THE EFFECT OF VITAMIN B-6 DEFICIENCY ON COPPER, ZINC AND IRON BALANCE IN THE RAT

by

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DATE SUBMITTED : 18 NOVEMBER 1988

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INTRODUCTION

Vitamin B-6 in its biologically active form as pyridoxal 5'-phosphate is a coenzyme for more than 60 enzyme systems. The diversity of this unique compound is illustrated by the involvement of pyridoxal 5'-phosphate in gluconeogenesis, amino acid metabolism, erythrocyte function, immune function, niacin formation, lipid metabolism, steroid hormone action and nervous system function (Leklem, 1988). Deficiency of vitamin B-6 leads to impairment in growth and immune responses, anaemia and skin disorders in both man and animals (Rutishauser, 1982). Deficiency during infancy can lead to impaired central nervous system development accompanied by convulsive seizures, hyperactivity and behavioural changes (Bamji, 1981).

In 1965, Hsu found decreased concentration of zinc in the plasma, liver, pancreas and heart tissue of rats fed on a diet deficient in vitamin B-6. It was suggested that vitamin B-6 deficiency caused impaired absorption of dietary zinc in rats due to decreased production of the zinc binding ligand,picolinic acid. Picolinic acid is a tryptophan metabolite and it was argued that decreased levels of vitamin B-6, a cofactor in the tryptophan to picolinic acid pathway, decreased the availability of picolinic acid to facilitate dietary zinc absorption (Evans and Johnson, 1980; 1981).

However, other studies reported increased tissue zinc concentrations and increased *in vitro* uptake of intestinal zinc in vitamin B-6 deficient rats (Ikeda *et al.*, 1979; Prasad *et al.*, 1982).

In view of these contradictory reports and taking into account the possibility of the biological interaction of zinc with copper and

iron during intestinal absorption (Yip et al., 1985), the following major objectives for this study were formulated.

1. To induce varying levels of vitamin B-6 deficiency in rats and to determine their vitamin B-6 nutritional status by means of suitable biochemical indices.
2. To assess the animals rate of growth and food intake, both of which are the main factors related to nutrient balance.
3. To concurrently evaluate the status of zinc, copper and iron in both vitamin B-6 deficient and control animals by means of a trace element balance study.

Although a trace element balance study does not provide information on true absorption, body distribution, metabolic roles, pool sizes or rate of turnover, it is still of value as it will provide information on the overall retention or excretion of the trace elements as affected by the levels of vitamin B-6 in the diet (Engels et al., 1984).

I. LITERATURE SURVEY

A) CHEMISTRY AND SOURCES OF VITAMIN B-6

In 1934, Paul György first used the term "vitamin B-6" to designate a yeast extract factor with which he cured a dermatitis in rats not due to a deficiency in either thiamin or riboflavin, the only then known B complex vitamins (Kutsky, 1981). When one form of vitamin B-6 was synthesized in 1939 by Harris and Folkers, György suggested that: "In accordance with the chemical nature of vitamin B-6, which is a pyridine derivative containing several methoxyl groups, the term 'pyridoxine' appears appropriate"(Sauberlich, 1981).

Since then it has been discovered that vitamin B-6 exists naturally as 6 physiologically significant forms or vitamers. These are pyridoxine (PN), pyridoxal (PL), pyridoxamine (PM) and their respective 5'-phosphate esters, pyridoxine 5'-phosphate (PNP),pyridoxal 5'-phosphate (PLP) and pyridoxamine 5'-phosphate (PMP) (Fig. 1). All these forms being interconvertible in the body (Reynolds et al., 1981).

Amongst the 6 recognized vitameric forms, only PLP and PMP function as coenzymes. They are firmly bound to most vitamin B-6 dependent apoenzymes and therefore are the principle B-6 vitamers in mammalian tissue (Lumeng et al., 1985).

All forms of the vitamin appears as water soluble, white crystals, insoluble in ether. Aqueous solutions are unstable in light and destroyed by heating. PN and PM are stable in hot dilute mineral acids and alkali while pyridoxal decomposes in hot dilute alkali (Labadarios et al., 1984).

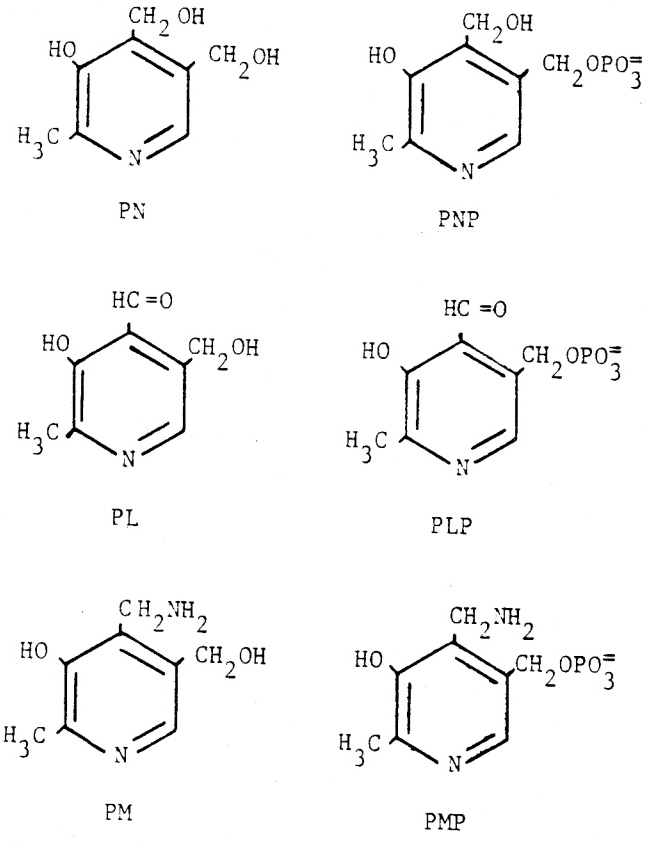


FIG. 1. Currently recognized forms of vitamin B-6.

Vitamin B-6 occurs widely in food. In animal products it is found largely as PLP and PMP and as PN in vegetable products - all 3 forms being considered equally effective in animal nutrition. However, indications are that the bioavailability of vitamin B-6 of plant derived food is lower than that in animal products. Stability data indicate that PN is the most stable form and although significant amounts of the vitamin are lost during thermal processing and during cereal grain processing, the remaining vitamers exhibit nearly complete bioavailability (Gregory et al., 1985). About 10 to 40% of the vitamin is lost during normal cooking procedures, mainly through the medium of evaporating water (Rutishauser, 1982).

B) PHYSIOLOGICAL ROLE OF VITAMIN B-6

1. Role of Vitamin B-6 in Amino Acid Metabolism

Over 60 essential enzyme systems involved in the intermediary metabolism of amino acids require vitamin B-6 in its coenzyme form as PLP which readily forms a Schiff base with amino acids and other nitrogen containing compounds. These enzymes include amino acid decarboxylases, transaminases, racemases and enzymes that effect reactions of amino acid side chains (Meisler, 1980).

(a) Transamination reactions

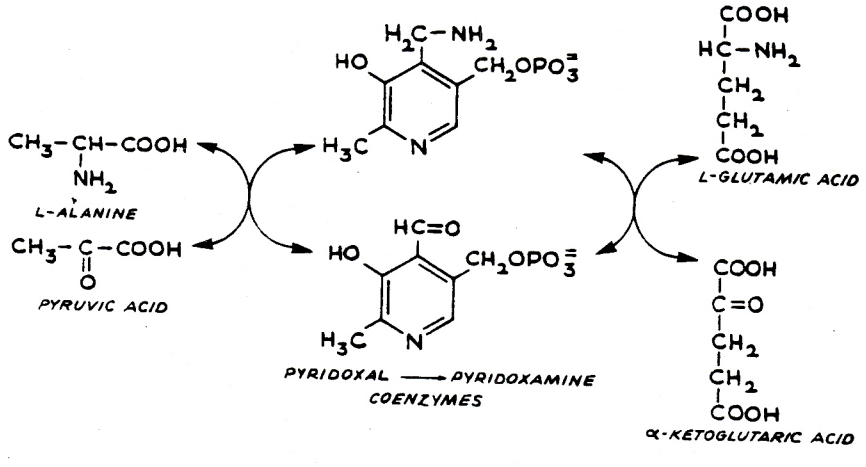


FIG. 2. Participation of PLP in transamination reactions.

In transamination reactions, there is reversible interconversion of amino acids and their corresponding keto acids allowing for both amino acid catabolism and biosynthesis of non-essential amino acids from their precursors. In Fig. 2 the transaminase bound PLP coenzyme reacts with L-alanine to yield the corresponding keto acid and enzyme bound

PMP. Since this reaction is freely reversible, the aldehyde form of the coenzyme is regenerated by the transfer of the amino group to α ketoglutarate to form glutamic acid, thereby the bound coenzyme also acting as a carrier of amino groups (Labadarios, 1984).

(b) Transulfuration reactions

PLP as a coenzyme is required by enzymes involved in the metabolism of the nutritionally essential amino acid methionine, from which adequate amounts of cysteine is biosynthesised via the transulfuration pathway, thereby sparing the latter amino acid from being an essential dietary requirement in mammal. Taurine is also produced via this pathway with PLP as a coenzyme and is an essential nutrient for the cat and kitten and perhaps for some primates, including man (Sturman, 1981).
Methionine is a principle methyl group donor and cysteine is required for the synthesis of coenzyme A and also as a precursor of taurine which conjugates with bile acids to form taurocholic acid.

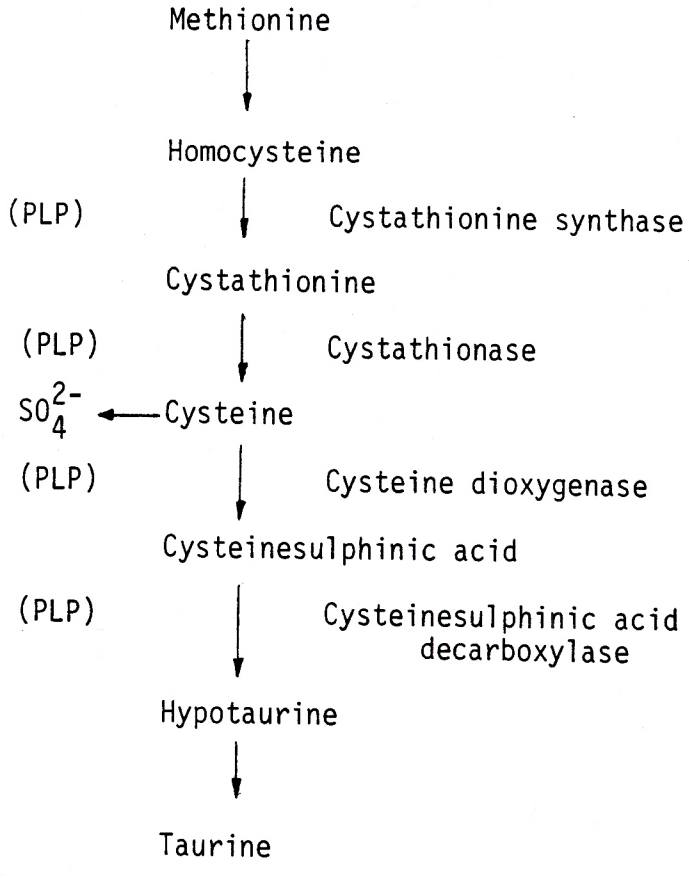


FIG. 3. The role of vitamin B-6 in methionine metabolism (Labadarios, 1984).

PLP is tightly bound to apocystathionine synthase therefore nutritional vitamin B-6 deficiency has little effect in enzyme activity. However, PLP is not tightly bound to apocystathionase nor apocysteinesulphinic acid decarboxylase and during vitamin B-6 deficiency, cystathioninuria with a reduction in holocystathionase is present. Homocysteinuria is a condition which is usually due to an inherited deficiency of cystathianine synthase.

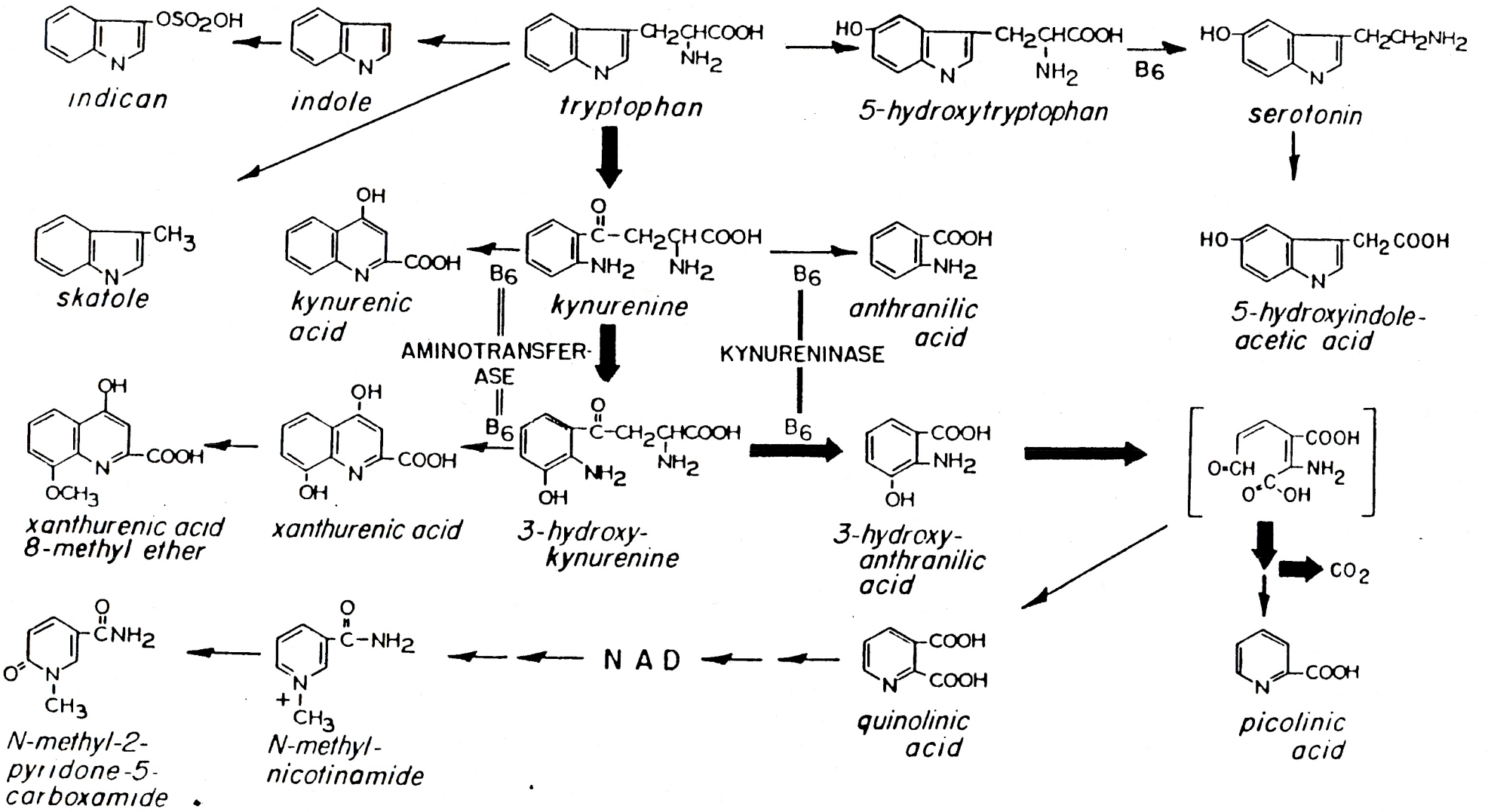


FIG. 4. Metabolic chart showing some of the metabolites of tryptophan. The large arrows indicate the pathway for degradation of the majority of ingested tryptophan. B-6 indicates the sites of action of enzymes requiring PLP as a cofactor.

(c) Tryptophan metabolism

Tryptophan, a nutritionally essential amino acid also requires PLP as a coenzyme in its metabolism. Tryptophan, in addition to being a vital amino acid component of most proteins and enzymes, is also an important precursor of the vitamin niacin. Serotonin, a biogenic amine having a variety of neuroendocrine factors is a quantitatively minor but a vital functional metabolite of tryptophan.

Fig. 4 shows major pathways of tryptophan metabolism and indicates sites of involvement of vitamin B-6 (as PLP), including the decarboxylase which forms serotonin, the aminotransferase that forms kynurenic and xanthurenic acid and kynureninase responsible for 3-hydroxyanthranilic acid formation.

The latter metabolite is a precursor for picolinic acid which is reported to be involved in Zn absorption and quinolinic acid which is necessary for NAD synthesis. The major portion of the daily tryptophan intake is metabolized via this pathway to CO_2, with only a few percent going to the serotonin and niacin routes which nevertheless are still vital to many processes in spite of their small amounts (Brown, 1985).

Measurement of the urinary excretion of xanthurenic acid, kynurenine and 3-hydroxykynurenine in response to a tryptophan load test have proved to be sensitive biochemical indices for functional vitamin B-6 deficiency and are reported to correlate well with other indices of vitamin B-6 status (Brown, 1981). The use of tryptophan metabolites to assess vitamin B-6 status will be further discussed later in this study.

2. Vitamin B-6 and Glucose Homeostasis

PLP is an essential coenzyme of glycogen phosphorylase which catalyses the first steps in glycogenolysis. Vitamin B-6 deficiency results in decreased activity of both liver and muscle glycogen phosphorylase. Vitamin B-6 also acts as a coenzyme for the transaminase enzyme involved in gluconeogenesis.

3. Nucleic Acid Synthesis and Protein Metabolism

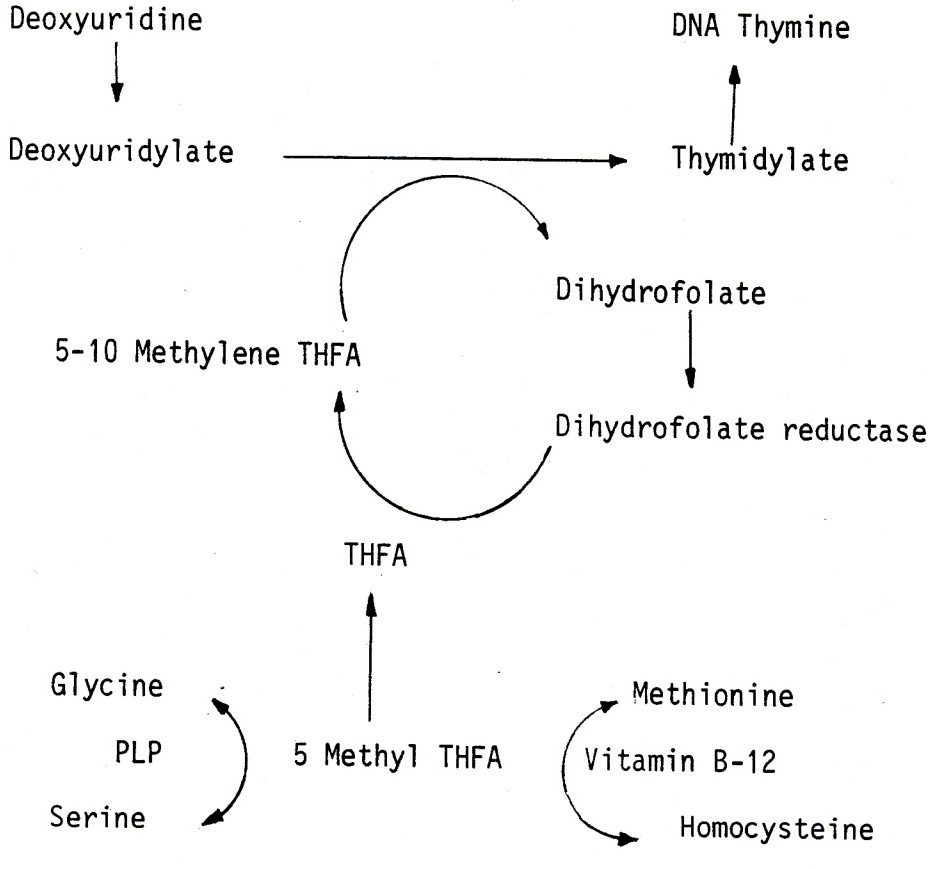


FIG. 5. Vitamin B-6, B-12 and folic acid interrelationship in DNA thymine synthesis.

Because of the vitamin B-6 function in nucleic acid synthesis and subsequent protein production, it is an important requirement for cell division, cell growth and repair. It has been reported on studies on rats that vitamin B-6 is required for the crosslinking of elastin to collagen fibres in vascular tissue and in the lung and a vitamin B-6

deficiency predisposes the animal to both pulmonary and vascular defects (Hegsted, 1986). Vitamin B-6 also plays an important role in the immune system with a deficiency leading to a decrease in lymphocyte numbers, reduced thymus size and an overall decrease in immunocompetance in rats, (Hegsted, 1976).

4. Haem Synthesis

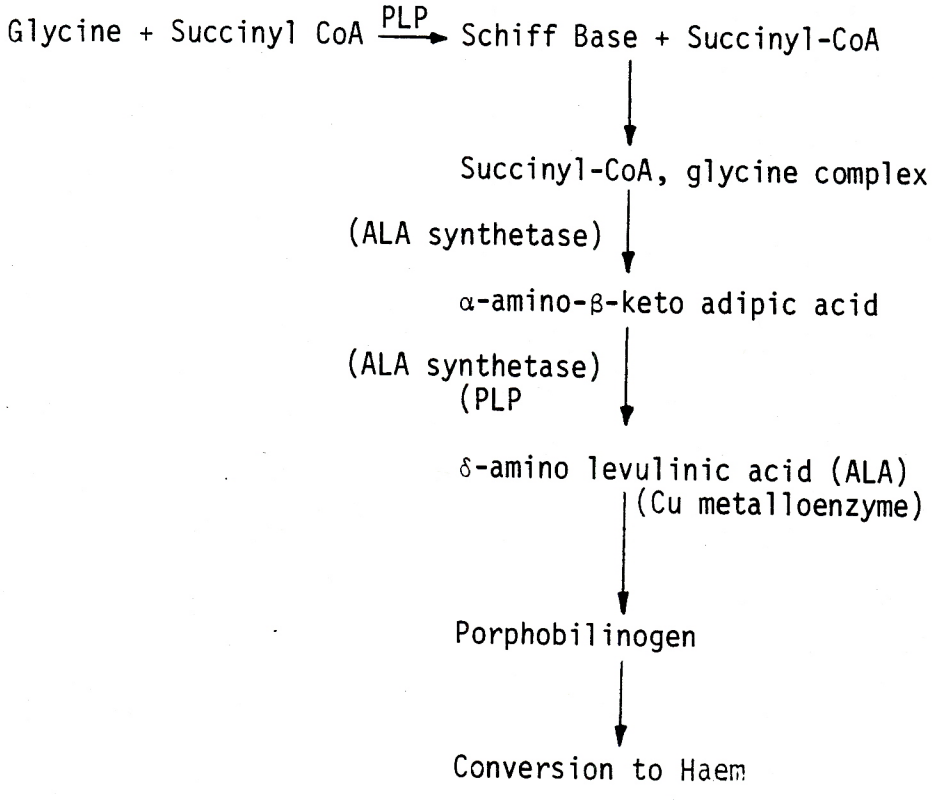


FIG. 6. The role of vitamin B-6 in haem synthesis.

Vitamin B-6 is essential for haem synthesis and for erythrocyte function and metabolism. Severe hypochromic, microcytic (pyridoxine responsive) anaemia with elevated serum iron occurs during vitamin B-6 deficiency. Evidence suggests that the block in haem synthesis occurs at an early stage in the deficiency at the glycine, succinyl CoA product level in the decarboxylation of α-amino-β-keto adipic acid (Labadarios, 1985).

5. Central Nervous System Function

Vitamin B-6 is vital for the development and maintenance of the integrity of the nervous system. Both myelination and the metabolism of neurotransmitters are affected during vitamin B-6 deficiency. Vitamin B-6 deficiency occurring in rats during both the preweaning period and postweaning period lead to convulsive seizures and EEG changes indicating the role in both development and maintenance of the CNS.

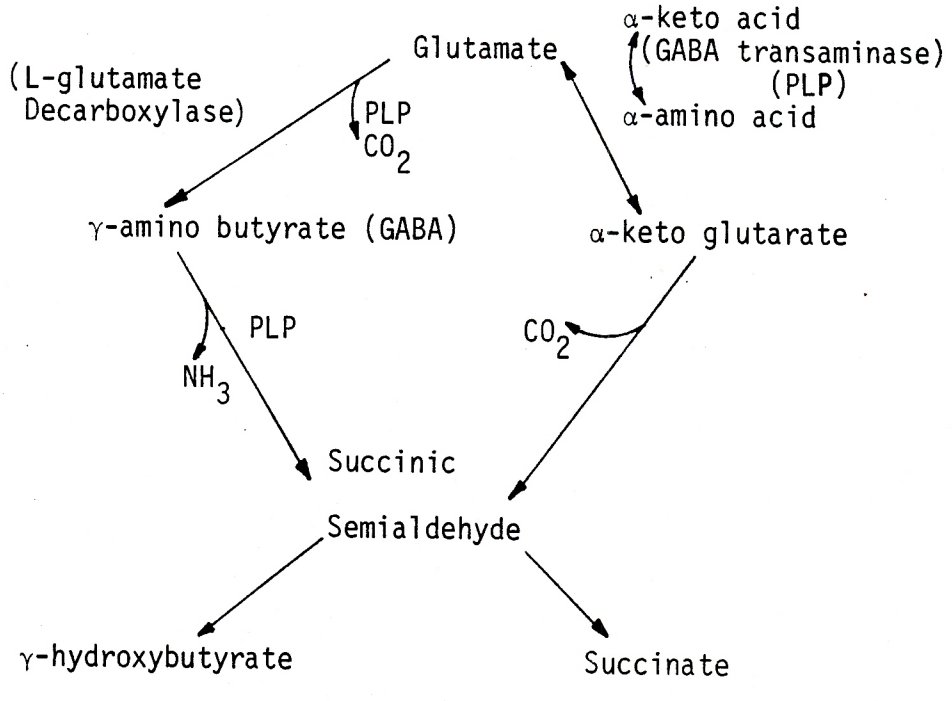


FIG. 7. Vitamin B-6 and GABA synthesis.

