma, tissues, and urine are lower in vitamin B-6 deficient rats. The hypothesis that a vitamin B-6 deficiency impairs carnitine synthesis has been suggested by results from two different studies. First, the results of the present study are consistent with the results of the in vitro study of Dunn et al. (1982), in which there was a lowered carnitine concentration and accumulation of several precursors in the carnitine biosynthetic pathway when the vitamin B-6 antagonist, 1-amino-D-proline, was added to a perfused liver system. Thus, the reduced activity of 3-hydroxy-4-N-trimethyllysine aldolase may decrease the carnitine synthesis. Second, the lowered concentrations in the tissues of vitamin B-6 deficient rats is consistent with the findings of Loo and Smith (1986a), who in abstract form reported a decreased concentration of TCN in the skeletal muscle and the heart muscle of vitamin B-6

deficient rats. They also observed a lowered activity of lysine methyltransferase, the first enzyme in the carnitine synthetic pathway. In a separate report, Loo and Smith (1986b) suggested that the activity of protein methyltranserase III (i.e., lysine methyltransferase) is regulated by modulation of the molar ratio of S-adenosylmethionine to S-adenosylhomocysteine (SAM: SAH ratio). Moreover, in an earlier report, Eloranta et al. (1976) observed that pyridoxine deficiency increased the level of S-adenosylhomocysteine. Thus, the reduced SAM: SAH ratio in vitamin B-6 deficient rats decreases the methylation of the ξ-amino group of lysine residues present in histone by protein methyltransferase III and may reduce the supply of the initial substrate for the biosynthesis of carnitine.

Because the liver is the site of carnitine biosynthesis (Hulse et al. 1978), the lower concentration of TCN in the liver of vitamin B-6 deficient as compared to the vitamin B-6 sufficient rats may, over time, result in a cumulative effect on the concentration of TCN in other tissues. This effect could then lead to a significant decrease in tissue concentrations of TCN, as observed for the vitamin B-6 deficient rats in this study. This cumulative effect was also evident when comparing TCN levels between plasma and urine as well as between plasma and tissues.

The amount of a constituent in a 24-hour urine collection may be considered as an integration of the circulating level of that constituent. Thus, although the difference in TCN concentration in plasma between +B6 rats and -B6 rats was 20%, the difference in TCN concentration in urine was greater (30%). The concentration of carnitine in skeletal muscles and heart muscles is influenced by the plasma carnitine concentration, reflecting the relative long-term dynamics of carnitine. Reduced tissue levels of carnitine due to lowered plasma carnitine concentrations have

also been reported in the hearts of diabetic animals (Vary & Neely, 1982). In the present study the relationship between the plasma TCN concentration and the tissue TCN concentration was also evident in the significant correlation observed between plasma and skeletal muscle TCN concentrations as well as between the plasma and heart muscle TCN concentration. Thus, while there was a 20% difference in plasma TCN concentration between +B6 pair-fed rats and -B6 ad libitum rats, a larger difference was observed in skeletal (35%) and heart (34%) muscles. Therefore, the above data support the hypothesis that vitamin B-6 deficiency impairs carnitine synthesis.

Further support for a vitamin B-6 requirement in the biosynthesis of carnitine is provided by the effect of repletion with the +B6 diet. Following repletion, the concentration of PLP in the plasma and liver of previously vitamin B-6 deficient rats was restored to those of the control animals. Thus, in a situation in which the vitamin B-6 status is similar, concentrations of TCN and FCN in the plasma, tissues, and urinary excretions of TCN and FCN returned to those observed for the rats fed the +B6 diets for 8 weeks.

A third line of evidence for a vitamin B-6 requirement in carnitine synthesis is derived from the results of the fasting study. In mammals, the sources of carnitine are either carnitine biosynthesis or dietary, and during periods of fasting, the only possible source of carnitine is via biosynthesis. Moreover, fasting could eliminate possible variations of carnitine concentration due to the effect of pair-feeding upon vitamin B-6 deficient rats (i.e., pair-fed control animals ate their food in a shorter period of time than vitamin B-6 deficient animals, thus their dynamics of energy metabolism may be different from that of the vitamin B-6 deficient

group). This elimination of the variation in carnitine concentration was observed in the liver, as reflected by the smaller standard deviation after fasting. Thus, with fasting a significantly lower mean TCN concentration was observed in the livers of -B6 rats. Similarly, the correlation between liver PLP and liver TCN became significant.

Nonetheless, the mechanisms which increase the carnitine content on a g liver basis during fasting cannot be entirely identified from the results of this study. Whereas Brass and Hoppel (1978) pointed out the loss of liver mass, others (Kispal et al., 1987; Sandor et al., 1985) found that the increased carnitine concentrations could be accounted for by a decreased rate of carnitine release from the liver or enhanced uptake of carnitine by the liver. Because the patterns of liver TCN increase were similar in both +B6 and -B6 rats during fasting periods, vitamin B-6 deficiency affected the synthesis per se, but not the increased concentration of liver TCN due to fasting. The concentrations and patterns of change for liver, plasma, skeletal muscle, and heart muscle TCN levels observed in pair-fed control rats during the 3-day period of fasting were similar to those observed in other studies in which rats were fasted (Brady et al., 1986; Brass & Hoppel, 1978; Kerner & Breber, 1983; Pearson & Tubb, 1967). Brass and Hoppel showed a conservation of carnitine during fasting, which was reflected by a decrease in the excretion of carnitine in urine. In the present study, there was a continued loss of carnitine in the urine. This discrepancy could be due to differences in the strains of rats and/or their feeding schedules. The pair-fed controls in this study, unlike ad libitum fed animals, could be considered as animals which were chronically partially starved since their food intake was restricted to the intake of the vitamin B-6 deficient animals. Thus, prior to the one or three-day fast

the energy intake of the animals in this study could have been similar to that of the animals in the study of Brass and Hoppel which were fasted for two to three days and which showed an increased excretion of TCN in their urine. Because vitamin B-6 deficiency does not appear to affect the renal clearance of nitrogen compounds (Okada & Suzuki, 1974), the increase urinary excretion of TCN may be due to either increased esterification of carnitine related to increased acetyl CoA levels or an increased degradation of protein from skeletal muscles during periods of fasting.

Because the patterns of change of TCN and FCN in the plasma and tissues of both groups during fasting periods were similar in this study, we suggest that there is no additional inhibitory effect of vitamin B-6 deficiency on the distribution of carnitine derivatives during periods of fasting. Considering the role of carnitine in fatty acid metabolism and the requirement for vitamin B-6 in the synthesis of carnitine, a lowered vitamin B-6 intake, in conjunction with a lowered caloric intake, may impair the adaptation of animals during metabolic events related to fatty acid oxidation during periods of fasting .

In summary, the three lines of evidence presented strongly suggest that vitamin B-6 is required in carnitine biosynthesis in vivo. While vitamin B-6 affects carnitine synthesis in the liver during fasting, there is no additional effect of vitamin B-6 deficiency on the distribution of carnitine derivatives in either tissues or urine.

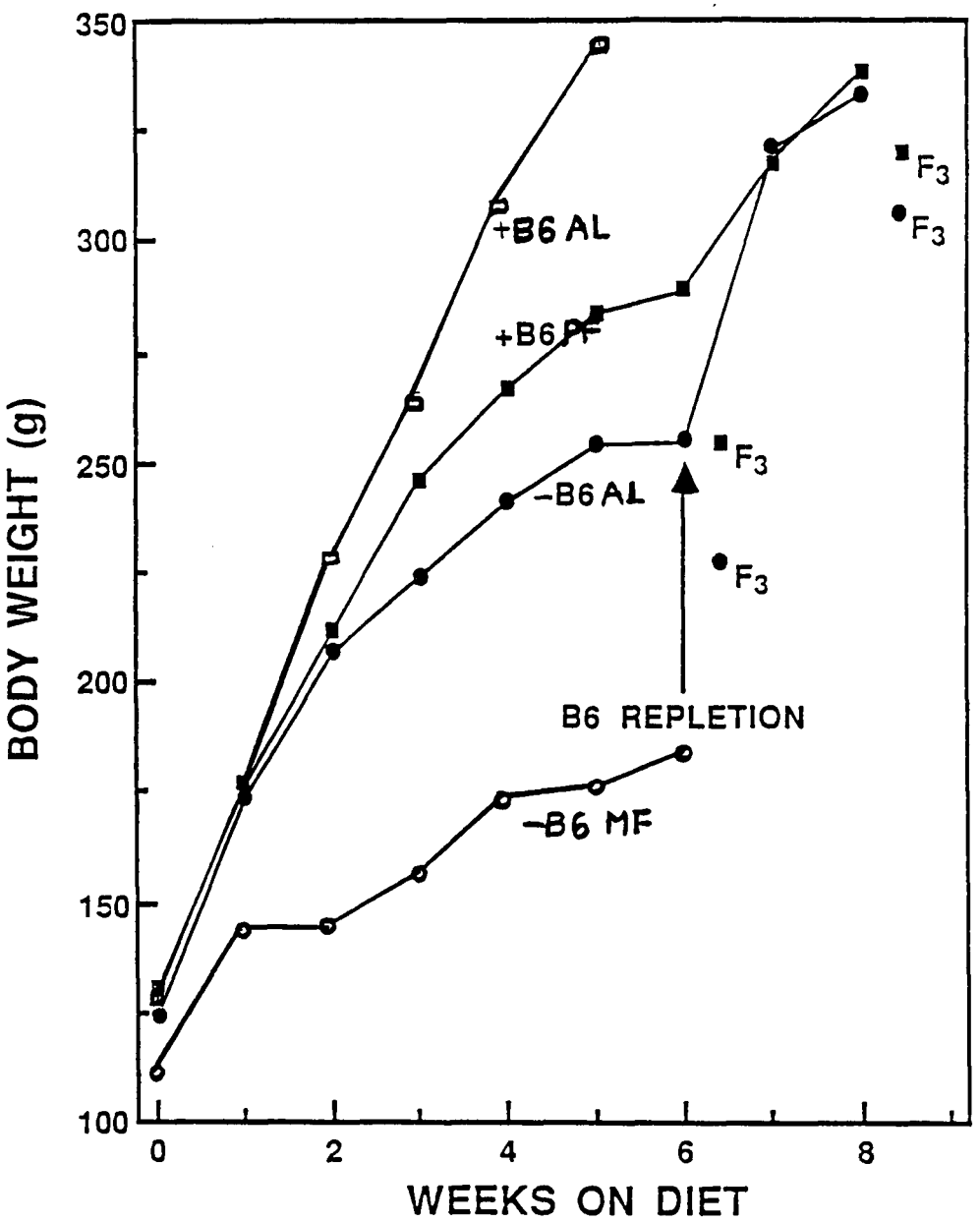

Figure 3-1. Effect of vitamin B-6 deficiency and feeding pattern on body weight (+B6 = control diet; -B6 = vitamin B-6 deficient diet; AL = ad libitum-fed; PF = pair-fed; MF = meal-fed; F_3 = 3-day fast).

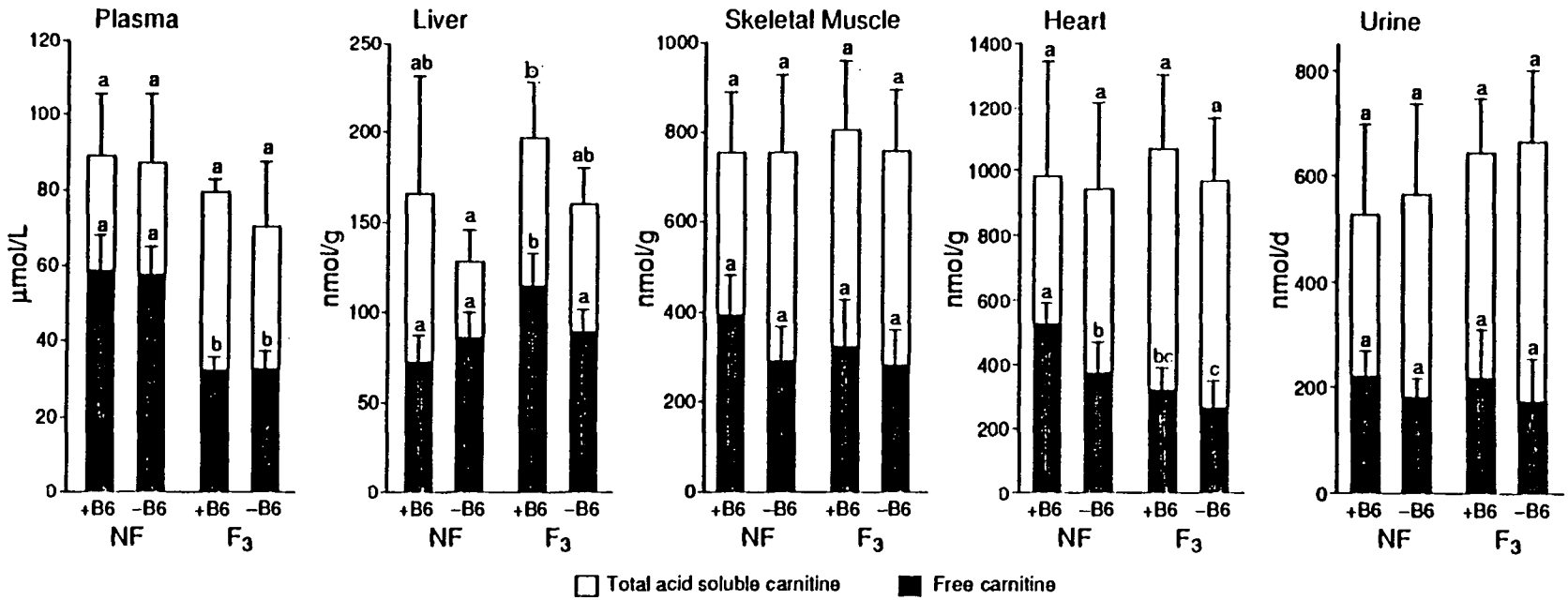

Figure 3-2. Effect of repletion of vitamin B-6 deficient rats on plasma and tissue concentrations and urinary excretion of total acid soluble carnitine and free carnitine. (Each bar with different letters is significantly different ($P < 0.05$); +B6 = control diet; -B6 = prior vitamin B-6 deficient diet; NF = non-fasted; F_3 = 3-day fast.)

