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

by

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CHAPTER ONE

INTRODUCTION

Pyridoxal-5-phosphate (PLP), the biologically active form of vitamin B-6, acts as a cofactor for enzymes catalysing transaminase, decarboxylase and synthetase reactions (among others) in pathways spanning carbohydrate metabolism spingolipid biosynthesis and degradation, amino acid metabolism, hemoglobin biosynthesis, neurotransmitter biosynthesis, and in other pathways for many other important biomolecules (Merrill and Henderson, 1987). The metabolic participation of vitamin B-6 represents a versatility probably not matched by any other coenzyme. Over one hundred enzymes are known to require PLP as an activating coenzyme (Sauberlich, 1985). Thus, in view of its important role in intermediate metabolism and diversity of function an increasing interest has arisen in this field.

Trace elements, such as copper, iron and zinc act as catalysts in a wide range of enzyme systems, either through substrate activation or in the form of metalloenzymes. Consequently it is not unexpected that diverse interactions exist between vitamin B-6 and trace elements.

Few studies have been conducted to investigate the link between vitamin B-6 deficiency and trace element status. Hsu, 1965 reported a decrease in zinc content of plasma, liver, pancreas and heart of vitamin B-6 deficient rats. Contrary to these findings, Gershoff, 1968 found an increase in the zinc levels of the rat pancreas and kidney. Prasad et al, 1982 attributed this increase to a direct effect of vitamin B-6 on the intestinal mucosa. They suggested that pyridoxine deficiency may result in impairment of nucleic acid synthesis with consequent inhibition of protein synthesis. Thus it is probable that an alteration in the brush border membrane will lead to non-specific metal-ion uptake. It is also known that picolinic acid facilitates zinc absorption. PLP is required for picolinic acid formation. Thus a deficiency of vitamin B-6 may impair zinc absorption (Evans and Johnson, 1980).

Pyridoxine deficiency has also been reported to have led to an elevation of Fe stores in the liver and kidney (Kirksey and Tabacchi, 1967, Ikeda et al, 1979). Increased liver stores have been attributed to that fact that less Fe is incorporated into hemoglobin in pyridoxine deficiency since PLP is involved in hemoglobin synthesis.

Copper is needed for iron mobilization (Peereboom, 1985) and is mutually antagonistic to zinc. The close biological interaction of copper with zinc and iron and the fact that several copper metalloenzymes such as amine oxidases (Gray and Daniel, 1981) require PLP also warrants an investigation of the effect of vitamin B-6 on copper status.

Thus, due to the lack of information and controversy surrounding the effect of vitamin B-6 on trace element status it seems necessary to investigate this issue further. Due to the importance of Cu, Fe and Zn as essential trace elements and because of their close biological interaction they were chosen for analysis.

The dietary intake of copper, iron and zinc are reflected in tissue content (Delves, 1985, Jackson et al, 1982). Therefore tissue content was chosen as a method for assessing trace element status. Due to the central role of the liver in trace element metabolism, it was selected for analysis. Because of their relatively high concentrations of Cu, Fe and Zn, skeletal muscle, kidney and heart were also chosen for study (Prasad, 1978).

The results of a balance study conducted by Channa, 1988 in our laboratories supported the idea of a possible link

between vitamin B-6 and trace elements. This type of study is important in that it provided an insight into the effect of vitamin B-6 on retention and excretion of trace elements (Beisel, 1979). Due to the limitations of this kind of assessment, however no information on body distribution of trace elements can be given. Therefore it was decided that the assessment of trace element status by measurement of tissue content be investigated.

Since it was found in the first study that the food intake of deficient rats was decreased, a second study was conducted including a pair-fed control group. Hence, a second study was conducted to distinguish between metabolic consequences arising solely from decreased food consumption and those related directly to vitamin B-6.

Thus the major objectives of this study were:

1) To induce varying levels of vitamin B-6 deficiency states in the rats.
2) To compare the food intake between the various groups of deficient and control rats and pair-feed the deficient group.
3) To compare the growth rate between all groups.
4) To assess trace element status by determining the levels of Cu, Fe and Zn in the tissues of the different groups of rats.

CHAPTER TWO

LITERATURE SURVEY

2.1 Historical background of vitamin B-6

In 1926, Goldberger and Lillie fed rats a diet deficient in what was considered to be a pellagra-preventitive factor; these animals developed dermatitis acrodynia. Gyorgy, in 1934, observed that this same factor prevented development of skin lesions in the rat. He was the first to demonstrate that besides vitamin B-1, and B-2, the vitamin B-complex contained a third factor which was named vitamin B-6 (See Labadarios and Shephard, 1984).

Vitamin B-6 was isolated in 1938 by three research groups working independantly and was synthesized by Harris and Folkers in 1939. Two other natural forms of the vitamin, pyridoxal and pyridoxamine, were shown to exist in 1945 (Driskell, 1984).

2.2 Nomenclature

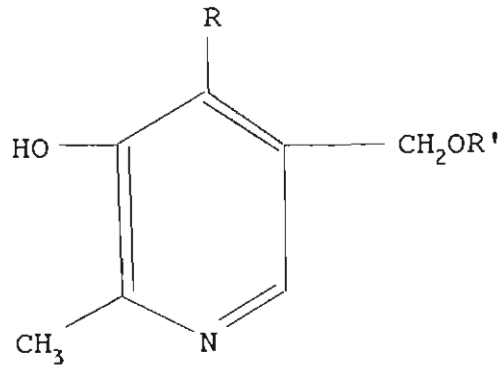
Vitamin B-6 is a collective term for three naturally occurring related compounds: pyridoxine (PN), pyridoxal

(PL) and pyridoxamine (PM). It is recommended as the generic descriptor for all 3-hydroxy-2-methylpyridine derivatives that exhibit the biological activity of pyridoxine in rats (Rutishauser, 1982).

2.3 Chemistry of vitamin B-6

The three naturally occurring forms of vitamin B-6 are pyridoxine, pyridoxal or pyridoxamine. In animal products, pyridoxal and pyridoxamine predominate while in plant products pyridoxine predominates (Driskell, 1984).

The different forms of vitamin B-6 may be characterized structurally as displayed in Figure 1:
(Labadarios and Shephard, 1984)



R' = H	R = CH_2OH	Pyridoxine (PN)
R' = H	R = CHO	Pyridoxal (PL)
R' = H	R = CH_2NH_2	Pyridoxamine (PM)
R' = PO_3^{2-}	R = CH_2OH	Pyridoxine-5'- phosphate (PNP)
R' = PO_3^{2-}	R = CHO	Pyridoxal -5'- phospate (PLP)
R' = PO_3^{2-}	R = CH_2NH_2	Pyridoxamine-5'- phosphate (PMP)

FIG 1. The chemical structure of vitamin B-6 vitamers.

All forms of the vitamin are readily soluble in water and are slightly soluble in ethanol and insoluble in ether. Aqueous solutions of the vitamin are unstable in visible and UV light and are destroyed by heating. Researchers working with this nutrient thus perform their analysis in darkened laboratories (Driskell, 1984). Pyridoxine and pyridoxamine are stable in hot dilute mineral acids and alkalis, while pyridoxal decomposes in hot dilute alkali (Labadarios and Shephard, 1984).

All forms of the vitamin occur as white crystals. Pyridoxine hydrochloride (PN.HCl) is the commonly available synthetic form of the vitamin. It is easily crystallizable and odorless and has a molecular weight of 205.6 and a melting point of 206^{0}C. This form of the vitamin is typically used in nutrient supplements and animal diets. Pyridoxine hydrochloride is used by pharmaceutical companies in drug preparation. A 10% loss of vitamin activity was found in several pharmaceutical products after storage for 1 year at 23^{0}C (Driskell, 1984).

2.4 Total Body Content of Vitamin B-6 in Man

In adult men and women the total body stores of vitamin B-6 have been estimated to be between 20 to 150mg of PN. A large share of body vitamin B-6 is contained in phosphorylase and in muscle most vitamin B-6 is contained in this enzyme. The total blood content of vitamin B-6 is about 0,5mg PN (Rutishauser, 1982).

2.5 Physiological Functions of Vitamins B-6

PLP, the active form of vitamin B-6, serves as a coenzyme in several metabolic reactions. The functions of vitamin B-6 will be reviewed from the perspective of the role of PLP.

The role of PLP can be viewed from a biochemical and a cellular viewpoint (Leklem, 1988). With respect to its biochemical function, pyridoxal-5-phosphate forms a Schiff's base with amino acids and other nitrogen containing compounds.

The functions of vitamin B-6 from a systems or cellular point of view are depicted in Figure 2 (Leklem, 1988). Most of the cellular processes affected by PLP are related to the role of PLP in amino-acid metabolism (Leklem, 1988).

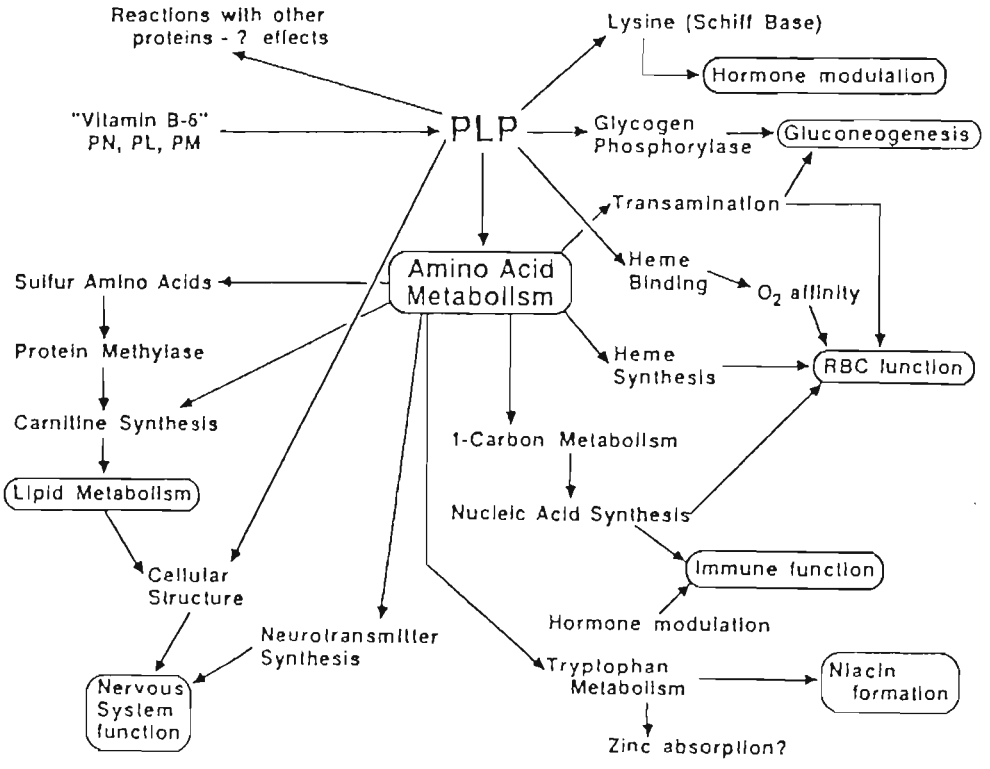


FIG. 2 : Cellular processes in which PLP acts as a coenzyme or binds with proteins and modifies the action of the protein.

2.5.1 Role of PLP in Transamination reactions

All known aminotransferases are PLP dependent. Most amino acids at some stage of their metabolism undergo transamination except for threonine, lysine, proline and hydroxyproline (Sauberlich, 1985). Transamination involves the interconversions of a pair of amino acids into their corresponding keto acids. In the initial step a PLP-enzyme complex is formed. The bound PLP undergoes a reaction with an amino acid to form a keto acid and enzyme bound PMP. (Fig. 3) (Labadarios and Shephard, 1984)

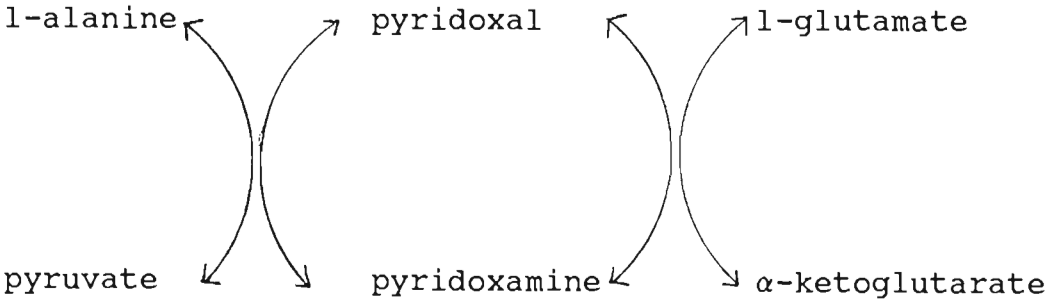


FIG. 3 : Role of vitamin B-6 in transamination reactions.

This general reaction permits catabolism, interconversion and synthesis of amino acids and α-ketoglutarate is more often the acceptor of the amino group. As a result of this

reaction amino acids such as alanine and serine are synthesized (Sauberlich, 1985).

2.5.2 GABA Synthesis

Vitamin B-6 is needed in the brain for the decarboxylation of glutamic acid to ϒ-aminobutyric acid (GABA) (Diskell, 1984) L-Glutamic acid decarboxylase 1, found in the brain is a PLP-dependent enzyme catalysing the formation of GABA (Fig. 4) (Labadarios and Shephard, 1984).

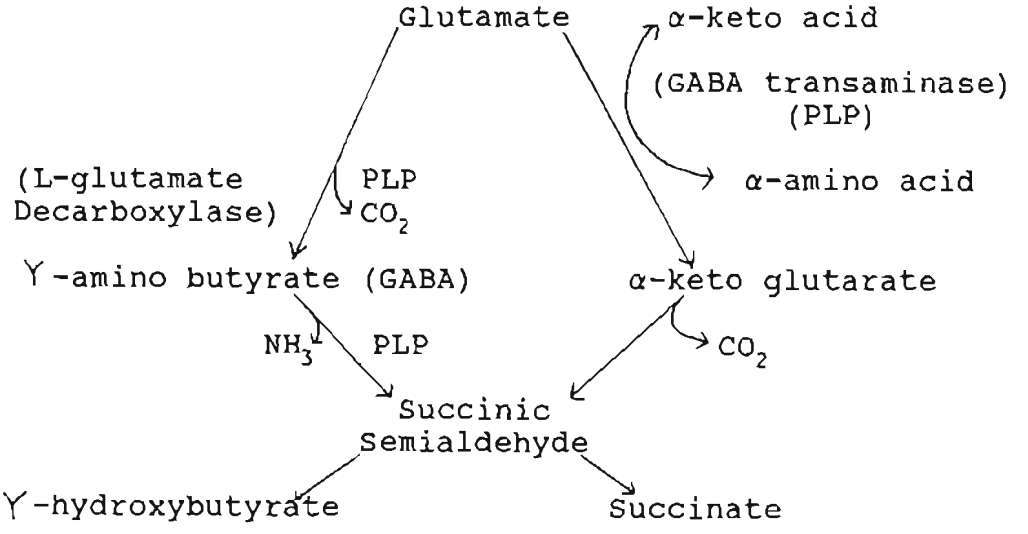


FIG.4 : Role of vitamin B-6 in GABA synthesis.

GABA is required as a normal regulator of neuronal activity and has an inhibiting effect on the central nervous system (CNS). Decreased levels of PLP may result in suppression of glutamate decarboxylase activity resulting in lower GABA

concentrations. This results in convulsions which can be corrected by vitamin B-6 administration (Labadarios and Shephard, 1984).

2.5.3 Serotonin Synthesis

Serotonin (5-Hydroxytryptamine) is produced by the action tryptophan decarboxylase on 5-hydroxytrytophan as illustrated in Figure 5 (Sauberlich, 1985).

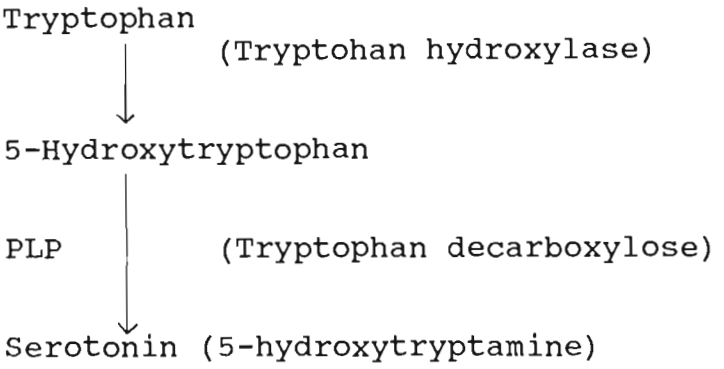


FIG. 5 : Role of vitamin B-6 in serotonin synthesis.

Serotonin is a potent vasoconstrictor and a stimulator of smooth muscle contraction and also functions in the same manner in the brain (Driskell, 1984).

2.5.4 Catecholamine Synthesis

PLP dependent enzymes are involved in the synthesis of catecholamine hormones viz., dopamine, norepinephrin and epinephrin as illustrated in Figure 6 (Sauberlich, 1985).