The synthesis of neurotransmitter GABA is PLP dependent. GABA is a normal regulator of neuronal activity and has an inhibitory effect on the central nervous system. Convulsive seizures are therefore not uncommon during vitamin B-6 deficiency and they can be corrected by the administration of pyridoxine (Labadarios, 1985).

6. Catecholamine Synthesis

Coenzyme PLP is required for the normal synthesis of the catecholamines adrenaline, noradrenaline and dopamine. It was also found that the activity of dopamine <u>in vivo</u> was increased by elevation of tissue PLP levels.

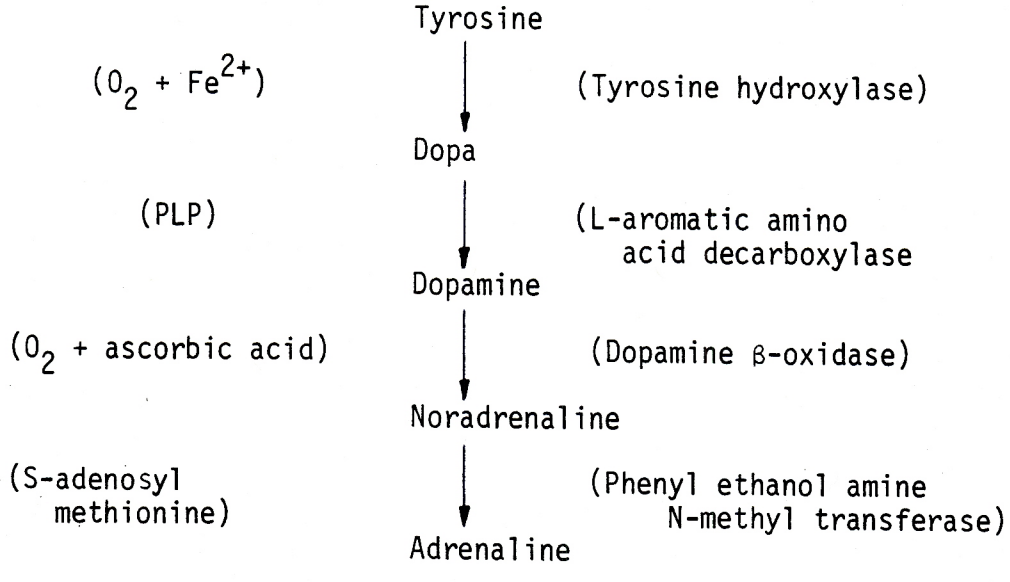


FIG. 8. Vitamin B-6 in catecholamine synthesis.

7. Steroid Function

At cellular level, PLP has been identified as acting in the area of steroid function. At physiological levels, PLP acts reversibily with the receptors for oestrogen, androgen, progesterone and glucocorticoids. During vitamin B-6 deficiency, an increased uptake and retention of steroids occurred in the target cells probably increasing the sensitivity of the target cell to the steroid. From this observation, the role of vitamin B-6 is presumably to inhibit the binding of steroid-receptor complex to DNA (Leklem, 1988).

In summary, the functional properties of vitamin B-6 are illustrated in Fig. 9. The various processes which PLP influences are shown, the majority of the processes being related to the role of PLP in amino acid metabolism.

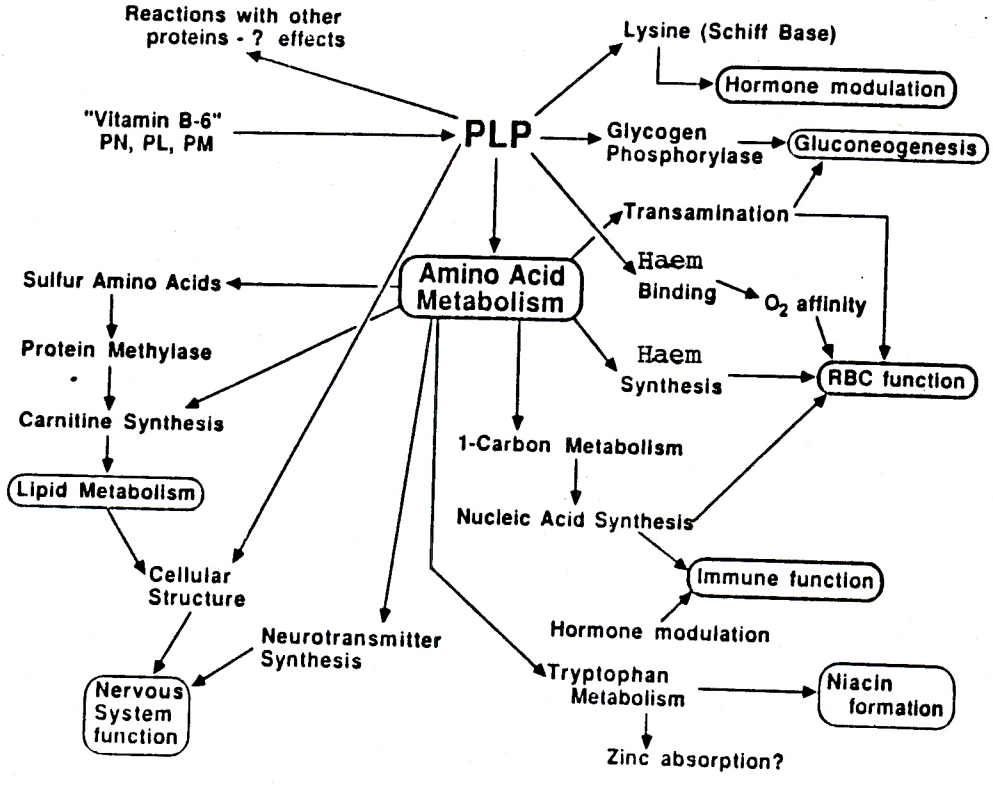


FIG. 9. Cellular processes in which pyridoxal 5'-phosphate (PLP) acts as a coenzyme or binds with proteins and modifies the action of the protein. The primary biological systems subsequently influenced are circled. Haem binding refers to PLP binding to haemoglobin (Leklem, 1988).

C) METABOLISM OF VITAMIN B-6

1. Digestion, Absorption and Cellular Metabolism

The dietary vitamin B-6 compounds are mainly PN, PLP and PMP. PLP and PMP are first hydrolysed to PL and PM by intestinal alkaline phosphatase before they can be absorbed. PL, PM and PN are then absorbed in the

intestine via a non-saturable passive process. They reach the circulation and are transported to the liver where uptake occurs by facilitated diffusion. Although the liver is the primary organ for the interconversion and metabolism of the 3 forms of vitamin B-6, interconversion can also take place in other tissues like the intestines, kidney, brain and lung. The metabolic steps involved are depicted in Fig. 10.

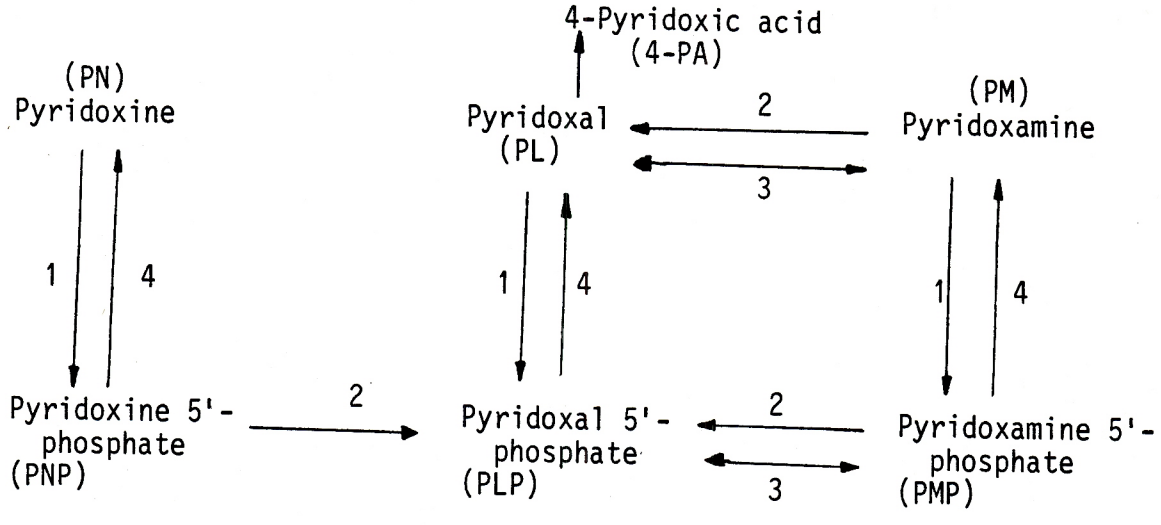


FIG. 10. General pathway of mammalian vitamin B-6 metabolism
1 - Pyridoxal (PL) kinase;
2 - Pyridoxine (pyridoxamine) 5'-phosphate oxidase (PNP (PMP) oxidase);
3 - Aminotransferases;
4 - Alkaline phosphatases;
5 - Aldehyde oxidase and dehydrogenase.

The 2 metabolic steps common to all 3 forms is the phosphorylation step in which a phosphate group is attached via PL kinase, and the dephosphorylation step where the phosphate group is removed by the action of a phosphatase. PNP and PMP are then converted to PLP through the action of PNP (PMP) oxidase. Tissues like skeletal muscle, pancreas, heart and erythrocytes (in the rat) lack the latter enzyme and have PL as the only form which serves as a source of PLP.

PL, taken up either directly or produced from the dephosphorylation of PLP is converted by aldehyde oxidase and/or an NAD dependent dehydrogenase to 4-PA. This is an irreversible reaction and serves as a major route for the metabolism of excess vitamin B-6.

As a result of interconversion of the forms of vitamin B-6 in the liver, PLP and PL are released into the plasma. The liver is regarded as, the primary source of plasma PLP but muscle glycogen phosphorylase, which serves as a reservoir for vitamin B-6, may be a source of PLP during conditions of caloric deficit. Thus plasma PLP and PL serve as the only source of PLP in tissues which lack oxidase enzymes.

PLP is the most abundant form of vitamin B-6 in the plasma, accounting for 60-70% of the total vitamin present and is bound primarily to albumin which protects PLP from hydrolysis. PL is the next most abundant form, followed by lower levels of PN and PM. PNP and PMP are very low or essentially absent under normal dietary conditions.

Although circulatory PLP accounts for 60-70% of the plasma vitamin B-6 and was long regarded as the transport form of the vitamin, it may not be the direct source of vitamin B-6 for many tissues. The binding of PL to haemoglobin and albumin is not as tight as PLP, suggesting that PL may be a major , immediate source of vitamin B-6 to the tissues. While PL can pass more easily into cells, PLP would need to be dephosphorylated first and only if the rate of dephosphorylation is sufficient, plasma PLP would serve as an adequate source of PL. Plasma PLP could also be regarded as a circulatory store, presumably helping to maintain an equilibrium between plasma and tissue levels of PLP.

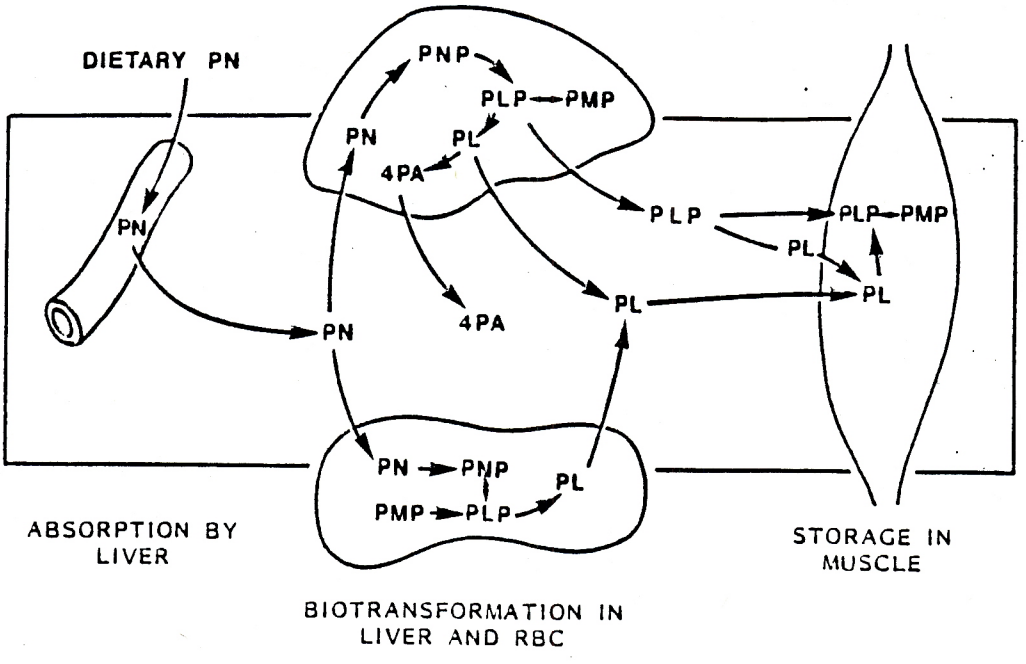


FIG. 11. A proposed pathway for the conversion and transport of vitamin B-6 compounds in plasma after a PN injection.

In conclusion, the liver plays an important role in interconverting the various vitamers and releasing PLP, PL and 4-PA into circulation (Fig. 11). In humans, the erythrocyte is an important source of plasma PL by its role in converting PN to PL but in the rat this is not possible as the rat erythrocytes lack the PNP (PMP) oxidase coenzymes. Plasma PL seems to be the immediate and major source of vitamin B-6 to tissues but the possibility of plasma PLP being an adequate source to tissues under certain conditions cannot be excluded (Leklem, 1988; Lumeng, 1985).

2. Storage and Excretion

Approximately 60% of the vitamin B-6 present in rat muscle is bound to the enzyme glycogen phosphorylase which in turn makes up nearly 5% of the soluble protein of muscle. An excess intake of vitamin

B-6 usually results in an increase in the total muscle glycogen phosphorylase. However, the muscle PLP bound to this enzyme serves as a source of vitamin B-6 during a general caloric deficit only and not during a specific vitamin B-6 deficiency. Erythrocytes may also serve as reservoirs for vitamin B-6 as both PLP and PL bind to haemoglobin and PLP binds to aminotransferases (Leklem, 1988, Labadarios, 1984).

The primary excretory form of vitamin B-6 is 4-PA formed from PL and which appears in the urine. The excretory product is formed from PL in many tissues by the action of aldehyde oxidase or by the action of NAD dependant alcohol dehydrogenase on PL in the liver.

In conclusion Ink (1984) appropriately states that "much of the fascination with vitamin B-6 is due to its simplicity of the chemistry, of the interchangeability of the various vitamers, their specific binding to nonenzymic proteins in the circulation and the regulation of coenzyme concentration in spite of the fact that there is little or no selective transport of the non-phosphorylated forms through membranes."

D) VITAMIN B-6 REQUIREMENTS IN RATS

In vitamin B-6 deficiency studies, supplements of the vitamin are usually given in the form of pyridoxine hydrochloride (PN.HCl). Beaton and Cheney (1965) reported a requirement of 40-80 µg/d of PN.HCl for maximum weight gain and maximal erythrocyte aspartate aminotransferase (AST) activity in rats. For maximal alanine amino transferase (ALT) activity the requirement was 80 µg or more per day. They suggested that rat diets should provide about 100 µg/d (equivalent to 6-7 mg/kg of diet) to ensure adequacy

of intake. This figure was in response to the Committee on Animal Nutrition of the National Research Council's conclusion that 12 µg/d of PN-HCl were adequate to meet the requirements of growing, pregnant or lactating rats (Beaton et al., 1965).

Although the American Institute of Nutrition Standards for Nutritional Studies report (1977) recommended a level of 7 mg PN.HCl per kg of diet (equivalent to 70-80 mg/d) to be adequate for growth, gestation and lactation of rats, a review of the literature shows that individual authors suggest PN-HCl requirements varying between 24 µg/d to 80 µg/d in rats (equivalent to 2,4 mg/kg and 7 mg/kg respectively).

Driskell et al. (1973), concluded that 45 µg/d of PN-HCl was adequate for maximal growth and maximal ALT activity in weanling and sexually mature rats.
Chen et al. (1975) concur with Driskell et al. by concluding that 4 mg/d of PN.HCl per kg of diet (equivalent to 40 µg/d) was adequate for growth and for maximal erythrocyte AST and ALT activity - contrary to Beaton and Cheney's report of 80 µg/d required for maximal ALT activity.

Lumeng et al. (1978) found that maximal growth and maximal erythrocyte AST activity in weanling rats occurred with 29 µg/d of PN-HCl, slightly lower than that of Beaton and Cheney. Van den Berg et al. (1982) also obtained maximal growth in rats at a level of 24 µg/d and Mercer et al. (1984) repeated that maximal growth and ALT activity was attained at a level of 75 µg/d in accordance with Beaton and Cheney.

One can conclude from this review that levels between 40 to 80 µg/d (4 to 7 mg/kg of diet) of PN.HCl can be regarded as the adequate upper or "safe" limit for maximal growth, enzyme activity, pregnancy and lactation in rats. 24 µg/d can be as the lower limit and any levels below this in the diet can be regarded as inadequate or deficient.

A high casein diet usually increases the request for B-6 in the animal. All the studies mentioned above had approx 20% casein in the diets and this therefore rules out the possibility of the variations in casein content leading to different levels of requirements of vitamin B-6.

One possible explanation for the variation in vitamin B-6 requirements is the role played by the GIT microflora in the synthesis of vitamin B-6. While the human colon may have very little absorption capacity of microflora synthesised vitamin B-6, this ability has been demonstrated in the rat (Gregory et al., 1986). It may be suggested that varying contributions of vitamin B-6 by the GIT microflora could lead to variations in dietary vitamin B-6 requirement in rats.

E) EFFECT OF VITAMIN B-6 DEFICIENCY IN THE RAT

As an animal develops a vitamin B-6 deficiency, alterations in pathways utilizing vitamin B-6 coenzymes ultimately lead to outward clinical signs of deficiency. Vitamin B-6 deficiency is capable of producing poor growth, muscular weakness, fatty liver, convulsive seizures, anaemia, reproductive impairment, oedema, nerve degeneration and impaired immune responses in the rat (Rutishauser, 1982). The most characteristic symptoms of vitamin B-6 deficiency is a typical acrodynia in which the region around the nose becomes erythematous and erodes, accompanied by a nasal discharge, red and swollen paws and very little growth. The survival period of rat deprived of PN rarely extends beyond 50 to 60 days and supplements of vitamin B-6 ranging from 50 to 100 µg/d given after the appearance of typical symptoms produce an immediate resumption of growth and healing of dermatitis within 10 days (McCoy, 1963).

At biochemical level, the developing vitamin B-6 deficiency progressively leads to reduced circulating plasma PLP levels accompanied by a loss in the activity of vitamin B-6 dependant enzymes in the blood, brain, kidney and liver. Loss of PLP is accompanied by the reduction of the PLP content of the tissue and an increased percentage of vitamin B-6 dependant enzymes in the apo form.

F) VITAMIN B-6 AND TRACE ELEMENTS - THE METABOLIC CONNECTION WITH ZINC (Zn), COPPER (Cu) AND IRON (Fe)

Trace elements, occurring in such small amounts in the body serve primarily in catalytic rather than structural functions in cells and tissues. They act as catalysts in a wide range of enzyme systems and have either weak ionic effects or highly specific associations via proteins known as metalloenzymes. In the latter, the metal is firmly attached to the protein in a fixed number of atoms per molecule which usually cannot be removed without the loss of enzyme activity (Underwood, 1981).

Since the metalloenzyme and vitamin B-6 are extensively involved as catalysts in numerous metabolic pathways, there will invariably be common pathways as shown in Table 1, where both have vital but different catalytic roles or where vitamin B-6 functions as a cofactor for the metalloenzymes.

TABLE 1. Vitamin B-6 and metalloenzymes/proteins. Functional interrelationships

METALLOENZYME/PROTEIN	FUNCTION/PATHWAY	ROLE OF VITAMIN B-6
COPPER		
1. δ-amino levulinate dehydrogenase	Haem synthesis	Co-catalyst
2. Lysyl oxidase	Collagen-elastin crosslinking	Cofactor
3. Spermine oxidase	Oxidative deamination	Cofactor
4. Diamine oxidase	Oxidative deamination	Cofactor
5. Dopamine β-hydroxylase	Catecholamine synthesis	Co-catalyst
ZINC		
1. Lactic dehydrogenase	Deamination	Cofactor
2. Glutamic dehydrogenase	Deamination	Cofactor
3. Alkaline phosphatase	Metabolism of PO_4^-	Cofactor
4. Nucleotidyl transferase	Nucleic acid synthesis and protein metabolism	Co-catalyst
5. Thymine kinase	Nucleic acid synthesis and protein metabolism	Co-catalyst
IRON		
1. Cytochrome C	Haem synthesis	Co-catalyst
2. Cytochrome oxidase	Haem synthesis	Co-catalyst
3. Ferritin	Iron storage	Cofactor required for synthesis
4. Hemosiderin	Iron storage	
5. Transferrin	Iron transport	
6. Tryptophan pyrrolase (oxygenase)	Tryptophan catabolism	B-6 required as cofactor

From the above vitamin B-6 trace element interrelationship, the following basic questions arise:

- Could a change in the animals vitamin B-6 status have any ramifications on its trace element status?
- Could changes in tissue vitamin B-6 levels influence changes in tissue metalloenzymes and/or trace element content of various tissues?
- If tissue changes do occur are they accompanied with/ without changes in trace element absorption, excretion and retention and vice versa?
- What would be the mechanisms underlying the above changes - if they do occur?

G) VITAMIN B-6 AND TRACE ELEMENTS - CURRENT VIEWS

Hsu (1965) reported that the Zn content of plasma, liver, pancreas and the heart of PN depleted rats was significantly lower than control animals fed on a diet supplemented with 20 mg/kg of PN-HCl. No significant difference in the Zn content of the brain, kidney and spleen was found. Increased uptake and retention of radioactive Zn-65 by the PN deficient tissue also occurred. This was expected due to their decreased Zn content. The amount of isotope measured in the GIT and feces of the deficient group also increased suggesting that a PN deficiency enhanced the rate of excretion of radiozinc from the body. Hsu's study cited previous reports of increased alkaline phosphatase activity and decreased activity of lactic and glutamic dehydrogenase, all zinc containing enzymes, during PN deficiency.
However, his study was unable to clarify whether the changes in Zn uptake, excretion and metalloenzyme activity were due to Zn deficiency or to vitamin B-6 deficiency.

Neal et al. (1962), studied absorption of Fe^{55-59} in rats fed PN and Fe deficient diets and reported that PN deficiency did not enhance the absorption of Fe when Fe intake was between 50-100 µg/d (levels considered to be physiological). If daily Fe intake exceeded 1 mg, PN deficient animals showed a greater iron absorption than control animals. This was contrary to the report of Yeh et al. (1962), which stated that Fe absorption was not affected by PN deficiency regardless of the severity of the deficiency or the dosage of Fe given.

Kirksey et al. (1967) investigated the possibility of PN as a factor in the regulation of Fe in body stores and reported that PN deficient pregnant rats had elevated levels of Fe in plasma, liver, spleen and duodenum but no major impairment in Fe absorption was evident.

Evans and Johnson (1980) proposed "the tryptophan-picolinic acid connection" in the role of Zn absorption. Picolinic acid (pyridine-2-carboxylic acid) found in human milk, rat intestine and pancreas is a product of tryptophan metabolism (with vitamin B-6 as a cofactor in the pathway from tryptophan to picolinic acid - see Fig. 4). Because picolinic acid is a strong bidentate metal chelating ligand with high association constants with Zn, Cu and Fe, it was suggested that there may be a possibility that this metabolite may function in facilitating the absorption of these divalent ions from the intestinal lumen and facilitate their transport to portal blood. Intestinal interaction or competition for binding with the ligand was not ruled out between these elements.

Evans and Johnson reported in this study that growth and the Zn concentration in the kidneys of rats fed diets supplemented with picolinic acid was greater than animals eating unsupplemented diets. The same was found in animals fed PN deficient diets supplemented with picolinic acid and compared to animals fed with unsupplemented PN deficient diets. It must be noted that these results contrast with that of Hsu (1965) who found no significant difference in the Zn content in kidney tissue between PN deficient and PN sufficient rats.
These results suggest that PN deficiency causes impaired absorption of dietary Zn resulting from a decreased production of picolinic acid.