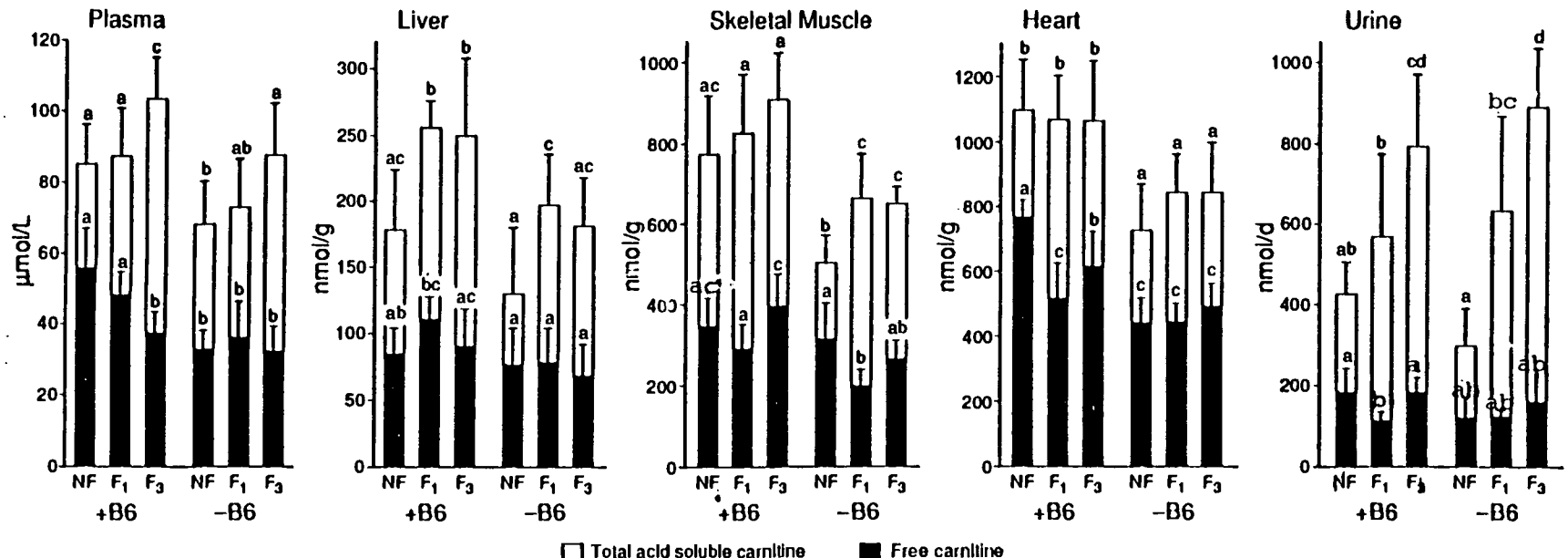

Figure 3-3. Effect of fasting on plasma and tissue concentrations and urinary excretion of total acid soluble carnitine and free carnitine in both control rats and vitamin B-6 deficient rats. (Each bar with different letters is significantly different ($P < 0.05$); +B6 = control diet; -B6 = vitamin B-6 deficient diet; NF = non-fasted; F_1 = 1-day fast; F_3 = 3-day fast.)

Table 3-1. Feed efficiency of rats fed a vitamin B-6 sufficient or a vitamin B-6 deficient diet.

Diet	Feeding Pattern	1 wk	3 wk	5 wk	6 wk
		g body weight/g food			
+B6[1]	Ad libitum	3.40±.15	2.27±.53	1.59±.20	1.15±.14
	Pair-fed	2.91±.31	2.23±.37	1.10±.31	.44±.18
-B6[1]	Ad libitum	3.22±.24	1.24±.34	.87±.49	.11±.41
	Meal-fed	3.57±.43	1.87±.52	.83±.44	.45±.53

[1] +B6 = control diet, -B6 = vitamin B-6 deficient diet

Table 3-2. Effect of vitamin B-6 deficiency on plasma and tissue concentrations of pyridoxal 5'-phosphate.

Diet:	+B6[1]		-B6[1]	
Feeding pattern	Ad libitum	Pair-fed	Ad libitum	Meal-fed
Plasma (μmol/L)	1160.2±195.2[a]	675.6±264.2[b]	14.3±8.6[c]	21.2±8.3[d]
Liver (nmol/g)	27.5±1.3[a]	33.3±2.7[b]	7.8±2.3[c]	11.5±1.9[d]
Skeletal muscle (nmol/g)	23.1±1.9[a]	21.5±2.6[a]	7.3±.6[b]	6.8±1.5[b]
Heart muscle (nmol/g)	7.9±.8[a]	7.0±.2[a]	5.0±.2[b]	4.8±.7[b]

Within a given row, those values with different letters are significantly different ($p < .05$).
[1] +B6 = control diet, -B6 = vitamin B-6 deficient diet

Table 3-3. Plasma tissue concentrations and urinary excretions of total acid soluble carnitine (TCN) and free carnitine (FCN) in rats fed a vitamin B-6 deficient diet or a vitamin B-6 sufficient diet.

Diet:		+B6[1]		-B6[1]	
Feeding pattern		Ad libitum	Pair-fed	Ad libitum	Meal-fed
Plasma (μmol/L)	FCN	68.7±12.3a	55.6±11.2b	33.Ø±5.Øc	15.1±5.8c
	TCN	94.1±21.9a	85.1±11.3a	68.2±12.1b	49.Ø±14.5c
Liver (nmol/g)	FCN	73.3±27.4a	84.8±18.8a	77.1±27.Øa	87.5±26.8a
	TCN	151.5±67.7a	177.9±46.Øa	13Ø.3±49.3a	157.Ø±31.3a
Skeletal muscle (nmol/g)	FCN	372.8±61.6a	348.4±69.3a	315.6±87.3a	2Ø5.1±27.1b
	TCN	698.6±95.3ab	773.8±146.Øb	5Ø1.3±69.4c	564.7±183.3ac
Heart muscle (nmol/g)	FCN	7Ø2.8±133.Øa	766.1±51.3a	444.4±77.Øb	417.1±79.4b
	TCN	1125.8±161.9a	1Ø96.Ø±156.9a	725.3±14Ø.9b	659.7±118.5b
Urine (nmol/d)	FCN	155.5±43.2ab	185.Ø±56.7a	121.4±48.3b	124.8±45.3ab
	TCN	4Ø4.1±84.9a	426.6±79.5a	299.9±91.9b	343.8±58.7ab

Within a given row, those values with different letters are significantly different ($p < .Ø5$).

[1] +B6 = control diet, -B6 = vitamin B-6 deficient diet

Table 3-4. Renal clearance of free carnitine (FCN) and total acid soluble carnitine (TCN) during fasting.

Diet:	+B6[1]			-B6[1]		
Feeding pattern	NF[2]	F1[2]	F3[2]	NF[2]	F1[2]	F3[2]
			ml/day			
FCN	3.5±1.2	2.4±Ø.6	5.Ø±1.3	3.7±1.4	3.8±1.5	5.Ø±3.Ø
TCN	5.2±1.4	6.8±3.Ø	7.8±2.3	4.6±2.1	8.9±4.Ø	1Ø.4±2.Ø

[1] +B6 = control diet, -B6 = vitamin B-6 deficient diet
[2] NF = nonfast; F1 = 1-day fast; F3 = 3-day fast

CHAPTER IV

EFFECT OF SHORT TERM FASTING ON URINARY EXCRETION OF 4-PYRIDOXIC ACID AND TISSUE CONCENTRATION OF PYRIDOXAL 5'-PHOSPHATE IN RATS

Abstract

The purpose of this study was to investigate the effect of fasting on vitamin B-6 (B6) metabolism. Rats (6 per group) were fed a B6 deficient (-B6) (0.04 mg pyridoxine [PN]/kg) diet (ad libitum, meal-fed) or a control (+B6) (5.7 mg PN/kg) diet (ad libitum, pair-fed). These diets were fed for 6 weeks and then the rats were repleted with the +B6 diet for 2 weeks. Rats were fasted for 1 and 3 days at week 6 and for 3 days after repletion. Pyridoxal 5'-phosphate (PLP) concentrations in the plasma, liver, skeletal muscle, and heart muscle and urinary 4-pyridoxic acid (4-PA) excretion were compared for each group. Also, the concentration of plasma glucose, liver glycogen, and plasma free fatty acid were compared. Fasting resulted in a significant increase in PLP concentration in the plasma, liver, and heart muscle of rats fed the -B6 diet. At day 1 and 2 of the fast, urinary 4-PA excretion increased. These changes are consistent with a redistribution of vitamin B-6 (as PLP) when there is a caloric deficit. Thus, with fasting, PLP is supplied by an endogenous source, possibly PLP bound to skeletal muscle glycogen phosphorylase.

Key words: vitamin B-6 metabolism, pyridoxal 5'-phosphate, fasting, vitamin B-6 deficiency, 4-pyridoxic acid, liver glycogen, free fatty acid, blood glucose.

Introduction

The metabolic processes that occur during fasting allow for the survival of mammalian organisms for a period of time in spite of lack of caloric intake. Among the tissues involved in these processes, adipose tissue is the major energy source. In addition to supplying energy, these processes involve the breakdown of glycogen and the transamination and the subsequent conversion of ketoacids to glucose.

In rats (Freminet, 1981) hepatic glycogen content is almost totally exhausted after a one-day fast. Despite the limited store of carbohydrates, a continuing supply of glucose is essential for the needs of the brain and other tissues. Research has suggested that vitamin B-6 is involved in fuel metabolism in three ways during fasting. First, pyridoxal 5'-phosphate (PLP), the biologically active form of vitamin B-6, acts as an integral part of glycogen phosphorylase (EC 2,4,1,1), which catalyzes the breakdown of glycogen to glucose 1-phosphate (Krebs and Fischer, 1964). Second, PLP is a cofactor for aminotransferases, which catalyze the conversion of certain amino acids to glucose (Plebani et al., 1980). Third, PLP is a cofactor for serine transhydroxymethylase, which may be identical to 3-hydroxy-6-N-trimethyllysine aldolase, an enzyme involved in carnitine synthesis (Hulse et al., 1978). Carnitine acts as a carrier of fatty acyl groups across the mitochondrial membrane for subsequent oxidation.

For many years it has been assumed that vitamin B-6, a water soluble vitamin, is not stored in the body. However, with a high intake of vitamin B-6 (10 x requirement of the rat), muscle phosphorylase and total muscle vitamin B-6 increased steadily (Black et al., 1978). Thus, in the rat, muscle phosphorylase acts as a reservoir for vitamin B-6. Caloric restriction, but not a vitamin B-6 deficiency per se, was shown to result in a decrease of muscle phosphorylase and the possible release of PLP. However, neither vitamin B-6 nor PLP was measured in the skeletal muscle of rats subjected to caloric restriction. This vitamin B-6 reservoir theory was tested indirectly in humans by Leklem and Shultz (1983) and Manore et al. (1987). Immediately after either long distance running by male adolescent trained athletes (Leklem and Shultz, 1983) or 20 min of exercise on an ergometer by young and postmenopausal women (Manore et al., 1987), the concentration of plasma PLP increased significantly. However, the effect of fasting on the temporal changes of PLP in the plasma and tissues and the urinary excretion of 4-PA has not been determined.

Therefore, the objective of this research was to investigate the effect of fasting on the concentration of PLP in the plasma, tissues, and the urinary excretion of 4-PA, with and without adequate dietary vitamin B-6. Also, to properly access the relationship between fuel use and vitamin B-6 metabolism, plasma free fatty acid (FFA), liver glycogen, and blood glucose were measured. The hypothesis was that with fasting PLP would be released from muscle glycogen phosphorylase, resulting in an increased concentration of PLP in the plasma and in an increase of the urinary excretion of 4-PA, the major metabolite of vitamin B-6.

Materials and Methods

Animals

Weanling male Wistar rats[1] of 60 to 80 g were divided into 4 groups: group 1 (6 rats, ad libitum control), group 2 (30 rats, pair-fed control), group 3 (30 rats, ad libitum vitamin B-6 deficient), and group 4 (6 rats, meal-fed vitamin B-6 deficient). Animals were housed individually and lighting was controlled (12 hrs on, 12 hrs off).

Diets

The animals were fed a semisynthetic diet[2] which conformed with the AIN-76 nutrient recommendation for the rat (AIN, 1977, 1980), with the exception of vitamin B-6. The diet contained 20% casein, 65% carbohydrate, and 5% fat by weight. Analysis of the diet prior to the start of the study indicated that the control diet contained 5.7 mg pyridoxine (PN)/kg diet and the vitamin B-6 deficient diet contained 0.04 mg PN/kg diet.

Feeding

All animals were adapted to the control diet for 1 week prior to feeding their respective diets. Training for the meal-fed animals was

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[1]From Charles River-Kinstar Laboratory, Wilmington, MA.

[2]Supplied by U.S. Biochem, Cleveland, OH. Provided per kg diet: casein, vitamin-free, 200 g; D.L.-methionine, 3 g; cornstarch, 150 g; sucrose, 511.1 g; fiber-celufil, 50 g; corn oil, 50 g; choline bitartrate, 2 g; $CaHPO_4$, 17.5 g; NaCl, 2.5 g; potassium citrate$\cdot H_2O$, 7.7 g; H_2SO_4, 1.82 g; magnesium carbonate (43-48% Mg), 123 mg; ferric carbonate (16-17% Fe), 210 mg; zinc carbonate (70% ZnO), 56 mg; cupric carbonate (53-55% Cu), 10.5 mg; KIO_3, 0.35 mg; $Na_2S_2O_3\cdot 5H_2O$, 0.35 mg; $CrK(SO_4)_2\cdot 12H_2O$, 19.25 mg; thiamin HCl, 6 mg; riboflavin, 6 mg; nicotinic acid, 30 mg; D-calcium panthothenate, 16 mg; folic acid, 2 mg; D-biotin, 0.2 mg; cyanocobalamin, 10 mg; retinyl palmitate, 8 mg; dl-d-Tocopheryl acetate, 200 mg; cholecalciferol; 25 mg, menaquinone, 50 mg.

begun during this period of adaptation and they had daily access to food for two hours, from 8:00 to 10:00 a.m. Each of the 4 groups were then fed their respective diets for 6 weeks. At the 6th week, 6 animals from the pair-fed control group and the ad libitum vitamin B-6 deficient group were fasted for 1 and 3 days, respecively. At the end of week 6, 12 animals from the deficient group were repleted with the control diet for 2 weeks and 12 animals from the pair-fed control group were continued on the vitamin B-6 sufficient diet. Pair feeding was continued. After repletion, 6 animals from each group were fasted for 3 days. Water was provided during fasting.

Collection of Samples

At the respective time points (nonfast, 1-day fast, 3-day fast at 6 weeks, nonfast and 3-day fast following 2 weeks repletion diet), animals were anesthetized with CO_2 and sacrificed by decapitation. Immediately following decapitation, blood was collected in a heparinized tube and centrifuged for 15 min to separate the plasma. The liver, heart muscle, and skeletal muscle (gastrocnemius) were rapidly removed and frozen in liquid nitrogen. In every case 24-hour urine samples were collected over acetic acid (as a preservative) prior to decapitation. The completeness of the urine collections was checked by determination of creatinine. The plasma, tissues, and urine samples were stored at -40°C until they were analyzed.