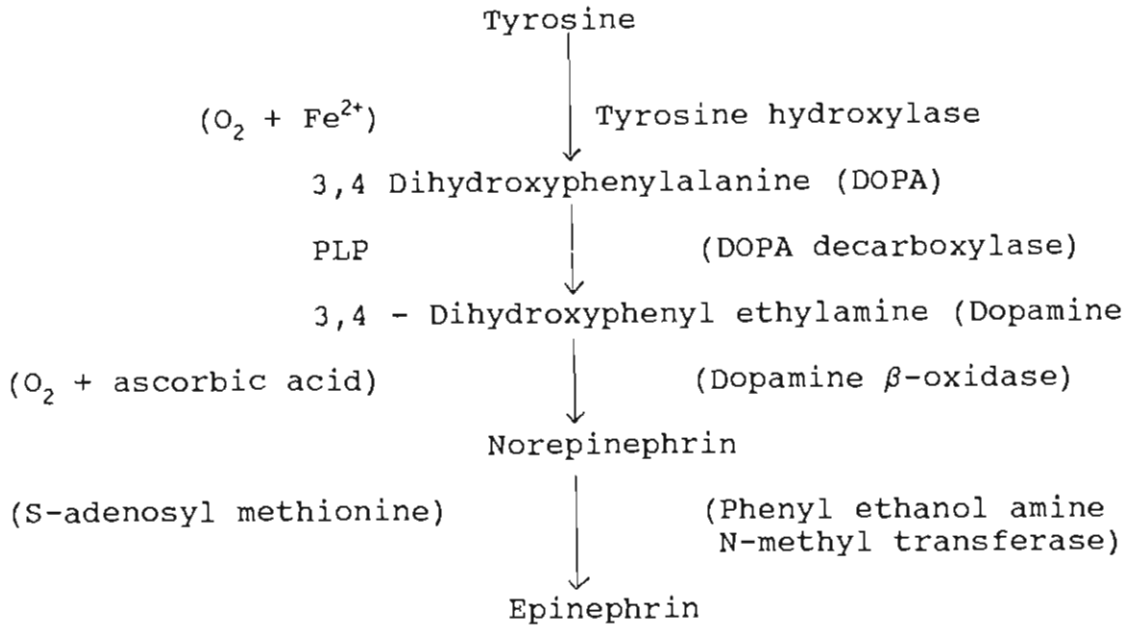


FIG. 6 : The role of vitamin B-6 in catecholamine synthesis

The decarboxylase activity is increased <u>in vitro</u> by the addition of PLP and <u>in vivo</u> by the elevation of tissue PLP levels. Dopamine levels were reported to be elevated by pyridoxine adminstration (Labadarios and Shephard, 1984).

2.5.5 Polyamine Synthesis

Ornithine is decarboxylated by the PLP dependent enzyme, ornithine decarboxylase to form putrescine. Putrescine serves as a precursor for the polyamines, spermine and spermidine. Ornithine may also be reacted upon by

ornithine-δ-transaminase which is PLP dependent to form glutamic acid semialdehyde. This may serve as a precursor for proline. Hyperornithemia, caused by a genetic defect in ornithine decarboxylase which gives rise to elevated ornithine in plasma may result in gyrate atrophy and impaired visual functions. Vitamin B-6 supplements have been encourgaging in stabilizing of visual functions (Sauberlich, 1985).

2.5.6 Vitamin B-6 and sulphur amino acids

Sulphur containing amino acids are metabolized through numerous pathways which require PLP (Labadarios and Shephard, 1984). The pathway for methionine metabolism is shown in Figure 7.

Methionine and cysteine are important in protein biosynthesis. Cysteine is involved in maintaining protein configuration through disulphide bond formation (Tryfiates, 1986). Taurine is an essential amino acid in man (Sturman, 1981). The enzymes involved in Figure 7 are affected by vitamin B-6 deficiency but not to the same extent. Nutritional vitamin B-6 deficiency has little effect on cystathionine synthase deficiency because of the tight bond between PLP and this enzyme. Homocystinuria due to

cystathionine synthase deficiency is inherited. The symptoms include optic lens dislocation, progressive skeletal deformities and severe thrombo-embolic disease. Biochemically it is characterized by high concentrations of homocysteine and methionine and abnormally low concentrations of plasma and urine cysteine (Labadarios and Shephard, 1984).

Vitamin B-6 is not tightly bound to apocystathionase or apocysteinesulphinic decarboxylase. A deficiency of vitamin B-6 causes a reduction in holocystathionase and cystathionuria which is reversed by pyridoxine supplements. Holocysteinesulphinic acid decarboxylase is reduced to zero during a vitamin B-6 deficiency but this has no biological significance because of the dietary intake of taurine (Labadarios and Shephard, 1984).

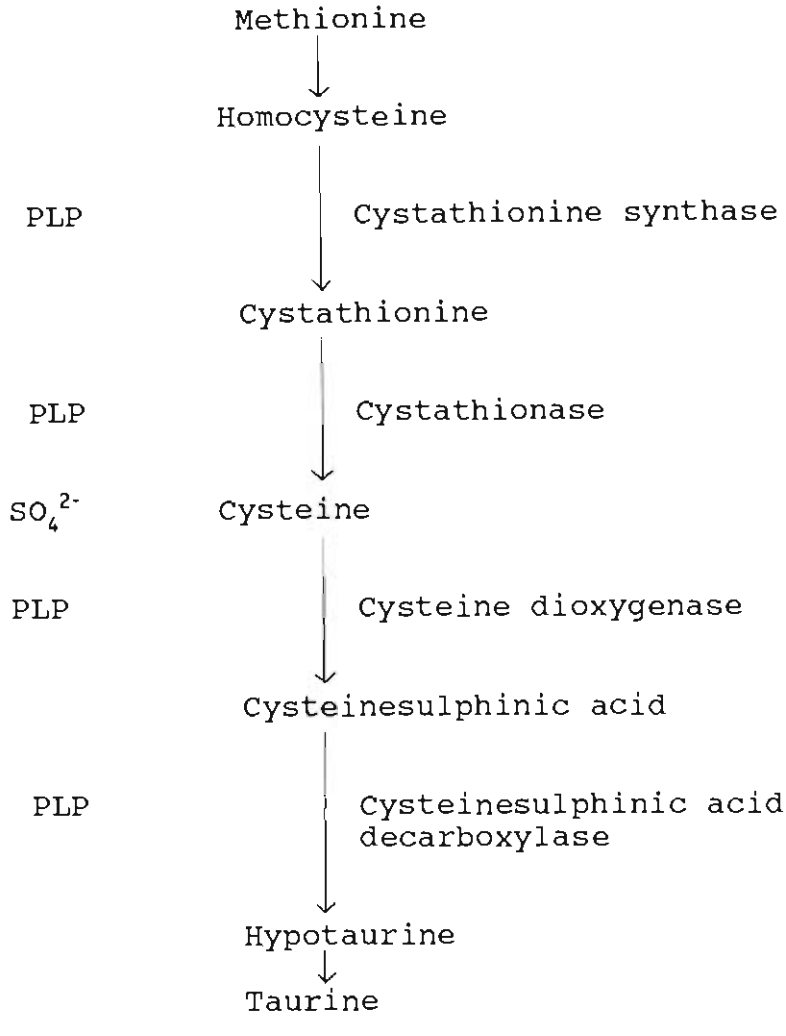


FIG. 7 : Role of vitamin B-6 in methionine metabolism

2.5.7. Tryptophan metabolism

Vitamin B-6 and tryptophan-niacin metabolism is one of the most extensive vitamin B-6 amino acid interrelationships as illustrated in Figure 8.

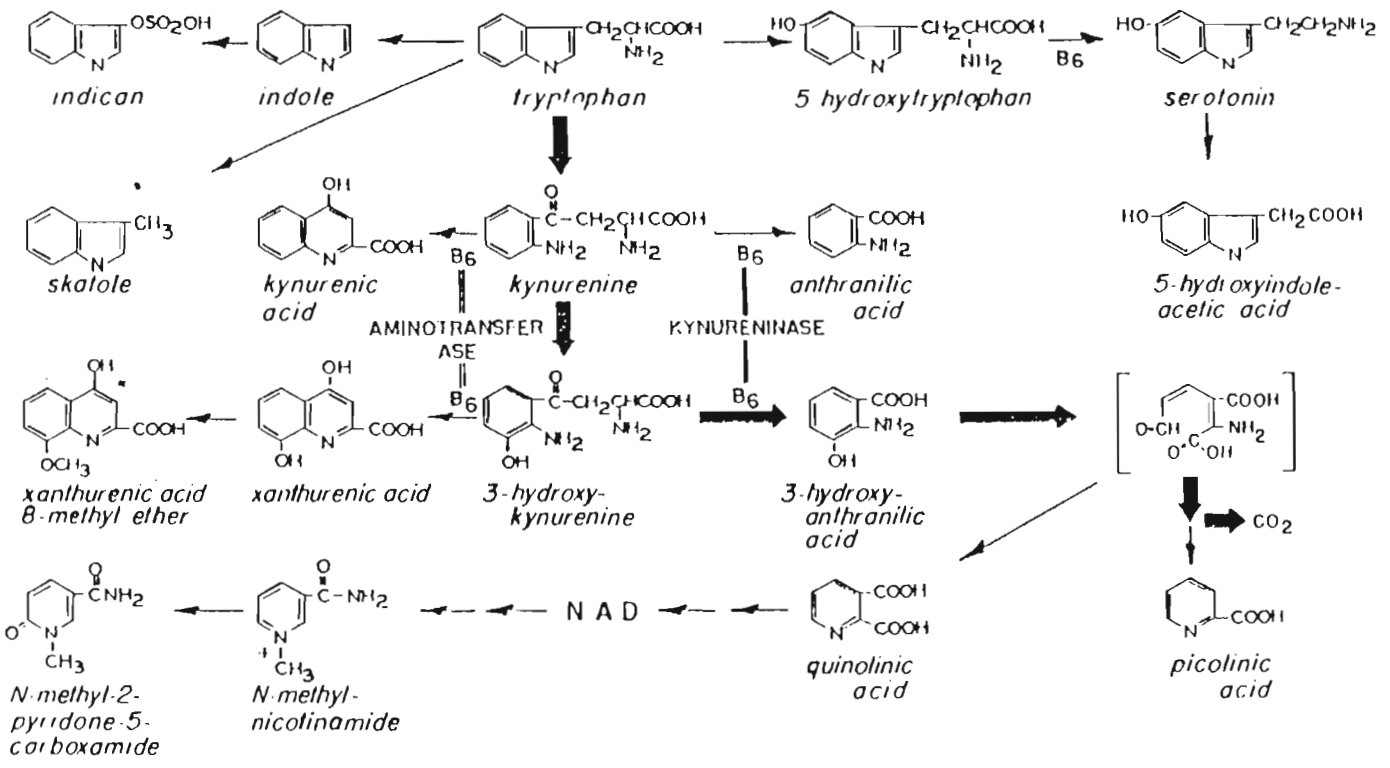


FIG. 8 : Metabolic chart showing some of the metabolites of tryptophan. The large arrows indicate the pathway for degradation of the majority of ingested tryptophan. B-6 indicates the sites of action of enzymes requiring PLP as a cofactor.

This is partly related to the use of the tryptophan load test to assess vitamin B-6 status (Leklem, 1988). The key enzymes involved in tryptophan metabolism that require PLP are shown in Figure 8 (Brown, 1981).

Kynureninase, a PLP dependent enzyme is very sensitive to a vitamin B-6 deficiency. If this enzyme is impaired an increased excretion of tryptophan metabolites in the urine is observed. The metabolites in particular are xanthurenic acid, kynurenic acid, kynurenine and 3-hydroxyanthranilic acid. Some of these metabolites possess carcinogenic properties and may induce bladder tumors.

The importance of the contribution of tryptophan metabolism to niacin needs has been recognised in diseases where the absorption and retention of tryptophan may result in a pellagra syndrome that responds to nicotinamide therapy (Sauberlich, 1985).

It was reported that low vitamin B-6 intake may have only a moderate effect on the tryptophan to niacin conversion (Leklem, 1988).

2.5.8 Role of vitamin B-6 in gluconeogenesis and glycogenolysis

Since PLP is a coenzyme for alanine aminotransferase, at cellular levels, PLP would be expected to influence the conversion of alanine to pyruvate. It has been demonstrated that under short-term fasting conditions, vitamin B-6 deficiency has not compromised gluconeogenesis to an extent to which glucose synthesis is affected (Leklem, 1988).

Subclinical vitamin B-6 deficiency has in some cases been associated with impaired glucose tolerance tests. This may be explained by the role of PLP in phosphorylase activity. Epinephrin, which is dependent on PLP for synthesis, stimultes phosphorolysis of glycogen. However, vitamin B-6 may have an effect on insulin metabolism (Sauberlich, 1988).

Glycogen phosphorylase, which catalyses the first step in glycogenolysis, contains PLP. The active form contains 4 mol PLP while the inactive form, 2 mol PLP. PLP appears not to act as a coenzyme for the enzyme but rather to affect its conformation (Driskell, 1984). Studies carried out by certain investigators indicate that PLP associated with muscle glycogen phosphorylase may serve as a source for

vitamin B-6 during caloric deficit. The mechanism for PLP release has not been explained (Leklem, 1988).

2.5.9 Vitamin B-6 and erythrocyte function

δ-aminolevulinate synthase requires PLP for the synthesis of δ-aminolevulinic acid (ALA). It is then acted upon by ALA dehydratase,a Zn containing enzyme to form porphobilinogen which is converted to heme (Fig. 9). Heme is incorporated during the formation of hemoglobin, myoglobin cyctochrome C_1 and other proteins requiring heme. (Sauberlich, 1985)

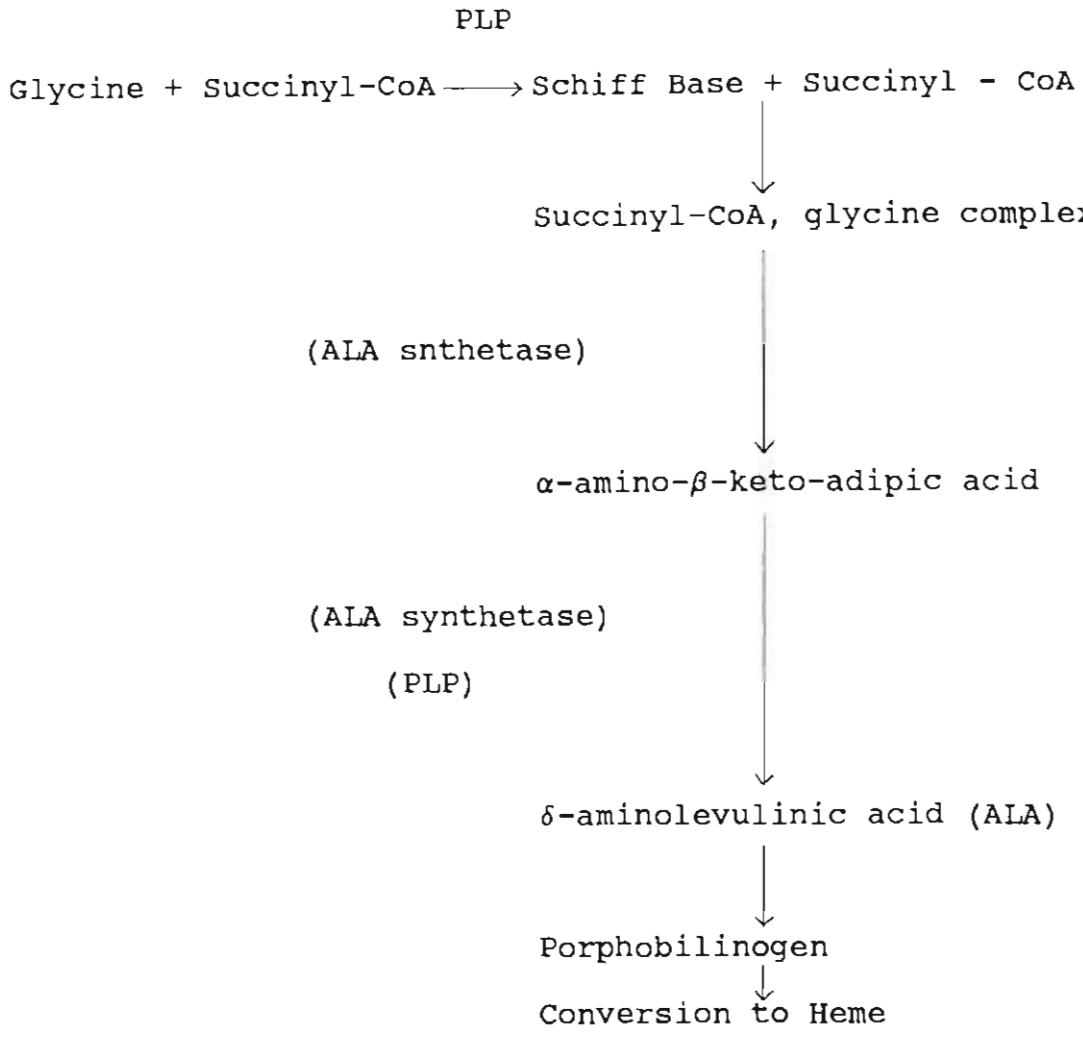


FIG. 9 : The role of vitamin B-6 in heme synthesis.

PLP and PL bind to hemoglobin (Hb) and erythrocyte aminotransferases. PL and PLP have different binding sites on Hb. While PLP lowers the O_2 binding affinity of Hb, PL has the opposite effect.