In another study carried out to substantiate the hypothesis that Fe and Zn may compete for a common carrier molecule (which may be picolinic acid) in the intestinal lumen, Evans and Johnson (1981) reported that the absorption of dietary Zn was significantly less in rats fed high Fe diets than in rats fed adequate Fe diets. (High Fe diets contained 220 ppm Fe, low Fe diets contained 30 ppm Fe. Both diets contained 16,5 ppm Zn).
Zn absorption in rats fed a high Fe diet supplemented with picolinic acid was markedly increased and did not differ from that in rats fed an adequate Fe diet. It was also reported that Zn absorption increased, Zn excretion decreased. Zn balance increased as the level of vitamin B-6 increased. This was due to the effects of increased production of endogenous picolinic acid, the levels of which were monitored in the pancreas.

This study concluded that high levels of Fe in the diet inhibit Zn absorption via competition for binding with picolinic acid and also provided further evidence to support the hypothesis that picolinic acid facilitates the absorption and retention of dietary Zn.

The hypothesis of Evans and Johnson was opposed strongly by Rebello et al. (1982) and Hurley et al. (1982) who disputed both the extent of the tissue levels and the role of picolinic acid as a Zn binding ligand. These authors claimed that picolinic acid is low in milk and apparently absent in pancreatic juice and the intestine and the compound described by Evans and Johnson (1980) is in fact citrate. They ascribe impaired Zn absorption to be due to a breakdown of intestinal mucosa rather than in a decreased picolinic acid production.

Prasad et al. (1982) reported a significant enhancement in the uptake of Zn and other ions (Ca and Cd) from the intestine of vitamin B-6 deficient rats, confirming the earlier observations of Ikeda et al. (1979) and Gershoff (1968) who demonstrated an increase in the Zn content of various tissues of B-6 deficient rats. This was contrary to the reports of Hsu (1965) and Evans and Johnson (1980; 1981) who reported decreased levels of tissue Zn during vitamin B-6 deficiency.

Prasad et al. suggested a mechanism for increased Zn absorption similar to, but with opposite effect as that proposed by Hurley et al. (1982). According to Prasad et al., vitamin B-6 is involved in catalysing a wide variety of enzymatic reactions almost entirely concerned with nitrogen metabolism. PN deficiency may result in an impairment of nucleic acid synthesis and consequently an inhibition of protein synthesis, cell division and repair. Therefore, marked alteration could be expected in the brush border membrane leading to a non-specific increase in metal ion intake.

The above reports answer some of the questions raised previously with regards to vitamin B-6 and trace element interrelationships. Regardless of whether picolinic acid plays a role in zinc uptake, the reports show that zinc uptake or tissue levels of zinc are directly related to

vitamin B-6 intake indicating that the nutritional status of zinc may be related to vitamin B-6 status. Indeed, Brown (1985) points out that many symptoms of zinc deficiency and vitamin B-6 deficiency are similar including effects on food consumption, growth, skin lesions and impaired immune factors.

The object of the present study is to conduct a trace element balance study, to shed light on the absorption, retention and excretion of trace elements during the course of a diet induced vitamin B-6 deficiency. Since trace elements rarely act or respond to changes in isolation from other trace elements, this study will concurrently evaluate the status of Zn, Cu and Fe during vitamin deficiency thereby accounting for at least one important nexus in trace element interaction.

Trace element "balance" is the difference between the intake and the excretion of a particular element and is indicative of how much of the trace element is retained or lost. Intake is usually measured by determining the level of the trace element in the diet and excretory losses are determined by analysing the feces and urine. Losses through sweat and skin debris are not measured due to them being relatively minor and difficult to measure. Thus, by determining how much of a trace element is being retained or lost, a good indication of the animals total trace element status is obtained.
Although the balance study does not provide information on true absorption, body distribution, pool sizes and the turnover rate of the assessed nutrient, it is useful in evaluating the minimal daily requirement of the nutrient and the magnitude of the nutritional response to illness, trauma, deficiency or any other physiological or pathological condition (Beisel, 1979). To the best of our knowledge, there have been

no previous reports on Zn, Cu and Fe balance during vitamin B-6 deficiency.

II. MATERIALS AND METHODS

A) STRUCTURE OF THE STUDY

As stated in the introduction, the major object of this study was to determine Zn, Cu and Fe balance in rats receiving varying amounts of vitamin B-6 in the diet. The 2 major factors related to nutrient balance, growth and food intake, were also to be determined. During the course of the study, suitable biochemical indices to determine the nutritional status of vitamin B-6 in the animal were to be used. With regards to the objectives, the study was structured as follows:

Preparation and analysis of diets for experimental and control group animals.

The induction of vitamin B-6 deficiency in the experimental groups with the concurrent monitoring of food intake and body mass.

Collection of urine and fecal samples at predetermined intervals to determine trace element balance and to evaluate the vitamin B-6 status of the animals.

Determining urinary xanthurenic acid (XA) and plasma PLP and PL levels as indices of vitamin B-6 status using high performance liquid chromatography (HPLC).

Trace element analysis of diet, feces and urine using flame atomic absorption spectroscopy (AAS).

Statistical analysis of data obtained from analytical procedures.

B) DIET PREPARATION AND ANALYSIS

The powdered pyridoxine deficient basal diet used in this study was commercially prepared (ICN Nutritional Biochemical, Cleveland, Ohio) according to the formulation by French (1966).

The composition of the diet was as follows:

Vitamin free casein	30,0%
Sucrose	28,0%
Corn starch	28,0%
Alphacel, non-nutritive bulk	2,0%
Vegetable oil (hydrogenated)	6,0%
Corn oil	2,0%
Salt mixture Hawk Oser	4,0%

Plus ICN vitamin diet fortification mixture except pyridoxine hydrochloride.

Hawk Oser Salt Mixture

Calcium carbonate	6,860%
Calcium citrate	30,830%
Calcium phosphate monobasic	11,280%
Ferric citrate (16-17% Fe)	1,532%
Magnesium carbonate	3,520%
Magnesium sulphate anhydrous	3,830%
Manganous sulphate.H_2O	0,020%
Potassium aluminium sulphate	0,009%
Potassium chloride	12,470%
Potassium iodide	0,004%
Potassium phosphate dibasic	21,880%
Sodium chloride	7,710%
Sodium fluoride	0,051%

ICN Vitamin Diet Fortification Mixture

Composition	Grams/Kilogram
Vitamin A acetate (500 000 IU/g)	1,800
Vitamin D_2 (850 000 IU/g)	0,125
DL-alpha-tocophenol acetate	22,000
Ascorbic acid	45,000
Inositol	5,000
Choline chloride	75,000
Menadione	2,250
p-aminobenzoic acid	5,000
Niacin	4,250
Riboflavin	1,000
*Pyridoxine HCl	0,000
Thiamine hydrochloride	1,000
Calcium pantothenate	3,000
Biotin	0,020
Folic acid	0,090
Vitamin B-12	0,00135

For the study, 3 diets differing only in vitamin B-6 levels were prepared from the basal diet.

Diet 1 contained 3,5 mg of vitamin B-6 (in the form of PN.HCl) per kg of diet - this diet was the diet sufficient in vitamin B-6 (supplying approximately 42 µg of PN per day) and was fed to the control group 1.

Diet 2 contained 1,75 mg of PN per kg of diet. This was the marginally deficient diet (supplying approximately 21 µg of PN per day) and was fed to the experimental group 2.

Diet 3 contained 0 mg of PN per kg of diet. This was the total vitamin B-6 deficient diet fed to the experimental group 3.

Diets 1 and 2 were prepared as follows:

35 mg of PN.HCl (BDH Chemicals, England) was added to 1 kg of the basal diet which was then mixed thoroughly for 15 minutes in an electrical homogenizer (Forster Equipment Co., England). This formed a stock diet containing 35 mg/kg of PN.HCl.

Stock Diet

35 mg/kg PN

250 g stock diet mixed with
+ 2250 g basal diet
= 3,5 mg/kg of PN in Diet 1

125 g of stock diet mixed with 2375 g of basal diet
= 1,75 mg/kg PN in Diet 2

After the addition of stock, both diets were mixed thoroughly for 15 minutes in the homogenizer to obtain homogenous distribution of the vitamin. Batches of 2,5 kg of diets 1, 2 and 3 were prepared, labelled and then refrigerated at 7°C.

Trace Element Analysis of Diet

Random samples from all 3 diets were taken, digested and analysed for Zn, Cu and Fe according to the same procedures for fecal analysis described in section E) 2.

The water content of the diet was 5,39%.

The analysed trace element content per gram of diet and the American Institute of Nutrition (AIN) recommendations for rat diets (1977) are given below:

	Zn	Cu	Fe
Analysed :	18,4 µg/g	3,2 µg/g	157,5 µg/g
AIN Recommended:	30 µg/g	6 µg/g	35 µg/g

Compared to these recommendations, the diet used in this study had more than the recommended amount of Fe. Although Zn levels were lower than recommended analysed level of 18,4 µg/g was still adequate for normal growth and functions of the rats. The AIN standards for nutritional studies report had increased the zinc requirements by 2,5 times the previously recommended levels in order to allow a safety margin if the mineral mixture was used with plant protein rather than casein.

In the case of Cu, the analysed amount in the diet was also lower than the recommended level. However, the levels of Cu in the diet used in the study were still adequate to provide approximately 36 µg/d of Cu, which has been found to be adequate for good growth and haemoglobin formation (Gray et al., 1973).

C) INDUCTION OF THE VITAMIN B-6 DEFICIENCY

Thirty-three young male Wistar strain rats with masses ranging from 96 to 102 grams were randomly divided into 3 groups of 11 rats each.

Group1 was the control group that was fed the adequate diet containing 3,5 mg/kg of vitamin B-6 (in the form of PN.HCl).
Group 2 was the experimental group that was fed the marginally deficient diet containing 1,75 mg/kg of vitamin B-6.

Group 3 was the experimental group that was fed the totally deficient diet containing 0 mg/kg of vitamin B-6.

All rats were housed individually in perspex metabolic cages (Techniplast) attached to stainless steel racks. The metabolic cages had raised stainless steel grid floors which prevented coprophagy and allowed the feces and urine to be separately funnelled and collected in plastic containers below the cage. Cages were regularly and thoroughly washed with detergent and then rinsed with deionized water to keep contamination by adventitious trace elements to a minimum. Furthermore, all materials which were used in contact with the rats or in diet preparation were made of stainless steel, glass or plastic to avoid trace element contamination. Arrangement of the plastic fecal and urine containers made it possible to collect all the feces and urine produced in a 24 hour time span.

The animals fed ad libitum from perspex food containers attached to the cage. The container was structured in a way that allowed the animal to insert only its head into the container thereby preventing food spillage or excess adherance to the animals body. Deionized water made from an Elgastat B114 water deionizer (Elga Products, England) was also supplied ad libitum through valve controlled water bottles attached to the cage.

All animals were weighed biweekly throughout the study to monitor growth. Food cups were weighed and replenished every 24 hours in order to determine daily food consumption from which daily intake of Zn, Cu and Fe were calculated. The animals were also observed daily for any external signs of disease or deficiency.

These procedures in addition to sample collection were carried out for the duration of 8 weeks (56 days).

D) SAMPLE COLLECTION

All 24 hour feces and urine were collected from each animal, starting from day 0 till the end of week 8 of the study. The 24 hour urine was transferred from the containers attached to the cage to new plastic containers washed in 20% nitric acid and rinsed in deionized water. During transfer, the volume of the urine was noted after which it was frozen. After 7 days, all urine collected from each animal was thawed, pooled and well mixed to provide a pooled sample for that animal for that week. From this sample, 3 ml were placed in Eppendorf vials and used for xanthurenic acid determination. The remainder was used to determine mean daily trace element excretion for that week for that particular animal.

The 24 hour feces that were collected were also transferred to acid washed plastic containers and then dried in an oven for 24 hours at 80°C. On removal from the oven, the feces were first cooled to room temperature in a dessicator with $CaCl_2$ to prevent rehydration and after weighing the feces were ground to a fine powder (Greger et al., 1985). As in the case of urine, all feces collected over 7 days were thoroughly mixed and combined into composites for each animal for each week over the period of 8 weeks. Samples from this composite were analysed to determine the mean daily trace element excretion for that particular animal for that week.

At the end of 8 weeks, all animals were anaesthetized with ether and blood samples removed from the abdominal aorta prior to them being sacrificed. The blood was spun down for 15 minutes at 150x g and the plasma was used to determine PLP and PL levels.

E) TRACE ELEMENT ANALYSIS OF FECAL AND URINE SAMPLES

1. Flame Atomic Absorption Spectrophotometry (AAS) as an Analytical Procedure

In flame AAS, a solution containing the substance to be analysed is passed under controlled conditions as a very fine spray into the air supply of a burner. A flame created by the ignition of a mixture of air-acetylene or nitrous oxide-acetylene evaporates the solution and the substance to be analysed is converted to the atomic state. In this state the electrons in the outermost shell are in the lowest energy state closest to the nucleus called the ground state. The atoms in the ground state if excited to different levels by light energy, will only absorb exactly defined amounts of energy corresponding to each level of excitation (i.e. light of definite frequency) and an absorption spectrum for that particular substance can be observed.

In atomic absorption spectroscopy, the atoms in the ground state are excited by light passing through the flame and whose wavelengths corresponds with lines in the absorption spectrum of that substance. The ground state atoms therefore absorb specific quanta of energy and if the light beam is focussed on a slit of a photoelectric instrument, the fall in intensity of the beam is measured. The physical laws governing the fall in intensity is the same as the Beer-Lambert law in colorimetry. Thus it is the extinction that measures the concentration of the absorbing atoms. This is the basis of atomic absorption spectroscopy (Varley et al., 1980), which is basically the measurement of the absorption of light energy by atoms. The specificity of this technique allows it to be used for the determination of most essential elements like Zn, Cu, Fe, Mn, Co, Cr, etc.

2. Sample Preparation

Both fecal and diet samples were prepared for analysis by wet ashing according to a modified method of Sherman et al. (1981) and Hill et al. (1984) in a micro-Kjeldahl apparatus (Gerhardt). The reagents used were:

Nitric acid - 65% - 'Suprapure' grade (Merck, Darmstadt, F.R.G.) containing as maximum impurities Zn -0,005 ppm; Cu -0,002 ppm and Fe 0,01 ppm.

Perchloric acid - 60% - 'Spectrosol' grade (BDH, Poole, England) containing as maximum impurities Zn -0,1 ppm; Cu -0,02 ppm and Fe -0,1 ppm.

Hydrochloric acid - 36% - 'Spectrosol' grade (BDH), Poole, England) containing as maximum impurities Zn -0,2 ppm; Cu -0,05 ppm and Fe -0,2 ppm

0,3 g of dried powdered fecal samples (or 1 gram of powdered dried diet sample) was weighed out in plastic containers and placed in an acid washed flask containing 5 ml of nitric acid and 2 boiling beads. The flask was heated just below boiling for 10 minutes until brown fumes were given off and a brown sediment formed at the sides of the flask. 3 ml of nitric acid and 1 ml of perchloric acid was added to the flask and further heating was continued until the solution turned a clear yellow without any sediment. 2 ml of nitric acid and 1 ml of perchloric acid was then added and the flask was heated until the solution turned clear. The solution was allowed to cool and then diluted to 100 ml with 0,36% hydrochloric acid.

For every 33 fecal samples ashed (for 1 week of the study), 1 reagent blank was prepared. Also, for every batch of 33 samples, 1 sample had 1 µg/ml of Zn, Cu and Fe added as an internal standard and was digested in duplicate (i.e. sample + internal standard and sample - internal standard) in order to determine trace element recovery. Reagent blanks and samples with internal standards were also prepared for the diets.

3. Fecal Sample Analysis by AAS

Zn, Cu and Fe content of the diet and feces was determined by using a Perkin-Elmer Model 2380 microprocessor controlled atomic absorption spectrophotometer (Perkin Elmer Corporation, Norwalk, C.T., U.S.A.). Prior to analysis, setting up procedures for the apparatus were carried out. The hollow cathode lamp for the element to be analysed was placed into the lamp compartment and the lamp control was turned on until the lamp current as specified on the lamp for continuous operation was reached. The lamp was allowed to warm up for 5 minutes. The slit width and wavelength setting were selected for the element to be analysed from the recommended standard conditions and operating parameters for that element as listed in the operations manual (Perkin Elmer, 1976a). The gain setting was used to optimize both the wavelength setting and the lamp current. All 3 elements were analysed using a lean blue oxidizing air acetylene flame. Prior to ignition, the air pressure was set to 280 kPa and the acetylene pressure was set to 140 kPa and after ignition this air-fuel mixture resulted in a flame with a temperature of approximately 2300°C. Deionized water was aspirated to clear the burner slot of any residue from previous samples prior to sample analysis.

After set up, the AAS was calibrated with standards made up on the day of analysis. Standards were prepared from commercially available 'spectrosol' grade, zinc nitrate, cupric nitrate and ferric nitrate stock standards having a concentration of 1 mg/ml (BDH, Poole, England). The instruments sensitivity limit, the linear working range and the concentrations of the standards prepared for each element are listed in Table 2.

TABLE 2. Some operating parameters for Zn, Cu and Fe analysis

ELEMENT	Zn	Cu	Fe
Sensitivity limit	0,018 µg/ml	0,09 µg/ml	0,12 µg/ml
Linear working range	1 µg/ml	5 µg/ml	5 µg/ml
Concentration of standards used	1 µg/ml	2,5 µg/ml	5 µg/ml
			10 µg/ml

In the case of Zn and Cu analysis, the analyte concentration of the samples to be analysed were within the linear working range (determined by preliminary analyses) and therefore only 1 standard and a reagent blank were used to calibrate the instrument. For Fe analysis, 2 standards were used as the analyte concentration exceeded the linear working range. The concentration of the second standard was twice that of the linear range.

To calibrate the instrument, the concentration of the standard or standards for a given element was keyed in and then both reagent blank and standard solution were aspirated. This enabled the instrument to determine the absorbance of the standard solution and plot a calibra-

tion curve for that element. Where only one standard and blank were used (for Zn and Cu), the linearity of the calibration curve was checked periodically by intermediate standards.

After the appropriate calibration curves was established, samples were aspirated and the instrument converted the absorbance readings of the samples directly into concentration (µg/ml) which was displayed on the instrument. The instrument was programmed to take five readings of each sample and to display the mean, standard deviation and coefficient of variation for each sample. The integration time was set to 1 second. During the course of analysis, standards were periodically reaspirated to check for drifts in the calibration curve. If this occurred, the instrument was recalibrated and the analysis resumed (Perkin Elmer, 1976b).

4. Urine Sample Analysis by AAS

Zn, Cu and Fe in urine was determined by directly aspirating urine samples after setting up and calibrating the instrument as done in fecal and diet analysis (Zettner et al., 1965; Hill et al., 1984).

Prior to analysis, 15 ml of each urine sample was placed in acid washed centrifuge tubes and spun down at 150x g for 10 minutes. For Zn and Fe analysis the urine was diluted 1:3 with deionized water. Because of Cu levels in the urine are usually very low, the urine for Cu analysis was aspirated undiluted to prevent the Cu levels of the sample from falling below the instruments sensitivity limit.

F) URINARY XANTHURENIC ACID (XA), PLASMA PLP AND PL AS BIOCHEMICAL INDICES FOR VITAMIN B-6 STATUS

Over the years, numerous methods for assessing an animal's vitamin B-6 status have been developed. From these, the plasma PLP levels, urinary 4-PA and urinary tryptophan metabolite excretion following tryptophan loading have been used extensively to evaluate vitamin B-6 status. Plasma PLP is actually a direct measure of the bodies vitamin B-6 status at that point in time and animal studies have shown that the tissue levels correlate quite well with blood levels (Leklem et al., 1981). Urinary 4-PA provides a measure of the major metabolic end product of vitamin B-6. Measurements of urinary xanthurenic acid or other tryptophan metabolites like urinary 3-hydroxykynureinine and kynurenine after a tryptophan load is an indirect measure of vitamin B-6 status because of the numerous steps in tryptophan metabolism that require PLP. The last method is a sensitive index for vitamin B-6 deficiency and correlates well with the other tests for vitamin B-6 status. According to Leklem et al. (1981), there is no one "best" method for status assessment. The choice of method ought to take into account the particular situation being studied.

In the present study, care was taken to prevent factors other than vitamin B-6 levels from having effects on the sensitive nutrient balance of the animals. Stressful procedures like the removal of arterial blood and oral tryptophan loading which affect nutrient balance were avoided. The periodic monitoring of urinary XA levels of spontaneously excreted XA through the course of the study and its use as an indicator of vitamin B-6 status was suited to the present study due to the non-invasive procedures required in order to obtain the samples.

Spontaneous urinary XA excretion in the absence of tryptophan loading are not as enhanced as in the case after tryptophan loading but nevertheless still are significant in vitamin B-6 deficient animals due to the development of abnormal tryptophan metabolism during deficiency (Milholland et al., 1971). Plasma PL and PLP were also determined but samples were taken only at the end of the study and therefore unlike urinary XA levels, plasma PL and PLP levels indicated the animals vitamin B-6 status at the end of the 8th week only, rather than for the entire duration of the study.

G) HIGH PERFORMANCE LIQUID CHROMATOGRAPHY (HPLC) - AS AN ANALYTICAL PROCEDURE

Chromatography techniques involve the separation of large and small mixtures into their various components due to differences in equilibrium distribution of these components between a stationary phase and a mobile phase. In liquid chromatography selective adsorption occurs from the solution onto the active surface of finely divided solids. Closely related substances exhibit different degrees of adsorption so that separation which may be difficult chemically may be achieved by chromotography. Normally the adsorbant is the stationary phase while the other is the mobile phase.

Paper, thin layer and open column chromatography are different forms of liquid chromatography where the sample molecules are transported through the stationary phase by a liquid mobile phase. Individual molecules are retained by the stationary phase as a result of interaction between the sample molecules, mobile phase and stationary phase. The length of time for which they are retained is dependant on the chemical interactions

of the different components. Since these vary, the retention times vary and separation is achieved.

HPLC is liquid chromatography done with sophisticated equipment. A constant flow pulse free pump drives injected solvent under high pressures through columns packed with an adsorbant to bring about a specific mechanism of separation. After separation, detectors "see" the various components as they elute and integrators collect, quantitate and interpret results.

Separation mechanisms in HPLC

1. Adsorption chromatography or liquid/solid chromatography where separation of polar compound molecules occurs between a stationary solid polar phase and a non-polar liquid mobile phase.
2. Partition chromatography or liquid/liquid chromatography where the stationary phase consists of an inorganic support coated with an organic liquid. Partitioning of the sample molecules occurs between a liquid mobile phase and the adsorbed liquid stationary phase.
3. Bonded-phase chromatography which is similar to liquid/liquid chromatography except that the organic liquid is chemically bonded to the support thereby providing a more stable stationary phase and separation occurs more by adsorption rather than partition.
4. Size separation where molecules are separated by a porous gel on the basis of molecular size. No chemical interaction takes place within the system.

5. Ion-exchange chromatography is used to separate ionic compounds in an aqueous media. The stationary phase consists of a gel or resin carrying ionic functional groups on its surface. The nature of these group determines the retention properties and selectivity for a given solute. The eluent contains ions of opposite charge to the functional groups on the stationary phase and form ion-pairs with the latter. Sample ions exchange places with eluent ions and remain on the column for a period of time, dependent on the relative affinity of the packing for the eluent or sample ions.
6. In paired ion-chromatography an ion modifier is included in the mobile phase and this forms an ion pair with ionizable compounds. Analysis may then be effected by adsorption or partition chromatography rather than by the complex ion-exchange process.

In all the differing methods of separation, the basic method of all forms of liquid chromatography still applies, viz.: there is a stationary phase, a mobile phase and as a result of the various affinities of the sample for each, a separation occurs and the sample components are detected as they emerge from the base of the column. The results are then presented as a series of peaks on a chart recording. Under standardised conditions the time from injection of a given compound to its detection is always the same and is termed the retention time. Unknown components in a sample can therefore be identified by comparing their retention time with that of known components analysed under identical conditions or by the use of internal standards (Williams et al., 1984).

1. Quantification of Urinary XA Levels by High Performance Liquid Chromatography (HPLC)

The method used was an isocratic HPLC method of Ubbink et al. (1988) with minor modifications to suit rat urine analysis. All reagents used were analytical grade and supplied by Merck (Darmstadt, F.R.G.) except for XA which was supplied by Fluka (Buchs, Switzerland). Prior to HPLC analysis, a XA purification procedure from urine was carried out using anion-exchange solid-phase extraction.

Urine purification

Disposable extraction columns packed with 100 mg of strong anion-exchange resin (trimethylaminopropyl groups bonded to silica; from Analytichem, Harbor City, CA, U.S.A.) were washed with 1,0 ml of methanol and 2 ml of water. 0,4 ml of pooled urine was diluted to 1,0 ml with 0,2 M potassium phosphate buffer (pH 8,0), and the diluted buffered urine sample was applied to the extraction column. The extraction column was then washed with 2,0 ml of 0,2 M phosphate buffer and subsequently with 0,2 ml of 0,1 M nitric acid. Bound XA was eluted from the anion exchange resin with 1,2 ml of 0,1 M nitric acid and the eluate was collected in a silanized 2,0 ml volumetric flask and diluted to 2,0 ml with water. 400 µl were directly injected for HPLC analysis. All solutions were eluted from the extraction column by applying vacuum to a twelve position Supelco vacuum manifold (Supelco, Bellefonte, U.S.A.).

HPLC analysis

A Beckman (Beckman Instruments, Berkley, CA, U.S.A.) Model 112 solvent delivery module was fitted with a Whatman (Clifton, N.J., U.S.A.) Partisphere C_{18} analytical column (110 mm x 4,7 mm ID; particle size 5 µm).