Analyses

The vitamin B-6 content of the diet was determined microbiologically, using *Saccharomyces uvarum* (ATCC No. 9080) as the assay organism (Association of Official Analytical Chemists, 1980). Pyridoxal 5'-phosphate was determined by a modified radiometric enzyme method

(Chabner & Livingstone, 1970). Control samples were analyzed with each assay. The within-assay coefficient of variation (CV) was 4.2%, n = 6. The between-assay CV was 8.5%, n = 21. Urinary 4-PA was measured by a HPLC method (Gregory, 1979), modified as follows: 25 cm length, 4.6 mm internal diameter column (Econsil C18 10μ), injection of 50 μl of internal standard pyridoxamine (20 μg/ml), and 50 μl sample injection. The limit of detection was 218 nmol 4-PA/L. The within-assay CV was 3.8%, n = 6, and the between-assay CV was 3.3%, n = 25. Urinary creatinine was measured by an automated procedure (Pino et al., 1965). Plasma glucose was measured by an enzymatic method (Pileggi and Szustkrewixz, 1974). The within-assay CV was 3.3%, n = 4, and the between-assay CV was 1.7%, n = 6. Liver glycogen was measured by a colorimetric procedure (Hassid & Abraham, 1957). The within-assay CV was 10%, n = 6, and the between-assay CV was 5%, n = 4. Free fatty acid was measured colorimetrically (Falholt et al., 1973). The within-assay CV was 7%, n = 6, and the between-assay CV was 7%, n = 6.

Statistical Analysis

All data were subjected to an analysis of variance and tested for significant differences by the least significant difference (LSD) test (Heintz, 1986). A p value < 0.05 was considered to be significant.

Results

The effect of the various feeding patterns and vitamin B-6 deficiency on body weight and food efficiency is shown in Table 4-1. The mean body weight of the vitamin B-6 deficient groups (ad libitum and meal-fed) at week 6 was significantly lower than the mean body weight

of the control groups (ad libitum and pair-fed). Although the mean body weight of the meal-fed vitamin B-6 deficient rats and the mean body weight of the pair-fed control rats were lower than the mean body weights of the respective ad libitum groups, the food efficiency (ratio of body weight change to food intake) was similar. After the vitamin B-6 deficient rats were repleted with the control diet for 2 weeks, the body weights of both groups were essentially identical.

Figure 4-1 shows the effect of vitamin B-6 deficiency on the mean PLP concentrations in plasma and tissues. The mean concentrations of PLP in the plasma, liver, skeletal muscle, and heart muscle of vitamin B-6 deficient rats were significantly lower than the respective concentrations in the control rats. In the vitamin B-6 deficient rats the mean PLP concentrations in the plasma and the liver of the ad libitum rats were significantly lower than respective concentrations in the meal-fed rats, while in the control rats the mean PLP concentration in the plasma of the ad libitum rats was significantly higher than the mean PLP concentration in the pair-fed rats.

Figure 4-2 shows the effect of fasting on the mean PLP concentration in plasma and tissues. In the vitamin B-6 deficient rats, the mean PLP concentration increased significantly in the plasma (130%) and the liver (71%) during the first day of the fast and remained at those levels the third day of the fast, while in the control rats the mean PLP concentration decreased slightly in the plasma (14%) and in the liver (13%), remaining at that level the third day of the fast. With the 3-day fast the mean PLP concentration in the control rats decreased in the skeletal muscle (14%), while the concentration in the vitamin B-6 deficient rats did not change. The mean PLP concentration in the heart muscle increased the

first day of fasting, remaining at that level the third day of the fast in both control rats and vitamin B-6 deficient rats.

The effect of fasting on the mean urinary excretion of 4-PA, with and without a vitamin B-6 deficiency and after vitamin B-6 repletion, is shown in Table 4-2. The urinary excretion of 4-PA for the control rats increased significantly the first day of fasting (mean of 91 nmol/day, n = 5). There was no 4-PA detected in the urine of the vitamin B-6 deficient rats prior to fasting, but on the first day of fasting 3 of 6 rats excreted detectible levels of 4-PA (13.9 to 21.8 nmol/d, n = 3). For both the control rats and the vitamin B-6 deficient rats the urinary excretion of 4-PA decreased on the third day of the fast. While the urinary 4-PA excretion increased in 3 of 6 control rats for the third day of the fast, no detectible 4-PA was excreted by the vitamin B-6 deficient rats. After repletion with vitamin B-6, the patterns of urinary 4-PA excretion with fasting were similar. Following vitamin B-6 repletion, the mean urinary 4-PA excretion of previously vitamin B-6 deficient rats and control rats was similar for the nonfasted condition. However, the mean increase of 4-PA excretion the first day of the fast in control rats was 174% greater than that of the previously vitamin B-6 deficient rats. On the third day of fasting the decrease in 4-PA excretion of the previously vitamin B-6 deficient rats was 51% greater than the 4-PA excretion observed for the control rats.

After the vitamin B-6 deficient rats were repleted with the control diet (Figure 4-3), the PLP concentrations in the plasma, liver, and heart muscle returned to concentrations comparable to those of the control rats. The PLP concentration in skeletal muscle increased but was significantly lower than the concentration observed in the control rats.

The effect of vitamin B-6 deficiency on the concentration of plasma glucose, plasma FFA, and liver glycogen is shown in Table 4-3. In both the control rats and the vitamin B-6 deficient rats the mean plasma glucose concentrations in the ad libitum groups were significantly higher than the respective concentrations in the pair-fed control rats or the meal-fed vitamin B-6 deficient rats. Between ad libitum groups, the mean plasma glucose concentration of the control rats was significantly higher than the glucose concentration of the vitamin B-6 deficient rats. The mean plasma FFA concentrations in the vitamin B-6 deficient groups (ad libitum, meal-fed) were significantly lower than the respective concentrations in the control groups (ad libitum, pair-fed). The mean liver glycogen concentration in ad libitum vitamin B-6 deficient rats was significantly higher than the respective concentrations in the ad libitum control group and pair-fed control group (ad libitum vitamin B-6 deficient, 280.1±50.3; vs ad libitum control, 177.9±37.2; and vs pair-fed control, 1.0±0.2, μmol glucose/g). Since food intake of the pair-fed controls was restricted and their food was consumed in a shorter period of time than was the case for the vitamin B-6 deficient animals, the control rats may be considered as chronically, partially starved animals. This feeding schedule appears to have affected the concentrations of plasma glucose, liver glycogen, and plasma FFA in the pair-fed control animals.

As shown in Table 4-4, the plasma glucose concentration in the control rats did not change with fasting, while the plasma glucose concentration in the vitamin B-6 deficient rats significantly decreased the first day of the fast and remained at that level the third day of the fast. Following the 1-day fast, liver glycogen was almost completely utilized in both the control and the vitamin B-6 deficient rats. Although the concentrations of

plasma FFA in the vitamin B-6 deficient rats were lower than those in the control rats on day 1 and day 3 of the fast, the absolute increase of plasma FFA from the nonfast time period to day 3 (75 mg/l in control rats vs 66 mg/l in vitamin B-6 deficient rats) was similar.

After the vitamin B-6 deficient rats were repleted with the control diet, the concentrations of plasma glucose, liver glycogen, and plasma FFA returned to concentrations comparable to those in the control rats (Table 4-5).

Discussion

This study demonstrates that short-term fasting resulted in redistribution of PLP and is most evident in the vitamin B-6 deficient rats. The evidence of PLP redistribution is based on the increase of PLP concentration in plasma and tissues and the increase of urinary 4-PA excretion with fasting. The results of this study are consistent with changes observed in humans in plasma PLP concentration (Leklem & Shultz, 1983) and urinary 4-PA excretion following exercise (Manore et al., 1987).

The mean body weight and the mean PLP concentration in plasma and tissues of rats fed the vitamin B-6 deficient diet (ad libitum, meal-fed) were significantly lower than those of the control rats. Thus, animals were severely vitamin B-6 deficient by 6 weeks. Following vitamin B-6 repletion, the mean PLP concentrations in the plasma, liver, and heart muscle in previously vitamin B-6 deficient rats was similar to the respective concentrations in the control animals. While PLP concentration in the skeletal muscle of previously vitamin B-6 deficient rats increased, it was significantly lower than that of control rats. Since the turnover time of skeletal muscle glycogen phosphorylase in mice is approximately 12 days

(Butler et al., 1985), and PLP is stored in skeletal muscle bound to glycogen phosphorylase (Black et al., 1977), 2 weeks repletion may not have been sufficient time to reach the PLP concentrations observed in the skeletal muscle of the control rats.

In the vitamin B-6 deficient rats, fasting significantly increased the PLP concentrations in the plasma, liver, and heart muscle. Black et al. (1977) reported that skeletal muscle glycogen phosphorylase serves as a storage depot for vitamin B-6 and the total amount of glycogen phosphorylase in muscle decreased when a caloric deficit was introduced. Thus, the source of increased PLP in the plasma and liver of the vitamin B-6 deficient rats with fasting may be skeletal muscle glygogen phosphorylase. However, in the present study, no difference in skeletal muscle PLP concentration of the deficient rats was observed with a 3-day fast. This discrepancy may be explained by the large mass of skeletal muscle. A small decrease of PLP concentration in skeletal muscle may result in a significant increase in PLP concentration in plasma and in other tissues.

In control rats, fasting resulted in a vitamin B-6 deprivation due to the lack of food. With vitamin B-6 deprivation, as a result of the fast, less PLP would be synthesized in the liver. This deprivation effect might be greater than any PLP increase due to fasting, in which case we would not observe an increase in plasma PLP concentration. In the present study, a drop in liver PLP concentration was observed the first day of fasting. If our study is consistent with that of Black et al. (1978), with fasting the amount of muscle glycogen phosphorylase would have decreased and PLP could possibly be released into the plasma. Support for this comes from the slight but significant decrease in skeletal muscle PLP concentration the third day of fasting. With a three-day fast after

repletion, a decrease in PLP concentration in skeletal muscle was not observed. The reason for this may be that repletion animals were basically ad libitum fed, while the 6-week animals were pair-fed which resulted in the animals being chronically, partially starved. If skeletal muscle acts as a reservoir for endogenous PLP, one might expect an increase of PLP concentration in plasma. However, there may be less PLP synthesis in the liver due to vitamin B-6 deprivation during fasting. If the amount of PLP released from the liver was decreased due to less synthesis of PLP and the release of PLP from skeletal muscle into plasma was increased due to fasting, then the net result might be little or no change in PLP concentration, which was observed for the control animals in this study. Further support for this was found in the results for the non-fasted vitamin B-6 deficient animals. These animals were already deprived of vitamin B-6. Thus, fasting would be expected to have no further effect on the synthesis of PLP in the liver. Therefore, other factors such as PLP release from skeletal muscle could explain the increase in plasma PLP concentration observed. With a one-day fast there was an apparent increase in PLP concentration in the liver of vitamin B-6 deficient rats. However, with the 3-day fast the liver weight decreased. At the end of the 3-day fast the percentage decrease in liver weight (74%) was essentially the same as the percentage increase in liver PLP concentration (72%).

Fasting increased urinary 4-PA excretion in both vitamin B-6 deficient and control animals. Since 4-PA is the major vitamin B-6 metabolite and there was no exogenous vitamin B-6 source during fasting, the increased urinary 4-PA excretion could be a reflection of vitamin B-6 redistribution (as PLP) associated with the caloric deficit present in the

animals. The increase in urinary 4-PA excretion was greater and lasted over a longer period of time in the controls than in the vitamin B-6 deficient rats. The skeletal muscle PLP concentration in the vitamin B-6 deficient rats was significantly lower than that of the control rats. If the muscle is a reservoir for vitamin B-6, the vitamin B-6 deficient animals would not have had a large reservoir of endogenous PLP and thus would excrete less 4-PA in the urine. This muscle-PLP and urinary 4-PA relationship is also consistent with the results observed in the repletion study. After repletion, the skeletal muscle PLP concentration of previously vitamin B-6 deficient rats was significantly lower than that of rats fed the control diet for eight weeks. In this case, the increase of urinary 4-PA excretion the first day of fasting in previously vitamin B-6 deficient rats was less than that of rats fed the control diet for eight weeks.

Additional support for muscle as a vitamin B-6 reservoir was observed in the results for the meal-fed animals. Because the food intake of meal-fed animals was 25% less than that of the ad libitum vitamin B-6 deficient animals, the meal-fed animals were more energy deprived. Given this state of relative energy deprivation, there may have been a redistribution of PLP occurring among the meal-fed rats at the time the animals were sacrificed. This was indicated by a higher PLP concentration in both the plasma and liver of the meal-fed animals compared to the respective concentrations of PLP in the ad libitum vitamin B-6 deficient animals. Compared to other tissues, the difference of heart PLP concentration between control rats and vitamin B-6 deficient rats was relatively small. With fasting, in both control rats and vitamin B-6 deficient rats, heart PLP was increased. Thus, heart muscle may sequester more PLP than other tissues when the body is under metabolic stress.

Plasma and tissue concentrations of PLP may affect fuel metabolism during periods of fasting. Since PLP is associated with liver glycogen phosphorylase (Krebs & Fischer, 1964), lowered PLP concentrations in the liver could result in impaired glycogenolysis, which would result in an accumulation of liver glycogen. Because the liver glycogen concentration in the ad libitum vitamin B-6 deficient animals was higher than that of the ad libitum control animals, the feeding schedule can be eliminated as a reason for the glycogen accumulation in the liver of the vitamin B-6 deficient rats. Following repletion, the liver PLP concentration of the vitamin B-6 deficient rats returned to that of the control rats and the liver glycogen concentration in both groups was similar. In contrast to the observation in the present study, Suzuki and Okada (1982a) reported a marked decrease in liver glycogen in pyridoxine deficient rats. A possible reason for this discrepancy may be the type of diet (70% casein vs 20% casein). Because the liver weight of rats fed the 70% casein-pyridoxine deficient diet was higher than that of rats fed the 20% casein-pyridoxine deficient diet (Suzuki et al., 1976), liver glycogen concentration in rats fed the 70% casein-pyridoxine deficient diet might be diluted.

Since Ribaya and Gershoff (1977) reported that the epididymal fat pads from vitamin B-6 deficient rats are more permeable to glucose in the presence of insulin, the uptake of glucose into fat cells would be increased, thus resulting in a decreased plasma glucose concentration in the vitamin B-6 deficient rats. In the present study the plasma glucose concentration in the ad libitum vitamin B-6 deficient rats was significantly lower than the plasma glucose concentration in the ad libitum control rats. Compared to the control rats, the plasma FFA concentration was lower in the vitamin B-6 deficient rats. However, with fasting the increase of

plasma FFA concentration was similar in both groups. This suggests that the release of FFA from adipose tissue into the plasma may not be impaired as a result of a vitamin B-6 deficiency.