2.5.10 Vitamin B-6 and nucleic acid synthesis

The involvement of vitamin B-6 in nucleic acid synthesis is depicted in Fig. 10 (Labadarios and Shephard, 1984)

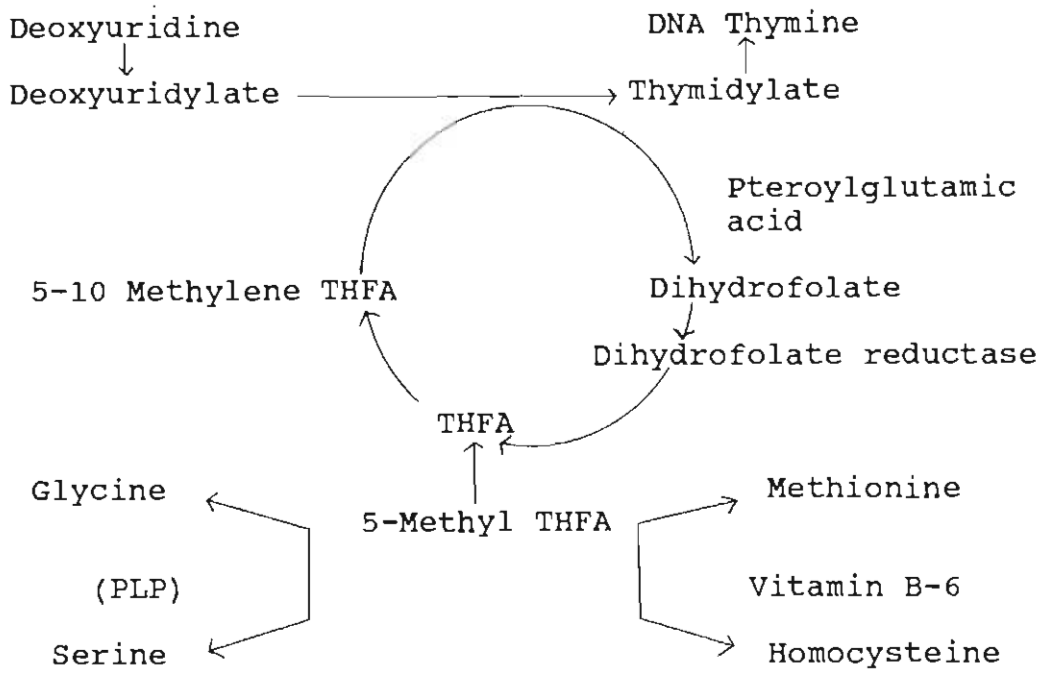


FIG. 10 : The involvement of vitamin B-6 in nucleic acid synthesis

Glycine is synthesized from serine in a step that requires PLP. The methylene group of N^5, N^{10} methylenetetrahydrofolate (methylene THFA) is the source of the methyl group for the synthesis of methionine and deoxythymidylate a key precursor for DNA synthesis (Tryfiates, 1986).

It has been concluded that a lack of vitamin B-6 impairs nucleic acid synthesis and results in a deleterious effect on protein production, cell division and repair. This impairment of nucleic acid synthesis is due to decreased c-1 fragment production from serine that is needed for DNA and RNA biosynthesis (Tryfiates, 1986).

PLP was also shown to inactivate certain tRNA synthetases, thus losing their ability to esterify amino acids to tRNA. It was also shown to modify tRNA causing it to lose the ability to accept amino acids and recognise mRNA codons. (Tryfiates, 1986)

2.5.11 Lipids

It was shown that vitamin B-6 deficiency in animals resulted in lower lipid content than controls. The symptoms of the deficiency include fatty liver, dermatitis, atherosclerosis and elevated plasma lipids (Driskell, 1984). The mechanism of vitamin B-6 involvement in lipid mtabolism has not been established. It is possible that the involvement of vitimin B-6 in lipid metabolism may represent the results of a secondary effects (Sauberlich, 1985). Driskell, 1984 states that the effects on lipids may be via its effects on hormones.

Pyridoxine may function in the utilization of unsaturated fatty acids. Studies using Aspergillus nidulans, a pyridoxine-less mutant, suggested a possible involvement of pyridoxine in fatty acid chain elongation and in the desaturation of fatty acids (Sauberlich, 1985).

2.5.12 Steriod function

The interaction of PLP with steroid function has recently been identified. Several studies have shown that PLP can be used to extract steroid receptors from nuclei (Leklem, 1988).

PLP binds to DNA binding domains and prevents interaction of steroid receptors with DNA (Litwak et al, 1985). Figure 11 shows the functional groups of the glucocorticoid receptor and sites at which PLP was shown to inhibit (Litwak, et al, 1985).

Several investigators have found, using rats, a greater retention of steriods at target cells during vitamin B-6 deficiency. This would presumably increase the sensivity of the end-target cell to the steriod. Studies on the significance of the interaction between PLP and steroids

receptors in humans still has to be determined (Leklem, 1988).

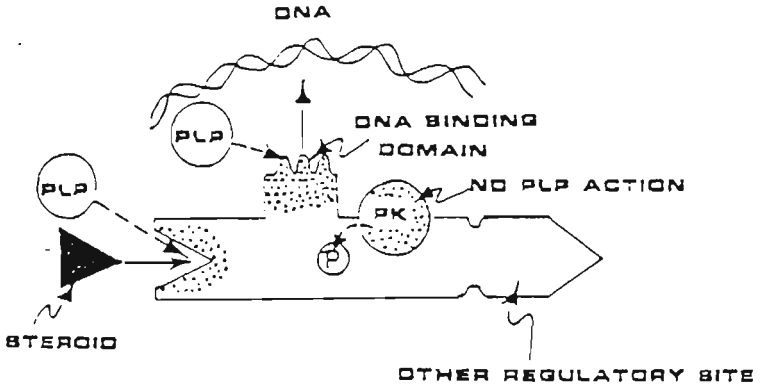


FIG.11 : PLP and glucocorticoid receptor functional sites

2.6 METABOLISM OF VITAMIN B-6

2.6.1 Absorption

In the rat intestinal absorption of pyridoxine and other forms occurs via a non-saturable passive process. However, Middleton (in Leklem, 1988) found a saturable component of uptake in the rat intestine. If this mechanism exists in humans, it would have implications for large supplemental

intakes. The uptake of phosphorylated forms occurs via hydrolysis by alkaline phosphatase. Phosphorylated forms can also be absorbed at high levels (Leklem, 1988).

2.6.2 Metabolism and Transport

PL, PN, and PM are forms in which most dietary vitamin B-6 reaches the circulation. They are rapidily transported into organs and tissues. (Ink and Henderson, 1984). The liver and the erythrocyte are important in defining the predominant vitamin forms found in blood plasma. Uptake into the liver occurs by facilitated diffusion. The liver is the primary organ for the interconversion and metabolism of the three forms of vitamin B-6. The metabolic steps involved are depicted in Fig. 12 (Leklem, 1988).

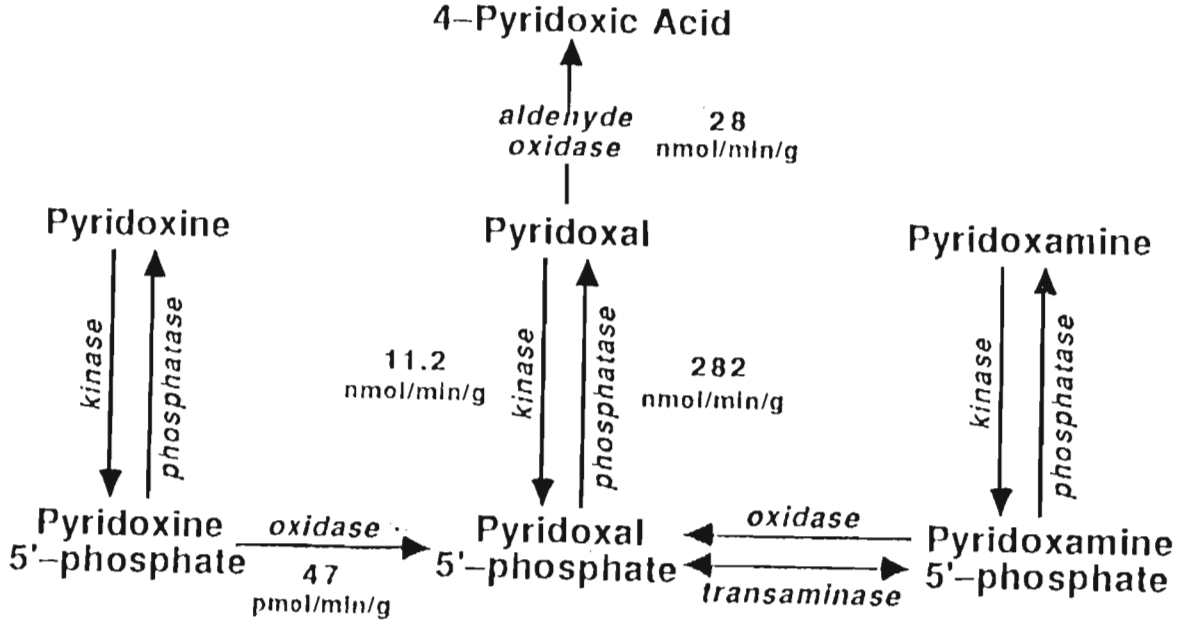


FIG. 12 : Interconversion of vitamin B-6 vitamers in the human liver.

The two metabolic steps common to all three forms are phosphorylation and dephosphorylation. In the phosphorylation step a phosphate group is attached at a 5' position via pyridoxal kinase and during dephosphorylation this group is removed by a phosphatase enzyme, probably alkaline phosphatase. PMP and PNP are then converted to PLP by pyridoxine (pyridoxamine)5-phosphate oxidase which requires flavin mononucleotide (FMN). Pyridoxal produced by either dephosphorylation of pyridoxal-5-phosphate or that which is taken up by the liver is converted to 4-pyridoxic acid (4-PA) by aldehyde oxidase and/or an NAD - dependant dehydrogenase. This is the process by which excess intakes of vitamin B-6 is metabolized (Leklem, 1988).

PLP formed in the liver is either utilized in the liver or released into the circulation. Although the liver was considered to be responsible for synthesis of the PLP found in plasma, it has been suggested that muscle may also release PLP into the plasma during caloric deficit (Leklem, 1988).

PLP in the plasma is tightly bound to albumin, a mechanism which protects it from hydrolysis and permits delivery to other tissues. Plasma also contains other forms of vitamin B-6. Under conditions of normal intake plasma PLP is found

in the highest concentration (60% to 70%) followed by PL, PN and PM in decreasing order. PMP and PNP are very low in concentration and may not be found (Leklem, 1988).

Thus PLP and PL (and perhaps PL in the erythrocytes) are the forms available to other tissues. However, PLP has to be hydrolysed by alkaline phosphatase first before uptake into the cells (Leklem, 1988).

A schematic diagram proposed by Lumeng et al, 1985 depicted in Fig. 13 shows the interconversion and transport of vitamin B-6 vitamers in plasma after pyridoxine ingestion. The importance of the liver and red blood cell in pyridoxine metabolism is clearly depicted by this diagram but essential differences occur. While pyridoxine is mainly metabolized to PLP, PL, and 4-PA in liver, the erythrocyte converts PN to PL. These end metabolites then appear in the plasma. This conversion of PN to PL and PLP by the liver and erythrocytes are very important for vitamin B-6 coenzyme synthesis in tissues that lack PMP (PNP) oxidase such as skeletal muscle, heart, lung, pancreas etc. (Lumeng, 1985). Thus skeletal muscle, which is the major repository of vitamin B-6 cannot effectively convert PN to PLP or PMP and it must rely on a supply of PLP or PL provided by other

tissues. This example emphasizes the importance of interorgan transport of B-6 vitamers in the overall scheme of mammalian vitamin B-6 metabolism (Lumeng, 1985).

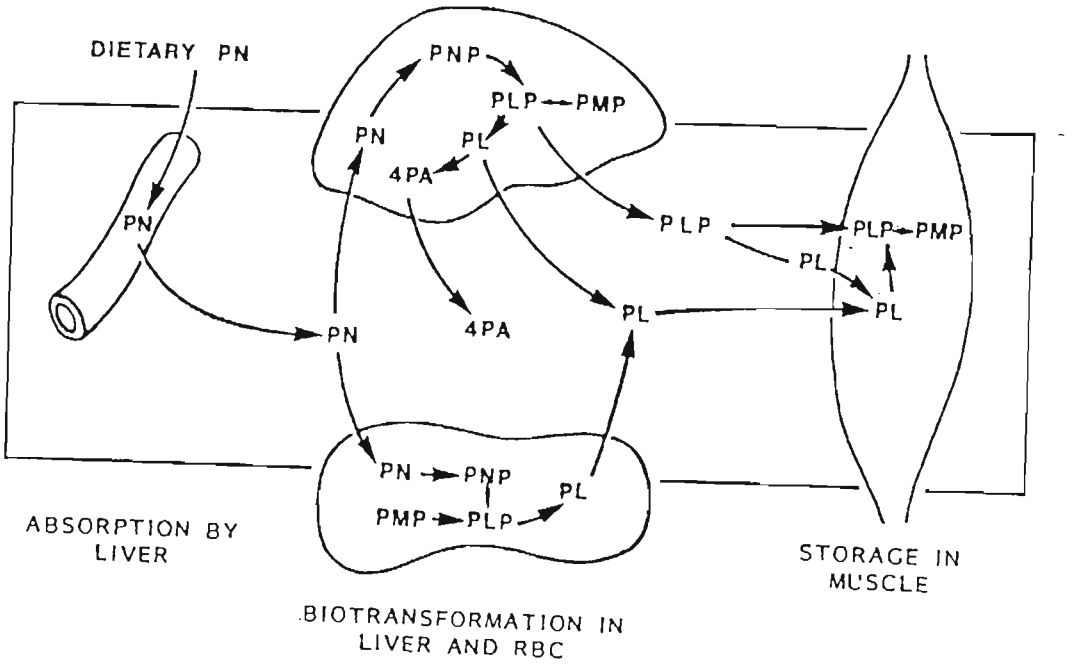


FIG.13 A proposed pathway for the conversion and transport of vitamin B-6 compounds in plasma after PN ingestion

2.7 ROLE OF THE ERYTHROCYTE IN VITAMIN B-6 METABOLISM

The pathway for conversion of PN to PLP is the same as in the liver. As illustrated in Figure 14, albumin bound PLP is incapable of entering the erythrocyte in the presence of plasma proteins. PN and PL enter by passive diffusion. PN is phosphorylated and 'metabolically trapped' within the erythrocyte and is rapidily oxidized by an FMN - dependent oxidase to PLP. The human erythrocyte has oxidase activity, while the rat lacks it (Reynolds and Natta, 1985).

PL which diffuses in the erythrocyte is rapidly and predominantly bound to the hemoglobin such that very little is phosphorylated to PLP. Thus PL undergoes 'metabolic trapping' by binding to a protein rather than phosphorylation. PL tends to remain in the erythrocyte because of its greater binding affinity to hemoglobin than albumin (Reynolds and Natta, 1985; Lumeng et al, 1985).

Complicating the role of the erythrocyte as a source of PLP and PL for other tissues is the binding of PL and PLP to hemoglobin. They bind at different sites and have different binding affinities.

2.7.1 Significance of Red-cell metabolism of vitamin B-6

PLP

Pyridoxal phosphate binds to the red cell at the same site as 2,3 diphosphogylcerate (DPG) and ATP bind. It also binds less firmly at another site. Pyridoxal phosphate was also found to lower the oxygen affinity of hemoglobin facilitating oxygen unloading as the above two compounds. Due to the large amount present, DPG plays a large part in lowering oxygen affinity. However on a molecular basis DPG and pyridoxal phosphate were equally effective in this function (Anderson, 1980).

PL

PL also binds to hemoglobin but to the α-chain and increases the oxygen binding affinity, whereas PLP binds to the β-chain of hemoglobin and decreases the oxygen binding affinity (Reynolds and Natta, 1985).

It has been shown after doses of pyridoxine that significant uptake by the red cell occurs *in vivo* and *in vitro* and pyridoxal present in blood is due to red cell conversion. The red cells, thus acts as a "factory" and a "reservoir" for pyridoxal, maintaining a constant ratio between red

cells and plasma whether pyridoxal is formed in the red cell or taken up from outside.

Pyridoxal phosphate is not able to enter red-cells because of its strong binding to albumin. This is probably also the reason for its inability to enter other cells. This is supported by its slow clearance from plasma. Thus pyridoxal may be the transport form of vitamin B-6 as it is able to enter cells (Anderson, 1980).

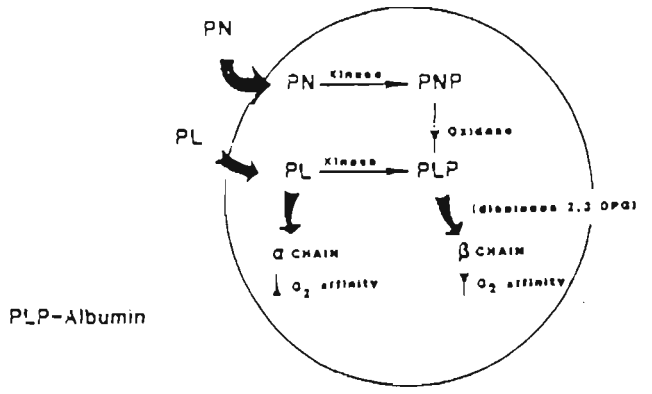


FIG. 14 : Metabolism of vitamin B-6 compounds in erythrocytes and their binding to hemoglobin chains

2.8 ASSESSMENT OF VITAMIN B-6 NUTRITIONAL STATUS

The four most commonly used tests to assess vitamin B-6 status in humans include:

2.8.1 Measurement of vitamin B-6 vitamers in serum or blood plasma. PLP is the most commonly used parameter to test for for adequacy of vitamin B-6 stores. It is a direct measure of the active coenzyme and is reflective of tissue levels (Leklem and Reynolds, 1981). Data collected by Lumeng et al, 1978 indicate that plasma PLP concentration is a valid indicator of vitamin B-6 nutrition as it increases with pyridoxine intake in the range between 0-100μg/day. It also correlates well with the PLP content of skeletal muscle, the major storage pool of vitamin B-6 compounds in the rat. However, Masse et al, 1988 suggested that the use of PLP alone to assess vitamin B-6 status maybe misleading. Measurement of the total concentration of vitamin B-6 aldehyde vitamers is suggested. Endogenous PLP is inversely related to alkaline phosphatase as demonstrated by other investigators. The hydrolytic action of this enzyme may be responsible for the elevation in plasma PL concentration. Hence PL was chosen to assess the vitamin B-6 status in rats in the present study.

2.8.2 Measurement of various PLP dependent enzymes in erythrocytes with and without added PLP. Pyridoxal phosphate is added *in vitro* to the incubation mixture and the percentage stimulation of transaminase activity in erythrocytes is measured. This is compared to the transaminase activity in an incubation mixture without PLP and is used to measure adequacy of PLP stores in tissues.