The column was protected by a Whatman reversed-phase guard cartridge installed between the injector and analytical column. A 0,025 M potassium dihydrogen-phosphate buffer (pH 5,5) containing 5% acetonitrile was used as a mobile phase at a flow rate of 1,2 ml/min. The column eluate was monitored at 340 nm with a Beckman Model 165 variable wavelength detector, coupled to a Spectra-physics (San Jose, CA, U.S.A.) 4270 integrator.

Standards: Standard solutions containing between 10 to 50 µg/ml XA were prepared and subjected to anion exchange solid phase extraction and HPLC analysis in the same manner as the urine samples. All results were expressed in µg/ml.

2. Determination of Plasma PLP and PL by HPLC

The method used to determine PLP and PL were those of Ubbink et al. (1985; 1986) where light and temperature stable PLP semicarbazone formed the basis for an isocratic HPLC method with fluorescence detection.

Reagents

PL and PLP were obtained from Merck (Darmstadt, F.R.G.). The internal standard, 6-methyl-2-pyridine carboxaldehyde (MPC) was obtained from Aldrich (Milwaukee, U.S.A.). Chromatography grade dichloromethane and acetonitrile and all other regents used (analytical reagent grade) were also obtained from Merck.

PLP and PL purification

Commercial PLP and PL preparations were purified by reversed phase HPLC (Whatman Partisil 10 OD S-3 column; mobile phase: 10% methanol and

0,1% glacial acetic acid in water) and then lyophilized. The purified PLP and PL were checked for impurities by reversed phase ion-pair chromatography, UV detection (290 nm) and wavelength scanning (210-360 nm) at different stages of peak elution. UV absorption spectra of the purified PLP and PL were determined in 0,1 M hydrochloric acid, 0,1 M sodium hydroxide and 0,1 M sodium phosphate buffer, pH 7,0.

Standards

Purified PLP and PL were used to prepare working standards containing 1-20 mg PLP and PL per ml. To prepare the internal standard, MPC was dissolved in 25 ml of 0,1 M sodium dihydrogen phosphate, 20 ml of 0,5 M semicarbazide solution was added and the mixture was heated for 20 mins at 40-45°C. After cooling, 0,1 M disodium hydrogen phosphate was added to adjust the pH to 7; the volume was adjusted to 500 ml using Na_2HPO_4-NaH_2PO_4 buffer (0,1 M; pH = 7,0). The 6-methyl-2-pyridine carboxealxehyde semicarbazone (MPCSc) solution was then divided into 2,0 ml aliquots, freezed dried, sealed under nitrogen and stored at -20°C.

Sample preparation

To 1 ml of plasma a mixture of 0,3 ml 10% TCA, 0,3 ml of 0,5 M semicarbazide and 50 µl MPCSC was added. The combination was mixed vigorously and then heated at 37°C for 30 mins. The clear supernatant obtained after centrifugation was washed once with diethyl ether and once with dichloromethane. A 30 µl aliquot of the water phase was analysed for PLP and PL using HPLC and results were expressed in ng/ml.

Instrumentation

A Perkin Elmer series 2 liquid chromatograph was slightly modified so that one pump was used for solvent delivery and the other pump for post

column reagent addition. An LS 4 Perkin Elmer fluorescence spectrometer (excitation wavelength: 367 nm; emission wavelength: 478 nm) was coupled to a Perkin Elmer Sigma 10 chromatography data station.

Columns

A Whatman (Clifton, NJ, U.S.A.) Solvecon pre-column (25 cm x 4,6 mm) installed on stream and before the injector, was followed by a Brownlee labs (Santa Clara, CA, U.S.A.) RP-18 Spheri-5 guard column (4 cm x 4,6 mm) and a Whatman Partisil-5 ODS-3 RAC II analytical column (10 cm x 4,6 mm 1-D; 5 µm particle size).

Chromatographic conditions

A solution of 0,05 M potassium dihydrogen phosphate (pH adjusted to 2,9 with concentrated orthophosphoric acid) containing 7% acetonitrile was used as mobile phase. Sodium hydroxide (4% w/v) was introduced for post column alkalinisation. The flow rates of the solvent delivery pumps and the post column reagent pump were 1,1 and 0,1 ml/mm respectively.

H) STATISTICAL ANALYSIS

The U-test of Mann-Whitney was applied to test for significance of differences between various measured parameters in 3 groups of animals receiving varying levels of dietary vitamin B-6 because of the small sample size and absence of proof in the normality of the data.
Simpson's rule to calculate the area under the curve was applied to the data on trace element balances and to XA and TE excretion.
All statistical tests and calculations using the raw data were done by the computer service provided by the Institute of Biostatistics of the South African Medical Research Council.

III. RESULTS

A) EFFECT OF VITAMIN B-6 DEFICIENCY ON GROWTH

Table 3 lists the mean percentage body mass gain of the 3 animal groups over the 8 week study period.

Percentage body mass gain for each animal was calculated by:

$$\frac{\text{Weekly animal mass (g)} \times 100}{\text{mass of animal on Day 0 of study (g)}}$$

Growth curves representing the data in Table 3 are shown in Fig. 12.

By applying the U test of Mann-Whitney, no significant difference in body mass gain was observed between Groups 1 and 2 over the 8 week period ($p > 0,05$). A significant difference was observed between Groups 1 and 3 and 2 and 3 over the entire 8 week period ($p < 0,05$).
The trend in the growth curves after week 8 indicates the levelling off in the growth of Groups 1 and 2.

Some characteristic symptoms of vitamin B-6 deficiency are shown in Fig. 13 and 14.

TABLE 3. Effect of vitamin B-6 deficiency on percentage body mass gained

GRP	WK 1	WK 2	WK 3	WK 4	WK 5	WK 6	WK 7	WK 8
1	164,27 ± 8,77[a]	212,11 ± 14,43[a]	246,45 ± 19,51[a]	268,41 ± 18,56[a]	291,55 ± 23,37[a]	322,22 ± 29,98[a]	345,07 ± 31,79[a]	362,03 ± 37,07[a]
2	158,98 ± 10,28[a]	202,07 ± 14,25[a]	233,62 ± 15,67[a]	256,70 ± 22,03[a]	280,67 ± 25,75[a]	303,30 ± 27,62[a]	324,03 ± 27,30[a]	337,60 ± 29,31[a]
3	144,23 ± 8,52[b]	160,78 ± 11,14[b]	170,06 ± 11,39[b]	176,61 ± 10,99[b]	176,92 ± 12,45[b]	176,36 ± 12,84[b]	179,56 ± 13,31[b]	177,65 ± 14,90[b]

Values are expressed as means ± SD for 11 rats per group. Means for each week not followed by the same superscript letter are significantly different ($P < 0,05$). Grp 1 received 3,5 mg/kg vitamin B-6 in diet; Grp 2 received 1,75 mg/kg vitamin B-6 in diet; Grp 3 received 0 mg/kg vitamin B-6 in diet. Individual weekly animal mass and P values are given in Appendix 1.

Vit. B-6 deficiency and growth

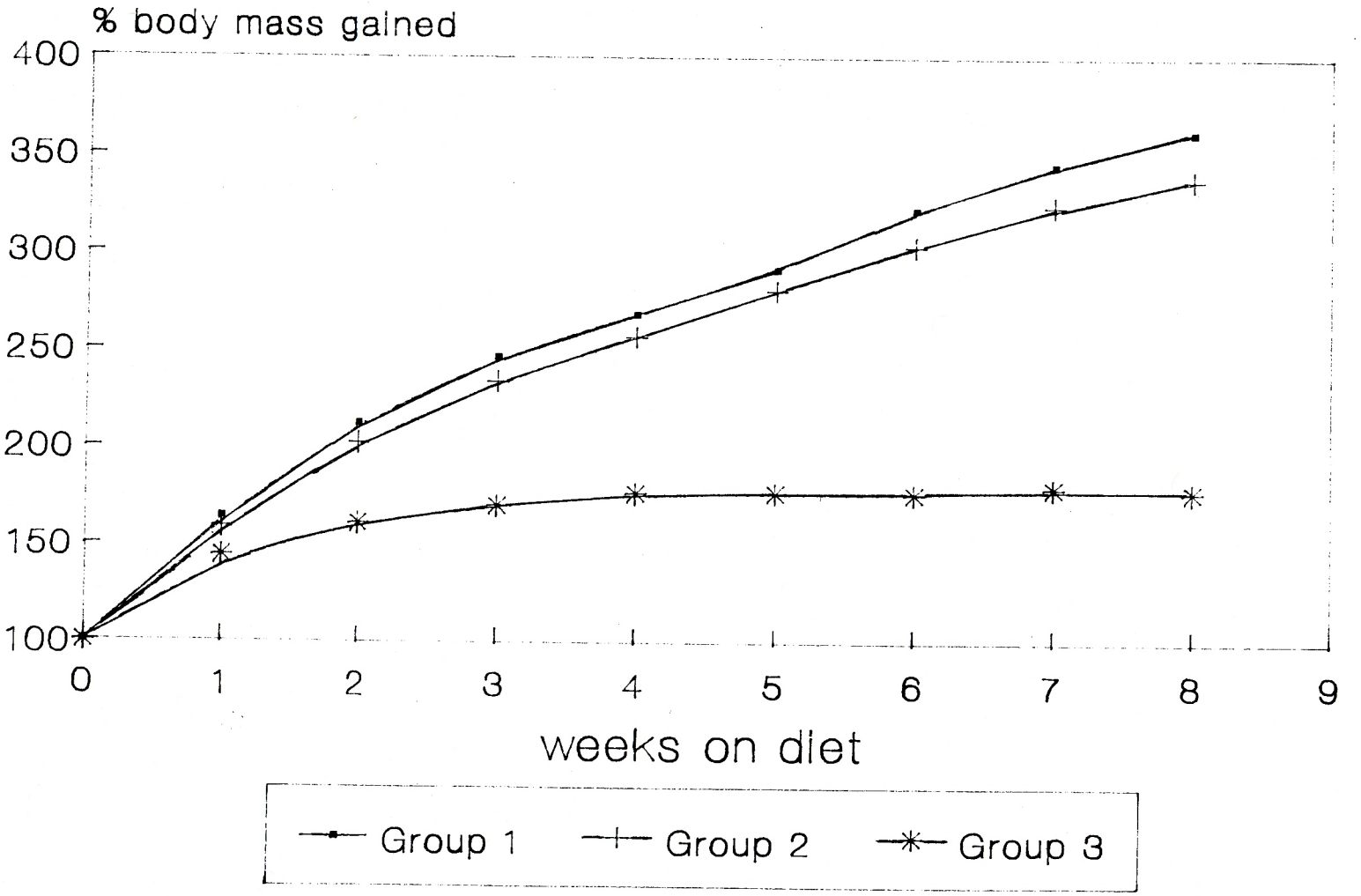
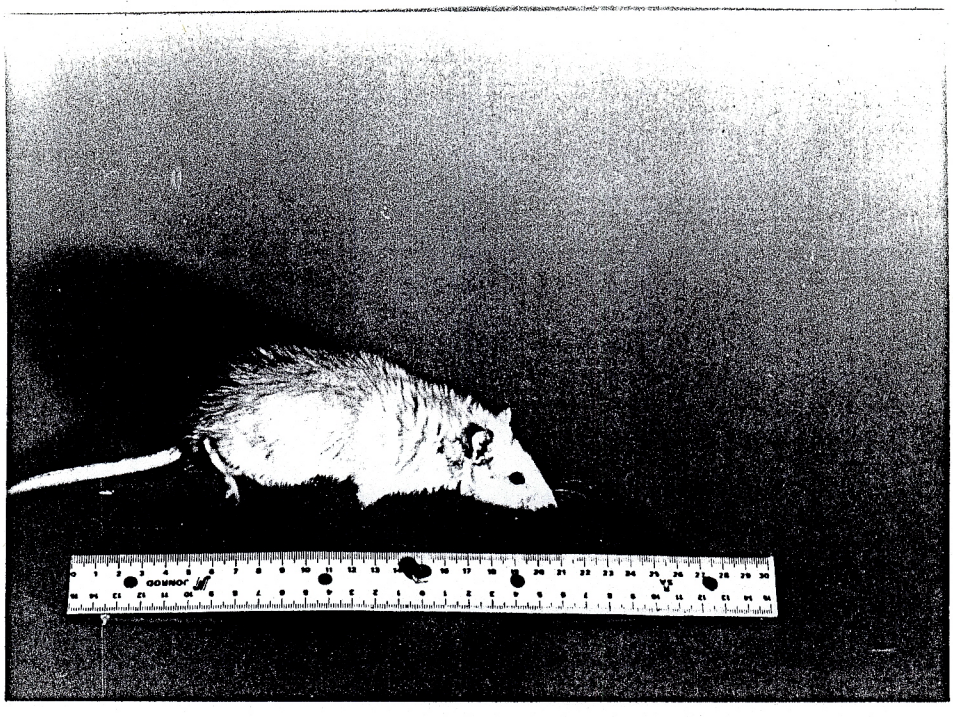


fig. 12

(a)



(b)

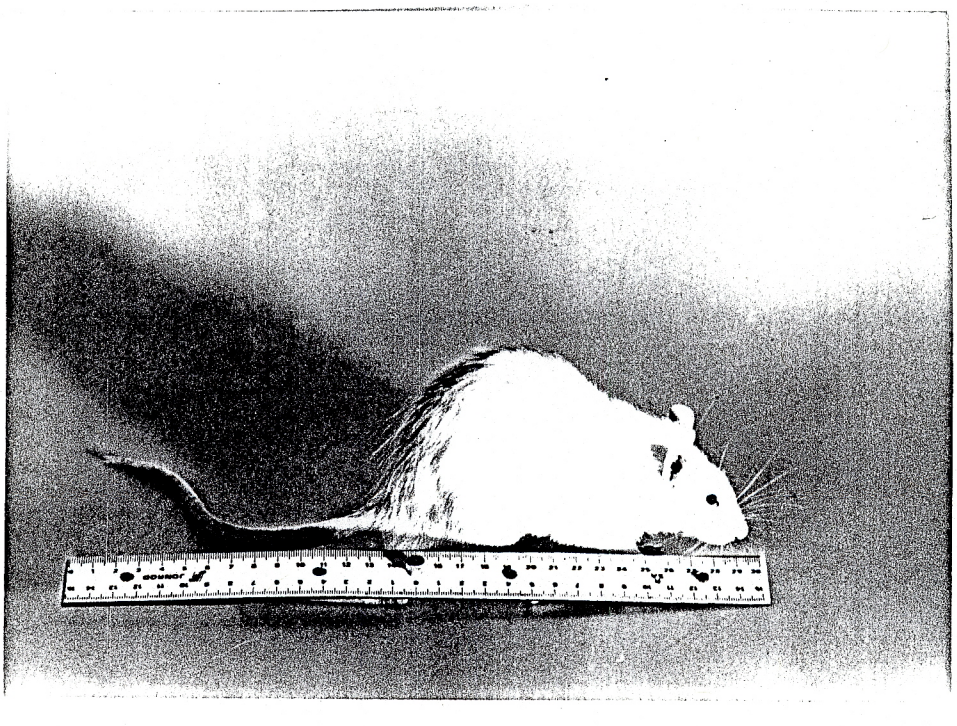


FIG. 13. Large differences in growth are observed between a vitamin B-6 deficient animal from Group 3 (13a) and a vitamin B-6 sufficient animal from Group 1 (13b).

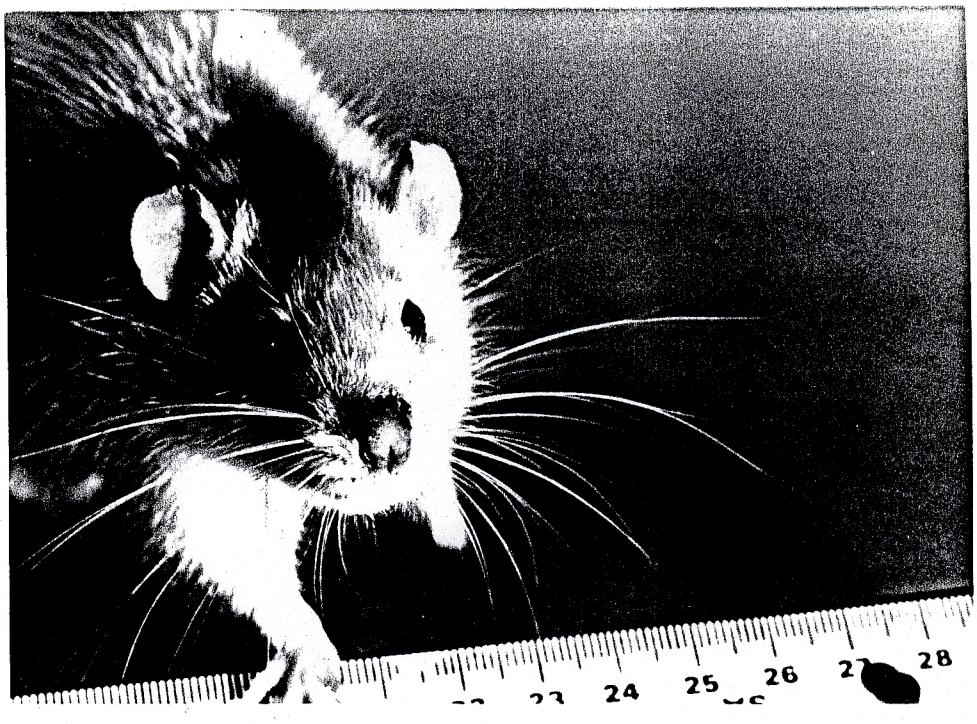


FIG. 14. Development of a typical acrodynia around the nasal area in a vitamin B-6 deficient animal from Group 3.

B) VITAMIN B-6 DEFICIENCY AND FOOD CONSUMPTION

Table 4 shows the mean daily (24 h) food consumption for each animal group over 8 weeks.

On application of the U test of Mann-Whitney, no significant differences in food consumption was observed between Groups 1 and 2. A significant difference was observed between Groups 1 and 3 and 2 and 3 over the 8 week period. The food consumption trends for each group are shown in Fig. 15 and they correspond with the growth patterns exhibited by each group. This trend is best illustrated in Group 3 where the food consumption pattern corresponds with the static growth shown in Fig. 12.

TABLE 4. Effect of vitamin B-6 deficiency on daily food consumption (g)

GRP	WK 1	WK 2	WK 3	WK 4	WK 5	WK 6	WK 7	WK 8
1	12,37 ± 1,57[a]	13,62 ± 2,18[a]	13,27 ± 2,16[a]	12,00 ± 1,14[a]	13,02 ± 1,41[a]	15,03 ± 2,10[a]	15,27 ± 2,17[a]	14,40 ± 1,98[a]
2	12,59 ± 1,38[a]	13,39 ± 1,65[a]	13,04 ± 1,60[a]	12,13 ± 1,54[a]	12,74 ± 1,12[a]	13,80 ± 1,23[a]	13,79 ± 1,72[a]	13,63 ± 0,86[a]
3	10,07 ± 1,19[b]	8,14 ± 0,96[b]	7,94 ± 0,88[b]	7,77 ± 0,74[b]	7,65 ± 1,17[b]	6,85 ± 0,69[b]	7,79 ± 1,37[b]	6,94 ± 1,39[b]

Values are expressed as means ± SD for 11 rats per group. Mean daily food consumption figures for each week not followed by the same superscript letter are significantly different ($P < 0,05$). Grp 1 received 3,5 mg/kg vitamin B-6 in diet; Grp 2 received 1,75 mg/kg vitamin B-6 in diet; Grp 3 received 0 mg/kg vitamin B-6 in diet. Individual rat food consumption and P values are given in Appendix 2.

vit. B-6 deficiency & food intake

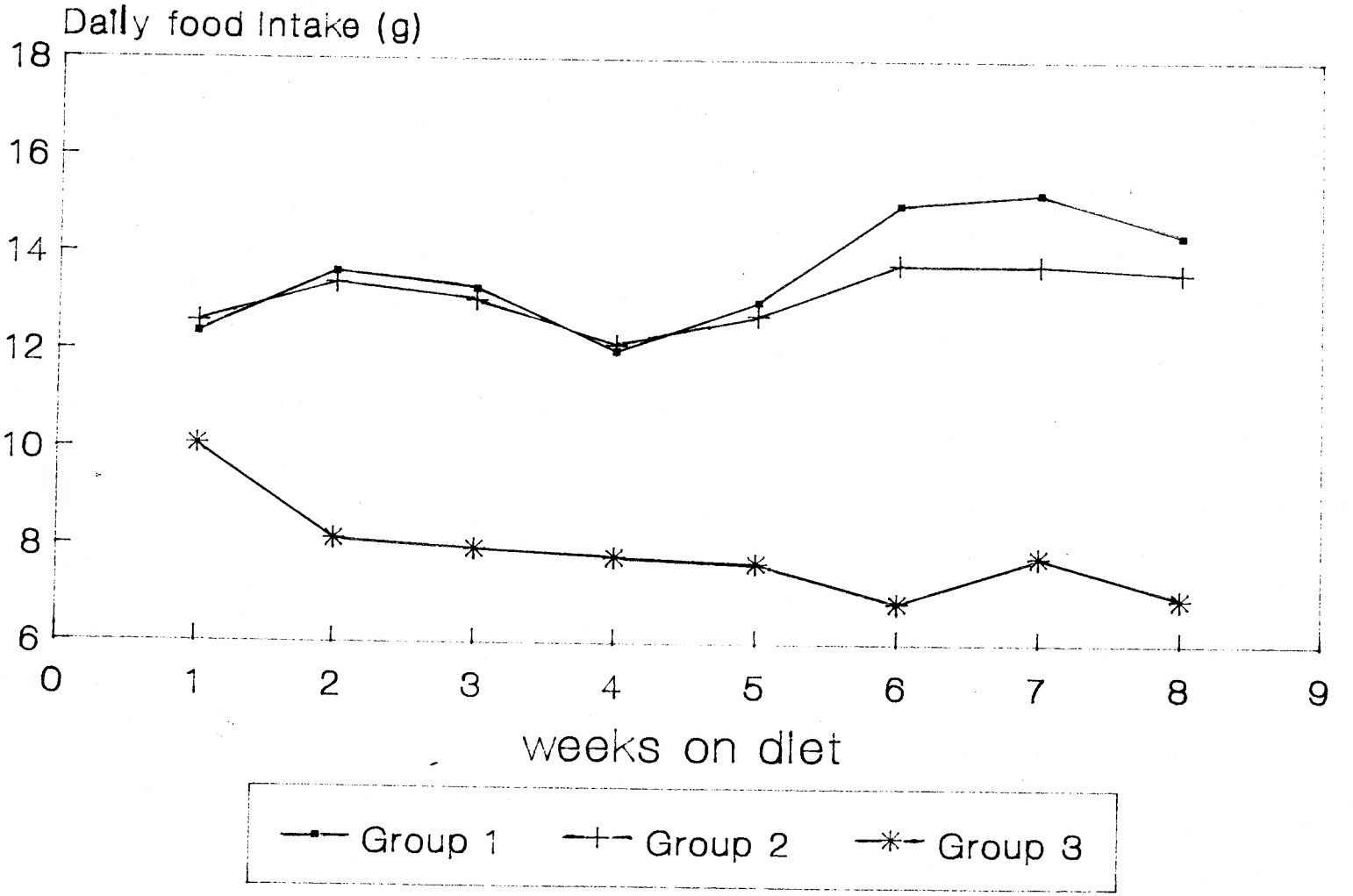


fig. 15

C) URINARY XANTHURENIC ACID (XA) EXCRETION AS AN INDEX OF VITAMIN B-6 DEFICIENCY

Table 5 shows the mean urinary XA excretion (n mol/24 h/g body mass) for the 3 groups of animals over the 8 weeks of study.
Urinary XA excretion for each animal in n mol/24 h/g body mass was calculated by:

$$\frac{\text{Urinary XA } (\mu g/ml) \times 1000 \times 24 \text{ h urine volume } (ml)}{\text{Molecular mass of XA} \times \text{animal mass } (g)}$$

The results in Table 5 are represented graphically in Fig. 16.
Using the U test of Mann-Whitney, no difference in XA excretion exists between the 3 groups on day 0 - i.e. the baseline level of XA excretion is the same for all groups ($p > 0,05$).

A significant difference in XA excretion exists between Groups 1 and 3 and 2 and 3 during weeks 1, 2 and 3 ($p < 0,05$). No significant difference exists between Groups 1 and 2 ($p > 0,05$).

A significant difference in XA excretion between Groups 1 and 2 and 1 and 3 exists from weeks 5 to 8 ($p < 0,05$). No significant difference exists between Groups 2 and 3 ($p > 0,05$) during the same period.

Whereas XA excretion remains fairly constant for Group 1 over 8 weeks, group 3 (subjected to a chronic vitamin B-6 deficiency) showed a dramatic rise in XA excretions during the first week. The levels then dropped as the deficiency progressed but still remain higher than Group 1.
Group 2 which had marginally deficient levels of vitamin B-6 in the diet when compared to Group 1, showed a significant difference in XA excretion only after week 4. Thereafter, there was no significant difference between Groups 2 and 3.