In summary, fasting results in an increased concentration of PLP in the plasma, liver, and heart muscle of rats fed a vitamin B-6 deficient diet. Urinary 4-PA excretions also increases during fasting. These changes are consistent with a redistribution of vitamin B-6 (as PLP) when there is a caloric deficit. The source of this vitamin B-6 may be skeletal muscle and, specifically, muscle glycogen phosphorylase.

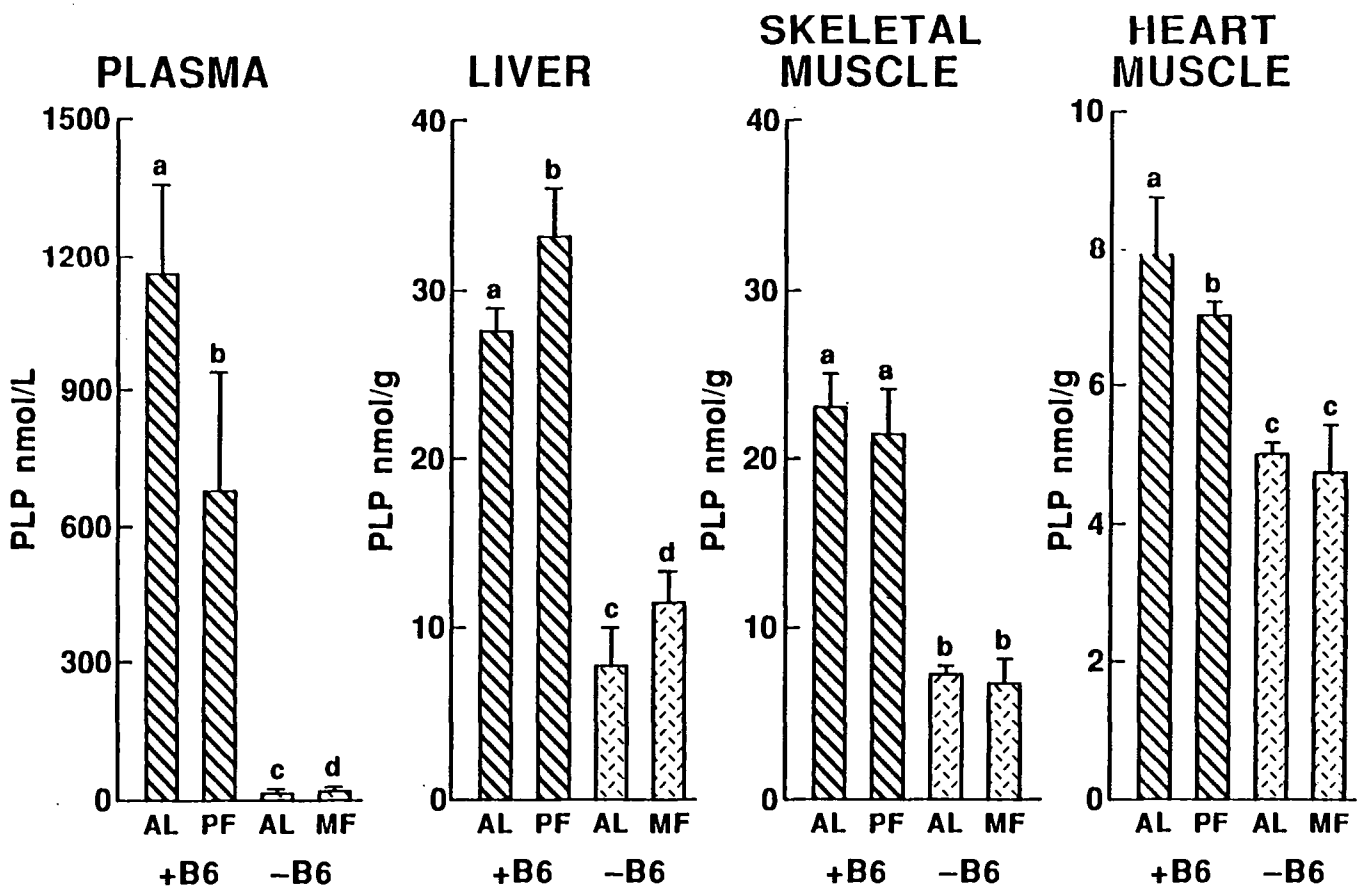

Figure 4-1. Effect of vitamin B-6 deficiency on the concentration of pyridoxal 5'-phosphate in plasma and tissue of rats. (Each bar with different letters is signficantly different ($P < 0.05$); +B6 = control diet; -B6 = vitamin B-6 deficient diet; AL = ad libitum; PF = pair-fed; MF = meal-fed.)

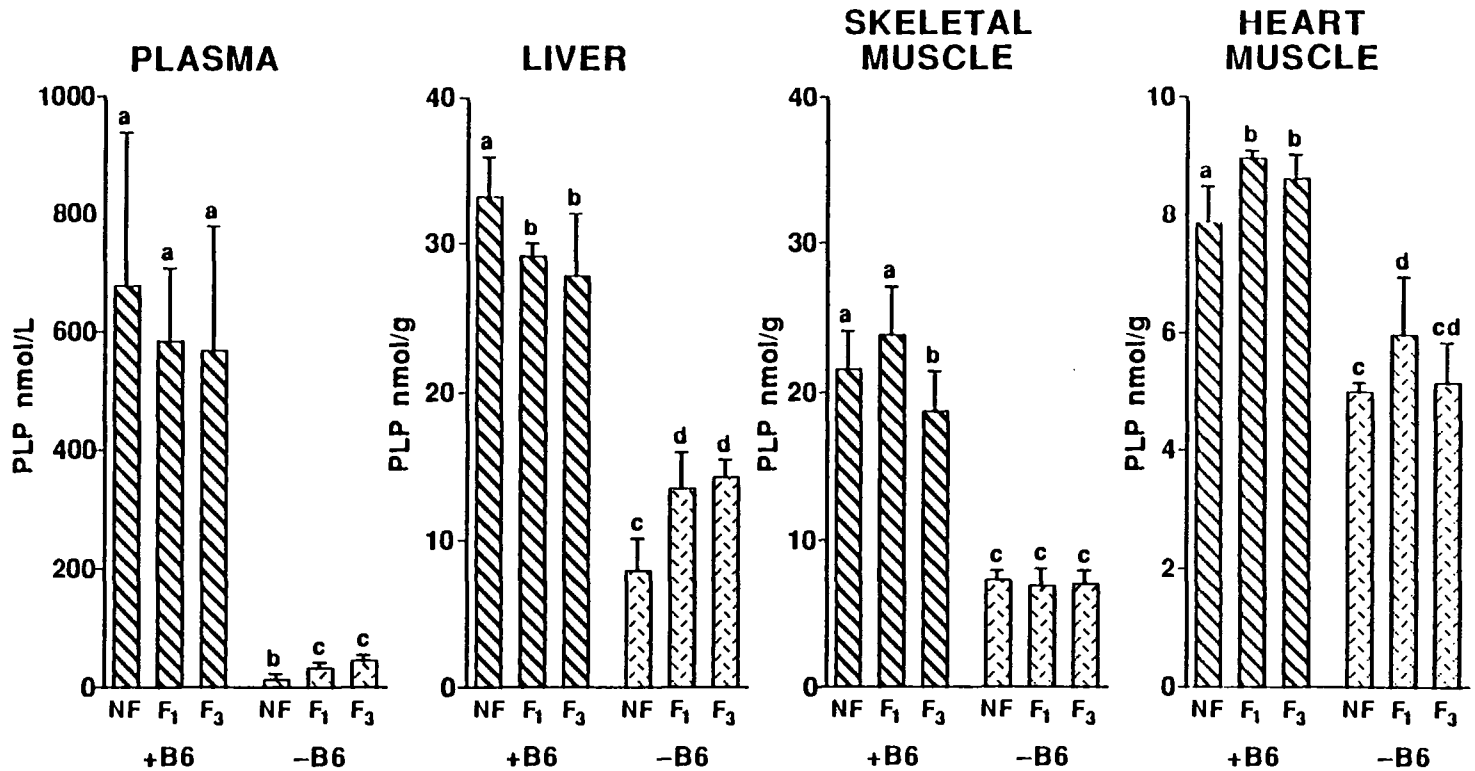

Figure 4-2. Effect of fasting on the concentration of pyridoxal 5'-phosphate in plasma and tissues of rats fed the control diet and vitamin B-6 deficient diet. (Each bar with different letters is signficantly different($P < 0.05$); +B6 = control diet; -B6 = vitamin B-6 deficient diet; NF = non-fasted; F_1 = 1-day fast; F_3 = 3-day fast.)

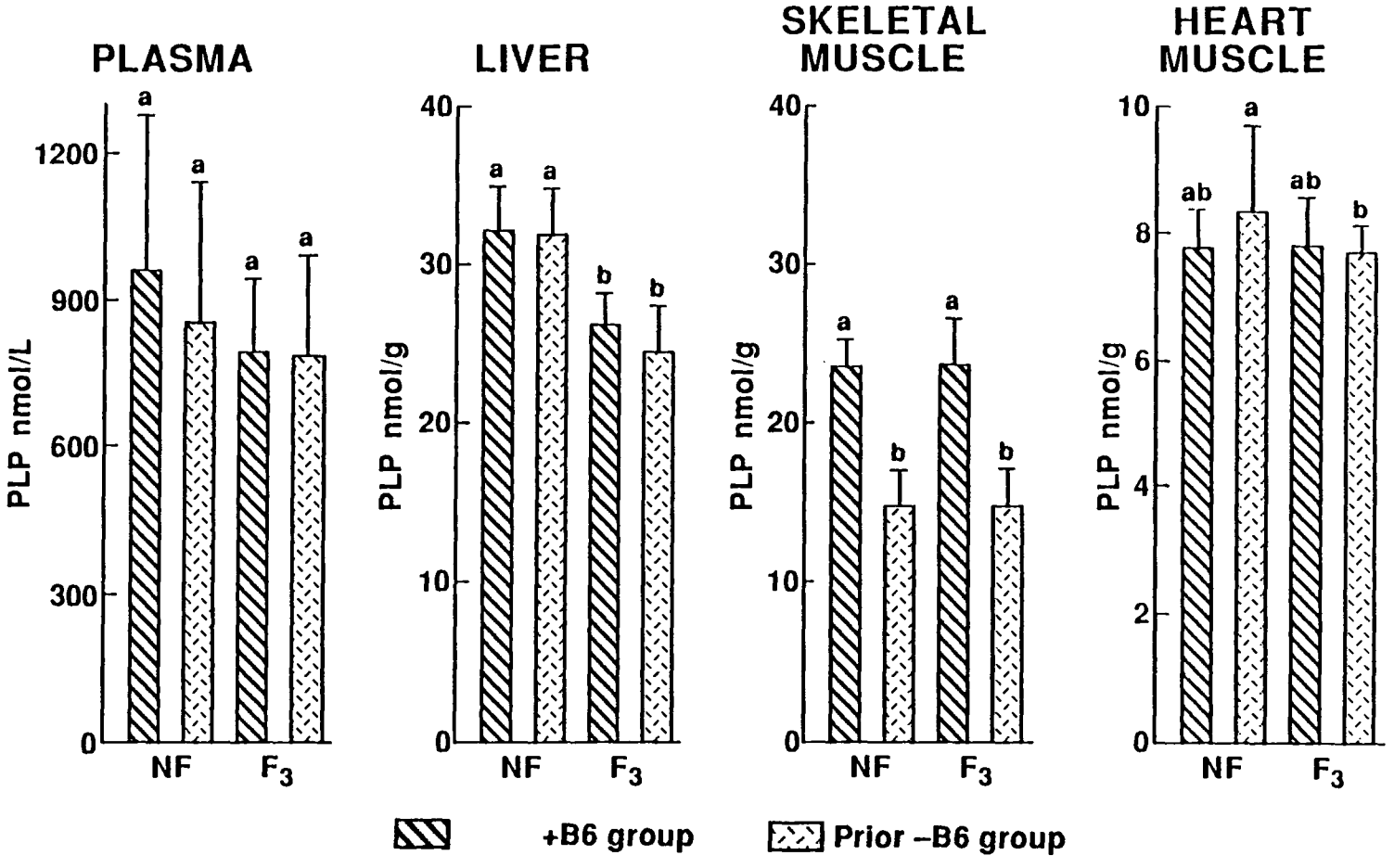

Figure 4-3. Effect of vitamin B-6 repletion and of fasting on pyridoxal 5'-phosphate concentration in plasma and tissue of rats. (Each bar with different letters is signficantly different($P < 0.05$); +B6 = control diet; -B6 = prior vitamin B-6 deficient diet; NF = non-fasted; F_3 = 3-day fast.)

Table 4-1. Effect of a 6-week vitamin B-6 deficiency on body weight and feed efficiency in rats.

Diet:	+B6[1]		-B6[2]	
Feeding pattern	Ad libitum	pair-fed	Ad libitum	meal-fed
Body weight (g)	364±29	289±9	254±12	180±38
ΔBW/Food intake (g body weight/ g food)	1.15±0.14	0.44±0.18	0.11±0.41	0.45±0.53

[1] control diet
[2] vitamin B-6 deficient diet

Table 4-2. Effect of vitamin B-6 deficiency and repletion on urinary 4-pyridoxic acid (4-PA) excretion and the effect of fasting on urinary 4-PA excretion (Δ).