2.8.3 Measurement of excretion of tryptophan metabolites in urine following a tryptophan load. This is also a useful indicator of vitamin B-6 status. This is a suitable technique for use in population surveys. It is however unsuitable in nutritional studies where the nutrient balance has to be maintained. Although widely used, results have to be interpreted with care as other metabolic and hormonal factors are known to be involved in tryptophan metabolism (Driskell, 1984).

2.8.4 Measurement of the excretion of pyridoxic acid, the inactive degradative metabolite of pyridoxal phosphate. Measurements of pyridoxic acid excretion are indicative of immediate dietary intakes of the vitamin and probably not indicative of body reserves (Driskell, 1984).

2.9 ASSAY PROCEDURES FOR VITAMIN B-6

2.9.1 Animal bioassay using either rats or chicks were used in the 1945-1955 era. The sample can be fed directly and the problems associated with other methods can be left to the enzyme machinery of the animal. However, accurate assay requires large numbers of animals and a time period usually involving weeks. Hence these methods are very slow and very expensive. These methods have been replaced by other techniques (Snell, 1981).

2.9.2 The standard method for the quantitation of vitamin B-6 in foods is via microbiological assay. According to Polansky, 1981, "the microbiological method using S. uvarum which was selected for its specific response to vitamin B-6, is still the most widely used method for the estimation of total vitamin B-6 in foods." The sample must be hydrolysed first as the yeast only grows on the free forms of vitamin B-6; generally the sample is suspended in a certain normality of HCl and autoclaved. The growth response of the organism is measured in a vitamin B-6 free basal medium to which either a sample or a standard is added (Driskell, 1984).

PN, PL and PM are equally active for Saccharomyces uvarum although certain strains have been reported to respond to a greater extent to PM. The procedures involved in such assays are time consuming with added disadvantages such as varialibility in the growth response of various micro-organism in the vitamin, mutation in micro-organisms, and growth inhibitory substances in the sample (Driskell, 1984).

2.9.3 Currently available chemical techniques have been reviewed by Dakshinamurti and Chauhan, 1981. Gas-liquid chromatography (GLC) has been used for the detection and quantitation of vitamin B-6. Prederivatization is essential for GLC analysis because of the low volatility of vitamin B-6 compounds. Williams, 1974 has reviewed in detail the GLC methodology for vitamin B-6 compounds. Some of the disadvantages of using this method are the sensivity of many of the derivitization methods to water or alcohol and the need to remove non volatile materials (Gregory and Kirk, 1981).

2.9.4 Recent developments concerning HPLC (high performance liquid chromatography) have made this technique competitive with GLC in speed and resolution of complex mixtures (Driskell, 1984). HPLC procedures were found to be reproducible, and speedy with minimal sample clean up.

2.10 VITAMIN B-6 REQUIREMENT OF THE RAT

Several studies have indicated that the level of protein in the diet affects the metabolism of vitamin B-6. Studies on rats have shown that when the vitamin B-6 intake is inadequate the lower levels of the vitamin is inversely proportional to protein intake. However, when vitamin B-6 intake was adequate, high dietary protein favoured liver storage of the vitamin. It was found that when rats were fed a higher ration of casein they developed acrodynia earlier and had a greater growth retardation. The increased vitamin B-6 requirement caused by the high protein intake may be due to an induction in the activities of amino acid metabolizing enzymes that need PLP (Olson, 1987).

Clarification of the role of intestinal microflora in the metabolism of vitamin B-6 is important in the determination of vitamin B-6 requirements in rats. Intestinal micro-organisms can synthesize vitamin B-6 in humans and other monogastric animals. The extent of synthesis in the rat is markedly influenced by diet composition. The extent of absorption and contribution to the metabolism of dietary vitamin B-6 by intestinal microflora is unclear. However recent data indicate that intestinal micro-organisms do not contribute significantly to qualitative and quantitative

aspects of vitamin B-6 metabolism in adequately nourished rats. The above has not been examined in marginal or overtly deficient animals (Rosenburg, 1989).

Beaton and Cheney, 1964 suggested that the pyridoxine hydrochloride requirement for maximal weight gain is at least 40ug/day and may approximate 80μg/day. He found that for maximal erythrocyte GOT activity the requirement range was in the 40 to 80μg/day range while erythrocyte GPT activity responded to dosages as high as 80μg/day. On the basis of his results he suggested that rats should take in about 100g of pyridoxine hydrochloride (PN HCl) per day. This would be equilavent to about 60 to 70 mg PN.HCl per 10g diet. This was confirmed by Mercer, et al, 1984.

Driskell et al, 1973 found that curiosity responses, pyridoxal phosphate, DNA, RNA and protein compositions of the brain reached a plateau at 30μg of PN intake daily. General activity responses and alanine aminotransferase activities reached a plateau at 45μg of PN intake daily. Based on the above parameters they suggested 45μg daily as the vitamin B-6 requirement for weanling and sexually mature male rats. Van den Berg et al, 1982 found that 24μg/day was needed for maximal growth of rats.

The American Institute of Nutrition (AIN) report on standards for nutritional studies (1977) recommended 7mg PN.HCl per kg of diet for adequate growth, gestation and lactation of rats.

Thus it can be concluded that the vitamin B-6 requirement of rats ranges between 24μg/day and 100μg/day. Anything above or below could lead to undesirable metabolic changes.

2.11 MANIFESTATIONS OF VITAMIN B-6 DEFICIENCY IN THE RAT

Vitamin B-6 deficiency in the rat results in a dermatitis resembling that of essential fatty acid deficiency. The dermatitis (also known as acrodynia) is observed on the tail, paws, nose, chin and upper thorax of vitamin B-6 deficient rats.In addition the deficiency produces poor growth, muscular weakness, fatty liver, convulsive seizures, anemia, reproductive impairment, oedema, nerve degeneration and impaired immune responses (Rutishauser, 1982).

Behavioural alterations in general activity curiosity scores and avoidance learning have also been reported (Driskell, 1984).

2.12 DISEASES ASSOCIATED WITH VITAMIN B-6 METABOLISM OR UTILIZATION

2.12.1 Atherosclerosis

Rinehart and Greenburg, 1949 were the first to associate vitamin B-6 deficiency with vascular disease. They found that sclerotic lesions had developed in arteries of monkeys fed a pyridoxine deficient diet. Few studies have been carried out to assess the implications of these findings in humans. However, Serfontein et al, 1985 showed in a pilot study that plasma PLP in myocardial infarction (Ml) patients was lower than healthy controls. It was also suggested that plasma PLP levels may be a far more sensitive indicator of myocardial infarction (MI) than plasma cholesterol or HDL values. Kok et al, 1989 found that all levels of vitamin B-6 indices except PL were lower in MI patients compared to the controls. It is suggested that the low levels may be implicated in the pathogenesis of the disease or may be as a consequence of MI.

Although the latter has not been ruled out, there are also several mechanisms whereby PLP could contribute to the progression of the disease. The mechanisms are as follows:

2.12.1.1 Sulphur amino acid metabolism

Atherosclerotic changes and thrombo-embolism commonly occur in homocysteinuric patients and infusion of homocysteine causes atherosclerotic lesions (Labadarios and Shephard, 1985). Several groups have proposed that elevated plasma levels of homocysteine due to marginal vitamin B-6 deficiencies could be a major contributory factor in atherosclerosis (Merrill and Henderson, 1987). The homocysteine theory of atherosclerosis is the most developed hypothesis of the three general mechanisms reviewed (Willet, 1985).

2.12.1.2 Lysyl Oxidase

Lysyl oxidase is an extracellular enzyme which is involved in elastin and collagen cross linking. It is Cu and PLP dependent.

Starcher, 1969 confirmed an earlier hypothesis that pyridoxine deficiency, like Cu deficiency, in chicks does reduce the rate of lysine oxidation in aortic elastin and the amount of desmosine and isodesmosine formed. He supported earlier suggestions that a pyridoxine cofactor is required for enzymatic lysine oxidation.

Elastin and collagen are the major fibrous proteins of the arterial wall. They are cross linked so that arterial structure may be restored after systole and diastole. Their cross linking is initiated by lysyl oxidase, which oxidatively deaminates lysine or hydroxylysine residues to their aldehydic derivatives. Levene and Murray, 1977 found strong evidence that lysyl oxidase, in addition to being Cu dependent, also required PLP. This claim has been refuted by Willett, 1985.

Levene and Murray, 1977 hypothesized that the earliest visible lesion of atherosclerosis found in human neonatal coronary arteries was a 'focal splitting' of the internal elastic lamina. They hypothesized that "this lesion is the result of imperfect cross linking of arterial elastin and collagen and is caused by a maternal deficiency of vitamin B-6 which is commonly found in pregnancy and which could thus impair the function of lysyl oxidase."

2.12.1.3 It has been suggested that low vitamin B-6 intake in rats and rabbits may increase the risk of coronary heart disease by raising serum cholestrol (Willet, 1985).

2.12.2 Hematological Disorders

The prototypical pyridoxine responsive anemia is characterized as having severe hypochromic, microcytic cells, elevated serum Fe and low erythrocyte protoporphyria. Most cases appear to have abnormalities in Fe metabolism suggestive of a genetically determined error in metabolism which results in increased dependance of erythropoiesis on vitamin B-6 (Labadarios and Shephard, 1985).

There is extensive literature regarding "pyridoxine responsive" anemias. Patients with sideroblastic anemia have shown an "optimal response" to pyridoxine therapy. While some patients responded others did not. The variable responsiveness to pyridoxine has not been explained reasonably (Merrill and Henderson, 1987).

One of the explanations for the response to pyridoxine therapy concerns a defective δ-aminolevulinate synthase (ALA-S) that results on a low affinity for PLP. Thus pyridoxine supplements are effective in patients with decreased ALA-S activity (Merrill and Henderson, 1987).

Bishop and Bethel, 1959 reported an observation on a man with hereditary hypochromic anemia and hemosiderosis who

demonstrated a therapeutic response to pyridoxine. He was not vitamin B-6 deficient. They suggested that the excess Fe stores and the increased iron concentration at the site of hemoglobin synthesis caused the formation of an Fe-PL complex that prevented the normal function of pyridoxal as a coenzyme in heme synthesis. The high doses of pyridoxine given may have prevented this inhibition and allowed heme synthesis to continue (Bishop and Bethel, 1959).

Sideroblastic anemia is a rare disorder in which varying numbers of hypochromic microcytic red cells are found in the blood and normoblasts containing an excess of Fe containing granules appear in the marrow. There are also increased Fe stores in the reticulo-endothelial system and in parenchymatous organs such as the liver, pancreas and heart, the serum cholestrol is raised and Fe binding protein is raised (Houston, 1985).

2.13 VITAMIN B-6 AND ITS INTERRELATIONSHIP WITH TRACE ELEMENTS

One of the most important functions of essential trace elements is their necessity for a large number of enzymes. Their association with enzymes is of two types, they may be an integral part of the enzyme protein molecule (metallo-enzymes) or they may be "activators" of the enzyme (metal ion activated enzymes).

Vitamin B-6 is also involved in many vital enzymatic and regulatory processes which have already have been discussed. Considering their wide involvement in metabolism, their involvement as catalysts in common pathways is inevitable. Consequently, it is not unexpected that diverse interactions exist between vitamin B-6 and trace elements.

2.13.1 Pyridoxine deficiency and Fe metabolism

Neal and Pearson, 1962 investigated the relationship between pyridoxine deficiency and Fe uptake at various levels of Fe intake. They found that pyridoxine deficient rats did not absorb increased amounts of Fe when their Fe intake was 100μg/day. They estimated the Fe requirement of rats to lie in the range of 50 to 100μg/day. When the Fe was increased

to 1mg/day pyridoxine deficient rats showed a significantly greater Fe absorption than the controls. They concluded that enhanced iron absorption in the pyridoxine deficient rat does not occur under physiological conditions.

Another study was carried out by Kirksey A. et al, 1967 to investigate:

- the effect of pyridoxine deficiency on normal increase in Fe absorption during late pregnancy.
- the effect Fe supplementation on reproductive performance and tissue stores. The diet given contained 200mg of Fe per day. The results relevant to the present studies were as follows:

Pyridoxine deficiency resulted in:

- impaired erythropoiesis including polycythemia, reduced mean corpuscular hemoglobin (MCH) and mean corpuscular volume (MCV).
- Liver Fe stores were elevated in the non-pregnant deficient rats.
- Spleen Fe stores were elevated.
- No major impairment in Fe absorption was apparent.

- A high level of Fe found in duodenum of pyridoxine deficient group was possibly part of a regulatory mechanism which blocked excessive absorption of iron as this group had elevated iron stores and therefore the need for Fe was less.

Kirksey et al, 1967 suggested that the elevation of Fe in tissues in the deficiency may be due to a decreased utilization of Fe in hematopoiesis. During a pyridoxine deficiency one would expect decreased availability of PLP which is required for the biosynthesis of hemoglobin. Although the hemoglobin concentration was not altered during the deficiency, polycythemia accompanied by significantly lower MCH and MCV were evidences of the effect of the deficiency on hematopoiesis (Kirksey and Tabacchi, 1967).

Kirksey et al, 1969 observed elevated plasma Fe and tranferrin in pyridoxine deficient mothers. Although they attributed this to less expansion of blood volume during the deficiency the possiblity of increased Fe absorption was not ruled out. The marked incease in tranferrin saturation in maternal circulation found are consistent with increased Fe absorption.

A confirmation of their former findings were reflected in elevated Fe content of the liver, spleen, and duodenum of pyridoxine deficient mothers (Kirksey, et al, 1969).

Ikeda et al, 1979 also found vitamin B-6 deficient conventional and germ free rats retained more Fe in their tissues than the control animals. The liver and kidney Fe were significantly increased in the conventional deficient group.

2.13.2 Vitamin B-6 and Zn metabolism

Hsu, 1965 found that a pyridoxine deficiency resulted in a decrease in Zn content in the plasma, liver, pancreas and heart tissue. No alterations were noted in the kidney and spleen.

He also observed an increase in Zn-65 uptake in the plasma and liver after an intramuscular injection of radiozinc. The gastrointestinal tract and faeces also displayed a highly significant increase in zinc radioactivity. This would suggest that a pyridoxine deficiency enhances the rate of excretion of radiozinc from the body.

Other investigators noted that certain zinc containing enzymes such as liver lactic acid dehydrogenase and plasma alkaline phosphatase displayed altered activities in pyridoxine deficient rats. Huber et al (in Hsu, 1965) indicated that less insulin activity existed in pyridoxine deficient rats. However it is not clear whether this was due to Zn deficiency or pyridoxine deficiency.

Gershoff's (1967) observations were contrary to Hsu's findings. He found that vitamin B-6 deficiency resulted in increased pancreatic, serum and kidney zinc levels in rats. When he fasted his control and deficient groups for 24 hours he found that fasting resulted in a considerable increase in Zn in the liver, serum, pancreas and kidney. Except for serum the effect of fasting on Zn levels was not significant in vitamin B-6 deficient rats. In addition to fasting he found that pair-feeding of the control group resulted in a significant rise in Zn content of the pancreas and kidney. Because of this there were no significant differences in the Zn levels between the deficient and pair-fed control groups, Gershoff, 1967 used his data to explain Hsu's conclusions. Hsu pair-fed and fasted his rats overnight before determining tissue Zn levels. Thus, according to Gershoff, Hsu's conclusion that vitamin B-6 deficiency in rats reduced

tissue Zn content was in greater part a reflection of the high tissue levels of Zn in his fasted controls.

The results of Gershoff was supported by Ikeda et al, 1979 who also found elevated Zn levels in the kidney of conventional rats and in the liver and spleen of germ free rats. No significant change was found in the liver, pancreas, spleen, lung and testis of the deficient conventional group compared to the control.

In a study undertaken by Prasad et al, 1982 it was found that a significant enhancement in the uptake of Ca, Zn, and Cd occurred in the intestine of vitamin B-6 deficient rats as compared to the control or pair-fed animals. A lack of specificity on the uptake of all three metals is highlighted by the fact that all three have a distinct mode of transport from the brush border. These observations support the increased Zn content of tissues in vitamin B-6 deficiency found by Gershoff and Ikeda.

Prasad et al, 1982, suggested that the lack of pyridoxine may result in impairment of nucleic acid synthesis leading to inhibition of protein synthesis, cell division and repair. The involvement of the coenzyme form in the catalysis of a wide variety of enzymatic reactions almost

entirely concerned with nitrogen metabolism is well established. Thus it would be probable that a marked alteration could be expected in the brush border membrane which may lead to a non-specific increase in metal-ion uptake.

2.13.2.1 The Effect of Vitamin B-6 and Tryptophan on Zn absorption

Evans, 1980 stated after nearly a decade of research that mammals have evolved an efficient method of transporting Zn through a barrier created by the absorptive cells of the intestine by a process that involves synergism among Zn, vitamin B-6 and tryptophan.

In 1973, Hahn and Evans (in Evans, 1980) detected a low-molecular weight (<1,500 daltons) zinc binding ligand (ZnBL) later identified as picolinic acid in the lumen wash and intestinal mucosal cells of rats. They also found that human milk and a pancreatic extract, both of which contain a substantial quantity of picolinic acid, have been effective in treating the symptoms of acrodermatitis enteropathica (resulting from impaired Zn absorption).

Evans, 1980 found that addition of picolinic acid to rat diets promoted growth and increased absorption and retention of dietary Zn. The production of picolinic acid from tryptophan is dependent on pyridoxal. Picolinic acid added to a vitamin B-6 deficient diet resulted in an increase in kidney Zn concentration compared to an unsupplemented diet. Therefore if endogenous picolinic acid facilitates dietary Zn absorption, a deficiency of either vitamin B-6 or tryptophan should impair Zn absorption. However, Hsu found no change in kidney Zn content between deficient and control animals.