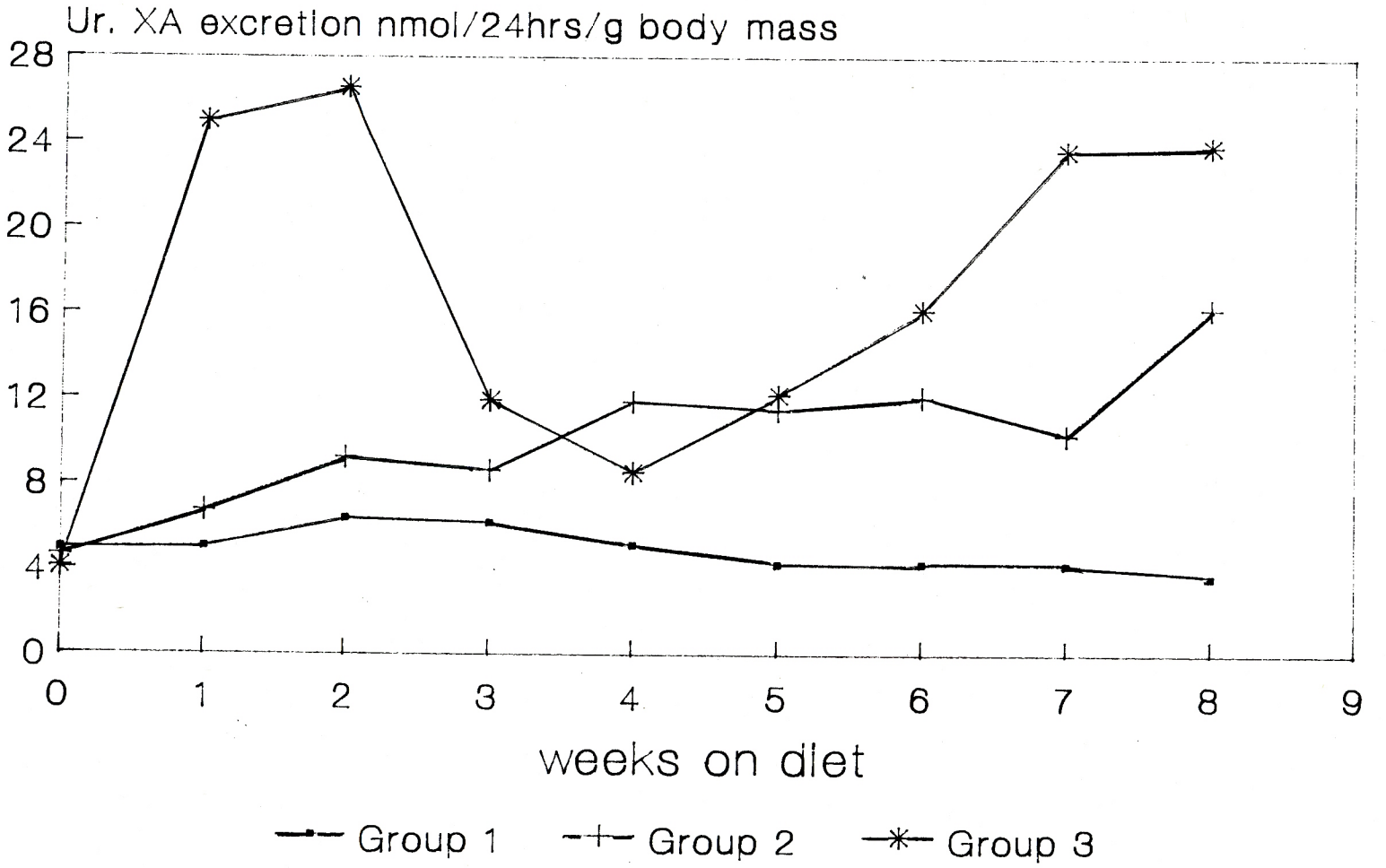
Using urinary XA excretion as an index of vitamin B-6 deficiency, one can conclude that the onset of deficiency for Group 3 was in the first week whereas for Group 2, the deficiency occurred from the fourth week onwards.

TABLE 5. Urinary xanthurenic acid excretion during vitamin B-6 deficiency

GRP	DAY 0	WK 1	WK 2	WK 3	WK 4	WK 5	WK 6	WK 7	WK 8
1	4,93 ± 6,57[a]	5,04 ± 3,17[a]	6,39 ± 2,86[a]	6,16 ± 3,72[a]	5,13 ± 2,45[a]	4,24 ± 2,03[a]	4,28 ± 2,18[a]	4,21 ± 2,35[a]	3,65 ± 3,10[a]
2	4,63 ± 1,83[a]	6,74 ± 3,90[a]	9,23 ± 4,91[a]	8,62 ± 3,68[a]	11,85 ± 7,33[b]	11,45 ± 6,01[b]	12,08 ± 5,74[b]	10,32 ± 5,99[b]	16,24 ± 9,04[b]
3	4,06 ± 2,31[a]	24,98 ± 12,03[b]	26,55 ± 15,82[b]	11,89 ± 6,82[b]	8,54 ± 5,94[a, b]	12,17 ± 7,29[b]	16,15 ± 11,04[b]	23,64 ± 18,70[b]	23,86 ± 23,52[b]

Values are means ± SD expressed in n mol/24 hrs/g body mass for 11 rats per group. Means for each week not followed by the same superscript letter are significantly different ($P < 0,05$). Grp 1 received 3,5 mg/kg of vitamin B-6 in diet; Grp 2 received 1,75 mg/kg vitamin B-6 in diet; Grp 3 received 0 mg/kg vitamin B-6 in diet. Urinary XA excretion for each animal and P values are given in Appendix 3.

Vit.B-6 deficiency & XA excretion



flg.16

D) PLASMA PLP AND PL LEVELS IN VITAMIN B-6 DEFICIENCY

Table 6 shows the mean plasma PLP and PL levels of the 3 groups after 8 weeks on their respective diets.

A statistically significant difference in PLP and PL levels occurs between all 3 groups ($p < 0,05$). Furthermore, a greater difference in PL rather than PLP levels exists between all 3 groups as shown in Fig. 17 and that little proportionality exists between the dietary and plasma levels of the vitamin for the 3 groups.

TABLE 6. Effect of vitamin B-6 deficiency on plasma PLP and PL levels

GRP	PLASMA PLP (ng/ml)	PLASMA PL (ng/ml)	PL/PLP RATIO
1	$46,86 \pm 16,46^{a}$	$63,04 \pm 16,34^{a}$	1,34
2	$11,14 \pm 5,87^{b}$	$12,04 \pm 5,29^{b}$	1,08
3	$3,31 \pm 0,86^{c}$	$2,71 \pm 0,92^{c}$	0,82

Values are expressed as means ± SD with 11 rats in Grp 1 and 2. Grp 3 reflects mean values for 10 rats. Means for each group not followed by the same superscript letter are significantly different ($P < 0,005$). Values are for samples taken from animals at the end of week 8. Grp 1 received 3,5 mg/kg vitamin B-6 in diet; Grp 2 received 1,75 mg/kg in diet; Grp 3 received 0 mg/kg vitamin B-6 in diet.
Using the Mann Whitney test, the two-tail P values for Grps 1 vs 2, 1 vs 3 and 2 vs 3 are all equal to 0,0001. A significant difference exists between groups when $P < 0,0167$.

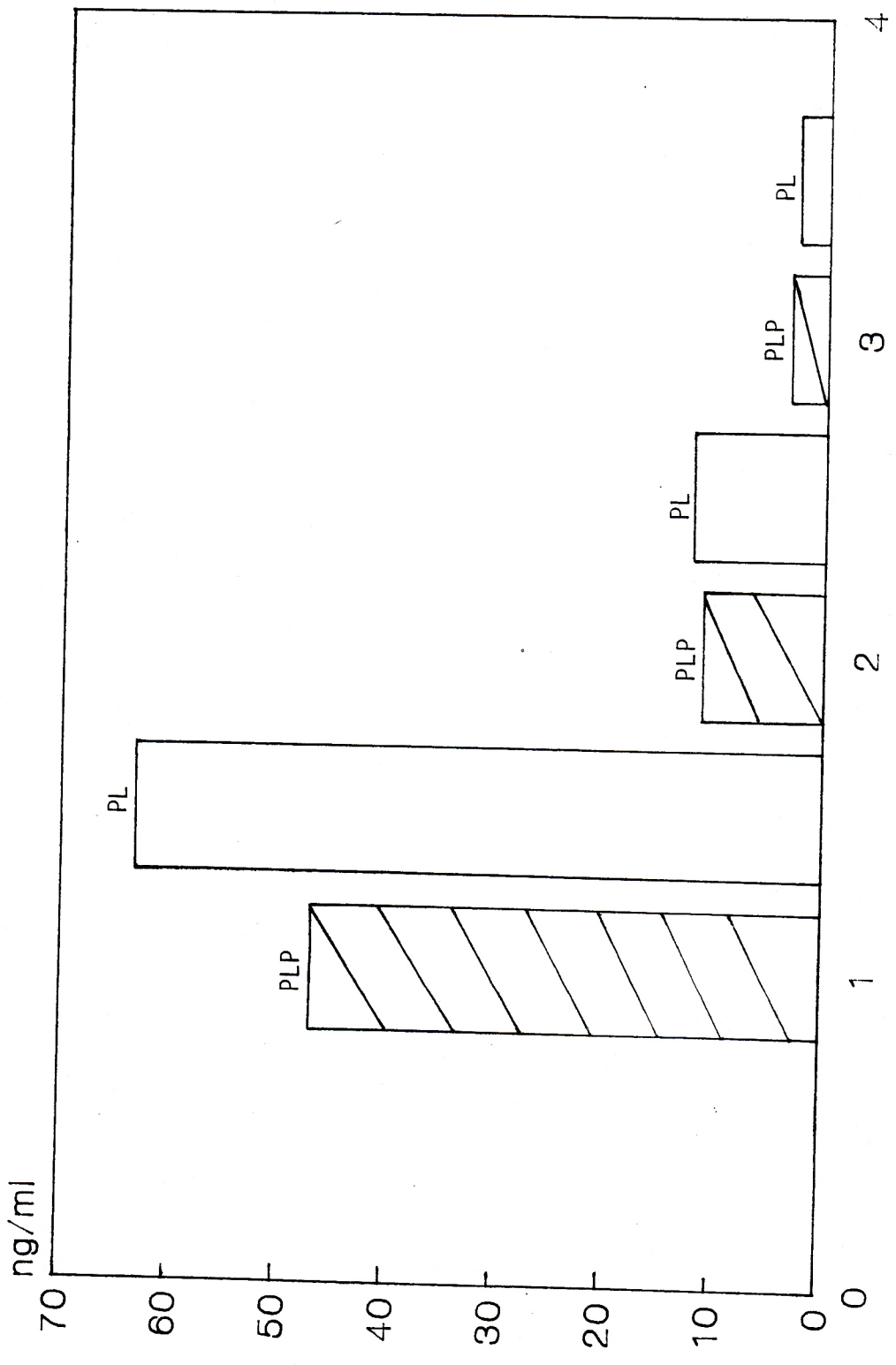

Plasma PLP & PL levels in Vit.B-6 defic.
ng/ml
70
60
50
40
30
20
10
0
PL
PLP
PL
PLP
PL
PLP
0
1
2
3
4
Animal Groups

fig. 17

E) Zn, Cu AND Fe BALANCE DURING VITAMIN B-6 DEFICIENCY

Tables 7, 8 and 9 show mean daily intake, fecal excretion, urinary excretion and balance for Zn, Cu and Fe, respectively over 8 weeks for the 3 groups of animals. These values are the means derived from the corresponding values for each animal over 8 weeks and calculated as follows:

a) Mean daily trace element (TE) intake for each week = Mean daily food consumption (g) (corrected for water content) x TE content of diet (µg/g)

b) Mean daily fecal TE excretion = Mean daily dried fecal mass (g) x TE content of fecal sample (µg/g)

c) Mean daily urinary TE excretion for each week = Mean daily (24 h) urine volume (ml) x TE content of urine sample (µg/ml)

d) Mean daily TE balance for each week = a - b - c

Figures for TE intake and excretion (both fecal and urinary) for each animal and P values are tabulated in Appendices 4, 5 and 6.

From Tables 7, 8 and 9, all animals were in positive balance for Zn, Cu and Fe over the 8 weeks. However, there was a significant difference in the degree of balance with Groups 1 and 2 having a significantly higher Zn, Cu and Fe balance than Group 3 ($p < 0,005$). There was no significant difference in the TE balance between Groups 1 and 2 ($p > 0,05$). Zn and Cu balances in Group 3 decreased from week 4 onwards whereas Fe balance remained more or less the same over 8 weeks.

Groups 1 and 2 exhibit only slight changes in Zn, Cu and Fe balance over the 8 weeks.

TABLE 7. Daily Zn intake, fecal and urinary excretion and balance during a vitamin B-6 deficiency (µg/d)

	WEEK 1				WEEK 2				WEEK 3				WEEK 4			
	INTAKE	FECAL	URINE 24/H	BALANCE	INTAKE	FECAL	URINE 24/H	BALANCE	INTAKE	FECAL	URINE 24/H	BALANCE	INTAKE	FECAL	URINE 24/H	BALANCE
Grp 1	215,55 ± 27,26	66,70 ± 16,48	4,48 ± 1,53	144,37 ± 29,30[a]	237,03 ± 38,11	39,43 ± 10,77	1,91 ± 0,38	195,69 ± 33,46[a]	230,76 ± 37,39	37,53 ± 9,52	1,95 ± 0,66	191,28 ± 32,14[a]	208,81 ± 19,91	44,66 ± 9,41	3,90 ± 1,74	160,25 ± 17,6
Grp 2	219,28 ± 23,88	61,76 ± 17,54	5,69 ± 2,29	151,83 ± 26,25[a]	233,28 ± 28,54	41,09 ± 9,95	2,74 ± 0,97	189,45 ± 25,23[a]	227,19 ± 27,85	40,41 ± 6,81	3,06 ± 0,83	183,72 ± 25,74[a]	210,92 ± 26,65	50,13 ± 10,49	4,80 ± 1,06	155,99 ± 27,
Grp 3	175,39 ± 20,81	54,79 ± 16,33	5,23 ± 1,16	115,37 ± 15,67[b]	141,79 ± 16,79	26,19 ± 10,54	3,38 ± 0,78	112,22 ± 21,74[b]	138,26 ± 15,16	26,96 ± 7,06	3,73 ± 1,10	107,57 ± 16,96[b]	135,52 ± 12,82	38,04 ± 7,30	5,84 ± 1,55	91,64 ± 10,

	WEEK 5				WEEK 6				WEEK 7				WEEK 8			
	INTAKE	FECAL	URINE 24/H	BALANCE	INTAKE	FECAL	URINE 24/H	BALANCE	INTAKE	FECAL	URINE 24/H	BALANCE	INTAKE	FECAL	URINE 24/H	BALANCE
Grp 1	226,83 ± 24,41	59,24 ± 10,82	3,33 ± 1,12	164,26 ± 25,46[a]	261,69 ± 36,49	61,41 ± 10,88	3,91 ± 1,34	196,37 ± 28,98[a]	265,91 ± 38,05	77,57 ± 15,44	3,46 ± 0,64	184,88 ± 34,25[a]	250,73 ± 34,47	85,63 ± 13,98	2,94 ± 0,47	162,16 ± 31,
Grp 2	221,54 ± 19,67	48,38 ± 8,04	3,27 ± 0,64	169,89 ± 15,33[a]	240,20 ± 21,63	55,79 ± 11,46	3,21 ± 0,90	181,20 ± 16,64[a]	240,03 ± 29,95	66,30 ± 13,70	4,01 ± 0,87	169,72 ± 25,14[a]	237,17 ± 14,48	70,45 ± 12,27	5,18 ± 0,23	161,54 ± 17,
Grp 3	133,27 ± 20,29	44,97 ± 7,12	4,51 ± 1,11	83,79 ± 18,52[b]	119,37 ± 12,08	47,18 ± 5,15	3,47 ± 0,93	68,72 ± 10,56[b]	135,87 ± 23,84	49,17 ± 9,91	4,26 ± 0,97	82,44 ± 17,60[b]	121,23 ± 24,01	50,64 ± 8,64	4,03 ± 0,93	66,56 ± 18,

Values are expressed as mean ± SD for 11 rats per group. Mean daily Zn balances for each week not followed by the same superscript letter are significantly different ($P < 0,05$). Figures for individual rats and P values for balances are given in Appendix 4.

TABLE 8. Daily Cu intake, fecal and urinary excretion and balance during a vitamin B-6 deficiency (µg/d)

	WEEK 1				WEEK 2				WEEK 3				WEEK 4			
	INTAKE	FECAL	URINE 24/H	BALANCE	INTAKE	FECAL	URINE 24/H	BALANCE	INTAKE	FECAL	URINE 24/H	BALANCE	INTAKE	FECAL	URINE 24/H	BALANCE
Grp 1	37,47 ± 4,73	11,96 ± 3,46	1,75 ± 0,60	23,76 ± 4,13[a]	41,21 ± 6,59	10,56 ± 1,94	1,49 ± 0,44	29,16 ± 5,71[a]	40,16 ± 6,48	8,09 ± 3,87	1,38 ± 0,68	30,69 ± 8,43[a]	36,30 ± 3,46	8,67 ± 2,54	1,70 ± 0,77	25,93 ± 3,02[a]
Grp 2	38,12 ± 4,14	13,44 ± 2,33	1,94 ± 0,75	22,4 ± 2,79[a]	40,54 ± 4,95	10,69 ± 2,94	1,49 ± 0,69	28,36 ± 3,55[a]	39,54 ± 4,81	10,56 ± 2,36	1,31 ± 0,64	27,67 ± 3,45[a]	36,67 ± 4,62	10,37 ± 2,71	1,72 ± 0,56	24,58 ± 3,92[a]
Grp 3	30,51 ± 3,63	10,94 ± 2,83	1,63 ± 0,25	17,94 ± 3,65[b]	24,64 ± 2,92	5,91 ± 1,90	0,80 ± 0,16	17,93 ± 3,84[b]	24,05 ± 2,64	6,97 ± 2,24	0,84 ± 0,16	16,24 ± 2,68[b]	23,54 ± 2,22	8,40 ± 2,21	0,91 ± 0,19	14,23 ± 1,69[b]

	WEEK 5				WEEK 6				WEEK 7				WEEK 8			
	INTAKE	FECAL	URINE 24/H	BALANCE	INTAKE	FECAL	URINE 24/H	BALANCE	INTAKE	FECAL	URINE 24/H	BALANCE	INTAKE	FECAL	URINE 24/H	BALANCE
Grp 1	39,44 ± 4,25	9,60 ± 2,72	2,10 ± 0,51	27,74 ± 3,29[a]	45,51 ± 6,35	12,07 ± 2,91	1,94 ± 0,45	31,50 ± 4,81[a]	46,19 ± 6,61	12,51 ± 2,85	1,44 ± 0,56	32,24 ± 5,41[a]	43,61 ± 6,01	11,28 ± 2,04	1,98 ± 0,85	30,35 ± 4,59[a]
Grp 2	38,54 ± 3,39	9,87 ± 2,68	1,90 ± 0,33	26,77 ± 2,27[a]	41,80 ± 3,75	13,01 ± 3,46	2,33 ± 0,72	26,46 ± 4,02[b]	41,74 ± 5,25	12,20 ± 2,70	1,47 ± 0,60	28,07 ± 4,08[a]	41,24 ± 2,52	11,18 ± 3,15	1,65 ± 0,60	28,41 ± 3,42[a]
Grp 3	23,20 ± 3,54	7,94 ± 2,11	1,01 ± 0,26	14,25 ± 2,36[b]	20,75 ± 2,11	6,90 ± 1,07	1,03 ± 0,18	12,82 ± 1,65[c]	23,66 ± 4,22	7,50 ± 2,14	1,00 ± 0,31	15,16 ± 3,39[b]	21,08 ± 4,18	6,89 ± 1,53	0,91 ± 0,42	13,28 ± 3,10[b]

Values are expressed as mean ± SD for 11 rats per group. Mean daily Cu balances for each week not followed by the same superscript letter are significantly different ($P < 0,05$). Figures for individual rats and P values for balances are given in Appendix 5.

TABLE 9. Daily Fe intake, fecal and urinary excretion and balance during a vitamin B-6 deficiency (µg/d)

	WEEK 1				WEEK 2				WEEK 3				WEEK 4			
	INTAKE	FECAL	URINE 24/H	BALANCE	INTAKE	FECAL	URINE 24/H	BALANCE	INTAKE	FECAL	URINE 24/H	BALANCE	INTAKE	FECAL	URINE 24/H	BALANCE
Grp 1	1844,99 ± 233,27	1011,56 ± 243,64	39,35 ± 24,11	794,08 ± 113,21[a]	2029,13 ± 325,92	1208,27 ± 216,59	24,91 ± 7,45	795,45 ± 155,31[a]	1975,32 ± 319,72	1126,93 ± 174,01	24,82 ± 8,77	823,57 ± 165,81[a]	1787,34 ± 170,42	1021,46 ± 138,02	22,09 ± 11,55	743,79 ± 108,88[a]
Grp 2	1876,76 ± 204,26	1062,52 ± 194,31	35,54 ± 13,03	778,70 ± 189,93[a]	1996,54 ± 244,20	1182,09 ± 169,30	33,10 ± 7,30	781,35 ± 142,40[a]	1944,64 ± 238,37	1074,20 ± 110,87	34,77 ± 8,16	835,67 ± 149,72[a]	1805,56 ± 228,07	1005,62 ± 154,46	24,90 ± 12,42	775,04 ± 178,26[a]
Grp 3	1501,59 ± 178,11	906,68 ± 149,73	24,79 ± 7,16	570,12 ± 110,04[b]	1213,76 ± 143,62	705,70 ± 86,42	32,93 ± 10,23	475,13 ± 141,65[b]	1183,75 ± 129,84	660,32 ± 188,19	27,66 ± 8,90	495,77 ± 141,87[b]	1159,76 ± 109,54	642,36 ± 106,31	24,06 ± 5,12	493,34 ± 76,76[b]

	WEEK 5				WEEK 6				WEEK 7				WEEK 8			
	INTAKE	FECAL	URINE 24/H	BALANCE	INTAKE	FECAL	URINE 24/H	BALANCE	INTAKE	FECAL	URINE 24/H	BALANCE	INTAKE	FECAL	URINE 24/H	BALANCE
Grp 1	1941,76 ± 208,63	1034,72 ± 331,89	39,94 ± 20,32	867,10 ± 334,96[a]	2240,01 ± 312,33	1240,44 ± 399,29	23,41 ± 11,80	976,16 ± 291,34[a]	2276,39 ± 325,95	1384,62 ± 237,34	23,98 ± 10,45	867,79 ± 219,33[a]	2146,09 ± 294,95	1309,89 ± 185,65	29,43 ± 4,06	806,77 ± 135,99[a]
Grp 2	1896,13 ± 168,21	1052,82 ± 148,27	29,85 ± 11,89	813,46 ± 96,22[a]	2056,13 ± 185,10	1188,48 ± 153,37	24,60 ± 8,06	843,05 ± 132,88[a]	2054,46 ± 256,57	1226,94 ± 170,75	22,35 ± 9,07	805,17 ± 215,38[a]	2029,53 ± 123,26	1160,74 ± 107,52	37,63 ± 9,12	831,16 ± 147,50[a]
Grp 3	1140,74 ± 173,59	654,83 ± 116,65	16,01 ± 4,03	469,90 ± 124,76[b]	1022,15 ± 103,51	583,54 ± 51,90	13,80 ± 5,63	424,81 ± 91,42[b]	1162,86 ± 203,91	655,15 ± 106,03	16,64 ± 4,61	491,07 ± 149,63[b]	1037,68 ± 205,75	576,34 ± 90,05	22,94 ± 3,47	438,40 ± 141,85[b]

Values are expressed as mean ± SD for 11 rats per group. Mean daily Fe balances for each week not followed by the same superscript letter are significantly different ($P < 0,05$). Figures for individual rats and P values for balances are given in Appendix 6.

F) Zn, Cu AND Fe EXCRETION DURING VITAMIN B-6 DEFICIENCY

An alternative approach to TE balance would be to express daily TE excretion as a percentage of the daily intake.

Table 10 shows the mean daily TE excretion as a percentage of the mean daily intake over 8 weeks for the 3 groups of animals.

For each animal the corresponding TE excretion was calculated as:

$$\frac{(\text{Mean daily fecal excretion } (\mu g) + \text{Mean daily urinary excretion } (\mu g)) \times 100}{\text{Mean daily intake } (\mu g)}$$

The data in Table 10 is graphically represented in Figs. 18a, b and c.

P values are given in Appendix 7.

A significant difference in Zn excretion occurs between Groups 1 and 3 and 2 and 3 from the fourth week onwards, with the vitamin B-6 deficient Group 3 having a higher level of Zn excretion than Groups 1 and 2 ($p < 0,05$). No significant difference in Zn excretion occurs between Groups 1 and 2 over the 8 weeks ($p > 0,05$). A similar profile from the fourth week onwards occurs for Cu excretion except that at week 7 there is no significant difference in Cu excretion between all 3 groups.

With regards to Fe excretion, no significant difference exists between the groups ($p > 0,05$), with all 3 groups showing a remarkably equal and consistent level of Fe excretion over the 8 weeks.