No	NF[1]	Δ F1-NF[2]	Δ F2-NF[2]	Δ F3-NF[2]
	6 Week +B6, nmol/d[3]			
25	183.3	+70.6	-19.2	-85.7
26	212.4	+76.6	+23.2	+13.4
27	138.8	+123.8	+60.2	+17.0
28	18.4[4]	+24.0[4]	+13.8[4]	+10.0[4]
29	177.0	+68.5	-23.9	-44.6
30	159.2	+117.3	+61.4	-65.0
x(n = 5)	174.9 ±27.9	91.4 ±26.9	19.3 ±37.0	-25.8 ±45.0
	6 Week, -B6, nmol/d[3]			
61	n.d.	+21.8	+18.8	n.d.
62	n.d.	+14.4	+14.2	n.d.
63	n.d.	n.d.	n.d.	n.d.
64	n.d.	n.d.	n.d.	n.d.
65	n.d.	+13.9	n.d.	n.d.
66	n.d.	n.d.	n.d.	n.d.
x(n = 3)[5]		16.7 ±4.4	16.5 (n = 2)	
	Repletion, P +B6, nmol/d[3]			
43	231.6	-61.6	+2.6	-58.0
44	263.6	-19.1	+69.3	-58.4
45	273.0	+43.8	-10.4	-79.6
46	30.6[4]	+13.4[4]	+11.4[4]	-1.5[4]
47	196.8	+206.1	+28.1	-5.8
48	344.4	+109.5	+50.6	-184.2
x(n = 5)	261.9 ±55.0	55.7 ±106.1	25.3 ±7.0	-77.2 ±65.7
	Repletion, P - B6, nmol/d[3]			
79	232.8	+62.2	+22.0	-69.4
80	265.0	-19.1	-104.2	-132.0
81	171.4	+21.2	-54.6	-85.2
82	224.8	+12.9	-74.7	-129.2
83	302.7	+23.5	-61.7	-157.5
84	276.0	+20.9	-43.5	-126.8
x(n = 6)	245.4 ±46.2	20.3 ±26.0	-57.8 ±42.1	-116.7 ±32.8

[1] Nonfast
[2] F1 = 1-day fast, F2 = 2-day fast, F3 = 3-day fast
[3] +B6 = control diet, -B6 = vitamin b-6 deficient diet, P +B6 = prior control diet, P -B6 = prior vitamin B-6 deficient diet
[4] Not included in calculation of mean, listed for information only
[5] Only 3 animals yielded detectible results; n.d. = non-detectible

Table 4-3. Effect of a 6-week vitamin B-6 deficiency on plasma, glucose, liver glycogen, and plasma free fatty acid concentrations.

Diet:	+B6[1]		-B6[2]	
Feeding pattern	Ad libitum	Pair-fed	Ad libitum	Meal-fed
Plasma glucose (mmol/L)	8.0±0.5[a]	5.3±0.3[b]	6.3±0.7[c]	5.3±0.2[b]
Liver glycogen (μmol glucose/g)	177.9±37.2[a]	1.0±0.2[b]	280.2±50.3[c]	1.4±0.4[b]
Plasma FFA (mg/L)	84±17[a]	92±10[a]	52±14[b]	56±16[b]

In a given row, those values with different letters are significantly different ($p < 0.05$).
[1] control diet; [2] vitamin B-6 deficient diet

Table 4-4. Effect of fasting on the plasma glucose, liver glycogen, and plasma free fatty acid in control and vitamin B-6 deficient rats.

Diet:	+B6[1]			-B6[2]		
Day of fasting	NF[3]	F1[4]	F3[5]	NF[3]	F1[4]	F3[5]
Plasma glucose (mmol/L)	5.3±0.3[a]	5.1±0.6[a]	5.4±0.9[a]	6.3±0.7[b]	4.6±0.6[a]	4.7±1.1[a]
Liver glycogen (μmol glucose/g)	1.0±0.2[a]	1.0±0.3[a]	1.0±0.3[a]	280.1±50.3[b]	1.2±0.4[a]	2.0±1.1[a]
Plasma FFA (mg/L)	92±10[a]	106±11[ac]	167±26[d]	52±14[b]	93±14[a]	118±18[c]

In a given row, those values with different letters are significantly different ($p < .05$).
+B6[1] = control diet; -B6[2] = vitamin B-6 deficient diet; NF[3] = Nonfasted, when at 6th week of experiment; F1[4] = 1-day fast; F3[5] = 3-day fast

Table 4-5. Mean concentration of plasma glucose, liver glycogen and plasma free fatty acids in rats repleted with vitamin B-6 and in control rats.

	NF[1]		F3[2]	
Feeding pattern	+B6[3]	P -B6[4]	+B6	P -B6
Plasma glucose (mmol/L)	7.2±Ø.4[a]	6.7±Ø.8[a]	4.5±Ø.6[b]	4.7±Ø.4[b]
Liver glycogen (μmol glucose/g)	182.6±58.7[a]	214.Ø±56.6[a]	Ø.9±Ø.1[b]	1.2±Ø.4[b]
Plasma FFA (mg/L)	88±28[a]	87±12[a]	166±31[b]	168±17[b]

In each given row, those values with different letters are significantly different ($P < Ø.Ø5$).

[1] NF = nonfasted; [2] F3 = 3-days fasted; [3] +B6 = control diet; [4] P -B6 = prior vitamin B-6 deficient diet

CHAPTER V

SUMMARY AND CONCLUSION

The objectives of this study were to (1) determine if there is a requirement for vitamin B-6 in carnitine synthesis in vivo and (2) investigate the effect of fasting on vitamin B-6 metabolism.

To meet these objectives, 72 male Wistar weanling rats (6 per group) were fed a vitamin B-6 deficient diet or a control diet. These diets were fed for six weeks and then the rats were repleted with the control diet for two weeks. Rats were fasted for one and three days at week six and after repletion. The concentrations of pyridoxal 5'-phosphate (PLP) and carnitine in the plasma, liver, skeletal and heart muscles, and urinary excretion of 4-pyridoxic acid (4-PA) and carnitine were compared for each group. In addition, the respective concentrations of plasma glucose, liver glycogen, and plasma free fatty acid were compared.

The data for PLP concentration and body weight indicate that the animals were severely vitamin B-6 deficient by six weeks. After repletion with the control diet, the mean concentrations of PLP in plasma, tissues, and urinary excretion of 4-PA returned to those of the control animals. The mean PLP concentration in skeletal muscle increased, but was lower than that of the control animals.

Evidence of a vitamin B-6 requirement in carnitine synthesis in vivo is based on three lines of evidence. First, after the animals were fed the

respective diets for six weeks, the mean total acid soluble carnitine (TCN) concentrations of the vitamin B-6 deficient rats (ad libitum, meal-fed) were significantly lower in plasma, skeletal muscle, heart muscle, and in urine compared to control rats (ad libitum, pair-fed). Second, following repletion, the concentrations of PLP in the plasma and liver of previously vitamin B-6 deficient rats were restored to that of the control animals. Thus, in a situation in which the vitamin B-6 status was similar, concentrations of TCN and free carnitine (FCN) in the plasma, tissues, and urinary excretion of TCN and FCN returned to those observed for the control rats. Third, with fasting, a reduction of the variation in carnitine concentration was observed in the liver, as reflected by a smaller standard deviation, and a significantly lower mean TCN concentration was observed in the liver of vitamin B-6 deficient rats as compared to the concentration in control rats.

With fasting, there were two lines of evidence for a redistribution of vitamin B-6. First, fasting increased the mean PLP concentrations in the plasma, liver, and heart muscle of vitamin B-6 deficient rats. In the control rats, there was no increase of the mean PLP concentration in the plasma and liver with fasting. The reason for this discrepancy may be that fasting resulted in a vitamin B-6 deprivation due to lack of food and this vitamin B-6 deprivation effect might have been greater than any increase of PLP concentration due to fasting. Second, urinary 4-PA excretion increased with fasting, especially during the first two days of the fast. Increased urinary 4-PA excretion was observed in both control and deficient rats. This increased 4-PA excretion is consistent with the increase in PLP concentration in plasma and tissues observed with fasting.

In conclusion, this study provides in vivo evidence for a vitamin B-6 requirement for carnitine synthesis. Considering carnitine's role in fatty acid metabolism and the requirement for vitamin B-6 in its synthesis, a lowered vitamin B-6 intake in conjunction with a lowered caloric intake may impair adaptation of metabolic events related to fatty acid oxidation. Short-term fasting results in redistribution of vitamin B-6 (as PLP). This redistribution may be favorable for animals experiencing a caloric deficit since PLP is needed in several metabolic steps, including glycogenolysis, gluconeogenesis, and synthesis of carnitine, the carrier for fatty acid oxidation.

From this study we found evidence for a vitamin B-6 requirement in carnitine synthesis. Thus, the next step would be to measure the activity of lysine methyltransferase, the first enzyme in the synthesis of carnitine and one which PLP appears to regulate indirectly. The activity of 3-hydroxy-4-N-trimethyllysine aldolase (for which PLP may act as a cofactor), the accumulation of carnitine precursors, and the decrease of certain intermediates in carnitine synthesis need to be measured under conditions of vitamin B-6 deficiency.

In this study we have observed the effect of short-term fasting on the redistribution of vitamin B-6 as PLP. In order to better understand the effect of short-term fasting on the redistribution of vitamin B-6, the other forms of vitamin B-6 (e.g., pyridoxine, pyridoxamine) should be measured. To avoid the problem of vitamin B-6 deprivation as a result of fasting, the injection or oral administration of vitamin B-6 during fasting is suggested.

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APPENDICES

Appendix 1. Composition of Diet.

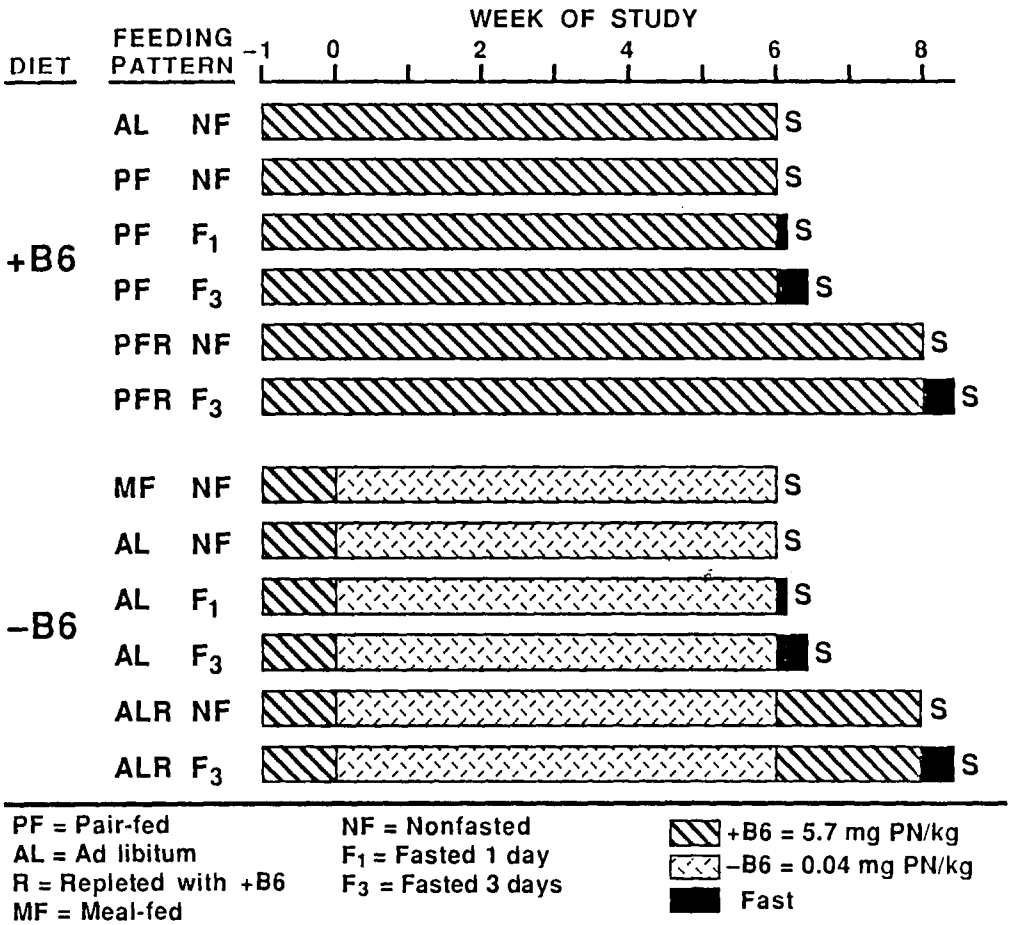
Components	Percent
Casein--Vitafree	20.0
DL Methionine	0.3
Cornstarch	15.0
Sucrose	50.0
Fiber--Celufil	5.0
Corn oil	5.0
Choline Bitartrate	0.2
AIN mineral mix*	3.5
AIN vitamin mix without pyridoxine**	1.0

*AIN-76 mineral mix (3.5% of total diet)	g/kg
Calcium phosphate dibasic ($CaHOP_4$)	500.0
Sodium chloride (NaCl)	74.0
Potassium citrate monohydrate ($HOC(COOK)(CH_2COOK)_2 \bullet H_2O$)	200.0
Potassium sulfate (H_2SO_4)	52.0
Magnesium carbonate (43 to 48% Mn)	3.5
Ferric carbonate (16 to 17% Fe)	6.0
Zinc carbonate (70% Zn)	1.6
Cupric carbonate (53 to 55% Cu)	0.3
Potassium iodate (KIO_3)	0.01
Sodium selenite ($Na_2SeO_35H_2O$)	0.01
Chromium potassium sulfate ($CrK(SO_4)_2 \bullet 12H_2O$)	0.55
Finely powdered sucrose	118.0

**AIN-76 vitamin mix	Per kg
Thiamin-HCl	600.0 mg
Riboflavin	600.0 mg
Pyridoxine HCl***	700.0 mg
Nicotinic acid	3.0 g
D-calcium panthothenate	1.6 g
Folic acid	200.0 mg
D-Biotin	20.0 mg
Cyanocobalamin (B_{12})	1.0 mg
Retinyl palmitate (vitamin A premix)	800.0 mg
dl-d-Tocopheryl acetate	20.0 g
Cholecalciferol (vitamin D_3)	2.5 g
Menoquinine (vitamin K)	5.0 g
Finely powdered sucrose	972.9 g

***Vitamin B-6 in the diet	mg pyridoxine/ kg diet
Control diet	5.691
Vitamin B-6 deficient diet	0.039

Appendix 2. Design of the experiment.



Appendix 3. Body weight (g), by week (Wk).

No	Ø Wk	1 Wk	2 Wk	3 Wk	4 Wk	5 Wk	6 Wk
	+B6 Ad libitum, nonfast						
7	109	159	202	258	306	335	361
8	140	205	255	315	360	390	414
9	105	157	200	245	298	330	355
10	106	163	205	245	288	323	343
11	121	173	208	237	283	313	332
12	130	188	233	270	316	354	379
x	118	174	217	261	308	340	364
	±14	±18	±20	±28	±27	±25	±29
	+B6 Pair-fed, nonfast						
19	136	177	214	255	278	295	298
20	129	178	212	248	271	282	288
21	141	182	216	254	267	276	279
22	120	167	202	233	261	278	286
23	127	177	216	251	277	295	301
24	133	176	211	235	255	275	283
x	131	176	211	246	267	283	289
	±7	±4	±4	±9	±9	±9	±8
	-B6 Ad libitum, nonfast						
55	120	171	199	216	230	250	250
56	123	173	205	222	240	249	259
57	127	174	212	235	254	260	264
58	135	185	221	231	254	270	265
59	116	167	197	216	228	232	232
60	127	173	206	222	240	261	259
x	124	173	206	223	241	253	254
	±6	±6	±8	±7	±11	±13	±12
	-B6 Meal-fed, nonfast						
91	86	135	145	163	179	188	193
92	102	149	149	166	175	183	186
93	119	166	159	163	190	198	202
94	84	118	92	104	100	105	103
95	119	154	160	173	182	201	206
96	113	156	146	162	178	180	192
x	103	146	141	155	167	175	180
	±15	±17	±25	±25	±33	±35	±38

+B6 = control diet; -B6 = vitamin B-6 deficient diet

Appendix 3 (continuation). Body weight (g), by week (Wk).