This should explain Hsu's, findings of a decreased Zn level in certain tissues during pyridoxine deficiency. Evans, 1980 found that rats fed 2, 4, 10 and 40mg PN.HCl per kilogram diet absorbed 150, 165, 195 and 233g Zn per day respectively. They also found that rats fed a diet low in tryptophan absorbed less zinc. Hence, because picolinic acid is a tryptophan metabolite and depends on pyridoxine for its production, these results provide strong evidence that endogenous picolinic acid is essential for normal Zn absorption.

In a another study undertaken by Evans and Johnson, 1980 they found that Zn absorption in rats fed a high iron diet

(300ppmFe, 16,5ppm Zn) was significantly less than in rats fed an adequate Fe (220ppm Fe, 16,5ppm Zn). When the high Fe diet was supplemented with picolinic acid, Zn absorption was increased and did not differ from that in rats fed the adequate Fe diet.

Zn absorption and pancreatic picolinic acid concentration increased as dietary level of vitamin B-6 was increased in the high Fe diet. Therefore, the inhibitory effect of high Fe on Zn absorption was decreased by the exogenous and endogenous production of picolinic acid.

They also found increased Zn retention in rats fed higher levels of vitamin B-6. This was attributed to increased endogenous picolinic acid production which increased the efficiency of Zn absorption.

Evans and Johnson, 1980 concluded that picolinic acid is the ligand, found in the pancreas, that facilitates Zn absorption. It is secreted from the pancreas into the intestine during digestion and may form a Zn-dipicolinate complex. This complex due to its specificity for receptor sites on the absorptive cell or because of its high association constant facilitates the transport of Zn and other divalent cations into and across the intestinal cell.

Another important conclusion was that high levels of dietary Fe inhibit Zn absorption via competition for binding with endogenous picolinic acid.
These results provide further evidence to support the hypothesis that picolinic acid facilitates the absorption of dietary Zn.

However Hurley and Lonnerdal, 1980 identified citrate as a zinc binding ligand in human milk and stated that the apparent effectiveness of Viokase (a commercial pancreatic extract) in increasing Zn absorption in patients suffering from acrodermatitis enteropathica maybe due to its citrate content. They also failed to identify picolinic acid in the pancreas. Furthermore it was found that picolinic acid is not a significant end product of tryptophan in the rat. Only 7,4% OF 3-OH anthranilic acid was converted to picolinate by rat liver extracts and only 4% was converted to urinary picolinate in vivo.

They did not agree with Evans' conclusions that impaired Zn absorption in protein deficiency is due to decreased levels of picolinic acid resulting from lack of adequate tryptophan. They hypothesize that the decrease in Zn absorption may be as a result of breakdown of intestinal mucosa caused by protein deficiency.

They agreed that picolinic acid is a good chelator of zinc. But state that increasing zinc absorption by adding a chelating agent in no way indicates that the chelating compound has a normal physiological role (Hurley and Lonnerdal, 1980).

2.13.3 VITAMIN B-6 AND COPPER METABOLISM

Although no studies have been found in the literature concerning the effect of vitamin B-6 on copper metabolism the likelihood of an interrelationship is very strong. The fact that certain Cu metalloenzymes need PLP as a cofactor supports this statement.

Amine oxidases of the plasma, kidney and liver are copper proteins. The amine oxidases of the plasma and kidney also contain firmly bound pyridoxal phosphate (Gray and Daniel, 1973).

Lysyl oxidase of the chick aorta was shown to be sensitive to a vitamin B-6 or copper deficiency (Starcher, 1969). Lysyl oxidase is responsible for the normal crosslinking of both collagen and elastin which is necessary for their functional integrity. Whether this enzyme is vitamin B-6

deplendent needs confirmation (Levene and Murray, 1977). This aspect has been reviewed under atherosclerosis.

Dopamine β-hydroxylase plays an important role in central nervous system functions as it is involved in noradrenaline synthesis. It is a copper metalloenzyme and is dependent on PLP.

The close interdependence of Cu and Fe also warrants an investigation of both trace elements in pyridoxine deficiency.

It has been shown that copper is needed to ensure the proper amount of hemoglobin in blood.

In the swine Cu deficiency leads to:

- defective absorption of Fe from the intestine.
- a restricted flow of Fe from the reticuloendothelial cells to the plasma.
- excessive retention of Fe in the liver and
- impaired production of haemoglobin (Prasad, 1978).

It has been shown that Cu deficiency results in a reduction of tissue content and a decrease in plasma Cu which leads to anemia - a hypochromic, microcytic anemia.

It appears that the Cu deficiency in animals results in impaired ability of the duodenal mucosa, the reticulo-endothelial system and the liver to release Fe to the plasma. This is suported by the hypothesis that ceruloplasmin (ferroxidase) is the enzyme thats needed for transfer of Fe from tissues to plasma (Peereboom, 1985; Prasad, 1978).

2.14 ASSESMENT OF TRACE ELEMENT STATUS

This section will briefly outline the methods used to assess trace element status. Although these tests are referred to as assessments of trace element status few, if any, provide measurements of or indications of total body content of trace elements. They simply "assess whether the activity (available concentration) of a given trace element is sufficient to maintain the rates and extent of the element dependent metabolic reactions within normal limits, or whether there is an excess or deficiency of this activity that could produce a biochemical defect and lead to a clinical abnormality" (Delves, 1985).

Some of the methods used to assess trace elements status given in order of importance are:

- Measurements of trace element concentrations in body

tissues, fluids, cellular and extracellular fractions and in serum proteins.

- Assay of element - specific activity in body tissues and fluids.
- *In vitro* uptake of radioisotopes by tissue.
- Trace element balance studies.
- Biochemical and clinical responses to administration of trace element. (Delves, 1985)

According to Delves, 1985 none of these methods are ideal and the method used will depend upon the element being investigated.

In the present study tissue content of Fe, Cu and Zn were analysed.

2.14.1. Zinc in body tissues

Jackson *et al*, 1982 have found that dietary depletion of zinc was found to result in a 30% decrease in total body zinc content in rats. In depleted animals he found no decrease in zinc content of hair, skin, heart or three different skeletal muscle. However levels of plasma, liver, bone and testes were significantly reduced when rats were put through a dietary treatment for 80 days. Several other

workers have shown that experimental zinc deficiency causes a decrease in zinc content of various animal tissues (Jackson et al, 1982).

Against this background and owing to the relatively high levels of zinc in rat muscle, heart and kidney. (Prasad, 1978) these tissues were chosen for analysis. However, after the completion of this study a relatively recent article by Golden, 1989 shows that the measurement of tissue content is not a valid assessment of Zn status.

He proposed that two types of nutritional deficiencies existed. During a Type 1 nutrient deficiency a reduction of tissue concentration is observed. However, during a Type II nutrient deficiency there is almost no reduction of the tissue element content. Zn deficiency falls into the latter category.

Golden, 1989 states that in both mild and severe experimental Zn deficiency, there is almost no reduction in the tissue Zn content. Muscle zinc is preserved. A small decrease in liver and pancreatic Zn is found in prolonged, profound deficiency. The only tissue with a reduction in zinc is bone.

2.14.1.1 Type II Nutrient Deficiency

Zn deficiency falls into this category. If the animal is at stage of development where sensitivity to Zn is high, it stops growing, without a reduction in tissue concentration when given a diet low in Zn. The clinical signs that develop is usually due to external factors such as stress or trauma. This makes this type of deficiency very difficult to diagnose.

The nutrient eg. Zn is locked in tissues and is fundamental to the composition, integrity and day-to day functioning of that tissue. Although Zn would be released if the whole tissue is catabolized, small amounts are released if there is metabolic re-adjustment. This re-adjustment may be totally unrelated to the deficiency of Zn eg. if there is a reduction in protein this will result in a alteration in Zn metalloenzyme activity leading to a slight decrease in functional tissue zinc concentration. These metabolic re-adjustments are proportionately small. The reduction in protein synthesis rate is probably the reason for the small changes in liver and pancreatic Zn concentrations observed in Zn deficiency. This change is non-specific (Golden, 1989).

Type II nutrient deficiencies will probably reveal negative Zn balances. This is because whole tissue is being broken down and the excess nutrient is liberated eg. Zn will have to be metabolized and excreted. A negative Zn balance is probably due to a dietary protein deficiency or to a metabolic re-adjustment of the tissue due to the Zn deficiency.

Zn deficiency (Type II nutrient deficiency) is characterized by a large fixed pool in the tissues and a small rapidly turning over, free pool which is sensitive to a deficiency.

Therefore plasma or serum Zn has most often being used for diagnosis of a deficiency. However, Solomons in Golden, 1989 states that its invalid as a measure as it does not reflect total body Zn. Golden argues that total body Zn bears little relationship to Zn status in a deficiency as it is not a Type I nutrient deficiency. Plasma is an unreliable indicator of Zn deficiency becuase it is subject to metabolic alteration. Research by Golden, 1989 has revealed that assay of plasma Zn and red blood cell metallothionein -1 can be used to diagnose a Zn deficiency.

2.14.2 Copper and Iron in body tissue

According to Golden, 1989 Cu and Fe deficiencies are Type I

nutrient deficiencies. This type of deficiency is characterized by a reduction in the tissue concentration of the nutrient and a defect in one or more specific pathways. This results in a loss of function so the deficiency has specific clinical signs.

Hence, unlike zinc, a dietary intake of Cu ad Fe is reflected in the tissue levels. Thus measurement of tissue content is a valid assessment of Cu and Fe status. The chain of events that follow this type of deficiency is as follows:

A diet with a low content of Fe or Cu will lead to a reduced tissue concentration and hence lead to an identifiable major defect in the metabolic pathway. This then leads to characteristic clinical signs and symptoms. All one has to do thereafter is diagnosis (Golden, 1989).

Hence it can be concluded that trace element analysis of body fluids and tissues is very a useful method of assessment of Cu and Fe status. The liver, being a major organ for the metabolism of Cu and Fe, is very useful in assessing the status of these trace elements (Bacon, 1984; Winge 1984). The heart, kidney and muscle were chosen because of the relatively high concentrations of Cu and Fe.

For the diagnosis of zinc deficiency, plasma Zn and red blood cell metallothionein-1 assay are recommended by Golden, 1989.

A balance study conducted by Channa, 1988 gave an insight into the quantity of Cu, Fe and Zn that entered and left the body during a given time in a vitamin B-6 deficient state. This type of study had no value for defining considerations such as internal body distributions, pool sizes, turnover rates, molecular uses or metabolic roles of the trace elements studied. Also one cannot calculate the true intestinal absorption as the magnitude of any endogenous contribution to fecal matter cannot be studied using the balance studies (Beisel, 1979).

Hence assessment of trace element status using measurement of tissue content was performed, to shed more light on the internal body distribution of trace elements during a vitamin B-6 deficiency.

CHAPTER THREE

MATERIALS AND METHODS

3.1 Structure of the Study

- Formulation of diets for experimental and control animal groups.
- Induction of various levels of vitamin B-6 deficiency in the experimental animals.
- Pair-feeding of 1 group of rats with the deficient group in the second experiment.
- Recording of food intake daily and body weight weekly.
- Biochemical confirmation of vitamin B-6 status (in all groups) by determining plasma pyridoxal levels.
- Digestion of heart, liver, kidney and muscle using a wet ashing technique.
- Determination of Cu, Fe and Zn levels in various tissues using atomic absorption spectrophotometry.
- Statistical analysis of data obtained.

3.2 MODEL SYSTEM

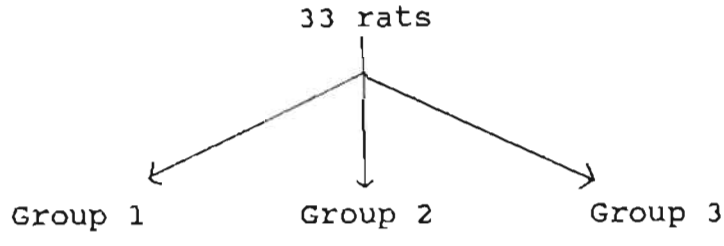
Two sets of experiments were carried out. In experiment 1 thirty-three young male Wistar rats with masses ranging from 96 - 102 grams were used.

In experiment 2 thirty-six young male, Wistar rats weighing approximately 80 grams were supplied by the Biomedical Resource Centre (BRC), University of Durban-Westville. These rats were housed individually in perspex metabolic cages (Techniplast) in an air-conditioned room, in the BRC away from laboratory equipment and personnel. The average temperature and humidity were 22°C and 55% respectively. Automatically controlled illumination with 12 hour light and dark cycle was maintained.

The cages were washed thoroughly using a disinfectant and rinsed with deionized water by the BRC. The cages had stainless steel grid bottoms that allowed faeces to collect in a container under the funnel thus eliminating the potential for corprophagy. Food and deionized water were available to the animals ad libitum in containers attached to the metabolic cages.

3.3 EXPERIMENTAL DESIGN

Experiment 1



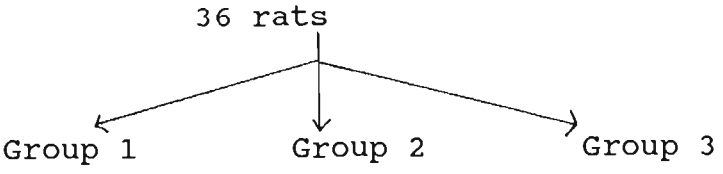
Rats were assigned three dietary treatments as follows:

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

Group 2 was fed the marginally deficient diet containing 1,75mg/kg of vitamin B-6 in the diet.

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

Experiment 2



12 rats	12 rats	12 rats
(Control)	(Pair-fed Control)	(Deficient)

Animal weights were used in assigning rats to the various dietary treatment groups to maintain similar group mean weights. A pair-fed control group for the deficient group was added in experiment 2 because it was anticipated that food consumption would be altered by the deficiency of PN.

The rats were assigned three dietary treatments as follows : the diets of groups 1, 2 and 3 contained 3,5; 3,5 and 0mg per kg of vitamin B-6 in the diet respectively.

On arrival all rats were fed the control diet for one day. A period of one week was allowed to establish the routine of pair-feeding before the start of the experimental period. Pair-fed rats were given an amount of vitamin B-6 adequate food equal to that consumed on the previous week by the rats to which it was paired.

3.4 DIET PREPARATION

The pyridoxine diet (ICN Nutritional Biochemicals, Cleveland, Ohio) used was based on the formulation by French S, 1966.

The diet used had the following composition:

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	

<u>Hawk Oser Salt Mixture</u>

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

The control (3,5mg/kg of vitamin B-6) and marginally deficient diet (1,75mg/kg of vitamin B-6) was prepared as follows (Channa, 1988):

3,5mg PN.HCl (BDH Chemicals, England) was added to 1kg of the basal diet to form the stock diet containing 35mg of PN.HCl per kg of diet. An electrical homogenizer (Forster Equipment Co., England) was used to mix the above for about 15 minutes.

Thereafter 250g of the stock diet was added to 2250g of the basal diet mixed as described above to yield 3,5mg/kg PN in the diet.

To prepare the marginally deficient diet, 125g of the stock diet was mixed with 2375g of basal diet to yield 1,75mg/kg PN in the diet.

3.5 ISOLATION OF TISSUES

At the end of eight weeks rats were held overnight in the metabolic cages without food but with free access to deionized water. On the following day rats were anesthetized with ether. While blood was being removed the anesthesia was maintained. Blood samples were obtained from the abdominal aorta into heparinized syringes. Samples were placed in labelled vials. Blood was centrifugated at 150x g for 15 minutes to separate the plasma for PL analysis. Plasma samples were frozen at -7°C.

Subsequent to the removal of blood; kidney, liver, heart and muscle were excised using stainless steel instruments, blotted and cleansed of adhering matter. Tissues were then placed immediately into labelled plastic vials individually. All vials were placed in crushed ice and then frozen at -7°C. All instruments (stainless steel) used were washed thoroughly in double deionized water to prevent contamination. All surgical procedures were carried out in a laboratory at the Biomedical Resource Centre, University of Durban - Westville.

3.6 REAGENTS AND GLASSWARE

The sources and purity of reagents used were as follows:

- Double deionized water (Elgastat water deionizer) from Elga limited.

- Nitric acid (Suprapure) from Merck, Darmstadt, F.R.G.

- Zinc (Spectrosol) from B.D.H. Chemicals, Poole, England.

- Copper (Spectrosol) from B.D.H. Chemicals, Poole, England.

- Iron (Spectrosol) from B.D.H. Chemicals, Poole, England.

All glassware and plasticware used during the experiment were cleaned by soaking in an aqueous solution of 20% nitric acid for 48 hours. Thereafter all glassware and plasticware were thoroughly rinsed several times using deionized water. After a brief period of drying they were stored in sealed particulate free plastic bags before being used.

3.7 PLASMA PL ANALYSIS BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

3.7.1 Principle of HPLC analysis

Chromatography involves the separation of mixtures into their various components due to the difference in equilibrium distribution of these components between two different phases.

Types of chromatography:
1. Paper
2. Thin layer (TLC)
3. Open column
4. Gas (G.C.)
5. High performance liquid (H.P.L.C.)

All except G.C. are forms of liquid chromatography i.e. 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 interactions between sample molecules, stationary phase and mobile phase. The length of time for

which they are retained is dependent on the unique chemical interactions of each component. Since these differ, the retention times differ and separation is achieved.

The basic method applies to all forms of liquid chromatography, viz., we have stationary phase, mobile phase, and as a result of the various affinities of the sample for each, a separation occurs (Williams amd Wilson, 1984).

In contrast to other chromatographic techniques, HPLC employs the principles of adsorption, partition, ion-exchange, exclusion and affinity chromatography. Being such a versatile technique it has emerged as the single most powerful chromatographic technique. The advantages of this technique lies in its high efficiency, speed of resolution and its use with minute amounts of material (Williams, and Wilson, 1984).