TABLE 10. Zn, Cu and Fe excretion during vitamin B-6 deficiency

	WK 1	WK 2	WK 3	WK 4	WK 5	WK 6	WK 7	WK 8
Zu Excretion								
Grp 1	33,38 ± 8,61[a]	17,53 ± 3,90[a]	17,17 ± 3,01[a]	23,26 ± 4,10[a]	27,85 ± 5,34[a]	24,98 ± 2,70[a]	30,64 ± 5,57[a]	35,65 ± 5,89[a]
Grp 2	30,86 ± 8,08[a]	18,78 ± 3,74[a]	19,26 ± 2,95[a]	26,33 ± 5,60[a]	23,29 ± 2,71[a]	24,49 ± 3,90[a]	29,39 ± 4,65[a]	31,94 ± 5,05[a]
Grp 3	34,04 ± 7,01[a]	21,29 ± 8,94[a]	22,52 ± 6,13[a]	32,38 ± 4,20[b]	37,66 ± 6,41[c]	42,63 ± 3,90[b]	39,51 ± 4,35[b]	45,81 ± 6,44[b]
Cu Excretion								
Grp 1	36,59 ± 7,49[a]	29,60 ± 4,64[a]	24,52 ± 10,32[a]	28,40 ± 6,62[a]	29,57 ± 5,17[a]	30,83 ± 4,09[a]	30,34 ± 4,43[a]	30,48 ± 2,76[a]
Grp 2	40,33 ± 4,16[a]	29,91 ± 4,32[a]	29,99 ± 3,12[a]	32,99 ± 6,21[a]	30,40 ± 4,73[a]	36,75 ± 7,48[a]	32,73 ± 4,99[a]	31,12 ± 7,14[a]
Grp 3	41,35 ± 9,46[a]	27,72 ± 8,96[a]	32,51 ± 8,09[a]	39,29 ± 6,93[b]	38,40 ± 6,07[b]	38,28 ± 3,58[b]	36,05 ± 6,19[a]	37,21 ± 5,59[b]
Fe Excretion								
Grp 1	56,40 ± 7,73[a]	60,86 ± 4,16[a]	58,43 ± 3,42[a]	58,33 ± 5,06[a]	55,46 ± 16,94[a]	55,94 ± 14,23[a]	62,03 ± 6,94[a]	62,46 ± 3,15[a]
Grp 2	58,52 ± 8,89[a]	60,88 ± 4,83[a]	57,25 ± 3,38[a]	57,29 ± 7,26[a]	56,98 ± 4,89[a]	58,99 ± 5,53[a]	61,19 ± 7,49[a]	59,18 ± 5,66[a]
Grp 3	62,00 ± 6,01[a]	61,32 ± 8,47[a]	57,74 ± 12,66[a]	57,31 ± 6,11[a]	59,05 ± 7,35[a]	58,74 ± 5,27[a]	58,29 ± 6,79[a]	58,56 ± 7,57[a]

Excretion as expressed as percentage of mean daily intake ± SD for 11 rats per group. Mean daily values for each week that are not followed by the same superscript letter are significantly different ($P < 0,05$). P values are given in Appendix 7.

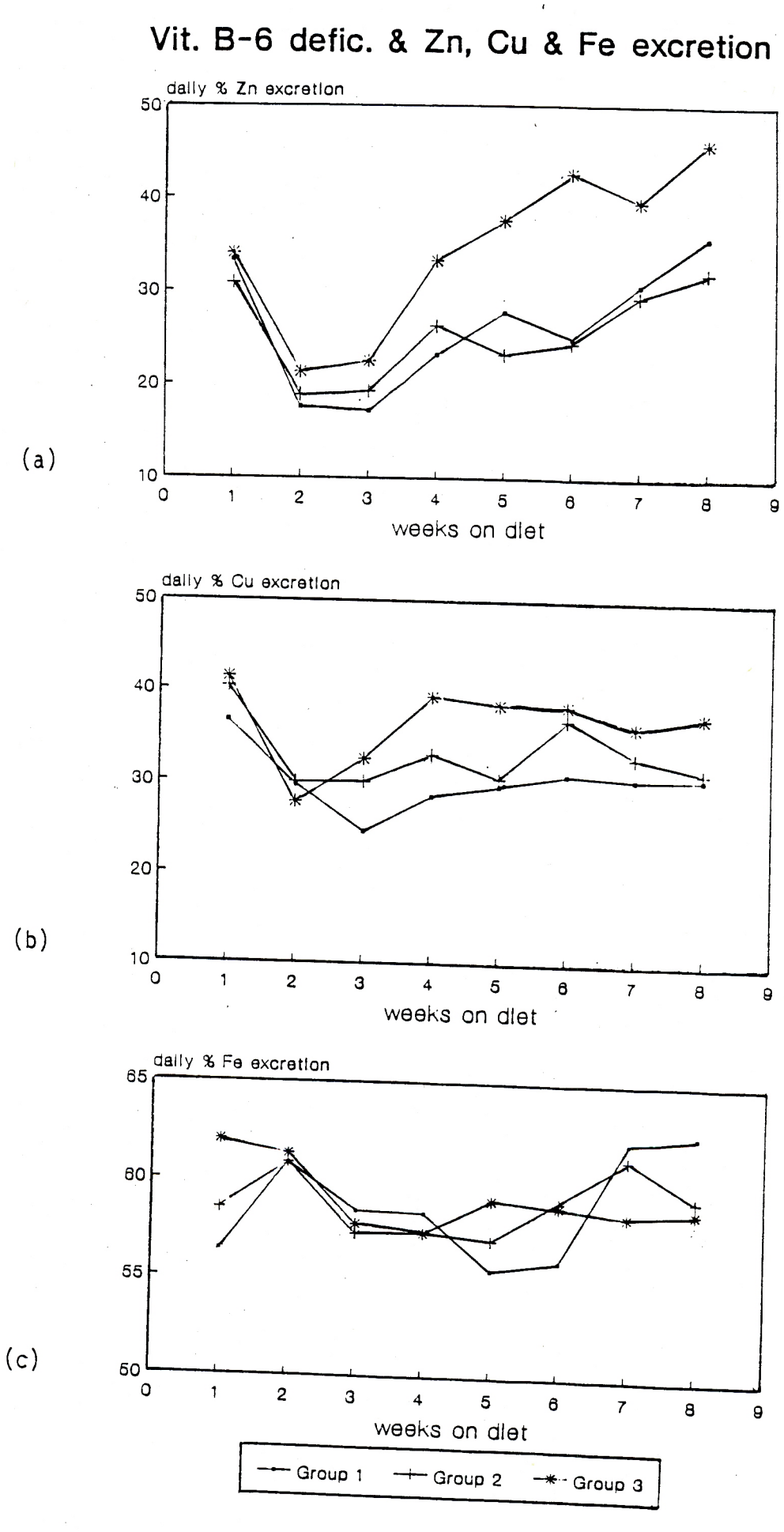

Vit. B-6 defic. & Zn, Cu & Fe excretion
dally % Zn excretion
50
40
30
20
10
0 1 2 3 4 5 6 7 8 9
weeks on diet
(a)
dally % Cu excretion
50
40
30
20
10
0 1 2 3 4 5 6 7 8 9
weeks on diet
(b)
dally % Fe excretion
65
60
55
50
0 1 2 3 4 5 6 7 8 9
weeks on diet
(c)
Group 1
Group 2
Group 3

FIG. 18.

G) CONFIRMATION OF THE STATISTICAL ANALYSIS

After the Mann-Whitney U test was applied to the different parameters to test for significant differences at specific points during the 8 weeks, Simpson's rules to calculate the entire area under each curve was applied to the same data.

Significant differences corresponding to that obtained by the Mann-Whitney tests were obtained for growth, food consumption, XA excretion, TE balance and TE excretion.

IV. DISCUSSION

A) GROWTH AND FOOD CONSUMPTION

Growth retardation and anorexia set in rapidly during the first week and acrodynia in the fourth week in Group 3 which was subjected to a total chronic vitamin B-6 deficiency. The marginally deficient group (Group 2) showed no signs of growth retardation, acrodynia or decreased food consumption throughout the 8 weeks on the diet when compared with the control group receiving the adequate vitamin B-6 diet (Group 1).

The speed of onset of the above deficiency symptoms depended also on whether the animals are in the weanling or the post-weaning stage before they are fed on the deficient diet. In this study the rats used were in the post-weaning stage with an average mass of 90 g. In a preliminary unpublished study with weanling rats with an average mass of 50 g, the deficiency symptoms in the totally deficient group appeared only after 6 weeks. Lumeng et al. (1978) also observed growth retardation after 4 weeks using weanling animals in a similar study. The time lag in the appearance of symptoms in the weanling rats is possibly due to them having higher body stores of vitamin B-6 as a result of the more recent access to the vitamin B-6 rich maternal milk when compared to the post-weaning animals. This store sustains them through the initial rapid growth spurt in the first few weeks even if the animals were deprived of dietary vitamin B-6.

Group 2 received approximately 21 µg/d of PN.HCl and exhibited no significant difference in growth when compared to Group 1 receiving approximately 42 µg/d of vitamin B-6. This is apparently in confirmation of the studies by Lumeng et al. (1978) and Van Den Berg et al. (1982)

who reported that a minimum of 29 µg/d and 24 µg/d respectively was required for maximal growth in rats.

With regards to food intake, Group 3 consumed about 8 g/d as compared to 13-14 g/d in the other 2 groups. These figures compare well with those obtained by Prasad et al. (1982) but contrast with those of Chang et al. (1981), where no significant difference in food intake was reported to exist between rats fed diets with 7,0, 1,0 or 0,6 mg of PN.HCl/kg of diet.

From the above discussion, it would appear that growth and food consumption can only be used as indicators of vitamin B-6 deficiency under conditions of chronic total deficiency rather than under conditions of marginal deficiency where no retardation of growth or decrease in food consumption is observed.

B) XA EXCRETION AND PLASMA PL AND PLP LEVELS

Although there was the expected considerable individual variation in the amount of XA excreted spontaneously (Milholland et al., 1971), urinary XA still provided a convenient indicator of the vitamin B-6 status of the animals. In Fig. 16, XA excretion increased dramatically in the totally deficient Group 3, increased gradually from the fourth week onwards in Group 2 and remained almost constant over the 8 weeks in control Group 1.

The inverse relationship between the plasma vitamer levels at the end of week 8 and the XA excretion for that week in Groups 2 and 3 confirmed that the deficiency was already firmly entrenched at that stage (Fig. 17). From the trends in the XA level excretion, it could be assumed that the PL and PLP levels in Group 2 were probably equivalent to that of Group 1

up to the fourth week after which they dropped to levels near to those of Group 3 and indicative of a vitamin B-6 deficiency.

The increased excretion of XA during vitamin B-6 deficiency may be seen as anomalous since PLP is also required for XA formation in the tryptophan metabolic pathway (Fig. 4). This anomaly is explained by enzyme studies which show that the first enzyme in the pathway to lose its PLP is kynureninase. This results in increased 3-hydroxy kynurenine concentration. Furthermore, during vitamin B-6 deficiency, the mitochondrial kynurenine aminotransferase binds its PLP more tightly than the cytosolic aminotransferase and the increased concentration of hydroxykynurenine results in enhanced XA formation and excretion (Brown, 1985). However, during severe chronic vitamin B-6 deficiency as seen in this study, the initially high XA excretion also decreased as the body stores of vitamin B-6 progressively become depleted.

The effect of stress or cortisol induced tryptophan pyrrolase activity must also be considered when XA excretion is used as an index of vitamin B-6 deficiency. Tryptophan pyrrolase (L-tryptophan-2,3-dioxygenase) gives rise to kynurenine (Fig. 4) and is an inducible enzyme which can increase many fold in response to its substrate or in response to induction by cortisol or by conditions which may increase cortisol. Therefore in animals under stress or illness, spontaneous XA excretion or excretion in response to tryptophan loading may be as a result of the induction of the enzyme rather than the result of vitamin B-6 deficiency (Brown, 1981). Millholland *et al.* (1971) however, have shown this possibility is unlikely in rats when vitamin B-6 deficient animals injected with cortisol reflected no increase in XA excretion.

Because invasive procedures on the animals during the study were excluded, blood samples were taken from the animal only at the end of the study and the plasma was analysed for PL and PLP. The B-6 vitamers are notorious for their instability and photosensitivity. This problem was overcome by the method of Ubbink et al. (1985) in which the semicarbazone forms of PL and PLP enabled HPLC analysis to be carried out under normal light and temperature conditions.

The PL/PLP ratio declined progressively from Group 1 to Group 3. Group 1 and 2 had higher PL levels than PLP whereas in Group 3 the opposite was true. The higher PLP levels in Group 3 may indicate the possibility that PL is ultimately the transport form of the vitamin to be taken up by the tissues even during deficiency.

Furthermore, on observing the levels of PL and PLP in the deficient animals, the possibility of the gut microflora contributing to any significant level to the vitamin B-6 status of the animals seemed very unlikely.

C) TRACE ELEMENT (TE) BALANCE

In this study all animals were in a positive balance with regards to Zn, Cu and Fe during the 8 weeks but Groups 1 and 2 were in greater positive balance for all 3 elements when compared to Group 3. There was no significant difference in TE balance between Groups 1 and 2.

Normally, in a well nourished adult animal which is not growing and which maintains approximately the same weight over long periods of time, body composition is usually constant and therefore the nutrient intake must approximate the amounts that is lost from the body (Hegsted, 1976b). However, in the same animal, nutrient retention must occur during growth or pregnancy. This accounts for the positive balances obtained for all

groups which were made up of animals undergoing their initial growth spurt.

The differences in the degree of the positive balances between the groups could at this stage be explained in terms of nutrient intake. Groups 1 and 2 showed no significant difference in TE intake and excretion and therefore had a similar level of TE balance. Group 3 had a lower intake with a correspondingly lower excretion and subsequently a less positive balance.
In most balance studies, the phenomenon that higher levels of dietary intake of a substance tend to produce increasingly positive balance values still remains unexplained (Beisel, 1979).

Under the best circumstances, balance studies can only define the quantity of an assayed substance that enters or leaves the body during a given period of time. The difference between these values is used to estimate how much of the assayed substance is retained or lost. Balance type data have no value for defining internal body distribution, pool sizes, turnover rate, molecular uses or metabolic roles of the substance being studied. Furthermore, the simple subtraction of measured fecal/urinary loss from oral intake does not allow one to calculate a true intestinal absorption value, as the magnitude of any endogenous contribution to the excreted material cannot be determined by balance measurements alone (Beisel, 1979). Nevertheless, in spite of some shortcomings, the balance study approach in the present study enabled TE balances to be followed longitudinally throughout the course of the vitamin B-6 deficiency and conclusively indicated that all animals retained rather than lost more Zn, Cu and Fe throughout the important growth phase in spite of the vitamin B-6 deficiency.

D) TRACE ELEMENT EXCRETION

Another more specific view of the animals TE status would be to determine an animal's daily TE excretion as a percentage of its daily intake. This procedure would enable one to determine to what extent the TE excretion contributed to final balance, especially if all the animals were in positive balance as in this study. In this study it was determined that Group 3 excreted a higher percentage of Zn and Cu in relation to its intake when compared to the other 2 groups which showed no significant difference in their percentage excretion. The implication is that the group with a less positive balance actually lost more Zn and Cu in relation to its intake when compared to the other 2 groups. The increased excretion,coupled with a decreased food intake, was the main factor that lowered the TE balance in Group 3. Furthermore, a marginal B-6 deficiency had no effect on Zn and Cu excretion as seen in Group 2. Percentage Fe excretion however, was remarkably constant in all 3 groups indicating the operation and intactness of some homeostatic control mechanism in all 3 groups.

1. Zn and Cu Excretion

The major excretory route for orally injested Zn and Cu is via the feces. Approximately 80% of the excreted Cu makes its way into the gut via the bile and the rest is via direct excretion into the bowel. Cu homeostasis is maintained almost exclusively by biliary excretion (Kay, 1981). Zn is secreted in the pancreatic juice and to a small extent in the bile and the feces is therefore, also the major route of excretion. As reflected in this study, only a small amount of Zn and Cu are excreted in the urine when compared to fecal excretion.

After dropping from an initially high level in week 1, mean daily Zn excretion in Groups 1 and 2 showed a small regular rise throughout the 8 weeks (Fig. 18). Compared to Groups 1 and 2, Group 3 however, showed a significant rise in Zn excretion from the fourth week onwards, from where it continued to increase from 32% of the daily intake until it reached approximately 45% at week 8. A similar but lower trend was observed for Cu with a maximum excretion of 37% at week 8.
Hsu (1965) reported an enhanced excretion of Zn from the body tissues into the gut during vitamin B-6 deficiency. This study also reports increased Zn excretion during vitamin B-6 deficiency, but the quantification of the endogenous zinc in relation to the unabsorbed zinc, was unfortunately beyond the scope of this study. The high levels of Zn and Cu excretion during the first week in all animals could be ascribed to the decreased efficacy of dietary absorption due to the animals going through the process of adapting to the diet. One can also conclude from this study that a marginal vitamin B-6 deficiency (Grp 2) has no effect on Zn and Cu excretion.

There are three possible explanations for increased Zn excretion during vitamin B-6 deficiency that could be considered.

(a) Fe-Zn interaction

Enhanced Zn excretion during vitamin B-6 deficiency could be due to the inhibitory effect of the high dietary Fe on intestinal Zn absorption due to Fe binding competitively with picolinic acid, the proposed zinc binding ligand (Evans et al., 1981). The diets used in this study had a Fe content of 157 µg/g, a level considerably higher than the normal physiological levels of approximately 35 µg/g of diet. But Zn excretion only increased after the animals were on the diet for 4 weeks! It

therefore seems unlikely that the direct effects of competitive binding of Fe to picolinic acid would take that long to manifest itself. Furthermore, Fe excretion in Group 3 remained constant even after Zn excretion increased. If competitive binding of Fe had any effects on Zn absorption, one would at least expect increased Fe absorption (or a decreased excretion) once Zn excretion increased.

(b) Alteration in the intestinal mucosa during vitamin B-6 deficiency

It is proposed that impaired Zn absorption in vitamin B-6 deficiency could be due to a breakdown in the intestinal mucosa (Hurley et al., 1982; Rebello et al., 1982), or as a result of alterations in the brush border membrane (Prasad et al., 1982), as a direct result of a vitamin B-6 deficiency. However, the above effect in the present study seem highly unlikely because Fe excretion (and therefore absorption) remained more or less constant for all 3 groups, indicating the presence of an intact control mechanism at intestinal level where the regulation of Fe absorption occurs. Alterations or breakdown in the intestinal mucosa would in all probability lead to a non-selective malabsorption of all elements.

(c) The tryptophan-picolinic acid connection

The most plausible explanation of increased Zn excretion during vitamin B-6 deficiency seems to be the "tryptophan-picolinic acid connection" (Evans, 1980; Evans et al., 1980; 1981). According to this hypothesis, picolinic acid, a tryptophan metabolite, is produced in the excocrine cells of the pancreas and then secreted into the lumen of the intestine, where it binds to zinc to form a complex that facilitates the passage of zinc through the luminal membrane across the absorptive cell and

through the basolateral membrane of the cell. Alternately at the basolateral membrane, ligands may bind to zinc which is then transferred to transferrin. In humans and animals consuming physiological levels of zinc, the quantity of zinc transported is directly related to the availability of picolinic acid which in turn depends upon the level of dietary tryptophan, pyridoxine and cations that compete with zinc for binding. In a vitamin B-6 deficiency, tryptophan metabolism is altered, leading to a decreased synthesis of picolinic acid. This in turn leads to a decreased absorption of dietary zinc as a result of which there is an increased level of unabsorbed zinc being excreted via the feces. It must be noted that this hypothesis accounts for only dietary zinc and does not explain the increase in endogenous zinc excretion or increased levels to tissue zinc during vitamin B-6 deficiency as reported by Hsu (1965) and Prasad et al. (1982), respectively.

There are two possible explanations for the increased Cu excretion accompanying increased Zn excretion during vitamin B-6 deficiency. Firstly, picolinic acid, in addition to its role in Zn absorption, may also be involved in Cu absorption since Cu also has a high association constant with picolinic acid (Evans et al., 1980). A deficiency of picolinic acid during vitamin B-6 deficiency will therefore impair both Cu and Zn absorption.

The second explanation involves the existence of a mechanism of mutual antagonism between Zn and Cu absorption in the intestinal lumen. It is well established that a dietary deficiency or excess of some micronutrient creates a secondary disorder in absorption, plasma or tissue levels and excretion of other minerals and metabolites (Sherman et al., 1981). It is reported that high concentrations of Zn in the lumen inhibit the intestinal uptake of Cu. Cu absorption in the intestine

may be inhibited when dietary or intraluminal Zn:Cu ratios range as high as 1000:1 or as low as 30:1 (Ostreicher et al., 1985). The high levels of unabsorbed luminal Zn may therefore be having an inhibitory effect on Cu absorption during vitamin B-6 deficiency.

In the light of the above discussion, the scheme in Fig. 19 summarizes the possible effects a vitamin B-6 deficiency may have on Zn and Cu absorption in the intestine.

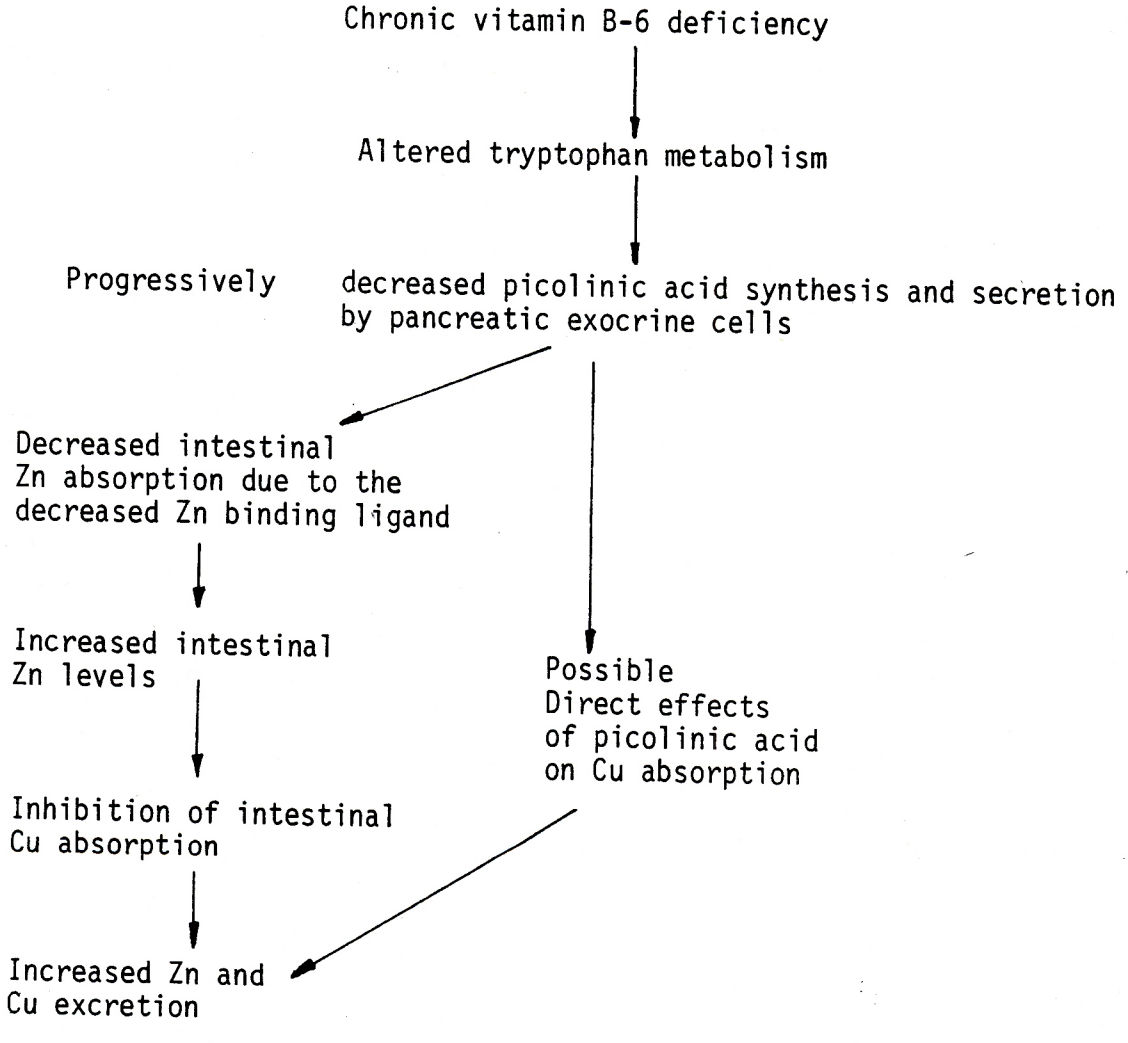


FIG. 19. A scheme of possible effects of a vitamin B-6 deficiency on intestinal Zn and Cu absorption.

2. Fe Excretion

Fe excretion is also mainly via the feces but a major part of this consists of Fe from unabsorbed food. True excretion of Fe occurs mainly by the sloughing off of intestinal and other Fe containing cell which are then incorporated into the feces. In normal rats, the ultimate regulator of iron absorption is the iron concentration of the epithelial cells of the upper intestine. In normal rats only a small part of the ingested iron taken up by the mucosal cells is transferred to the blood in accordance with the bodies Fe needs. The remainder stays in the mucosal cells and is lost into the gut lumen when the cells are sloughed off from the tip of the intestinal villi (Prasad, 1978).

With regards to Fe excretion in this study, no significant difference in excretion was found between all 3 groups with each group excreting almost the same percentage of its Fe intake. In other words, although Group 3 ingested proportionally less iron than Groups 1 and 2, the percentage of excreted iron in relation to the ingested iron was the same as in the other 2 groups (Fig. 18). From these findings one can tentatively agree with other reports that no impairment in Fe absorption occurs during vitamin B-6 deficiency (Yeh et al., 1962; Kirksey et al., 1967).