No	0 Wk	1 Wk	2 Wk	3 Wk	4 Wk	5 Wk	6 Wk	F1 6 Wk	F3 6 Wk
	+B6 Pair-fed, F1 day								
31	128	170	218	241	264	282	287	286	
32	129	169	221	243	262	275	280	280	
33	110	164	211	235	263	282	292	295	
34	120	164	211	239	261	279	285	286	
35	105	157	202	234	255	274	286	288	
36	135	179	225	241	256	270	275	280	
x	121	167	214	238	260	277	284	285	
	±11	±8	±8	±3	±3	±4	±15	±5	
	-B6 Ad libitum, F1 day								
67	129	179	213	239	250	257	261	256	
68	120	170	204	236	256	269	285	277	
69	119	174	212	250	280	299	315	311	
70	143	196	229	250	258	269	268	263	
71	119	164	194	223	235	243	228	207	
72	134	189	230	262	268	279	267	207	
x	127	178	213	243	257	269	270	263	
	±9	±11	±14	±13	±15	±19	±28	±33	
	+B6 Pair-fed, F3 day								
25	118	166	209	232	251	264	271		240
26	134	178	235	256	277	296	306		271
27	144	187	239	258	277	296	307		280
28	107	154	194	225	244	263	273		238
29	104	157	173	206	230	253	263		231
30	121	170	222	249	275	287	295		264
x	121	168	212	237	259	276	285		254
	±15	±12	±25	±20	±20	±18	±19		±20
	+B6 Ad libitum, F3 day								
61	116	171	213	236	240	252	249		227
62	118	174	215	242	250	290	302		262
63	116	172	209	246	275	286	292		252
64	113	158	193	208	217	223	225		189
65	115	172	203	222	245	254	255		221
66	117	171	206	236	255	258	254		214
x	115	169	206	231	247	260	262		227
	±1	±5	±7	±14	±19	±24	±28		±26

+B6 = control diet; -B6 = vitamin B-6 deficient diet

F1 = 1-day fast; F3 = 3-day fast

Appendix 3 (continuation). Body weight (g), by week (Wk).

No	0 Wk	1 Wk	2 Wk	3 Wk	4 Wk	5 Wk	6 Wk	7 Wk	8 Wk	F3 8 Wk
	+B6 Pair-fed, repletion, nonfast									
37	111	149	185	212	229	244	238	295	316	
38	119	166	211	242	263	283	292	345	379	
39	114	150	280	221	241	256	265	299	279	
40	139	184	240	256	266	273	279	322	351	
41	102	156	210	239	260	273	281	331	363	
42	108	155	200	219	240	257	266	317	345	
x	114	161	207	231	249	264	270	318	338	
	±12	±12	±18	±16	±15	±14	±18	±19	±36	
	-B6 Ad libitum, repletion, nonfast									
73	114	164	199	215	228	225	220	294	325	
74	114	161	190	217	248	263	277	320	333	
75	123	181	207	224	238	242	248	323	352	
76	114	165	197	221	235	240	236	309	323	
77	115	174	206	239	263	278	289	355	382	
78	126	175	208	234	251	264	265	324	343	
x	118	170	201	225	244	252	256	321	334	
	±5	±8	±7	±10	±13	±20	±26	±20	±22	
	+B6 Pair-fed, repletion, F3 day									
43	112	161	205	235	258	283	285	340	373	335
44	110	159	206	232	257	276	273	323	352	320
45	127	172	211	236	258	277	284	326	349	312
46	116	161	214	235	247	262	273	318	349	315
47	115	158	211	234	252	263	271	319	352	314
48	144	178	229	257	268	281	291	338	364	323
x	121	162	213	238	257	274	280	327	357	320
	±13	±5	±9	±9	±7	±9	±8	±20	±11	±8
	-B6 Ad libitum, repletion, F3 day									
79	148	198	240	274	283	304	313	357	383	340
80	117	160	185	206	216	228	227	275	296	256
81	112	163	196	207	217	217	223	297	322	280
82	113	158	203	231	238	238	239	306	328	285
83	138	191	221	238	258	278	280	353	377	336
84	138	185	224	258	277	298	297	365	387	340
x	128	176	212	236	248	261	263	326	349	306
	16	18	20	27	29	38	39	38	39	37

+B6 = control diet; -B6 = vitamin B-6 deficient diet; F3 = 3-day fast

Appendix 4. Food intake (g), by week (Wk).

No	1 Wk	3 Wk	5 Wk	6 Wk	No	1 Wk	3 Wk	5 Wk	6 Wk	No	1 Wk	3 Wk	5 Wk	6 Wk	8 Wk
+B6 Ad lib., NF					+B6, PF, F1					+B6, PF, Repl., NF					
7	15	19	20	19	31	15	16	13	13	37	16	15	13	13	19
8	18	21	22	22	32	15	16	13	13	38	15	16	13	13	22
9	16	20	20	20	33	16	16	13	13	39	16	16	13	13	22
10	16	20	21	21	34	15	15	14	13	40	15	16	13	13	20
11	16	18	20	19	35	15	16	12	13	41	15	15	13	13	20
12	17	19	22	22	36	15	16	13	12	42	15	16	13	13	22
x	16	20	21	21	x	15	16	13	13	x	16	16	13	13	21
	±1	±1	±1	±1		±0	±0	±1	±0		±1	±1	±0	±0	±1
-B6 PF, NF					-B6 Ad lib., F1					-B6 Ad lib., Repl., NF					
19	15	15	14	13	67	16	16	12	12	73	15	16	13	13	20
20	15	16	14	13	68	16	16	14	15	74	16	17	13	13	17
21	15	15	14	13	69	16	16	17	15	75	15	16	13	13	18
22	14	16	14	13	70	17	16	14	14	76	15	16	13	13	21
23	15	16	14	13	71	17	14	11	10	77	15	16	13	13	23
24	16	14	14	13	72	16	17	12	13	78	15	15	13	13	23
x	15	15	14	13	x	17	16	13	13	x	15	16	13	13	20
	±1	±1	±0	±0		±1	±1	±2	±2		±0	±1	±0	±0	±3
-B6 Ad lib., NF					+B6 PF, F3					+B6 PF, Repl., F3					
55	16	14	14	12	25	15	16	13	13	43	16	13	10	9	22
56	15	14	16	13	26	15	16	13	13	44	15	15	13	14	21
57	13	13	13	10	27	16	15	13	13	45	17	14	13	10	20
58	16	14	15	13	28	16	16	12	13	46	15	14	11	12	20
59	16	13	13	12	29	15	15	13	13	47	17	16	13	12	22
60	16	15	15	13	30	15	15	13	13	48	16	14	12	14	20
x	15	14	14	12	x	15	16	13	13	x	16	14	12	12	21
	±1	±1	±1	±1		±1	±1	±0	±0		±1	±1	±1	±2	±1
-B6 MF, NF					-B6 Ad lib., F3					-B6 Ad lib., Repl., F3					
91	12	10	10	10	61	16	12	13	13	79	18	13	13	11	21
92	14	10	12	10	62	16	15	14	13	80	15	14	12	12	19
93	14	9	10	10	63	16	17	14	13	81	16	12	13	12	19
94	9	7	6	6	64	15	15	11	12	82	15	14	14	13	19
95	12	9	12	10	65	16	15	17	13	83	18	13	12	12	23
96	11	7	9	9	66	16	14	10	11	84	16	17	12	10	21
x	12	9	10	9	x	16	15	13	13	x	16	14	13	12	20
	±2	±1	±2	±2		±0	±2	±2	±1		±0	±2	±1	±1	±1

+B6 = control diet; -B6 = vitamin B-6 deficient diet; F1 = 1-day fast; F3 = 3-day fast; MF = Meal-fed; PF = Pair-fed; NF = Nonfast; Ad lib = Ad libitum; Repl = Repletion

Appendix 5. Concentration of free carnitine (FCN), total acid soluble carnitine (TCN), pyridoxal 5'-phosphate (PLP), free fatty acid (FFA), and glucose (GLU) in plasma and the weight (WT) of the heart and concentration of free carnitine, total acid soluble carnitine, pyridoxal 5'-phosphate in the heart.

	PLASMA					HEART			
No	FCN µmol /L	TCN µmol /L	PLP nmol /L	FFA mg/L	GLU mmol /L	WT g	FCN nmol /g	TCN nmol /g	PLP nmol /g
				+B6 Ad libitum, nonfast					
7	62.9	77.6	1071.7	112	7.2	1.03	653.5	1284.0	7.7
8	72.5	103.2	1371.4	92	7.9	1.21	715.4	1099.4	8.7
9	59.6	130.4	1288.8	70	8.0	1.14	531.7	836.4	8.6
10	85.6	98.1	835.1	84	8.6	0.97	774.3	1272.1	8.6
11	78.4	86.6	1278.1	63	8.7	0.87	916.1	1134.1	6.7
12	53.3	68.6	1116.1	80	7.5	1.02	626.0	1218.6	7.4
x	68.7	94.1	1160.2	84	8.0	1.04	702.8	1125.8	7.9
	±12.3	±21.9	±195.2	±17	±0.5	±0.12	±133.0	±161.9	±0.8
				+B6 Pair-fed, nonfast					
19	58.1	78.9	677.0	104	5.7	0.95	771.4	1185.0	7.4
20	62.0	96.0	354.3	96	4.9	0.87	770.3	947.4	7.0
21	38.1	99.6	416.7	99	5.3	0.83	786.3	1190.1	6.8
22	63.0	77.5	841.0	87	5.1	0.87	669.8	864.1	6.9
23	45.8	70.7	1063.9	77	5.1	0.83	774.7	1222.2	7.0
24	66.7	87.8	700.5	87	5.3	0.78	824.2	1248.3	7.1
x	55.6	85.1	675.6	92	5.3	0.86	766.1	1096.0	7.0
	±11.2	±11.3	±264.2	±10	±0.3	±0.06	±51.3	±156.9	±0.2
				-B6 Ad libitum, nonfast					
55	36.1	87.7	16.0	76	5.7	1.00	409.8	813.9	5.3
56	31.9	62.7	18.0	49	5.8	0.76	542.4	889.4	1.3*
57	34.3	54.8	10.7	38	7.3	0.80	335.1	636.4	5.0
58	32.8	59.4	8.9	54	6.4	0.74	404.7	546.5	5.0
59	24.1	68.2	3.7	34	7.0	0.59	439.3	621.9	4.9
60	38.8	76.2	28.6	59	5.8	0.69	517.3	843.6	4.9
x	33.0	68.2	14.3	52	6.3	0.76	441.4	725.3	5.0
	±5.0	±12.1	±8.6	±14	±0.7	±0.14	±77.0	±140.9	±0.2
				-B6 Meal-fed, nonfast					
91	7.6	35.2	12.1	83	5.2	0.55	501.6	645.7	4.7
92	14.7	52.3	17.0	42	5.3	0.53	348.4	678.6	4.8
93	8.9	28.7	35.7	69	5.5	0.66	454.0	843.3	5.2
94	21.6	50.9	21.2	51	5.5	0.45	323.6	473.0	3.9
95	18.3	66.1	22.0	49	5.1	0.59	371.2	681.9	5.8
96	19.3	60.5	22.4	45	4.9	0.56	503.4	635.8	4.3
x	15.1	49.0	21.2	56	5.3	0.56	417.1	659.7	4.8
	±5.8	±14.5	±8.3	±16	±0.2	±0.06	±79.4	±118.5	±0.7

+B6 = control diet; -B6 = vitamin B-6 deficient diet; * = for information only, not included in calculation of mean

Appendix 5 (continuation). Concentration of free carnitine (FCN), total acid soluble carnitine (TCN), pyridoxal 5'-phosphate (PLP), free fatty acid (FFA), and glucose (GLU) in plasma and the weight (WT) of the heart and concentration of free carnitine, total acid soluble carnitine, pyridoxal 5'-phosphate in the heart.

	PLASMA					HEART			
	FCN	TCN	PLP	FFA	GLU		FCN	TCN	PLP
	µmol	µmol	nmol		mmol	WT	nmol	nmol	nmol
No	/L	/L	/L	mg/L	/L	g	/g	/g	/g
				+B6 Pair-fed, F1 day					
31	53.0	99.0	604.3	101	5.7	0.90	495.9	994.4	9.1
32	51.0	92.5	424.2	118	5.0	0.84	529.9	1135.0	9.1
33	42.7	82.4	484.2	98	4.3	0.78	436.7	1207.6	8.9
34	50.8	102.2	585.9	92	5.7	0.78	395.1	1156.9	9.0
35	53.3	65.6	778.2	108	4.8	0.86	551.0	1071.8	8.8
36	37.3	83.2	622.4	120	4.8	0.78	700.2	828.7	8.0
x	48.1	87.5	583.2	106	5.1	0.82	518.4	1065.7	9.0
	±6.5	±13.4	±122.6	±11	±0.6	±0.05	±106.4	±137.5	±0.1
				-B6 Ad libitum, F1 day					
67	32.3	61.9	25.7	104	5.5	0.74	459.3	668.9	5.5
68	45.0	86.9	40.2	89	3.0	0.70	469.2	985.3	6.1
69	37.0	68.5	31.1	76	4.5	0.87	352.1	955.6	5.9
70	45.3	93.2	33.6	96	4.6	0.70	414.6	834.1	5.9
71	17.8	59.8	45.9	114	4.2	0.59	482.2	864.2	7.7
72	39.7	67.8	21.9	81	4.8	0.69	497.0	753.3	4.8
x	36.2	73.0	32.9	93	4.6	0.72	445.8	843.6	6.0
	±10.3	±13.8	±8.7	±14	±0.6	±0.09	±53.7	±119.8	±1.0
				+B6 Pair-fed, F3 day					
25	31.3	88.4	360.0	131	6.6	0.77	630.3	1233.5	8.3
26	31.6	97.8	467.7	197	5.6	0.79	695.5	1012.9	9.1
27	33.9	104.2	603.4	161	3.9	0.93	661.7	1131.4	8.2
28	44.3	108.1	944.3	144	5.4	0.72	417.3	742.4	8.5
29	44.7	99.3	601.0	173	5.0	0.71	605.4	1059.2	9.0
30	38.2	122.3	429.6	194	5.9	0.77	690.0	1214.4	8.2
x	37.3	103.4	567.7	167	5.4	0.78	616.7	1065.6	8.6
	±6.1	±11.6	±208.0	±26	±0.9	±0.08	±103.6	±179.9	±0.4
				+B6 Ad libitum, F3 day					
61	31.9	92.6	40.5	113	3.8	0.70	444.2	1080.3	3.8
62	28.0	69.0	62.1	99	4.6	0.72	571.9	743.6	5.5
63	44.8	72.6	48.0	98	3.5	0.73	559.8	864.3	5.7
64	33.7	100.0	35.4	121	5.7	0.54	522.5	944.1	5.5
65	27.5	105.8	35.8	135	4.0	0.67	410.0	773.2	5.0
66	30.2	86.6	45.7	140	6.4	0.66	459.6	645.5	5.2
x	32.7	87.8	44.6	118	4.7	0.67	494.7	841.8	5.1
	±6.4	±14.7	±10.0	±18	±1.1	±0.07	±66.2	±155.4	±0.7

+B6 = control diet; -B6 = vitamin B-6 deficient diet; F1 = 1-day fast; F3 = 3-day fast

Appendix 5 (continuation). Concentration of free carnitine (FCN), total acid soluble carnitine (TCN), pyridoxal 5'-phosphate (PLP), free fatty acid (FFA), and glucose (GLU) in plasma and the weight (WT) of the heart and concentration of free carnitine, total acid soluble carnitine, pyridoxal 5'-phosphate in the heart.