Basically, separation of the sample components is achieved by interaction between the sample components and the stationary and mobile phase on the column. Detection occurs as the sample components emerge from the column, by means of a detector system. Results are presented as a series of peaks on a chart recording. Retention time is used to

identify unknown compenents in the sample. It is defined as time from injection of a given compound to its detection. The time is the same for a given compound under standard conditions. Identification of an unknown sample can be achieved by comparing components analysed under identical conditions, or by the use of an internal standard (Williams and Wilson, 1984).

3.7.2 Quantification of Plasma PL levels by HPLC

Ubbink et al devised a novel HPLC method for the simultaneous quantificantion of plasma PLP and PL levels. It comprises pre-column semicarbazone (SC) derivitization, HPLC to separate PLPSC and PLSC from each other and from unidentified background components, post column pH adjustment and fluorescence detection (Ubbink et al, 1985). The semicarbazone derivatization was done in the presence of trichloroacetic acid used for deproteinizing (Ubbink et al, 1986). This is a modification of a previous method described by Ubbink et al, 1985.

The HPLC separation is a simple, isocratic technique. An on-line flow pH meter was installed behind the detector cell to monitor pH fluctuations during analysis.

Reagents used

- PL and PLP from Merck (Darmstadt, F.R.G)
- Chromatography grade acetonitrile from Merck
- All other chemicals were of analytical - reagent grade and were obtained from Merck

Purification of PL

The commercial PL preparations were purified by reversed-phase HPLC (Whatman Partisil 10 ODS-3 column; mobile phase : 10% methanol and 0,1% glacial acetic acid in water) and then lyophilized. Purified PL was checked for impurities by reversed-phase ion-pair chromotography, UV detection (290nm) and wavelength scanning (210-360nm) at different stages of peak elution. UV absorption spectra of the purified PL was determined in 0.1 M hydrochloric acid, 0.1 M sodium hydroxide and 0,1 M sodium phosphate buffer, pH 7.0.

Standard preparation

Purified PL was used to prepare working standards containing 1-20ng PL. Equal volumes of standard solution and (50ml TCA, 30ml semicarbizide) precipitation reagent was added to

labelled vials. The mixture was incubated at 45° for 40 minutes.

Sample Preparation

An equal volume of precipitation reagent was added to 0,5ml plasma. The mixture was shaken thoroughly for even protein precipitation. The mixture was incubated for 40 minutes at 45°C. Prior to injection, the samples were centrifuged at 4000rpms for 15 minutes. Transferred 200µl of the supernatant to a microvial.

Instrumentation

A Beckman (Beckman Instruments, Berkeley, CA, U.S.A.) Model 112 solvent delivery module was slightly modified so that the pump was used for solvent delivery. An LS 4 Perkin-Elmer flourescence spectrophotometer (excitation : 367nm, emission : 478nm) was coupled to a Spectra Physics (San Jose, CA, USA) 4270 integrator.

Columns

A Whatman (Clifton, NJ, USA) Partisphere C_{18} analytical column (110 x 4.7mm I.D.; particle size 5µm) was used. To

protect the analytical column, a Whatman reversed-phase cartridge was installed between the injector and the analytical column. The guard cartridge was replaced every two weeks.

Chromotographic Conditions

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

3.8 ACID DIGESTION OF SAMPLES

In order to use atomic absorption spectroscopy samples must usually be in liquid form, often requiring digestion. Hence, the wet ashing technique described by Clegg et al, 1981, was modified and employed for sample digestion. They found that nitric acid proved to be the most effective wet ashing agent. With nitric acid, mean concentrations for iron, copper and zinc differed from NBS certified values by less than 1,5%.

Frozen samples were first thawed and weighed thereafter. Tissue weights used were approximately 0,5g. Liver, kidney, muscle and heart samples from all 69 rats were wet digested in 50ml Kjeldahl flasks containing 3 glass beads in nitric acid. The volume of nitric acid used was 6mls. All samples were predigested at room temperature for approximately 12 hours before heating began. Samples were subsequently heated to maintain constant boiling until evolution of nitric acid fumes (Clegg et al, 1981).

Batches of 4 samples including a blank and blank containing a standard were digested for 4 hours at a session. At the end of the digestion period, samples had a very slight clear yellow tint. After completion of digestion , the contents

of the flasks were cooled and thereafter transferred to 25ml volumeric flasks. The inside of the flask was rinsed several times with deionized water to ensure complete transfer of acid digest. Digested samples were brought to volume with deionized water. A blank and blank containing a known quantity of STD were included with a batch of 4 samples.

Recovery Studies

12 male, adult Wistar rats were used to standardize the ashing and atomic absorption methods. Liver, heart, kidney and muscle were wet ashed as described previously. Standard addition of known concentration of Fe, Zn and Cu to samples were used to calculate percentage recoveries.

3.9 TISSUE ANALYSIS BY ATOMIC ABSORPTION SPECTROPHOTOMETRY (AAS)

Cu, Fe and Zn levels of the heart, liver, muscle and kidney were determined by atomic absorption spectrophotometry. A Perkin-Elmer Model 2380 microprocessor controlled atomic absorption spectrophotometer (Perkin-Elmer Corporation, Norwalk, C.T., U.S.A.) was used. The results were expressed in microgram/gram (μg/g) wet weight.

3.9.1 Principle of AAS

Three types of photon-atom interactions form the basis of the three main branches of atomic spectroscopy, namely:

atomic absorption spectroscopy (AAS)
atomic emission spectroscopy (AES) and
atomic fluorescence spectroscopy (AFS)

All techniques require an atom cell, a monochromator, detector and readout. The differences lie in the light source and geometry. Briefly, AES does not require a light source. The other techniques require a light source but while in AAS the light must pass straight through the atom cell to the detector, in AFS the detector is placed at right angles to the light source. Trace element analysis was

performed by flame atomic absorption spectrophotometry in this study hence only the principle of atomic absorption will be discussed (Metcalf, 1987).

In flame AAS a beam of radiation with a very narrow band width is produced by a radiation source (usually a hollow cathode lamp). Nebulization converts the sample which contains the element to be analysed, to a more amenable form for rapid atomization as follows:
The sample is drawn up through a capillary tube at high velocity. The necessary suction is provided by using high flows of the oxidant gas (air) and the venturi effect. The high speed of the gas flow tends to break up the solution through turbulence as it emerges from the capillary tube. The nebulizer also functions as a mixing chamber for the sample, oxidant and the fuel. The flame created by the ignition of air-acetylene atomizes the liquid aerosol from the nebulizer (Metcalf, 1987).

AAS measures the absorption of a beam of monochromatic light by atoms in a flame. The energy absorbed is proportional to the number of atoms present on the optical path. The external source emits the spectral line corresponding to the energy required for an electronic transition from the ground state to the excited state. Thus the electrons of an atom

move from its energy level in the ground state to an excited state. The energy of the electron is raised by the absorption of a discrete quantum of energy, exactly equivalent to that involved in transition. Before analysis of the absorbed light, a monochromator is necessary for isolation of the spectral line of interest before it reaches the detector (Williams and Wilson, 1984).

The absorption of light in the atom results in a light intensity, I_t, which is measured by the detector and amplifier. The incident light intensity, Io, is the signal obtained when a blank sample is aspirated and the readout is adjusted to give 100% transmission (zero absorbance). When the sample containing the element to be analysed is aspirated the transmitted intensity is reduced. This reduction in intensity can be related to the concentration of the analyte (Metcalfe, 1987).

3.9.2 Analytical Technique

Procedures involved in set-up:

The hollow cathode lamp was installed as specified by the instruction manual (Perkin Elmer, 1976b). The lamp current used was as specified on the lamp label.

The analytical wavelength, appropriate slit settings and other parameters are listed in table 1 (Perkin Elmer, 1976a).

Element	(nm)	Slit setting	Sensitivity Limit (μg/ml)	Linear Working range (μg/ml)
Cu	324,8	0,7	0,09	5
Fe	248,3	0,2	0,12	5
Zn	213,9	0,7	0,018	1

Table 1 : OPERATING PARAMETERS IN AAS

The gain setting was used to optimise current. A warm-up period of about 10 minutes was allowed. Ignition of the flame followed. The primary support gas used was air and the fuel was acetylene. The tanks were equipped with pressure regulators which were checked regularly to see if the pressures were the same as set previously (namely 60 psig for air; 70 psig for acetylene). A lean (oxidizing) air-acetylene mixture gives a blue hot flame. A rich (reducing air-acetylene) mixture gives a yellow cool flame.

Either the oxidant flow or fuel flow was adjusted to provide optimal flame conditions (a lean, oxidizing air-acetylene flame was used).

Calibration

At least two dilutions of daily working standard for Cu, Fe, and Zn were prepared from certified atomic absorption standards (1000μg/ml Cu, Fe and Zn). The concentrations of standards used to establish linear standard curves daily were as follows:

Cu	Fe	Zn
2.5μg/ml	5μg/ml	1μg/ml
	10μg/ml	

The appropriate calibration standards were analysed after 5 sample solutions as signals drifted over a period of time. Recalibration was performed if variations from the known concentrations were >5%. In the case of Fe, when the concentration range of the sample standard exceeded that of the sample a higher standard concentration was used.

Before shut-down and before commencing analysis deionized water was aspirated for a few minutes. This was performed to remove contaminants.

The operating parameters that were used are listed as follows:

Sample introduction rate	:	4ml/min
Integration time	:	1 second
Number of readings	:	3

3.9.3 Optimizing Procedures

The burner head was adjusted relative to the light path of the instrument to obtain maximum sensitivity. The nebulizer was adjusted for optimal sensitivity as specified by the operations manual (Perkin Elmer, 1976a)

3.10 STATISTICAL ANALYSIS

The nonparametric U-test of Mann-Whitney was applied to test for significance of differences between the parameters analysed as this was ideally suited for the sample size. The Bonferroni correction for multiple comparisons was made when several groups were compared. All statistical analysis was performed in collaboration with the Institute of Biostatistics of the South African Medical Research Council.

CHAPTER FOUR

RESULTS

4.1 THE EFFECT OF VITAMIN B-6 DEFICIENCY ON FOOD INTAKE

Experiment 1

The data in table 2 shows the mean daily food consumption for each animal over the eight week period. During this period a significant difference was found to exist between the control group (group 1) and the deficient group (group 3) ($p<0,05$). The food consumption of the marginally deficient group was also found to be significantly different from group 3 ($p<0,05$).

Experiment 2

The data on food consumption in both groups is given in table 3. The food consumption was significantly different between group 1 and group 3 ($p<0,05$).

Food intake declined significantly ($p<0,05$) by the first week of study in the vitamin B-6 deficient group. They often left food in the food containers and, in comparison to

the vitamin B-6 adequate animals, virtually always ate slowly. In contrast the control animals almost invariably ingested all their food leaving very little in the food containers.

The food consumption trends for each group in both experiments is shown in figures 15 and 16.

Table 2 : EFFECT OF VITAMIN B-6 DEFICIENCY ON DAILY FOOD CONSUMPTION (g) : Experiment 1

GROUP	WEEK 1	WEEK 2	WEEK 3	WEEK 4	WEEK 5	WEEK 6	WEEK 7	WEEK 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]	7,85 ±1,17[b]	6,85 ±0,69[b]	7,79 ±1,37[b]	6,94 ±1,39[b]

Food consumption is 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 ($P<0,05$).
Group 1 received 3,5mg/kg vitamin B-6 in diet
Group 2 received 1,75mg/kg vitamin B-6 in diet
Group 3 received 0mg/kg vitamin B-6 in diet.
Individual rat food consumption and P values are given in Appendix 1.

Table 3 : EFFECT OF VITAMIN B-6 DEFICIENCY ON DAILY FOOD CONSUMPTION : Experiment 2

GRP	WEEK 1	WEEK 2	WEEK 3	WEEK 4	WEEK 5	WEEK 6	WEEK 7	WEEK 8
1	14,27 ±1,09[a]	15,14 ±1,46[a]	16,54 ±1,92[a]	18,22 ±2,05[a]	17,67 ±2,15[a]	16,68 ±1,54[a]	17,13 ±1,39[a]	17,57 ±2,00[a]
3	11,41 ±1,15[b]	10,49 ±1,16[b]	9,41 ±1,01[b]	8,89 ±0,94[b]	8,86 ±1,05[b]	8,74 ±1,50[b]	8,75 ±1,42[b]	8,41 ±1,63[b]

Data are presented as mean ±SD and were obtained from 12 rats per groups. Means for each week not followed by the same superscript letter are significantly different ($P<0,025$). Groups 1 and 3 were fed 3,5 and 0mg/kg vitamin B-6 in the diet. Individual rat food intake and P values are given in Appendix 2.

FIGURE 15
VITAMIN B-6 AND FOOD INTAKE : EXPERIMENT 1

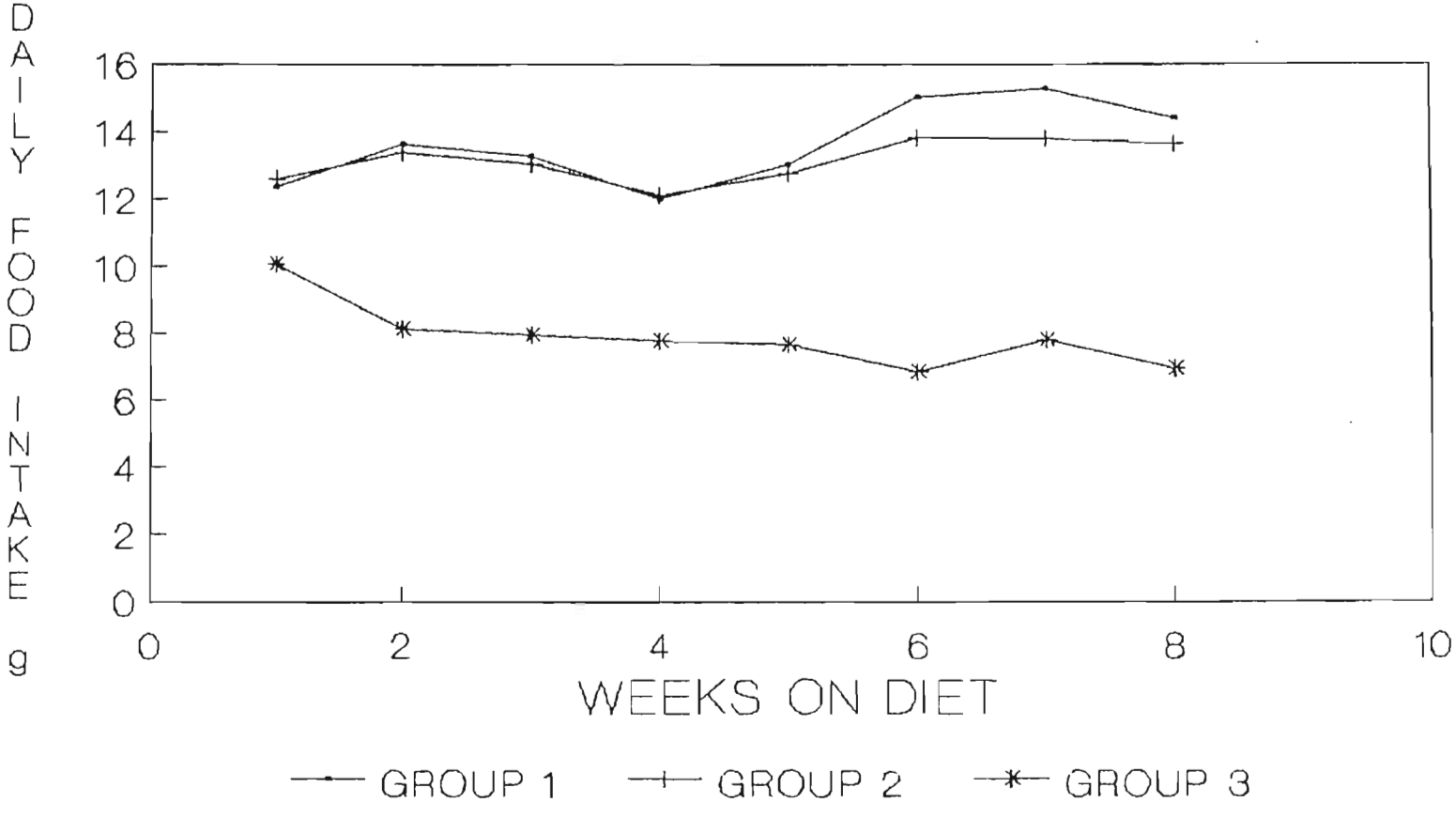
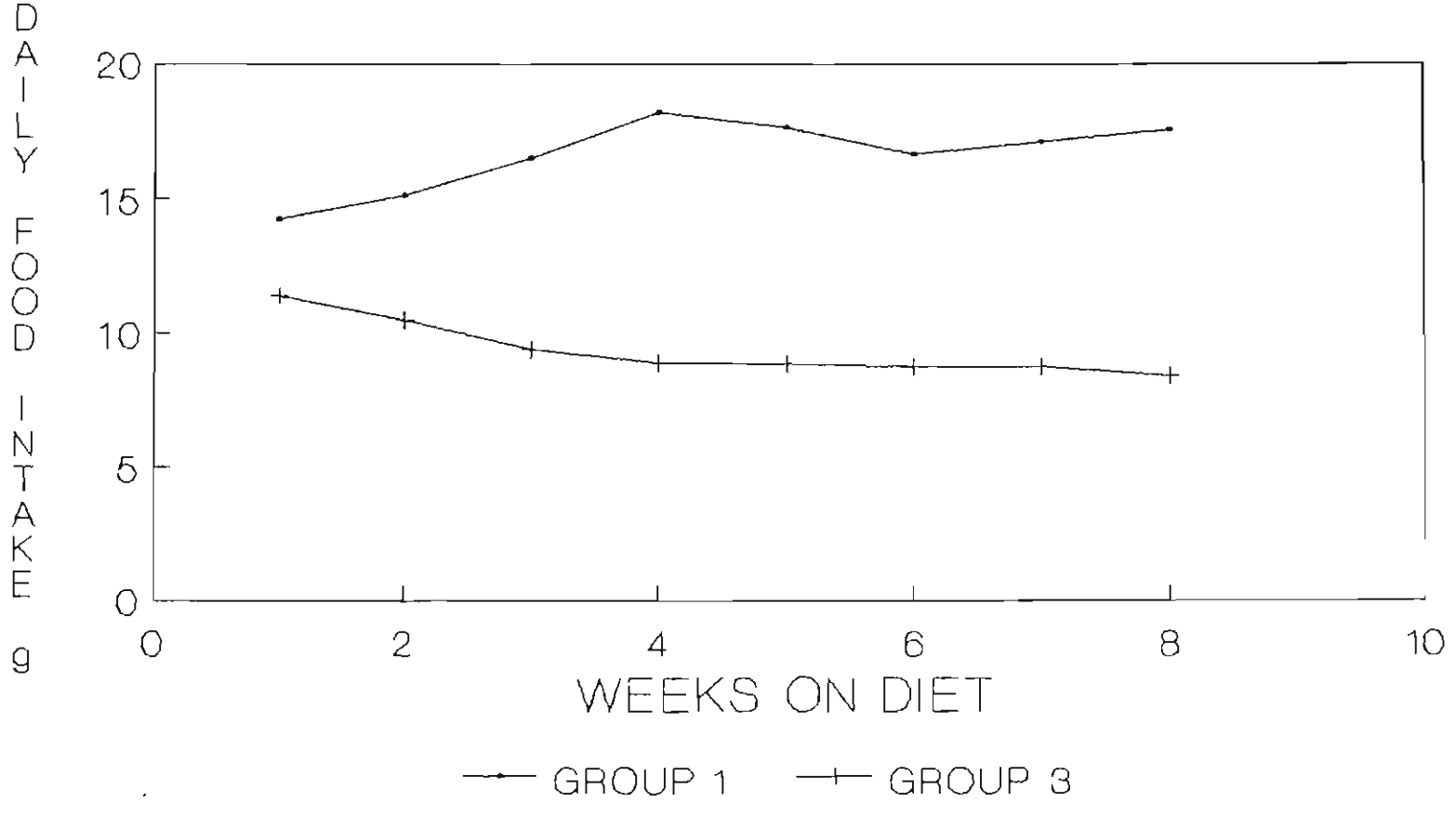


FIGURE 16
VITAMIN B-6 AND FOOD INTAKE: EXPERIMENT 2



4.2 THE EFFECT OF VITAMIN B-6 DEFICIENCY ON GROWTH

Experiment 1

The data on the mean percentage body mass gain of groups 1, 2 and 3 are given in table 4.