E) CONCLUSION

Although all 3 animal groups remained in positive trace element balance throughout the study, Group 3 animals showed a lower positive balance when compared to the other 2 groups. Although decreased food intake contributes to a lower balance, this study showed that increased excretion of Zn and Cu with regards to the intake also contributed to the

lowering of the TE balance in Group 3. There was no difference in Fe excretion between all 3 groups. The marginally deficient Group 2 showed no significant difference in TE balance or excretion when compared to the control group indicating that even subminimal levels of the vitamin still exert important beneficial effects. Regardless of the mechanism, the present study as well as previous ones strongly suggest that the nutritional status of TE, especially Zn and Cu are related to the vitamin B-6 status of the animal (Brown, 1985).

The findings in the present study do not by any means present a complete picture of the TE or tissue vitamer status during a vitamin B-6 deficiency in the rat. It is recommended that the following aspects need to be investigated if studies similar to the present ones are initiated in the rat:

1. The extent of the contribution of endogenous Zn, Cu and Fe to the total TE excretion during a vitamin B-6 deficiency;
2. The various tissue levels of Zn, Cu and Fe during a vitamin B-6 deficiency;
3. The fate of ingested PN-HCl during a vitamin B-6 deficiency;
4. Longitudinal profiles of tissue and plasma PL and PLP during a vitamin B-6 deficiency.

SUMMARY

In order to address the contradictory reports on the rat trace element status during a vitamin B-6 deficiency, Zn, Cu and Fe balance was assessed over 8 weeks in 3 groups of young male rats.
Group 1 was the control group fed on a diet supplemented with 3,5 mg/kg of vitamin B-6. Group 2 was the marginally deficient group, fed a diet supplemented with 1,75 mg/kg of vitamin B-6 and Group 3 was the totally deficient group without any vitamin B-6 in the diet.
Diet, urine and fecal samples were analysed to determine the mean daily Zn, Cu and Fe balance for each group during each week of the study. Urinary xanthurenic acid (XA), plasma pyridoxal (PL), and plasma pyridoxal 5'-phosphate (PLP) were also analysed in order to determine the vitamin B-6 nutritional status of each animal group.

The totally deficient Group 3 showed decreased growth and food consumption when compared to the control and marginally deficient groups. There was a significant difference in XA excretion and plasma PLP and PL levels between all 3 groups with a progressive increase in XA excretion and a progressive decrease in PLP and PL levels from Group 1 to Group 3. Although all groups were found to be in a positive balance for Zn, Cu and Fe, the totally deficient group was in a less positive balance compared to Groups 1 and 2. Furthermore, the percentage excretion of Zn and Cu, when compared to the intake, was greater in this group. This increased excretion, coupled with a decreased food intake, accounted for the lowered Zn and Cu balances in the totally deficient group. Fe excretion did not differ significantly between all 3 groups. The marginally deficient Group 2 showed no significant difference in growth, food consumption, trace element balance and excretion when compared to the control group, indicating the beneficial effects of

even subminimal levels of vitamin B-6. This study confirms that the nutritional status of trace elements, especially that of Zn and Cu, is related to the vitamin B-6 status of the animal.

ACKNOWLEDGEMENTS

I would like to express my sincere thanks to the following colleagues and friends who made this study possible:

Professor F.J. Burger of the Department of Human Physiology, University of Durban-Westville, whose ideas and help both initiated and sustained this study;

Professor J.B. Ubbink of the Department of Chemical Pathology, University of Pretoria who provided the much needed knowledge, advice, training and facilities for the vitamin B-6 and xanthurenic acid analysis;

Dr S.J. Reinach of the Institute of Biostatistics, Medical Research Council, who was responsible for the computerised statistical analysis;

Mr A. Raghavan and Miss Irene Mackraj for expert help with the animals;

Mr Anand Nadar who so willingly provided the extra pair of hands when needed the most;

My colleagues in the Department of Human Physiology, Miss Kogi Moodley, Miss Kogie Raidoo, Messrs K. Annamalai, M. Allee, M. Manjoo, S. Reddy and G. Muthuveran who helped, supported and advised throughout the study;

Mrs P.V. Subramony for the typing;

My family for their patience, encouragement and moral support.

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APPENDIX 1 : GROWTH : WEEKLY INDIVIDUAL ANIMAL MASS (g)

GRP	NO.	DAY 0	WK 1	WK 2	WK 3	WK 4	WK 5	WK 6	WK 7	WK 8
1	1	76	123	154	180	197	213	236	264	280
	2	78	134	177	204	219	236	269	276	286
	3	84	123	165	194	214	234	259	278	286
	4	96	168	220	259	276	300	332	372	392
	5	88	146	190	226	247	276	306	316	338
	6	86	150	200	234	244	270	310	336	360
	7	78	130	164	194	212	232	256	272	290
	8	88	144	190	218	240	258	282	296	308
	9	99	158	200	232	256	276	300	319	329
	10	94	144	175	192	210	220	236	257	266
	11	92	155	198	228	257	278	298	318	330
2	1	98	150	188	222	240	266	284	302	315
	2	100	148	186	228	226	250	269	292	304
	3	76	123	158	175	195	217	227	246	262
	4	82	126	160	179	202	218	244	265	277
	5	100	156	202	236	269	296	319	340	358
	6	80	137	169	200	225	242	264	282	298
	7	102	158	200	228	257	280	298	318	331
	8	102	148	185	218	232	244	268	284	290
	9	98	155	201	228	258	280	302	324	336
	10	97	162	208	234	248	274	298	317	326
	11	79	142	183	213	238	264	286	298	306
3	1	90	125	140	148	154	150	151	158	163
	2	96	140	162	173	178	184	178	173	166
	3	82	120	124	132	138	139	134	138	132
	4	86	116	138	150	157	158	152	152	155
	5	84	124	140	150	154	150	150	151	154
	6	80	110	122	130	134	132	134	136	136
	7	84	136	148	157	162	164	171	177	182
	8	90	137	160	160	168	175	169	174	153
	9	95	131	137	144	152	154	154	154	155
	10	79	118	131	140	144	136	144	146	143
	11	91	122	136	142	148	152	150	158	159

APPENDIX 1 : CONTINUED

Two-tailed P-values for Table 3 - Effect of vitamin B-6 deficiency on percentage body mass gained

GRP	WK 1	WK 2	WK 3	WK 4	WK 5	WK 6	WK 7	WK 8
1 vs 2	0,1678	0,0709	0,0527	0,1228	0,1783	0,0613	0,0613	0,0940
1 vs 3	0,0004	0,0001	0,0001	0,0001	0,0001	0,0001	0,0001	0,0001
2 vs 3	0,0028	0,0001	0,0001	0,0001	0,0001	0,0001	0,0001	0,0001

A significant difference exists between groups if the P value is < 0,0167 $(\frac{0,05}{3})$.

APPENDIX 2 : FOOD CONSUMPTION : MEAN DAILY INDIVIDUAL FOOD CONSUMPTION (g) OVER 8 WEEKS

GRP	NO.	WK 1	WK 2	WK 3	WK 4	WK 5	WK 6	WK 7	WK 8
1	1	10,4	10,2	10,3	10,9	12,7	11,7	12,2	12,9
	2	11,6	13,0	13,2	10,8	11,9	14,0	14,3	12,8
	3	10,0	11,2	11,9	11,1	13,9	13,9	14,4	13,0
	4	15,0	17,4	17,2	12,7	14,1	17,8	20,5	18,6
	5	12,7	14,3	14,8	12,9	15,1	17,6	16,1	16,2
	6	13,4	16,1	14,9	12,4	13,4	17,1	16,3	15,7
	7	10,7	11,2	11,4	10,4	12,1	13,0	14,6	12,7
	8	12,4	14,0	13,7	12,7	12,0	15,1	13,5	12,9
	9	13,0	13,5	13,7	13,1	13,8	16,6	16,6	15,1
	10	12,8	13,6	10,2	11,2	10,1	12,8	14,0	12,8
	11	14,1	15,3	14,7	13,8	14,1	15,7	15,5	15,7
2	1	13,1	12,7	13,5	12,4	12,9	13,3	13,7	13,3
	2	12,1	13,4	11,2	10,8	11,9	13,2	10,7	14,1
	3	10,4	11,0	10,3	9,1	11,8	12,0	11,7	12,6
	4	10,3	11,8	11,7	10,3	11,9	12,4	12,4	12,4
	5	12,9	14,2	15,1	13,9	12,9	15,8	15,5	14,6
	6	12,0	11,7	11,8	13,1	13,2	13,3	12,3	13,9
	7	13,7	15,1	13,5	12,1	13,5	14,6	15,3	14,9
	8	12,5	12,6	13,7	12,3	12,2	13,1	13,7	12,7
	9	12,6	13,5	13,8	13,6	11,8	14,1	14,7	14,4
	10	14,3	16,4	15,4	11,9	12,4	14,3	16,5	13,9
	11	14,6	14,9	13,5	13,9	15,6	15,7	14,2	13,1
3	1	9,9	8,6	7,7	7,9	7,7	7,0	8,8	8,0
	2	11,7	7,4	8,8	7,6	8,6	7,0	6,7	5,9
	3	9,7	6,7	5,9	7,2	5,9	6,1	6,6	5,2
	4	8,2	8,7	8,1	8,7	8,3	6,3	7,8	6,9
	5	10,3	8,9	8,7	7,8	6,7	6,3	6,4	6,8
	6	8,5	7,8	7,2	7,4	6,8	6,7	6,1	6,5
	7	10,2	8,3	8,8	6,1	8,8	6,6	9,7	9,6
	8	11,6	10,2	8,7	7,9	8,3	6,0	8,2	4,8
	9	11,6	7,2	8,2	8,8	9,2	7,9	10,1	8,1
	10	9,7	7,7	7,8	7,9	5,8	7,8	6,9	6,8
	11	9,4	8,1	7,4	8,2	8,0	7,7	8,4	7,9

APPENDIX 2 : CONTINUED

Two-tailed P-values for Table 4 - Effect of vitamin B-6 deficiency on daily food consumption

GRP	WK 1	WK 2	WK 3	WK 4	WK 5	WK 6	WK 7	WK 8
1 vs 2	0,7928	0,6934	0,8434	0,8436	0,3078	0,2370	0,1148	0,4301
1 vs 3	0,0018	0,0001	0,0001	0,0001	0,0001	0,0001	0,0001	0,0001
2 vs 3	0,0004	0,0001	0,0001	0,0001	0,0001	0,0001	0,0001	0,0001

A significant difference exists between groups if the P value is < 0,0167 ($\frac{0,05}{3}$).

APPENDIX 3 : MEAN DAILY INDIVIDUAL URINARY XANTHURENIC ACID EXCRETION (UXA) OVER 8 WEEKS (µg/ml)

GRP	NO.	UXA0	UXA1	UXA2	UXA3	UXA4	UXA5	UXA6	UXA7	UXA8
1	1	16,3	23,4	40,6	15,7	14,0	8,8	7,0	38,2	32,8
	2	18,4	32,9	36,2	30,0	17,3	8,8	3,2	5,0	4,9
	3	14,6	28,6	39,4	27,3	18,0	14,7	31,0	19,0	12,0
	4	80,5	45,3	32,8	25,1	19,1	20,1	48,5	27,5	11,6
	5	13,5	26,3	28,6	11,3	9,1	12,7	15,8	7,6	4,2
	6	13,2	15,5	40,5	16,9	13,8	13,5	23,8	27,1	19,5
	7	19,1	12,4	19,9	10,8	12,5	15,6	35,0	27,9	27,1
	8	16,0	11,0	26,8	40,5	23,8	23,5	17,2	22,2	43,2
	9	34,7	29,5	29,8	26,2	24,2	20,5	22,1	11,5	10,9
	10	15,6	10,0	31,9	30,0	13,6	12,1	17,9	11,2	13,7
	11	10,6	6,0	10,2	13,7	8,9	11,5	14,8	12,3	11,6
2	1	25,7	11,4	28,7	61,4	65,3	33,5	44,1	63,6	113,5
	2	13,9	9,0	45,5	65,6	30,0	18,1	38,2	38,0	252,5
	3	21,8	80,5	128,5	51,1	70,7	90,1	97,0	51,6	30,9
	4	16,0	7,4	84,6	22,0	10,5	27,3	128,0	152,5	149,7
	5	36,7	48,2	29,2	24,2	39,6	57,4	74,5	46,0	191,5
	6	16,9	12,4	50,9	60,7	53,3	75,2	57,7	83,7	113,1
	7	20,2	8,2	29,6	36,9	40,6	46,5	80,4	54,6	102,8
	8	40,7	61,2	36,2	79,2	160,5	123,3	89,0	91,6	79,8
	9	38,8	57,3	41,7	83,5	127,4	140,4	88,5	78,3	198,7
	10	19,4	30,0	48,2	21,5	18,1	27,3	95,5	32,7	40,4
	11	10,4	26,9	39,2	9,5	7,3	38,5	50,2	20,2	18,4
3	1	14,4	146,3	155,2	74,3	56,7	48,8	53,4	51,4	120,7
	2	20,2	109,6	113,7	72,4	67,8	102,5	342,0	358,4	355,3
	3	20,7	114,6	126,7	65,6	35,6	24,7	57,7	46,9	33,4
	4	62,7	114,6	126,7	65,6	35,6	24,7	57,7	46,9	33,4
	5	7,7	96,9	211,9	11,5	15,4	135,0	86,8	163,7	446,3
	6	5,1	51,0	100,7	85,5	58,1	165,4	198,1	264,5	267,7
	7	30,5	83,5	61,6	39,1	68,3	50,0	43,3	26,0	34,3
	8	7,6	167,8	235,5	72,1	41,3	41,0	79,9	71,8	87,0
	9	10,1	20,9	12,6	18,9	19,0	27,8	126,9	114,9	164,8
	10	22,6	167,1	210,0	153,2	186,9	208,0	202,7	190.4	221,3
	11	20,9	106,5	103,4	50,6	28,7	109,2	33,5	168,0	171,1

APPENDIX 3 : CONTINUED : MEAN DAILY INDIVIDUAL URINE VOLUME (UrV) OVER 8 WEEKS (ml)

GRP	NO.	UrV0	UrV1	UrV2	UrV3	UrV4	UrV5	UrV6	UrV7	UrV8
1	1	4,0	4,9	5,0	8,9	12,0	11,4	10,4	11,0	10,0
	2	4,0	6,9	7,4	9,4	8,4	13,6	10,3	14,0	16,6
	3	4,0	4,9	5,7	9,4	11,3	12,6	10,7	12,6	10,3
	4	6,0	8,7	10,9	20,9	28,1	24,6	11,6	21,4	22,3
	5	4,0	9,1	10,6	14,1	20,7	21,3	17,4	16,6	15,9
	6	2,0	8,7	11,9	12,1	12,1	10,1	12,3	11,9	10,6
	7	2,0	4,9	5,3	7,6	10,3	12,3	8,7	11,1	11,7
	8	2,0	12	13,4	16,3	20,8	18,0	18,4	16,9	17,3
	9	2,0	5,7	4,3	7,0	11,3	8,0	10,0	11,9	9,6
	10	4,0	7,7	5,0	11,3	18,4	13,1	9,9	10,7	10,7
	11	4,0	14,6	9,9	13,3	22,6	21,4	12,1	14,3	14,9
2	1	5,0	9,3	7,1	8,6	14,6	12,0	7,9	9,7	10,7
	2	4,0	10,9	7,1	9,0	12,1	8,9	7,0	10,3	9,0
	3	4,0	4,0	4,7	5,4	8,3	7,0	8,7	10,3	16,6
	4	4,0	6,9	6,9	11,4	17,9	12,6	9,1	9,3	9,6
	5	4,0	7,3	6,7	9,9	11,6	11,4	8,6	9,1	6,1
	6	2,0	6,6	4,9	5,3	10,9	9,6	8,3	9,7	9,1
	7	4,0	12,9	9,0	9,4	11,9	9,4	9,3	10,0	8,6
	8	4,0	4,0	4,7	7,0	8,0	6,3	7,9	8,1	7,7
	9	2,0	6,0	7,6	8,3	7,6	9,3	6,3	6,6	7,0
	10	4,0	8,3	10,4	11,9	13,9	10,4	12,0	14,7	14,3
	11	6,0	10,0	8,7	15,4	24,9	23,4	10,9	12,4	12,6
3	1	4,0	6,3	6,7	5,4	5,7	5,1	5,3	6,6	5,0
	2	6,0	5,7	5,1	4,7	5,4	5,7	4,0	4,4	3,1
	3	4,0	5,4	3,6	3,9	3,1	3,0	3,6	4,4	3,6
	4	2,0	5,4	4,6	4,4	4,1	4,3	4,3	5,3	4,6
	5	2,0	5,6	6,9	6,9	4,4	3,7	4,0	3,9	4,9
	6	6,0	4,3	5,9	6,0	4,6	3,6	4,0	3,6	3,7
	7	4,0	4,9	8,6	11,4	6,0	5,9	4,9	5,6	4,9
	8	6,0	6,6	7,6	5,3	4,3	5,0	4,3	4,7	3,9
	9	2,0	7,1	2,6	4,0	3,7	3,6	3,7	4,0	3,4
	10	4,0	5,9	3,8	4,7	3,4	3,3	3,7	3,9	3,6
	11	4,0	5,7	4,1	4,0	3,7	3,4	2,9	4,6	3,6

APPENDIX 3 : CONTINUED

Two-tail P-values for Table 5 - Urinary Xanthurenic acid excretion during vitamin B-6 deficiency

GRP	DAY 0	WK 1	WK 2	WK 3	WK 4	WK 5	WK 6	WK 7	WK 8
1 vs 2	0,0940	0,3410	0,1783	0,0940	0,0115	0,0028	0,0003	0,0018	0,0003
1 vs 3	0,5327	0,0003	0,0012	0,0452	0,2244	0,0043	0,0012	0,0006	0,0004
2 vs 3	0,4905	0,0007	0,0053	0,2244	0,3088	0,7676	0,4502	0,0613	0,2004

A significant difference exists between groups if the P value is < 0,0167 $(\frac{0,05}{3})$.

APPENDIX 4 : MEAN DAILY INDIVIDUAL Zn INTAKE AND EXCRETION (FECAL AND URINARY) OVER 8 WEEKS (µg/d)

GRP	NO.	WEEK 1			WEEK 2			WEEK 3		
		INTAKE	FECAL	URINARY	INTAKE	FECAL	URINARY	INTAKE	FECAL	URINARY
1	1	181,9	92,3	2,8	177,1	34,6	1,9	180,0	31,9	1,3
	2	203,0	47,6	3,5	226,0	27,3	1,5	229,3	34,3	1,7
	3	173,4	55,7	3,4	194,7	32,7	1,9	206,6	36,3	1,9
	4	261,6	81,6	4,4	302,9	60,5	2,6	298,9	56,4	2,5
	5	220,9	73,7	4,1	249,1	29,4	1,9	258,4	38,8	1,7
	6	233,4	56,4	4,6	279,7	42,8	1,4	258,7	35,4	1,5
	7	186,9	59,9	3,2	195,0	40,0	2,5	198,4	30,4	2,7
	8	215,3	52,6	5,8	243,9	34,0	1,6	238,1	25,7	3,4
	9	226,6	55,0	3,6	235,0	31,4	1,9	237,6	33,3	1,8
	10	222,4	95,1	6,0	236,9	55,4	1,7	177,1	35,8	1,3
	11	245,7	63,8	7,9	267,0	45,6	2,1	255,3	54,5	1,6
2	1	227,4	97,3	6,7	221,3	51,6	3,6	234,7	36,3	4,4
	2	210,9	52,3	6,8	233,4	55,8	1,7	195,6	35,8	1,8
	3	181,9	52,7	2,8	192,1	33,1	1,9	179,3	34,3	2,3
	4	180,0	43,5	4,9	205,9	27,2	2,5	203,4	49,2	2,1
	5	224,3	56,3	4,4	246,9	53,5	1,8	263,7	38,9	2,7
	6	209,0	92,2	5,7	204,0	29,8	3,2	206,1	37,9	3,6
	7	237,9	69,8	8,9	262,3	47,9	4,3	235,0	37,6	3,9
	8	217,7	49,7	4,3	219,3	32,6	2,9	238,1	42,1	2,7
	9	219,3	57,8	3,2	235,3	39,1	1,8	240,0	32,1	2,9
	10	248,4	52,9	4,7	286,0	39,5	2,2	267,9	54,2	3,6
	11	255,3	54,9	10,2	259,6	41,9	4,2	235,3	46,1	3,7
3	1	170,3	50,2	4,9	149,0	30,6	3,6	133,9	33,4	4,1
	2	203,4	84,2	6,5	129,3	19,5	2,3	153,0	29,3	4,1
	3	169,6	34,4	4,9	116,4	22,5	3,4	103,6	32,1	2,7
	4	142,7	49,3	7,0	151,4	31,5	3,7	140,4	14,3	2,5
	5	179,0	47,8	6,0	155,3	22,6	2,5	150,6	30,8	3,3
	6	148,1	51,4	5,3	135,4	20,6	4,7	125,4	14,4	5,9
	7	177,1	44,4	3,6	144,9	17,5	4,6	154,0	23,5	2,7
	8	202,9	86,5	4,3	178,0	21,6	2,5	151,4	32,1	2,5
	9	202,6	59,6	6,6	125,4	55,0	3,2	143,6	24,5	4,7
	10	169,3	43,5	4,6	134,0	20,3	3,5	136,0	33,7	3,9
	11	164,3	51,4	3,8	140,6	26,4	3,2	129,0	28,5	4,6

Percentage recovery for Zn = 94%.

APPENDIX 4 : CONTINUED : MEAN DAILY INDIVIDUAL Zn INTAKE AND EXCRETION (FECAL AND URINARY) OVER 8 WEEKS (µg/d)

GRP	NO.	WEEK 4			WEEK 5			WEEK 6		
		INTAKE	FECAL	URINARY	INTAKE	FECAL	URINARY	INTAKE	FECAL	URINARY
1	1	189,6	40,5	2,2	221,0	81,2	1,7	203,1	43,1	2,8
	2	188,4	49,2	2,3	207,8	51,5	1,6	243,7	58,1	2,7
	3	193,1	36,3	2,4	243,1	59,6	3,0	242,4	58,2	4,2
	4	220,3	57,3	7,6	245,6	45,4	5,2	309,1	59,7	3,5
	5	224,0	46,0	6,2	262,6	62,9	4,5	306,0	76,6	2,6
	6	215,6	59,2	3,3	232,8	75,0	3,0	299,1	66,1	8,8
	7	180,6	39,2	3,4	210,3	48,8	3,7	226,6	47,5	4,7
	8	220,3	32,4	5,0	209,4	51,8	4,3	262,1	57,3	3,3
	9	228,4	40,2	2,7	240,6	56,9	3,1	289,4	70,1	3,3
	10	195,6	35,4	4,4	176,6	61,0	2,7	223,7	60,5	3,5
	11	241,0	55,6	3,4	245,3	57,6	3,8	273,4	78,3	3,6
2	1	215,3	55,3	4,4	224,3	45,4	3,2	232,1	53,7	4,7
	2	187,7	42,2	6,2	206,1	40,4	3,7	229,7	38,2	2,7
	3	159,0	38,1	2,7	205,3	45,1	3,1	208,7	45,1	2,3
	4	178,7	56,8	5,9	207,1	50,2	2,6	215,0	58,2	2,5
	5	242,0	58,9	4,2	224,7	53,2	2,4	275,0	71,2	2,8
	6	226,7	39,9	4,2	229,3	44,8	3,4	231,6	50,7	3,9
	7	211,3	41,3	4,9	235,9	50,2	3,4	254,1	62,9	3,9
	8	214,4	62,3	3,8	212,4	59,2	2,8	227,4	61,9	2,8
	9	236,0	40,9	5,2	204,7	39,9	4,4	245,3	40,2	2,3
	10	207,4	68,1	5,4	215,8	39,5	2,8	249,7	59,3	2,8
	11	241,6	47,6	5,9	271,3	64,3	4,2	273,6	72,3	4,6
3	1	138,6	45,3	6,0	133,9	56,1	4,8	122,4	54,6	3,6
	2	131,7	32,1	6,3	149,6	45,5	4,3	121,4	48,9	3,2
	3	125,9	45,2	5,5	103,3	38,5	2,6	105,4	44,9	2,9
	4	152,0	39,9	5,5	145,4	46,4	4,5	109,8	47,4	2,6
	5	135,6	26,1	8,7	116,7	32,1	6,7	110,1	40,6	4,3
	6	128,6	30,8	7,4	117,7	35,2	5,9	117,0	42,9	5,9
	7	107,0	31,4	3,7	152,7	49,1	3,9	114,6	46,5	3,5
	8	137,4	36,1	4,6	145,4	46,4	4,6	104,6	43,6	2,7
	9	153,6	49,3	5,7	160,3	44,6	4,4	137,4	42,0	3,1
	10	137,0	40,1	3,6	101,7	50,2	3,3	135,8	51,3	3,4
	11	143,3	42,1	7,2	139,3	50,6	4,6	134,6	56,3	3,0

APPENDIX 4 : CONTINUED : MEAN DAILY INDIVIDUAL Zn INTAKE AND EXCRETION (FECAL AND URINARY) OVER 8 WEEKS (µg/d)

GRP	NO.	WEEK 7			WEEK 8		
		INTAKE	FECAL	URINARY	INTAKE	FECAL	URINARY
1	1	211,9	48,1	2,9	224,4	64,7	3,0
	2	248,1	71,2	2,5	222,1	104,2	2,0
	3	250,7	88,3	3,8	226,9	86,9	3,7
	4	357,4	91,3	3,8	323,6	95,8	2,7
	5	280,1	103,2	2,9	282,6	108,9	3,3
	6	283,9	62,3	2,8	273,1	70,7	2,5
	7	254,1	91,9	4,3	220,9	74,0	2,8
	8	235,3	77,3	3,5	224,4	79,4	3,1
	9	289,1	69,7	3,6	262,9	94,9	3,4
	10	244,0	75,4	4,5	223,7	80,2	3,2
	11	270,4	74,6	3,4	273,4	82,2	2,7
2	1	239,0	61,1	4,4	231,6	75,8	5,8
	2	185,9	56,9	4,0	244,4	57,2	4,3
	3	204,0	64,2	4,6	219,7	68,2	6,9
	4	234,0	48,8	3,1	216,6	64,2	3,2
	5	269,4	60,6	3,6	253,4	71,9	6,6
	6	213,7	65,1	3,5	242,1	64,3	5,2
	7	266,9	83,8	4,5	258,7	94,8	5,9
	8	239,1	67,4	2,7	220,9	70,1	5,3
	9	255,3	53,9	4,3	250,6	50,7	4,4
	10	286,4	96,9	3,5	241,9	85,7	3,4
	11	246,6	70,6	5,9	229,0	72,1	6,0
3	1	153,6	62,9	3,9	139,3	60,3	5,1
	2	116,3	50,9	3,4	103,9	53,0	4,1
	3	114,8	34,5	4,2	91,3	44,0	5,1
	4	136,4	51,1	3,5	121,1	42,5	2,7
	5	112,3	40,5	4,9	118,9	44,9	4,4
	6	106,1	35,8	6,0	112,7	28,9	5,4
	7	168,3	61,7	5,5	167,4	58,9	3,6
	8	143,6	54,3	3,2	84,4	41,4	3,1
	9	176,1	49,1	4,6	140,6	63,7	4,3
	10	120,1	41,9	4,7	116,1	52,6	2,9
	11	147,0	58,2	3,0	137,8	56,9	3,6

APPENDIX 4 : CONTINUED

Two-tail P values for Table 7 - Daily Zn balance during vitamin B-6 deficiency

GRP	WK 1	WK 2	WK 3	WK 4	WK 5	WK 6	WK 7	WK 8
1 vs 2	0,6695	0,5327	0,5113	0,8182	0,6695	0,2505	0,5767	0,5767
1 vs 3	0,0165	0,0001	0,0001	0,0001	0,0001	0,0001	0,0001	0,0001
2 vs 3	0,0035	0,0001	0,0001	0,0001	0,0001	0,0001	0,0001	0,0001

A significant difference exists between groups if the P value is < 0,0167 $(\frac{0,05}{3})$.