	PLASMA					HEART			
	FCN	TCN	PLP	FFA	GLU		FCN	TCN	PLP
	µmol	µmol	nmol		mmol	WT	nmol	nmol	nmol
No	/L	/L	/L	mg/L	/L	g	/g	/g	/g
				+B6 Pair-fed, repletion, nonfast					
37	74.6	102.7	830.2	121	6.5	1.00	567.2	818.0	7.6
38	53.6	59.9	979.6	64	7.0	1.01	539.6	1025.9	7.6
39	56.8	89.5	902.0	56	7.3	0.81	444.8	685.1	6.9
40	59.9	103.5	757.9	115	7.2	0.92	617.1	1222.1	8.4
41	59.5	82.0	705.0	69	7.2	1.05	468.4	1556.7	7.6
42	47.1	97.8	1578.1	104	7.9	1.01	530.0	603.4	8.6
x	58.6	89.2	958.8	88	7.2	0.97	527.9	985.2	7.8
	±9.1	±16.6	±318.9	±28	±0.4	±0.09	±63.4	±360.1	±0.6
				-B6 Ad libitum, repletion, nonfast					
73	65.8	125.2	548.6	88	7.3	0.90	413.7	850.3	8.2
74	64.4	75.5	805.5	108	5.8	1.01	534.7	1138.9	8.6
75	48.4	80.3	585.4	90	8.1	0.93	250.3	622.3	8.7
76	54.4	80.7	781.7	75	6.3	1.01	376.3	1336.9	8.3
77	52.6	79.3	1209.4	87	6.2	1.01	327.2	688.2	8.6
78	59.2	82.3	1195.6	74	6.4	1.00	343.7	1022.1	7.8
x	57.5	87.2	854.3	87	6.7	0.98	374.3	943.1	8.4
	±6.9	±18.7	±288.5	±12	±0.8	±0.05	±95.7	±274.3	±0.4
				+B6 Pair-fed, repletion, F3 day					
43	33.4	60.0	884.2	144	5.2	0.97	242.6	819.7	8.7
44	35.2	79.7	595.1	217	4.7	0.93	238.2	1009.7	6.6
45	31.0	75.9	712.7	141	4.5	0.89	367.2	1171.6	8.3
46	26.9	91.8	982.5	167	3.5	0.87	395.8	774.3	8.3
47	28.1	66.0	898.7	140	4.9	0.96	373.1	1248.8	7.7
48	35.0	101.5	690.7	188	4.1	1.10	309.5	1376.6	7.2
x	31.6	79.1	794.0	166	4.5	0.95	321.1	1066.8	7.8
	±3.6	±15.6	±149.3	±31	±0.6	±0.08	±68.7	±240.8	±0.8
				-B6 Ad libitum, repletion, F3 day					
79	33.4	69.2	1072.1	163	4.3	1.15	391.6	1024.6	7.1
80	35.8	48.3	516.0	136	4.4	0.80	202.9	708.7	7.6
81	23.5	71.5	982.2	182	4.6	0.92	247.2	1147.0	8.3
82	33.7	61.3	679.8	177	5.4	0.91	182.3	1078.9	7.7
83	34.9	72.3	735.6	182	4.3	0.95	329.6	1128.1	7.9
84	33.7	99.8	712.5	171	5.2	0.91	265.7	742.6	7.5
x	32.5	70.4	783.0	168	4.7	0.94	269.9	971.6	7.7
	±4.5	±17.0	±206.1	±17	±0.4	±0.11	±78.8	±195.5	±0.4

+B6 = control diet; -B6 = vitamin B-6 deficient diet; F3 = 3-day fast

Appendix 6. Concentration of free carnitine (FCN), total acid soluble carnitine (TCN), pyridoxal 5'-phosphate (PLP), and glycogen (GLY) in the liver; weight (WT) of the liver.

No	WT g	FCN nmol /g	TCN nmol /g	PLP nmol /g	GLY μmol glu /g
	+B6 Ad libitum, nonfast				
7	13.6	86.9	125.5	17.3	190.0
8	15.8	53.6	64.4	29.4	219.7
9	16.0	40.7	99.4	26.3	202.5
10	14.6	71.3	209.5	28.5	191.6
11	11.4	118.9	242.5	25.8	128.7
12	11.1	68.2	167.5	27.5	135.1
x	14.0	73.3	151.5	27.5	177.9
	±2.1	±27.4	±67.7	±1.3	±37.2
	+B6 Pair-fed, nonfast				
19	8.7	69.8	150.1	32.8	0.9
20	8.1	85.3	270.9	36.2	0.8
21	8.1	68.5	157.2	32.7	1.3
22	8.3	81.1	167.4	36.5	0.9
23	8.2	120.2	159.9	29.0	1.0
24	7.9	83.7	161.8	32.7	0.8
x	8.2	84.8	177.9	33.3	1.0
	±0.3	±18.8	±45.9	±2.7	±0.2
	-B6 Ad libitum, nonfast				
55	13.2	117.8	142.3	9.0	346.5
56	11.3	96.7	216.9	9.7	234.9
57	11.4	66.6	100.2	5.3	217.5
58	12.1	73.5	105.1	9.9	280.7
59	13.9	68.3	140.7	4.7	328.0
60	12.2	39.7	76.9	8.4	273.3
x	12.4	77.1	130.3	7.8	280.1
	±1.0	±27.0	±49.3	±2.3	±50.3
	-B6 Meal-fed, nonfast				
91	6.7	76.1	112.3	11.7	1.5
92	6.4	122.5	147.3	13.3	1.3
93	7.6	78.5	135.6	12.1	0.6
94	3.6	104.3	187.3	11.1	1.6
95	6.5	97.8	165.8	12.9	1.8
96	5.8	45.5	193.8	8.1	1.5
x	6.1	87.5	157.0	11.5	1.4
	±1.4	±26.8	±31.3	±1.9	±0.4

+B6 = control diet; -B6 = vitamin B-6 deficient diet; glu = glucose

Appendix 6 (continuation). Concentration of free carnitine (FCN), total acid soluble carnitine (TCN), pyridoxal 5'-phosphate (PLP), and glycogen (GLY) in the liver; weight (WT) of the liver.

No	WT g	FCN nmol /g	TCN nmol /g	PLP nmol /g	GLY µmol glu /g
	+B6 Pair-fed, F1 day				
31	6.5	111.4	250.9	29.3	1.4
32	6.7	138.4	287.9	28.8	0.8
33	6.5	112.1	261.8	29.9	0.6
34	6.3	97.2	228.0	30.1	1.5
35	6.8	119.2	262.3	27.4	0.7
36	6.8	95.0	247.6	29.2	1.0
x	6.6	111.0	256.43	29.1	1.0
	±0.2	±16.6	±19.9	±1.0	±0.3
	-B6 Ad libitum, F1 day				
67	6.8	61.2	153.7	11.9	1.6
68	7.45	72.3	242.5	13.6	0.9
69	6.9	82.6	226.1	18.1	1.0
70	6.8	69.3	198.7	12.8	0.7
71	5.9	127.4	213.9	12.8	1.8
72	8.5	61.5	147.4	11.0	1.2
x	7.1	79.0	197.1	13.4	1.2
	±0.9	±25.0	±38.9	±2.5	±0.4
	+B6 Pair-fed, F3 day				
25	7.5	108.0	300.3	35.5	1.0
26	6.8	115.2	327.2	27.5	0.7
27	7.0	118.8	254.4	25.2	0.6
28	7.3	86.9	193.5	26.3	1.3
29	7.1	70.3	248.1	23.1	0.9
30	6.9	47.5	178.0	29.4	1.5
x	7.1	91.1	250.3	27.8	1.0
	±0.3	±28.2	±58.1	±4.3	±0.3
	+B6 Ad libitum, F3 day				
61	9.3	88.2	213.1	13.6	1.1
62	8.2	56.4	189.9	15.0	0.7
63	9.5	99.0	228.1	16.0	2.8
64	7.7	44.8	132.6	13.4	7.3*
65	6.0	80.9	174.8	14.8	3.5
66	7.8	45.9	147.6	12.6	1.8
x	8.1	69.2	181.0	14.2	2.0
	±1.3	±23.2	±37.0	±1.2	±1.1

+B6 = control diet; -B6 = vitamin B-6 deficient diet; F1 = 1-day fast; F3 = 3-day fast; glu = glucose; for information only, not included in calculation of the mean

Appendix 6 (continuation). Concentration of free carnitine (FCN), total acid soluble carnitine (TCN), pyridoxal 5'-phosphate (PLP), and glycogen (GLY) in the liver; weight (WT) of the liver.

No	WT g	FCN nmol /g	TCN nmol /g	PLP nmol /g	GLY μmol glu /g
	+B6 Pair-fed, repletion, nonfast				
37	12.6	60.0	143.1	30.5	199.6
38	13.6	71.5	215.2	35.7	163.2
39	8.35*	93.1	229.9	29.2	92.5*
40	12.0	61.0	89.2	26.4	182.8
41	12.9	87.4	225.7	34.6	182.5
42	14.4	64.5	94.0	36.2	274.6
x	12.3	73.1	166.2	32.1	182.6
	±2.1	±14.0	±65.9	±2.9	±58.7
	-B6 Ad libitum, repletion, nonfast				
73	16.7	80.8	128.6	33.9	280.9
74	12.8	73.5	118.1	33.9	134.0
75	14.3	81.1	139.3	28.5	227.8
76	12.5	105.5	146.9	30.8	158.8
77	14.7	84.7	99.9	35.5	229.4
78	14.7	64.2	129.0	28.7	255.0
x	14.3	86.6	128.6	31.9	214.3
	±1.5	±13.8	±17.3	±3.0	±56.6
	+B6 Pair-fed, repletion, F3 day				
43	7.6	139.1	246.0	24.3	1.0
44	7.7	120.2	217.4	28.2	1.1
45	7.3	95.1	206.1	26.0	1.0
46	7.3	92.5	169.7	26.1	0.8
47	7.7	115.2	161.6	24.4	0.9
48	7.7	127.8	179.9	28.5	0.7
x	7.5	115.0	196.8	26.3	0.9
	±0.2	±18.3	±32.2	±1.8	±0.1
	-B6 Ad libitum, repletion, F3 day				
79	8.5	100.7	140.4	25.7	0.9
80	7.2	107.3	196.9	21.4	1.3
81	7.2	85.9	155.4	23.1	1.7
82	8.3	88.5	167.3	28.8	1.7
83	8.4	81.4	150.9	26.2	0.8
84	8.2	75.5	152.3	21.6	0.8
x	8.0	89.9	160.5	24.5	1.2
	±0.6	±12.0	±19.8	±2.9	±0.4

+B6 = control diet; -B6 = vitamin B-6 deficient diet; F3 = 3-day fast; glu = glucose; for information only, not included in calculation of the mean

Appendix 7. Concentration of free carnitine (FCN), total acid soluble carnitine (TCN), and pyridoxal 5'-phosphate (PLP) in skeletal muscle.

No	FCN nmol /g	TCN nmol /g	PLP nmol /g	No	FCN nmol /g	TCN nmol /g	PLP nmol /g	No	FCN nmol /g	TCN nmol /g	PLP nmol /g
+86 Ad lib., NF				+86, PF, F1				+86, PF, Repl., NF			
7	342.9	672.4	26.0	31	311.8	943.4	18.0	37	459.0	725.9	25.1
8	371.3	697.0	22.7	32	264.9	785.7	23.0	38	315.6	816.3	22.6
9	344.0	796.2	24.6	33	330.4	643.5	23.9	39	373.3	995.2	20.2
10	295.7	553.7	21.0	34	347.3	900.9	24.1	40	273.8	693.9	24.0
11	471.9	660.7	23.0	35	306.5	694.6	26.9	41	485.6	696.7	24.5
12	411.4	811.7	21.5	36	185.2	1003.0	26.9	42	460.7	602.5	24.5
x	372.8	698.6	23.1	x	291.0	828.5	23.8	x	394.7	755.1	23.6
	±61.6	±95.3	±1.9		±58.8	±143.4	±3.3		±87.3	±136.2	±1.7
-B6 PF, NF				-86 Ad lib., F1				-86 Ad lib., Repl., NF			
19	362.7	810.2	20.1	67	230.9	685.1	7.4	73	336.3	601.4	15.1
20	271.5	893.5	19.5	68	207.0	783.6	8.8	74	272.0	761.5	13.7
21	306.2	541.4	22.4	69	176.5	489.1	7.2	75	254.0	691.4	15.0
22	453.3	650.6	24.5	70	147.6	766.0	5.5	76	205.8	954.7	16.5
23	297.8	904.4	18.4	71	183.6	659.4	6.1	77	285.3	966.3	10.6
24	399.2	842.7	24.1	72	259.5	589.6	6.0	78	415.7	540.9	17.2
x	348.4	773.8	21.5	x	200.8	662.1	6.8	x	294.8	752.7	14.7
	±69.3	±146.0	±2.6		±40.3	±110.7	±1.2		±72.8	±177.7	±2.3
-B6 Ad lib., NF				+B6 PF, F3				+86 PF, Repl., F3			
55	327.5	590.1	6.8	25	322.4	1010.3	17.3	43	215.1	705.9	27.4
56	165.9	583.8	8.0	26	456.6	1001.1	17.3	44	507.9	1058.3	21.6
57	320.2	462.4	7.7	27	289.9	859.1	23.5	45	285.7	858.6	20.8
58	300.3	464.5	6.4	28	403.3	846.6	16.8	46	349.4	864.9	24.2
59	436.3	484.7	7.5	29	469.9	1003.5	16.6	47	338.2	613.9	26.7
60	343.1	422.3	7.3	30	425.1	732.8	20.0	48	256.6	726.3	21.7
x	315.6	501.3	7.3	x	397.9	908.9	18.6	x	325.5	804.6	23.7
	±87.3	±69.4	±0.6		±76.8	±114.1	±2.7		±102.5	±156.9	±2.8
-86 MF, NF				-B6 Ad lib., F3				-B6 Ad lib., Repl., F3			
91	243.3	604.9	4.1	61	211.8	599.0	6.3	79	433.7	835.7	10.7
92	225.9	877.9	8.28	62	236.6	657.0	7.0	80	260.0	796.2	15.4
93	179.5	424.6	7.7	63	234.3	667.9	7.8	81	238.7	952.4	17.6
94	209.1	492.0	6.7	64	307.8	590.0	8.4	82	283.5	655.3	15.0
95	205.9	365.0	7.4	65	301.2	693.2	6.7	83	240.0	553.1	16.3
96	170.0	524.1	6.5	66	313.0	687.0	5.9	84	252.5	759.6	13.5
x	205.1	564.7	6.8	x	267.4	649.0	7.0	x	284.7	758.7	14.8
	±27.1	±183.3	±1.5		±44.8	±44.3	±0.9		±74.8	±139.9	±2.4

+86 = control diet; -B6 = vitamin B-6 deficient diet; F1 = 1-day fast; F3 = 3-day fast; MF = Meal-fed; PF = Pair-fed; NF = Nonfast; Ad lib = Ad libitum; Repl = Repletion

Appendix 8. Urinary excretion of creatinine (Cr), urea N, free carnitine (FCN), total acid soluble carnitine (TCN), and 4-pyridoxic acid (4-PA).