Percentage body mass gain was calculated as follows:

$$\frac{\text{Mean weekly animal mass (g)} \times 100}{\text{mass of animal on day 0}}$$

The percentage body mass gain of group 2 was not significantly different from group 3 ($p>0,05$). However a significant difference existed between groups 1 and 3 and groups 2 and 3 over the 8 week period ($p<0.05$).

Figure 17 shows the growth curves of the three different groups.

Experiment 2

Data on the mean percentage body weight gain of the 3 groups are listed in table 5. At the start of the study, the

weights were similar in all three groups with the average initial weights being approximately 80g.

However, by the 2nd week of the study, the B-6 deficient rats weighed significantly less than the control and remained lower throughout the rest of the study. A significant difference was observed between all three groups ($p<0,05$) after the second week of study. The percentage weight gain of the pair-fed group was not significantly different from group 3 in week 6 ($p>0,05$).

Figure 18 shows the growth curves of the three different groups. Growth patterns were similar for all groups for the first 4 weeks of dietary treatment. Group 1 and 2 showed a progressive increase in body weight gain.

Table 4: EFFECT OF VITAMIN B-6 DEFICIENCY ON PERCENTAGE BODY MASS GAINED : Experiment 1

GROUP	WEEK 1	WEEK 2	WEEK3	WEEK 4	WEEK 5	WEEK 6	WEEK 7	WEEK 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 ($P<0,05$).
Group 1 received 3,5mg/kg vitamin B-6 in diet.
Group 2 received 1,75mg/kg vitamin B-6 in diet.
Group 3 received 0mg/kg vitamin B-6 in diet.
Individual weekly animal mass and P values are given in Appendix 3.

Table 5 : EFFECT OF VITAMIN B-6 DEFICIENCY ON PERCENTAGE INCREASE IN MASS : Experiment 2

GROUP	WEEK 1	WEEK 2	WEEK 3	WEEK 4	WEEK 5	WEEK 6	WEEK 7	WEEK 8
1	170,47 ±19,38[a]	223,83 ±25,98[a]	268,91 ±30,01[a]	325,91 ±32,55[a]	365,80 ±34,52[a]	395,75 ±31,76[a]	423,83 ±31,92[a]	443,63 ±37,69[a]
2	159,59 ±5,96[a]	185,91 ±6,31[b]	219,48 ±8,33[b]	235,03 ±12,50[b]	239,48 ±11,79[b]	239,38 ±11,37[b]	256,47 ±11,46[b]	267,46 ±12,97[b]
3	161,89 ±14,75[a]	187,79 ±15,91[b]	196,84 ±17,18[c]	206,95 ±17,78[c]	209,37 ±24,87[c]	216,84 ±27,58[b]	221,37 ±28,08[c]	225,79 ±31,47[c]

Data are presented as means ±SD and were obtained from 12 rats per group.
Means for each week not followed by the same superscript letter are significantly different ($P<0,05$). Group 1 (control group) received 3,5mg/kg vitamin B-6 in diet. Group 2 is the pair-fed control group. Group 3 received 0mg/kg vitamin B-6 in the diet.
Individual weekly animal mass and P values are given in Appendix 4.

FIGURE 17
VITAMIN B-6 AND GROWTH : EXPERIMENT 1

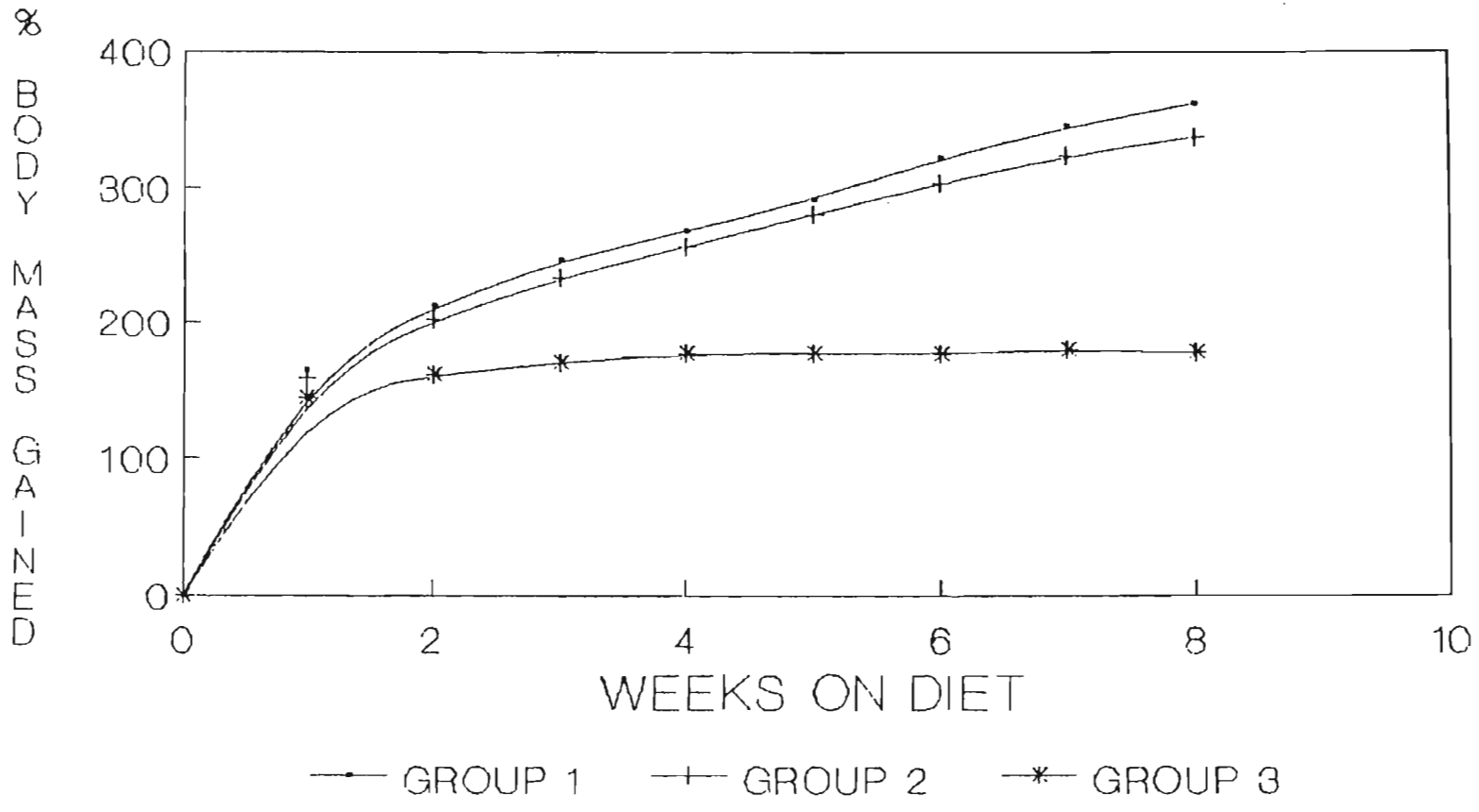
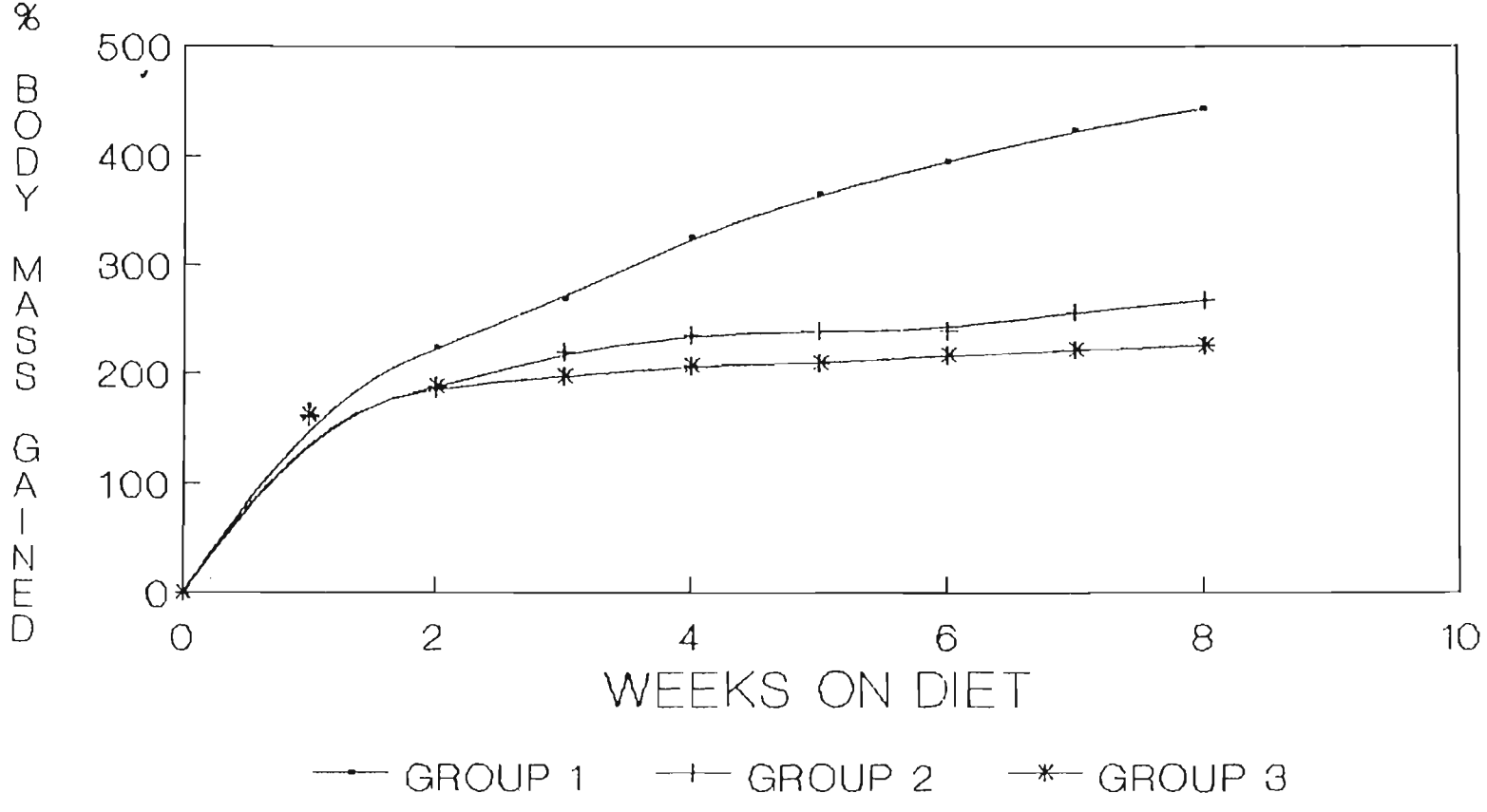


FIGURE 18
VITAMIN B-6 AND GROWTH : EXPERIMENT 2



4.3 PLASMA PL IN VITAMIN B-6 DEFICIENCY

Experiment 1

At the end of 8 weeks of dietary treatment blood was drawn and analysed for plasma PL levels. (Table 6 and 7) Using the U-test of Man-Whitney, there were significant differences between groups 1 and 2, 1 and 3 and 2 and 3 in both experiments 1 and 2 ($p<0,05$)

Figure 19 shows the plasma PL levels in both experiments.

FIGURE 19
PLASMA PL LEVELS IN VITAMIN B-6 DEFICIENCY

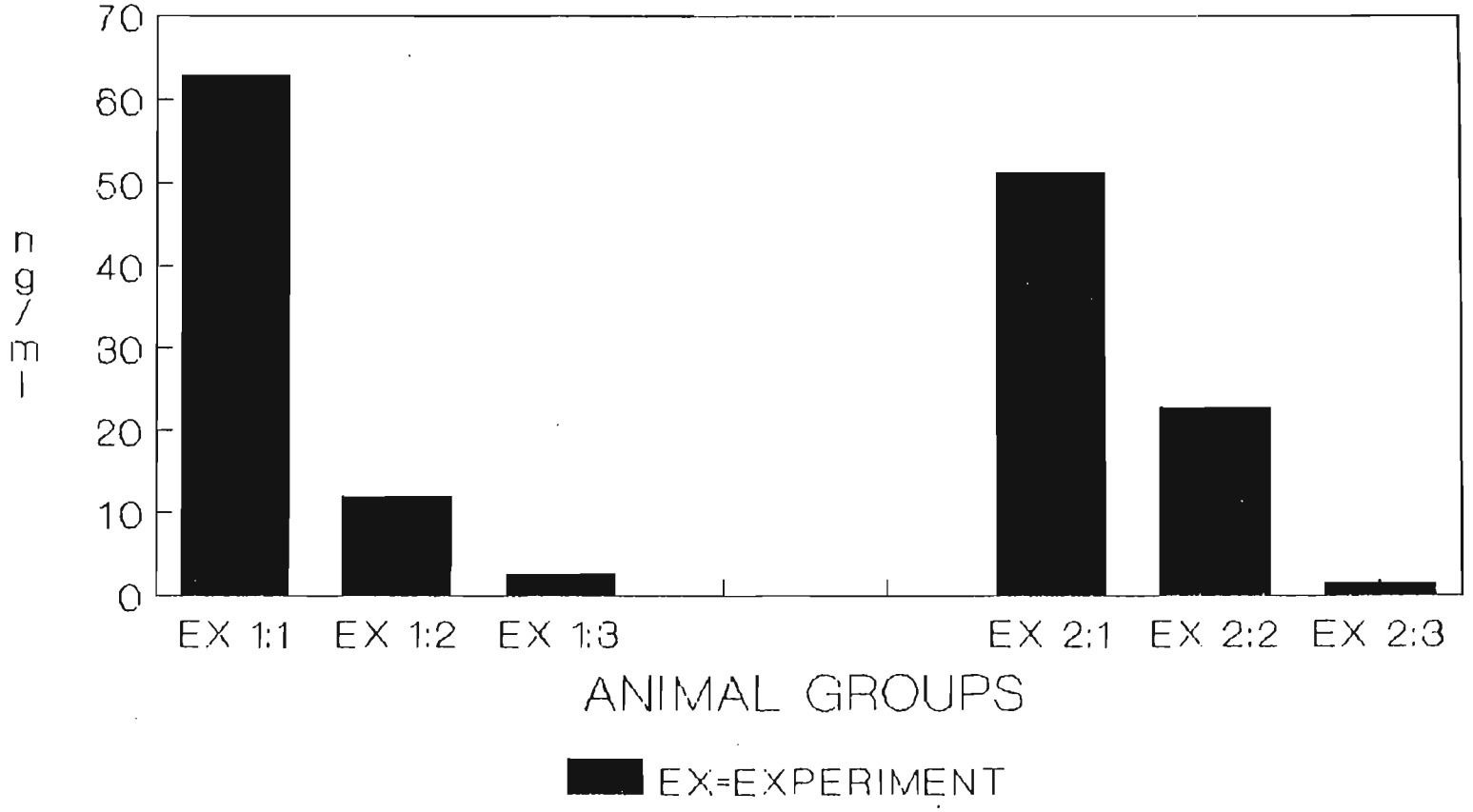


TABLE 6 : THE EFFECT OF VITAMIN B-6 DEFICIENCY ON PLASMA PL LEVELS : EXPERIMENT 1

GROUP	PLASMA PL (ng/ml)
1	63,04 ±16,39[a]
2	12,04 ± 5,29[b]
3	2,71 ± 0,92[c]

Plasma PL values are given as means ± SD and were obtained from 11 rats in group 1 and 2 and 10 rats in group 3. Means for each group not followed by the same superscript letter are significantly different. ($p<0,05$). PL values were obtained at end of an eight week period following an overnight fast. Groups 1, 2 and 3 received 3,50; 1,75 and 0mg/kg vitamin B-6 in the diet respectively. The two-tailed P values for groups 1 vs 2 , 1 vs 3 and 2 vs 3 were equal to 0,0001 . A significant different exists between groups when $p<0,0167$.