APPENDIX 5 : MEAN DAILY INDIVIDUAL Cu INTAKE AND EXCRETION (FECAL AND URINARY) OVER 8 WEEKS (µg/d)

GRP	NO.	WEEK 1			WEEK 2			WEEK 3		
		INTAKE	FECAL	URINARY	INTAKE	FECAL	URINARY	INTAKE	FECAL	URINARY
1	1	31,6	8,7	1,6	30,9	10,5	1,3	31,3	8,7	1,2
	2	35,3	8,4	1,9	39,3	9,6	1,6	39,9	10,3	1,6
	3	30,1	9,9	1,0	33,9	9,8	1,4	36,0	8,7	1,4
	4	45,4	15,0	1,0	52,7	13,7	1,8	52,0	13	1,7
	5	38,4	18,0	0,9	43,3	11,4	1,4	45,0	11,7	0,8
	6	40,6	12,0	2,5	48,5	13,5	1,1	45,0	13,0	0,6
	7	32,6	11,7	1,7	33,9	10,0	0,5	34,6	11,2	1,7
	8	37,4	9,9	1,7	42,4	8,0	1,5	41,4	7,4	3,1
	9	39,4	8,3	2,6	40,9	8,3	2,1	41,3	10	1,2
	10	38,7	17,3	2,2	41,1	12,3	1,8	30,9	7,9	1,1
	11	42,7	12,4	2,2	46,4	9,1	1,9	44,4	7,8	0,8
2	1	39,6	11,7	3,6	38,4	9,2	2,4	40,9	9,1	1,9
	2	36,7	11,5	1,2	40,6	10,3	2,3	34,0	8,1	1,1
	3	31,6	9,2	2,0	33,4	6,3	1,7	31,4	7,8	1,2
	4	31,3	12,4	1,2	35,9	8,0	1,9	35,4	11,3	0,9
	5	39,0	14,5	2,0	42,9	16,5	1,0	45,9	13,6	0,8
	6	36,3	12,7	1,9	35,4	7,8	1,9	35,9	9,5	2,3
	7	41,4	12,3	1,9	45,6	9,9	2,2	40,9	7,2	2,6
	8	37,9	15,5	1,2	38,1	11,4	0,7	41,4	12,3	0,9
	9	38,1	16,5	1,3	40,9	11,7	0,8	41,7	11,3	0,9
	10	43,1	15,1	2,4	49,7	13,2	0,8	46,6	14,1	0,9
	11	44,3	16,5	2,7	45,1	13,3	0,7	40,9	11,9	0,9
3	1	29,6	12,2	1,8	25,9	7,1	0,9	23,3	8,1	0,7
	2	35,4	15,6	1,9	22,4	6,8	0,6	26,6	10,5	0,8
	3	29,4	9,4	1,5	20,3	5,4	0,8	18,0	6,4	0,6
	4	24,9	13,2	1,7	26,3	8,4	0,9	24,4	5,0	0,8
	5	31,1	7,2	1,6	27,0	5,4	0,4	26,1	6,9	0,8
	6	25,7	9,1	1,5	23,6	2,9	0,9	21,9	3,7	1,0
	7	30,9	9,7	1,3	25,1	6,4	0,8	26,9	10,6	0,8
	8	35,3	14,9	2,0	31,0	3,9	0,9	26,3	4,3	1,1
	9	35,3	10,8	1,9	21,8	8,7	0,9	25,0	6,4	0,8
	10	29,4	7,1	1,4	23,3	6,5	0,8	23,7	7,9	0,7
	11	28,6	11,1	1,3	24,4	3,5	0,9	22,4	6,9	1,1

Percentage recovery for Cu = 99%.

APPENDIX 5 : CONTINUED : MEAN DAILY INDIVIDUAL Cu INTAKE AND EXCRETION (FECAL AND URINARY) OVER 8 WEEKS (µg/d)

GRP	NO.	WEEK 4			WEEK 5			WEEK 6		
		INTAKE	FECAL	URINARY	INTAKE	FECAL	URINARY	INTAKE	FECAL	URINARY
1	1	33,0	6,7	1,1	38,4	8,6	2,2	35,3	10,4	1,9
	2	32,7	6,1	1,3	36,1	7,6	2,2	42,4	9,1	2,3
	3	33,6	6,9	1,0	42,3	6,3	1,9	42,1	10,9	1,9
	4	38,3	12,5	3,4	42,7	9,4	3,4	53,7	14,9	1,9
	5	39,0	11,5	2,1	45,7	15,2	2,1	53,3	17,3	1,2
	6	37,4	12,2	1,4	40,4	7,7	1,9	52,0	12,4	2,6
	7	31,3	6,9	1,2	36,6	8,8	1,9	39,4	10,6	1,7
	8	38,3	5,9	2,7	36,4	8,6	2,3	45,6	9,8	1,8
	9	39,7	10,5	1,2	41,8	11,8	2,0	50,3	16,4	1,9
	10	34,0	8,8	1,3	30,7	8,2	1,3	38,9	12,1	1,4
	11	41,9	7,4	2,0	42,7	13,4	1,9	47,6	8,9	2,7
2	1	37,4	8,9	1,7	39,0	8,0	2,0	40,4	12,9	2,3
	2	32,6	7,4	2,5	35,9	6,9	2,1	40,0	8,8	2,5
	3	27,7	5,6	1,4	35,7	6,4	2,4	36,4	6,7	2,9
	4	31,1	10,3	2,9	26,0	8,4	2,3	27,4	13,6	2,9
	5	42,1	10,5	1,7	39,1	12,4	1,9	47,9	12,5	3,6
	6	39,4	8,3	1,6	39,9	8,3	1,7	40,3	13,1	2,2
	7	36,7	9,9	2,0	41,0	10,8	2,2	44,1	11,4	2,9
	8	37,3	13,6	1,3	37,0	11,8	1,3	39,6	19,5	1,3
	9	41,0	12,6	1,1	35,6	10,3	1,7	42,7	12,9	1,3
	10	36,1	13,9	1,2	37,6	9,8	1,7	43,4	16,4	2,0
	11	42,0	13,1	1,5	47,1	15,5	1,6	47,6	15,3	1,7
3	1	24,1	8,8	0,9	23,3	8,9	1,1	21,3	6,8	1,0
	2	22,9	7,5	1,1	26,1	10,6	1,1	21,1	6,9	1,2
	3	21,5	7,5	0,5	18,0	4,9	0,8	18,3	5,6	1,1
	4	26,4	12,9	1,2	25,3	7,7	1,4	19,1	5,7	1,1
	5	23,6	5,0	1,2	20,3	4,3	1,4	19,1	5,5	1,3
	6	22,4	9,1	0,9	20,4	7,6	1,0	20,3	7,5	0,8
	7	18,6	5,9	0,9	26,6	10,6	1,1	20,0	7,1	1,1
	8	23,9	8,2	0,9	25,3	10,2	1,1	18,1	6,8	1,2
	9	26,7	10,7	0,7	27,9	8,2	0,6	23,9	6,8	0,7
	10	23,8	7,1	0,6	17,7	6,6	0,7	23,6	8,9	0,9
	11	24,8	9,7	0,8	24,3	7,8	0,8	23,4	8,3	0,9

APPENDIX 5 : CONTINUED : MEAN DAILY INDIVIDUAL Cu INTAKE AND EXCRETION (FECAL AND URINARY) OVER 8 WEEKS (µg/d)

GRP	NO.	WEEK 7			WEEK 8		
		INTAKE	FECAL	URINARY	INTAKE	FECAL	URINARY
1	1	36,8	11,2	1,8	39,0	11,1	1,7
	2	43,1	10,3	1,7	38,6	9,6	1,3
	3	43,6	12,6	1,6	39,4	10,9	0,9
	4	62,1	18,3	1,1	56,3	13,3	3,1
	5	48,4	17,6	0,7	49,1	14,2	1,9
	6	49,4	11,1	1,9	47,6	12,9	0,9
	7	44,1	12,8	1,5	38,4	8,4	2,2
	8	40,9	11,9	0,7	39,0	9,7	2,8
	9	50,3	9,6	1,2	45,7	13,9	1,4
	10	42,4	11,3	1,1	39,0	11,2	2,2
	11	47,0	10,9	2,6	47,6	8,9	3,4
2	1	41,6	11,6	1,2	40,3	9,9	0,8
	2	32,3	9,8	1,2	42,6	8,4	1,1
	3	35,4	6,9	2,2	38,1	6,9	2,6
	4	40,7	11,0	1,3	27,7	10,4	2,3
	5	46,9	12,5	1,5	44,0	9,8	1,4
	6	37,1	10,8	0,8	42,1	11,0	2,1
	7	46,4	13,3	1,2	45,0	17,2	1,8
	8	41,6	15,8	1,1	38,4	11,4	1,2
	9	44,4	13,5	1,0	43,6	12,2	1,1
	10	49,9	12,4	2,8	42,0	9,3	2,3
	11	42,9	16,6	1,9	39,9	16,5	1,5
3	1	26,7	12,3	0,9	24,3	9,6	1,0
	2	20,1	6,9	1,1	18,0	4,9	0,5
	3	20,0	4,3	1,3	15,9	5,3	0,5
	4	23,7	6,7	1,3	21,1	6,1	0,6
	5	19,6	5,7	1,3	20,7	6,0	1,1
	6	18,4	5,9	1,2	19,6	5,9	1,0
	7	29,3	8,1	0,6	29,1	8,1	0,7
	8	25,0	9,0	1,2	14,7	5,8	1,0
	9	31,0	8,6	0,4	24,4	8,4	0,8
	10	20,9	6,5	0,9	20,1	7,4	0,8
	11	25,6	8,6	0,8	24,0	8,3	2,0

APPENDIX 5 : CONTINUED

Two-tail P values for Table 8 - Daily Cu balance during vitamin B-6 deficiency

GRP	WK 1	WK 2	WK 3	WK 4	WK 5	WK 6	WK 7	WK 8
1 vs 2	0,5767	0,5767	0,5767	0,4306	0,3750	0,0165	0,0818	0,4118
1 vs 3	0,0053	0,0003	0,0001	0,0001	0,0001	0,0001	0,0001	0,0001
2 vs 3	0,0053	0,0003	0,0001	0,0001	0,0001	0,0001	0,0001	0,0001

A significant difference exists between groups if the P value is < 0,0167 $(\frac{0,05}{3})$.

APPENDIX 6 : MEAN DAILY INDIVIDUAL Fe INTAKE AND EXCRETION (FECAL AND URINARY) OVER 8 WEEKS (µg/d)

GRP	NO.	WEEK 1			WEEK 2			WEEK 3		
		INTAKE	FECAL	URINARY	INTAKE	FECAL	URINARY	INTAKE	FECAL	URINARY
1	1	1557,0	684,0	30,2	1516,6	923,9	37,3	1541,3	944,2	17,8
	2	1737,0	951,1	34,6	1935,0	1090,2	23,4	1962,0	1187,9	20,6
	3	1485,0	658,0	22,6	1667,3	1051,7	25,7	1768,6	981,2	13,9
	4	2238,7	1516,3	23,0	2590,0	1618,1	28,3	2558,3	1392,0	23,2
	5	1890,0	1078,1	21,7	2133,0	1145,6	9,5	2211,7	1318,4	30,5
	6	1998,0	1090,8	37,3	2394,0	1393,1	17,4	2214,0	1167,2	13,1
	7	1599,7	922,9	22,6	1669,6	1022,2	26,5	1698,7	1037,8	24,1
	8	1842,7	862,5	33,8	2088,0	1219,5	19,3	2038,6	1194,5	41,0
	9	1939,6	1076,1	52,1	2011,6	1075,5	30,9	2034,0	1149,7	31,0
	10	1903,6	1251,8	50,5	2027,3	1248,9	26,1	1516,6	792,2	22,7
	11	2103,7	1035,1	104,5	2286,0	1507,8	29,6	2184,7	1231,1	35,1
2	1	1946,3	1382,3	68,2	1894,6	1229,9	22,1	2009,3	1139,3	31,6
	2	1804,6	841,2	35,2	1998,0	1286,7	30,6	1674,0	975,8	26,7
	3	1557,0	894,4	29,5	1644,7	975,1	27,2	1534,6	870,3	27,5
	4	1541,3	892,9	38,2	1761,7	999,8	27,7	1741,6	1019,5	33,9
	5	1919,3	1037,1	34,7	2112,7	1386,1	29,8	2256,7	1173,6	40,2
	6	1788,7	1279,0	33,9	1746,0	950,6	32,6	1764,0	1045,5	39,2
	7	2036,6	1225,1	23,9	2245,4	1258,6	28,3	2011,6	1120,5	51,5
	8	1863,0	873,2	18,4	1876,4	1023,9	36,8	2038,6	1160,8	37,8
	9	1876,6	1096,7	27,5	2013,7	1311,6	43,2	2054,3	1075,6	23,4
	10	2126,3	1181,9	36,5	2448,0	1414,9	42,9	2293,0	1259,6	29,5
	11	2184,7	1083,9	45,0	2220,7	1165,8	42,9	2013,4	975,7	41,2
3	1	1458,0	760,6	26,2	1275,7	805,7	36,5	1145,3	671,3	28,0
	2	1741,6	1096,2	37,4	1107,0	665,5	21,4	1309,6	941,5	15,3
	3	1451,3	765,4	24,7	996,7	546,1	26,6	886,6	558,3	12,9
	4	1221,7	793,2	32,6	1296,0	710,4	33,6	1201,6	456,0	21,1
	5	1532,3	948,1	21,7	1329,7	677,3	35,0	1289,3	867,1	33,1
	6	1269,0	737,8	28,8	1158,7	758,8	33,3	1073,3	345,0	24,8
	7	1516,6	934,0	16,6	1239,7	722,5	54,5	1318,6	902,6	36,7
	8	1737,0	1208,8	28,2	1523,3	723,0	43,4	1296,0	738,7	33,9
	9	1734,7	946,4	16,3	1073,3	853,4	16,5	1228,6	554,5	25,6
	10	1449,0	821,5	26,0	1147,6	598,3	29,9	1167,7	627,8	30,9
	11	1406,3	961,5	14,2	1203,7	701,7	31,6	1104,7	600,7	42,0

Percentage recovery for Fe = 91%.

APPENDIX 6 : CONTINUED : MEAN DAILY INDIVIDUAL Fe INTAKE AND EXCRETION (FECAL AND URINARY) OVER 8 WEEKS (µg/d)

GRP	NO.	WEEK 4			WEEK 5			WEEK 6		
		INTAKE	FECAL	URINARY	INTAKE	FECAL	URINARY	INTAKE	FECAL	URINARY
1	1	1622,3	853,6	25,2	1892,3	112,3	26,1	1739,3	1022,8	17,5
	2	1613,3	963,3	8,7	1779,9	1037,0	30,5	2085,7	1248,4	42,6
	3	1653,7	971,8	31,1	2081,3	1052,8	41,5	2074,6	1190,3	29,2
	4	1885,6	1203,2	49,8	2101,6	981,2	87,7	2646,0	1704,2	32,6
	5	1917,0	1155,5	24,2	2247,7	1263,0	68,3	2619,0	1663,5	12,5
	6	1845,0	1146,3	14,9	1993,4	1296,9	43,2	2560,6	1442,9	29,5
	7	1545,7	905,7	16,8	1800,0	968,2	28,7	1939,6	1134,2	9,4
	8	1885,6	926,5	18,1	1793,2	1093,4	32,4	2243,3	1227,8	14,4
	9	1955,3	1085,4	11,5	2058,7	1286,9	30,2	2477,3	1511,7	31,5
	10	1674,0	834,6	14,9	1512,0	1021,2	20,5	1914,7	1107,9	6,2
	11	2063,3	1190,2	27,8	2099,3	1269,0	30,2	2340,0	1391,1	32,1
2	1	1842,7	963,1	37,1	1919,3	884,6	28,8	1986,7	1271,9	24,5
	2	1606,7	719,7	15,7	1764,0	895,2	27,4	1966,5	935,6	14,1
	3	1361,3	809,3	5,7	1757,1	948,4	23,9	1786,6	1023,2	20,9
	4	1530,0	1048,6	14,5	1773,0	985,6	27,9	1840,6	1179,8	35,9
	5	2072,3	1310,3	19,8	1923,7	1163,5	20,9	2353,6	1404,4	30,3
	6	1939,6	935,3	13,3	1962,0	1070,6	22,9	1982,3	1044,2	27,3
	7	1809,0	1074,5	32,0	2108,3	1232,9	33,7	2175,7	1274,6	25,4
	8	1836,0	1091,8	31,2	1818,0	1068,0	26,6	1946,3	1117,2	30,2
	9	2020,6	1041,4	24,1	1752,7	1033,4	36,5	2099,3	1127,6	8,1
	10	1775,3	1063,9	32,8	1847,3	940,8	17,9	2137,6	1384,7	22,3
	11	2067,7	1003,9	47,7	2322,0	1358,0	61,9	2342,3	1310,1	31,6
3	1	1185,7	654,6	20,9	1145,3	676,5	15,4	1048,6	572,3	18,6
	2	1127,3	592,7	25,1	1280,3	825,5	23,6	1039,6	631,1	23,9
	3	1077,7	597,8	20,1	884,3	464,5	10,4	902,3	514,3	20,7
	4	1300,4	894,8	32,4	1244,3	667,4	23,1	940,6	546,3	14,0
	5	1161,0	627,9	28,4	999,0	545,4	14,6	942,7	547,6	7,8
	6	1100,3	677,6	24,5	1008,0	647,5	17,2	1001,3	618,4	10,6
	7	915,7	477,1	16,7	1307,0	853,5	15,5	981,0	586,1	9,3
	8	1176,7	615,3	24,2	1244,3	737,3	12,7	895,6	568,8	12,3
	9	1314,0	684,9	24,6	1372,4	622,4	15,4	1176,7	523,6	7,5
	10	1172,3	542,5	17,1	870,7	581,8	14,5	1163,3	684,9	9,3
	11	1226,3	700,8	30,7	1192,6	581,3	13,7	1152,0	625,6	17,8

APPENDIX 6 : CONTINUED : MEAN DAILY INDIVIDUAL Fe INTAKE AND EXCRETION (FECAL AND URINARY) OVER 8 WEEKS (µg/d)

		WEEK 7			WEEK 8		
GRP	NO.	INTAKE	FECAL	URINARY	INTAKE	FECAL	URINARY
1	1	1813,6	1100,5	25,7	1921,6	1126,0	26,4
	2	2124,0	1386,9	13,9	1901,3	1258,1	33,8
	3	2146,4	1459,1	38,5	1941,7	1206,1	28,1
	4	3060,0	1935,2	43,1	2769,7	1729,6	30,8
	5	2398,6	1646,3	21,9	2418,7	1494,7	34,3
	6	2430,0	1278,7	28,8	2337,7	1342,2	26,6
	7	2175,7	1365,1	29,7	1890,0	1102,1	21,2
	8	2013,7	1349,6	9,1	1921,6	1230,6	26,5
	9	2475,0	1147,4	20,3	2250,0	1430,7	33,9
	10	2088,0	1194,9	16,1	1914,7	1162,5	30,9
	11	2315,3	1367,1	16,7	2340,0	1326,2	31,2
2	1	2045,3	1233,5	10,5	1982,3	1224,7	43,4
	2	1590,7	1046,5	26,2	2092,6	1049,7	39,4
	3	1746,0	1173,5	31,8	1881,0	1077,8	20,9
	4	2002,6	1005,2	16,2	1854,0	1161,7	31,0
	5	2306,3	1055,8	15,4	2169,0	984,2	42,4
	6	1829,3	1266,5	14,6	2072,3	1204,6	29,9
	7	2283,6	1340,9	21,6	2209,6	1391,8	51,2
	8	2047,4	1224,9	12,2	1890,0	1148,4	34,7
	9	2184,7	1221,8	34,3	2144,3	1125,1	47,5
	10	2452,6	1623,2	34,4	2070,0	1225,7	43,7
	11	2110,6	1304,6	28,7	1959,7	1174,5	29,8
3	1	1314,0	843,1	10,8	1192,6	655,3	24,5
	2	994,6	638,8	12,7	888,6	399,3	20,4
	3	983,3	469,3	18,2	780,7	478,1	22,3
	4	1167,7	658,0	26,6	1037,3	515,6	18,4
	5	960,7	606,3	12,9	1017,0	611,7	22,9
	6	909,0	561,4	11,9	965,3	573,7	25,6
	7	1440,0	771,4	15,0	1433,3	692,2	30,6
	8	1228,6	721,7	19,7	722,3	507,7	22,2
	9	1507,6	644,4	17,9	1203,8	641,7	21,3
	10	1028,3	565,9	17,5	994,6	620,8	18,9
	11	1257,7	726,4	19,9	1179,0	643,6	25,3

APPENDIX 6 : CONTINUED

Two-tail P values for Table 9 - Daily Fe balance during vitamin B-6 deficiency

GRP	WK 1	WK 2	WK 3	WK 4	WK 5	WK 6	WK 7	WK 8
1 vs 2	0,9738	0,9215	0,8955	0,4905	0,8696	0,2786	0,7180	0,8182
1 vs 3	0,0009	0,0004	0,0006	0,0001	0,0004	0,0001	0,0004	0,0002
2 vs 3	0,0138	0,0003	0,0007	0,0007	0,0001	0,0001	0,0009	0,0001

A significant difference exists between groups if the P value is < 0,0167 ($\frac{0,05}{3}$).

APPENDIX 7

Two-tail P values for Table 10 - Zn, Cu and Fe excretion during vitamin B-6 deficiency

GRP	WK 1	WK 2	WK 3	WK 4	WK 5	WK 6	WK 7	WK 8
Zn								
1 vs 2	0,3410	0,4502	0,1228	0,2505	0,0138	0,9738	0,5327	0,1580
1 vs 3	0,8182	0,2505	0,0197	0,0002	0,0043	0,0001	0,0007	0,0028
2 vs 3	0,2004	0,7180	0,1077	0,0165	0,0001	0,0001	0,0006	0,0001
Cu								
1 vs 2	0,1077	0,8696	0,1580	0,0940	0,7180	0,0452	0,3410	0,7180
1 vs 3	0,2004	0,7180	0,0940	0,0018	0,0043	0,0003	0,0235	0,0035
2 vs 3	0,6695	0,8696	0,3410	0,0452	0,0115	0,3752	0,2786	0,0328
Fe								
1 vs 2	0,5767	0,9738	0,4905	0,9215	0,3754	0,9738	0,7676	0,2244
1 vs 3	0,0940	0,5767	0,6224	0,4118	0,9215	0,8696	0,1783	0,1077
2 vs 3	0,2244	0,9215	0,5327	0,9215	0,4905	0,9738	0,2244	0,6695

A significant difference exists between groups if the P value is < 0,0167 ($\frac{0,05}{3}$).