No	Vol ml/d	Cr µmol /d	Urea N µmol /d	FCN nmol /d	FCN nmol /µmol Cr	FCN CL	TCN nmol /d	TCN nmol /µmol Cr	TCN CL	4-PA nmol /d	4-PA nmol /µmol Cr
					+B6 Ad libitum, nonfast						
7	30	41.7	660	123.6	2.9	2.0	525.3	12.6	6.8	194.6	4.7
8	30	44.7	950	170.0	3.8	2.3	415.2	9.3	4.0	337.2	7.5
9	30	38.2	850	99.4	2.6	1.7	455.3	11.9	3.5	191.8	5.0
10	30	36.3	1007	224.6	;6.2	2.6	352.6	9.7	3.6	172.0	4.7
11	30	41.5	593	150.6	3.6	1.9	277.8	6.7	3.2	204.2	4.9
12	30	40.5	978	165.3	4.1	3.1	398.6	9.8	5.8	222.3	5.5
x		40.5	840	155.5	3.9	2.3	404.1	10.0	4.5	220.3	5.4
		±2.7	±125	±43.2	±1.3	±0.5	±84.9	±2.1	±1.5	±59.6	±1.1
					+B6 Pair-fed, nonfast						
19	30	46.9	850	255.6	5.5	4.4	565.0	12.1	7.6	200.0	4.3
20	30	44.5	703	91.5	2.1	1.5	335.8	7.6	3.5	189.0	4.3
21	30	39.9	778	186.5	4.7	4.9	415.2	10.4	4.2	201.0	5.0
22	30	42.6	635	229.1	5.4	3.6	426.5	10.0	5.5	22.1*	0.5*
23	30	46.3	771	165.4	3.6	3.6	367.1	7.9	5.2	22.1*	0.5*
24	30	47.6	718	180.9	3.8	2.7	450.0	9.5	5.1	184.0	3.9
x		44.6	743	185.0	4.2	3.5	426.6	9.6	5.2	193.5	4.4
		±2.6	±75	±56.7	±1.3	±1.2	±79.5	±1.7	±1.4	±8.3	±0.5
					-B6 Ad libitum, nonfast						
55	40	49.3	789	171.8	3.5	4.8	256.4	5.2	2.9	n.d.	n.d.
56	40	48.8	500	61.9	1.3	1.9	212.8	4.4	3.39	n.d.	n.d.
57	40	66.8	571	178.8	2.7	5.2	460.1	6.9	8.4	n.d.	n.d.
58	40	58.1	596	131.9	2.3	4.0	353.4	6.1	6.0	n.d.	n.d.
59	40	57.1	653	107.3	1.9	4.4	277.5	4.9	4.1	n.d.	n.d.
60	40	42.5	493	76.9	1.8	2.0	239.1	5.6	3.1	n.d.	n.d.
x		53.7	600	121.4	2.2	3.7	299.9	5.5	4.6		
		±7.9	±111	±48.3	±0.8	±1.4	±91.9	±0.9	±2.1		
					-B6 Meal-fed, nonfast						
91	40	36.3	507	55.5	1.5	7.3	367.6	10.1	10.5	n.d.	n.d.
92	40	42.5	421	106.8	2.5	7.3	318.6	7.5	6.1	n.d.	n.d.
93	40	49.7	482	144.5	2.9	16.2	329.1	6.6	11.5	n.d.	n.d.
94	40	22.9*	286	150.4	6.6*	6.7	432.4	18.8*	8.50	n.d.	n.d.
95	40	40.7	564	105.6	2.6	5.8	255.9	6.3	3.9	n.d.	n.d.
96	40	41.3	518	185.8	4.5	9.6	359.2	8.7	5.9	n.d.	n.d.
x		42.1	464	124.8	2.8	8.8	343.8	7.8	7.7		
		±4.9	±100	±45.3	±1.1	±3.9	±58.7	±1.6	±2.9		

+B6 = control diet; -B6 = vitamin B-6 deficient diet; CL = clearance; n.d. = no detectable; * = for information only, not included in calculation of the mean

Appendix 8 (continuation). Urinary excretion of creatinine (Cr), urea N, free carnitine (FCN), total acid soluble carnitine (TCN), and 4-pyridoxic acid (4-PA).

No	Vol ml/d	Cr µmol /d	Urea N µmol /d	FCN nmol /d	FCN nmol /µmol Cr	FCN CL	TCN nmol /d	TCN nmol /µmol Cr	TCN CL	4-PA nmol /d	4-PA nmol /µmol Cr
					+B6 Pair-fed, F1 day						
31	40	62.9	339	135.3	2.2	2.6	370.3	5.9	3.7	257.9	4.1
32	40	63.5	471	111.7	1.8	2.2	516.4	8.1	5.6	289.0	4.6
33	50	61.1	286	83.4	1.4	2.0	942.8	15.4	11.5	262.6	4.3
34	40	65.9	361	122.4	1.9	2.5	412.9	6.3	4.0	42.0*	0.6*
35	40	56.0	314	107.1	1.9	2.0	577.8	10.3	8.8	245.5	4.4
36	40	54.2	353	129.7	1.8	3.5	582.7	8.1	7.0	276.5	5.1
x		63.5	353	114.9	1.8	2.4	567.2	9.0	6.8	266.3	4.5
		±5.1	±64	±18.7	±0.3	±0.6	±203.3	±3.5	±3.0	±16.9	±0.4
					-B6 Ad libitum, F1 day						
67	40	50.1	353	112.6	2.2	3.5	367.9	7.3	5.9	21.8	0.4
68	40	59.8	328	103.1	1.7	2.3	731.0	12.2	8.4	14.4	0.2
69	40	73.7	271	154.7	2.1	4.2	332.0	4.5	4.9	n.d.	n.d.
70	40	65.9	218	118.2	1.8	2.6	670.3	10.2	7.2	n.d.	n.d.
71	40	56.0	318	114.4	2.0	6.4	943.5	16.8	15.8	13.9	0.2
72	40	62.0	453	138.4	2.2	3.5	747.6	12.1	11.0	n.d.	n.d.
x		61.3	321	123.6	2.0	3.8	632.0	10.5	8.9	16.7	0.1
		±8.1	±71	±17.5	±0.2	±1.5	±237.3	±4.3	±4.0	±9.3	±0.2
					+B6 Pair-fed, F3 day						
25	50	63.5	343	187.2	2.9	6.0	922.4	14.5	10.4	101.6	1.6
26	40	66.3	461	141.0	2.1	4.5	1002.6	15.1	10.3	225.8	3.4
27	40	62.2	250	226.7	3.6	6.7	848.0	13.6	8.1	155.8	2.5
28	40	57.9	325	143.1	2.5	3.2	817.0	14.1	7.6	28.4*	0.5*
29	40	59.8	303	175.6	2.9	3.9	517.8	8.7	5.2	132.4	2.2
30	40	57.6	318	222.3	3.9	5.8	658.4	11.4	5.4	94.2	1.6
x		61.3	336	182.7	3.0	5.0	794.3	12.9	7.8	142.0	2.3
		±3.4	±79	±37.1	±0.7	±1.3	±177.8	±2.4	±2.3	±53.0	±0.7
					+B6 Ad libitum, F3 day						
61	40	57.4	186	87.1	1.5	2.7	888.0	15.5	9.6	n.d.	n.d.
62	40	56.4	186	281.4	5.0	10.0	908.4	16.1	13.2	n.d.	n.d.
63	40	62.9	218	229.8	3.7	5.1	846.4	13.5	11.7	n.d.	n.d.
64	50	53.3	321	99.7	1.9	3.0	758.7	14.2	7.6	n.d.	n.d.
65	50	56.8	196	65.1	1.1	2.4	1162.3	20.4	11.0	n.d.	n.d.
66	40	44.8	375	196.47	4.4	6.5	792.1	17.7	9.2	n.d.	n.d.
x		55.3	246	159.9	2.9	5.0	892.6	16.2	10.4		
		±6.0	±82	±88.2	±1.6	±3.0	±143.6	±2.5	±2.0		

+B6 = control diet; -B6 = vitamin B-6 deficient diet; F1 = 1-day fast; F3 = 3-day fast; CL = clearance; n.d. = no determination; * = for information only, not included in calculation of the mean

Appendix 8 (continuation). Urinary excretion of creatinine (Cr), urea N, free carnitine (FCN), total acid soluble carnitine (TCN), and 4-pyridoxic acid (4-PA).

No	Vol ml/d	Cr µmol /d	Urea N µmol /d	FCN nmol /d	FCN nmol /µmol Cr	FCN CL	TCN nmol /d	TCN nmol /µmol Cr	TCN CL	4-PA nmol /d	4-PA nmol /µmol Cr
					+B6 Pair-fed, repletion, nonfast						
37	40	58.2	964	253.9	4.4	3.4	766.2	13.2	7.5	231.6	4.0
38	40	58.2	932	252.1	4.3	4.7	670.2	11.5	11.2	263.9	4.5
39	40	52.3	960	267.4	5.1	4.7	368.6	7.0	4.1	273.0	5.2
40	40	54.8	939	162.9	3.0	2.7	579.0	10.6	5.6	30.6*	0.6*
41	40	50.7	835	161.3	3.2	2.7	376.0	7.4	4.6	196.8	3.9
42	40	54.2	800	236.9	4.4	5.0	403.1	7.4	4.1	344.4	6.4
x		54.7	907	222.4	4.1	3.9	527.2	9.5	6.2	261.9	4.8
		±2.7	±71	±47.7	±0.8	±1.1	±169.5	±2.6	±2.8	±55.0	±1.0
					-86 Ad libitum, repletion, nonfast						
73	40	60.4	796	186.7	3.1	2.8	828.8	13.7	6.6	232.8	3.9
74	40	49.2	907	167.4	3.4	2.6	462.8	9.4	6.1	265.0	5.4
75	40	57.9	710	233.0	4.0	4.8	644.1	11.1	8.0	171.4	3.0
76	40	60.4	675	142.2	2.4	2.6	520.1	8.6	6.4	224.8	3.7
77	40	53.8	1039	209.3	3.9	4.0	617.3	11.5	7.8	302.7	5.6
78	40	61.3	978	170.8	2.8	2.9	322.0	5.2	3.9	276.0	4.5
x		57.2	850	184.9	3.3	3.3	565.9	9.9	6.5	245.4	4.3
		±4.3	±146	±32.4	±0.6	±0.9	±173.3	±2.9	±1.5	±46.2	±1.0
					+B6 Pair-fed, repletion, F3 day						
43	40	87.8	364	173.4	2.0	5.2	629.0	7.2	10.5	173.6	2.0
44	40	61.0	243	316.9	3.6	6.2	704.7	11.6	8.9	205.5	3.4
45	50	76.3	314	280.4	3.7	9.0	807.7	10.6	10.6	193.4	2.5
46	40	68.2	246	67.7	1.0	2.5	646.7	9.5	7.1*	28.1	0.4
47	40	86.8	396	272.5	3.1	9.7	569.9	6.6	8.6	191.0	2.2
48	40	75.9	218	309.3	4.1	8.8	502.5	6.6	5.0	160.2	2.1
x		76.0	296	220.0	2.9	6.9	643.4	8.7	8.5	184.7	2.4
		±9.5	±71	±89.2	±1.1	±2.8	±106.0	±2.2	±2.2	±17.8	±0.6
					-86 Ad libitum, repletion, F3 day						
79	50	87.2	336	186.0	2.1	5.6	829.2	9.5	12.0	163.4	1.9
80	40	62.9	293	65.0	1.0	1.8	518.2	8.2	10.7	133.0	2.1
81	40	58.2	203	282.0	4.8	12.0	818.0	14.1	11.4	86.2	1.5
82	40	67.5	375	232.9	3.4	6.9	712.6	10.6	11.6	95.6	1.4
83	40	79.4	314	109.0	1.4	3.1	543.4	6.8	7.5	145.2	1.8
84	40	87.2	407	184.7	2.1	5.5	564.5	6.5	5.7	149.2	1.7
x		73.7	321	176.6	2.5	5.8	664.3	9.3	9.8	129.0	1.7
		±11.5	±71	±79.3	1.4	±3.5	±140.8	±2.8	±2.6	±31.1	±0.3

+86 = control diet; -B6 = vitamin B-6 deficient diet; F3 = 3-day fast; * = for information only, not included in calculation of the mean

Appendix 9. List of abbreviations.

+B6	control group
-B6	vitamin B-6 deficient group
4-PA	4-pyridoxic acid
Ad lib	ad libitum
Cr	Creatinine
F1	fasted 1-day
F3	fasted 3-days
FCN	free carnitine
FFA	free fatty acid
MF	meal-fed group
NF	nonfasted
PF	pair-fed group
PLP	pyridoxal 5'-phosphate
Repl	Repleted with control (+B6) diet
TCN	total acid soluble carnitine
WT	weight