TABLE 7 : EFFECT OF VITAMIN B-6 DEFICIENCY ON PLASMA PL LEVELS : EXPERIMENT 2

GROUP	PLASMA PL (ng/ml)
1	51,32 ±12,42[a]
2	22,87 ± 4,64[b]
3	1,51 ± 0,5[c]

Results (mean ±SD) were obtained from 12 rats per group at the end of 8 weeks. Means not followed by the same superscript letter are significantly different ($p<0,05$)

Group 1 received 3,5mg vitamin B-6 per kg diet.

Group 2 (pair-fed control) received 3,5mg/kg vitamin B-6 in the diet.

Group 3 received 0mg/kg vitamin B-6 in diet.

The two-tailed p-values for groups 1 vs 2, 1 vs 3 and 2 vs 3 were equal to 0,0001. A significant difference exists between groups when $p<0,0167$.

4.4 TRACE ELEMENTS ANALYSIS

The mean of the trace element levels of the various tissues of experiment 1 and experiment 2 are given in Tables 8,9 and 10.

Trace element concentration in $\mu g/g$ = Trace element concentration in $\mu g/ml$ x 25 (dilution factor) divided by weight of tissue

The percentage recoveries of trace elements after digestion were in the following range :

Cu : 90-113

Fe : 90-112

Zn : 90-113

The individual values for each element in each tissue is given in Appendix 8.

4.4.1 Copper

Experiment 1

The copper levels of the various tissues examined are summarized in table 8.

TABLE 8 : **MEAN OF COPPER LEVELS IN TISSUES IN µg/g WET WEIGHT**

Experiment 1

GROUP	LIVER ±SD	KIDNEY ±SD	HEART ±SD	MUSCLE ±SD
1	4.28 ±0,83[a]	5,72 ±0,73[a]	3,48 ±1,17[a]	1,08 ±0,43[a]
N	11	10	11	11
2	3,59 ±1,02[a]	5,13 ±0,95[a]	3,44 ±1,13[a]	1,25 ±0,27[a]
N	11	10	10	11
3	3,27 ±0,47[b]	6,24 ±1,43[a]	4,96 ±1,79[a]	0,94 ±0,40[a]
N	11	9	11	11

Experiment 2

GROUP	LIVER ±SD	KIDNEY ±SD	HEART ±SD	MUSCLE ±SD
1	3,95 ±0,46[a]	4,40 ±0,74[a]	3,42 ±0,53[a]	1,59 ±0,42[a]
N	12	12	11	12
2	4,27 ±0,60[a]	4,61 ±0,85[a]	4,39 ±0,56[b]	1,06 ±0,18[b]
N	12	12	12	12
3	3,73 ±1,17[a]	3,99 ±0,19[a]	3,72 ±0,71[a]	0,78 ±0,26[b]
N	12	12	12	12

Values are expressed as mean ±SD. N = no of rats per group. Values not followed by the same superscript letter are significantly different (P<0,05). In experiments 1 and 2, groups 1 and 3 received 3,5mg/kg and 0mg/kg vitamin B-6 in the diet. Group 2 (experiment 1) received 1,75mg/kg vitamin B-6 in the diet. Group 2 (experiment 2) was the pair-fed control receiving 3,5mg/kg vitamin B-6 in the diet. Figures for individual rats and P-values are given in Appendix 5.

Group 3 had a significantly lower liver Cu content than group 1 ($p<0,05$). Group 3 Cu content of kidney, heart and muscle were not significantly different from group 1 ($p>0,05$).

Group 2 tissue Cu content were not significantly different from group 1 and 3 ($p>0,05$).

Experiment 2

Group 3 Cu content of tissues were not significantly different from group 1 ($p>0,05$) except for muscle which was significantly reduced ($p<0,05$).

Group 2 (pair-fed) Cu content of tissues were not significantly different from group 1 ($p>0,05$) except for the heart which was significantly elevated and muscle which was significantly reduced ($p<0,05$).

4.4.2 Iron

Experiment 1

The iron content of the various tissues examined are summarized in table 9. There were no significant differences in the liver, kidney, heart and muscle Fe content between group 1 and group 2 ($p>0,05$).

A significant increase was observed in the liver and heart of group 3 when compared with group 1 and group 2 ($p<0,05$). A significant decrease in muscle Fe content was found in group 3 when compared to groups 1 and 2 ($p<0,05$).

Liver Fe content was significantly higher in group 3 than group 2 ($p<0.05$).

Experiment 2

The data in table 9 indicate that the Fe content in the kidney of the pair-fed control group was significantly higher than the control group (group 1) ($p<0,05$).

The deficient group (group 3) had a significantly higher Fe content in the liver when compared to group 1 while the muscle Fe content was significantly decreased ($p<0,05$). No significant differences were observed in the kidney and heart between group 3 and group 1 ($p<0,05$).

The Fe content of the liver, kidney and muscle of group 3 were significantly different from the pair-fed group ($p<0,05$).

TABLE 9 : **MEAN OF IRON LEVELS IN TISSUES IN µg/g WET WEIGHT**

Experiment 1

GROUP	LIVER ±SD	KIDNEY ±SD	HEART ±SD	MUSCLE ±SD
1	167,67 ±17,06[a]	69,04 ±7,91[a]	85,80 ±6,26[a]	17,44 ±2,23[a]
N	11	11	11	11
2	154,46 ±30,79[a]	70,73 ±8,74[a]	82,81 ±5,31[a]	17,41 ±3,59[a]
N	11	11	11	11
3	295,99 ±55,36[b]	69,32 ±7,94[a]	98,07 ±6,46[b]	9,65 ±1,43[b]
N	11	11	10	11

Experiment 2

GROUP	LIVER ±SD	KIDNEY ±SD	HEART ±SD	MUSCLE ±SD
1	177,92 ±32,58[a]	55,52 ±5,70[a]	73,22 ±13,37[a]	14,49 ±1,81[a]
N	12	12	12	12
2	166,45 ±21,75[a]	85,14 ±25,54[b]	86,83 ±17,52[a]	14,66 ±2,03[a]
N	12	11	12	12
3	316,59 ±31,85[b]	62,42 ± 8,59[a]	77,98 ± 4,80[a]	11,14 ±1,57[b]
N	12	12	12	12

Results are expressed as mean +SD. N=no of animals used. Values not followed by the same superscript letter are significantly different (P<0,05). In experiments 1 and 2, groups 1 and 3 received 3,5 and 0,mg/kg vitamin B-6 in the diet. In experiment 1, group 2 received 1,75mg/kg of vitamin B-6 in the diet. In experiment 2, group 2 which was pair-fed with the deficient rats, recieved 3,5mg of vitamin B-6 in the diet. Figures for individual rats and P values are given in appendix 6.

4.4.3 Zinc

Experiment 1

From table 10 Zn content of the liver, kidney, heart and muscle of group 1 was not significantly different from group 3 ($p>0,05$). Zn levels of all tissues when not significantly different when group 2 was compared to group 3 ($p>0,05$).

Experiment 2

The Zn content of the liver, kidney, heart and muscle of group 1 was not significantly different from 3 ($p>0,05$).

Group 1 Zn levels were not significantly different from the pair-fed group ($p>0.05$). Group 2 Zn tissue levels were not significantly different from group 3 ($p>0.05$).

TABLE 10 : **MEAN OF TISSUE Zn LEVELS IN µg/g WET WEIGHT**

Experiment 1

GROUP	LIVER ±SD	KIDNEY ±SD	HEART ±SD	MUSCLE ±SD
1	32,58 ±1,70[a]	31,41 ±2,96[a]	20,12 ±5,34[a]	9,00 ±2,14[a]
N	11	11	11	11
2	32,78 ±1,98[a]	35,07 ±7,85[a]	19,12 ±5,01[a]	11,67 ±1,50[a]
N	11	11	10	11
3	32,75 ±3,48[a]	31,50 ±1,79[a]	26,26 ±8,73[a]	11,21 ±2,15[a]
N	11	11	10	10

Experiment 2

GROUP	LIVER ±SD	KIDNEY ±SD	HEART ±SD	MUSCLE ±SD
1	30,61 ±1,97[a]	24,18 ±4,11[a]	17,99 ±3,51[a]	9,50 ±2,32[a]
N	12	12	11	12
2	32,44 ±1,99[a]	23,21 ±2,49[a]	18,99 ±1,08[a]	8,73 ±1,62[a]
N	12	12	12	12
3	30,95 ±1,94[a]	26,36 ±5,74[a]	18,97 ±0,99[a]	9,50 ±1,97[a]
N	12	12	11	1

Values are expressed as means ±SD. N = number of animals used. Means not followed by the same supescript letter are significantly different ($P<0.05$). In experiment 1, groups 1, 2 and 3 received 3,5; 1,75 and 0mg/kg vitamin B-6 in the diet respectively. In experiment 2, groups 1 and 3 recieved 3,5 and 0mg/kg vitamin B-6 in the diet, group 2 was the pair-fed control. Figures for individual rats and P values are given in Appendix 7.

CHAPTER FIVE

DISCUSSION

5.1 APPEARANCE OF RATS

Deficient animals in both experiments developed variable degrees of vitamin B-6 deficiency symptoms. All deficient animals developed an unkempt fur appearance compared to the controls. Around the 4th week some deficient animals developed acrodynia.

5.2 FOOD CONSUMPTION AND GROWTH

Experiment 1

In this series group 1 received approximately 42µg/d of PN.HCl while group 2 received 21µg/d of PN.HCl. Despite the differences in PN.HCl intake/day no significant difference ($p>0,05$) in growth was observed between group 1 (control) and group 2 (marginally deficient). This finding supports earlier reports that 24µg/d (van den Berg *et al*, 1982) and 29µg/d (Lumeng *et al*, 1978) of vitamin B-6 were sufficient for maintaining maximal growth of the rat.

The food consumption of groups 1 and 2 were approximately 13-14 g/d as compared to the consumption of approximately 8g/d in group 3.

Hence it can be concluded that growth and food consumption are not affected during a marginal deficiency of vitamin B-6.

Experiment 2

The vitamin B-6 deficient group (group 3) ate approximately 9g of diet per day while the vitamin B-6 adequate group (group 1) consumed almost twice the amount; approximately 16g per day. It was found that group 2 animals (pair-fed) which were fed the control diet usually quickly ate all the food offered to them. However, the vitamin B-6 deficient group often ate slowly and reduced their food intake after the first week. This suggests that either vitamin B-6 deficiency induces anorexia or that a diet deficient in vitamin B-6 may be less palatable to a rat.

The growth rates of group 1 was found to be significantly different from group 2 and group 3 ($p<0,05$).

In this study group 1 took in approximately 58μg/d of PN.HCl while the group 2 animals took in approximately 32μg/d of PN.HCl. The significant difference in growth rate between group 1 and 2 is due not only to the different intakes of PN but also probably due to restricted calorie intake, where deficiencies of other nutrients also play a role.

The percentage body mass gained was found significantly different between group 2 and 3 except in the 1st and 6th week. ($p<0,05$).

Hence, it can be concluded that the reduced weight gain of the deficient group could not have solely resulted from lower food intake because group 2 consumed the same quantity of food.

From the above discussion of growth rates in both experiments it was found that although the pair-fed group consumed 32μg/d of PN.HCl this was not sufficient to maintain maximal growth. However, the group receiving 21μg/d of PN.HCl showed no significant difference in growth when compared to the control ($p<0,05$). Thus it can be concluded that lower intakes of vitamin B-6 will only result in maximal growth if deficiencies of other nutrients do not exist.

5.3. PLASMA PL LEVELS

Plasma samples drawn at the end of eight weeks were analysed for PL using a method described by Ubbink et al (1985, 1986). Several methods based on HPLC have been described but were unsuitable for routine measurement of blood levels and suffered from the disadvantage that analysis had to be carried out in a specially equipped darkroom. Ubbink et al, 1985 developed a method whereby the semicarbazone PL derivatives were stable enough to allow analysis to be carried out in under normal light and temperature.

In both experiments deficient groups were found to have a significantly lower PL level ($p<0,05$) than groups 1 and 2. Although the pair-fed group's PL levels were much lower than the control group, they had a considerably higher level than the deficient group. This indicates that restricted feed does have an effect on lowering the level of PL.

5.4 COPPER CONTENT OF TISSUES

Abnormal copper metabolism was noted in the vitamin B-6 deficient rats. Although the liver and muscle Cu were significantly lower in group 3 than controls in experiments 1 and 2 respectively a similar trend was noticed in both

experiments, however, this did not achieve statistical significance.

Although group 3 consumed less food and deionized water than group 1 the reduction in copper is not likely to be an effect of a decreased dietary supply. This is due to the fact that the pair-fed control group took in the same amount of trace elements but had higher Cu levels than Group 3. However pair-feeding did result in an increase in heart copper levels.

Data on the effect of vitamin B-6 on Cu metabolism has been lacking. However, it is well established that several Cu metalloenzymes need PLP as a cofactor eg. the amine oxidases of the liver which contain firmly bound PLP are copper proteins. (Gray and Daniel, 1973) The possiblity of decreased Cu content being a reflection of changed activity of special Cu dependent proteins in vitamin B-6 deficiency cannot be totally ruled out.

A more plausible explanation for the present findings may be the observations of Channa, 1988. It was found that vitamin B-6 deficient rats exhibited a significantly greater rise in Cu excretion than control and marginally deficient rats in a balance study.

A significantly greater rise in Zn excretion was also noticed in vitamin B-6 deficient rats than in the control. The increased excretion of Cu was attributed to two factors:

i) the decreased availability of picolinic acid in vitamin B-6 deficiency. Evans and Johnson, 1980 reported that Cu has a high association constant with picolinic acid. Thus, in addition to its postulated role in zinc absorption it may also have an effect on Cu absorption.

ii) The mutual antagonism of Cu and Zn. Channa, 1988 also found a greater excretion of zinc in vitamin B-6 deficiency. The high intraluminal content of Zn may have inhibited the absorption of Cu (Channa, 1988). This phenomenon has been widely reported. This type of interaction may result in lower copper concentrations in various organs.

It is also possible that more Cu was utilized for ceruloplasmin synthesis due to the need for iron mobilization. But this is not likely as elevated Fe stores were observed.

Liver copper concentrations in the fetus and newborn is physiologically elevated and declines during maturation. (Versieck, 1985) It is possible that the normal process of mobilization of hepatic copper concentration which occurs during

maturation is augmented during pyridoxine deficiency. This would obviously lead to a further reduction in liver copper content.

5.5 IRON CONTENT OF TISSUES

Vitamin B-6 deficient rats were observed to have elevated liver and heart Fe levels compared to rats receiving adequate PN.HCl (3,5mg/kg vitamin B-6 in diet) and marginally deficient rats (1,75mg/kg vitamin B-6 in diet). Deficient rats were shown have a statistically significant reduction in muscle Fe content than control and marginally deficient rats.

Similar observations have been reported by Kirksey et al, 1967 and Ikeda et al, 1979. They found elevated levels of Fe in liver and kidney of pyridoxine deficient rats. However, the reduction in muscle Fe has not been previously reported.

Elevated Fe content in liver could not be attributed to differences in food intake. The liver Fe content of the pair-fed group receiving the same amount of food as the deficient group, was significantly different from the deficient rats. However, pair-feeding did result in significantly increased kidney Fe content. The reasons underlying the change in the Fe metabolism during pair-feeding is not known. However,

it is well known that the type of feeding pattern adopted by control animals, known as meal eating, results in a number of anatomical, biochemical and physiological modifications. While other animals were given food ad libitum, pair-fed animals eat their food rapidly leaving empty trays and starved for the remainder of the day. Possible mechanisms underlying changes in trace element metabolism caused through pair-feeding will not be dealt with in this study. The introduction of the pair-fed group in the second study was to distinguish between changes arising solely from decreased food consumption and those directly related to pyridoxine deficiency.However, it is possible that once Fe stores were repleted excess Fe may have been excreted via the kidneys in the pair-fed group. This could partially explain the increased Fe noted in the kidneys. In experiment 2 a similar trend was noted in the deficient group but this did not achieve statistical significance.

Generally, it is clear pyridoxine deficiency has led to elevation of liver Fe stores and a reduction in muscle Fe stores. Mechanisms underlying these changes are not tenable as several other metabolic pathways and parameters have to be tested to confirm these hypotheses.

The liver serves an important function in the maintenance of whole body iron homeostasis through it roles as a major storage

organ for Fe and is the principal site for transferrin synthesis (Bacon and Tavill, 1984). Its central role in Fe metabolism makes it a reliable means for assessing Fe status. Transferrin synthesised by the hepatocytes delivers Fe to storage sites and sites of utilization predominantly muscle and liver (Bacon and Tavill, 1984).

Possible mechanical leading to changes of the tissue Fe in vitamin B-6 deficiency are outlined.

1. Underwood, 1971 reported that young animals are more susceptible to loss of myoglobin than mature animals. Also skeletal muscle appears to be more susceptible to this loss than cardiac or diaphragmatic muscle . Because young animals were used in this study and no Fe reduction in other tissues were observed it is possible that a loss of myoglobin from muscle occurred. It is possible that Fe from muscle was mobilized during pyridoxine deficiency-induced abnormal Fe metabolism. This however only explains the decline in Fe content. Further research is needed to explain the mechanism and biological significance of the differences in Fe metabolism during vitamin B-6 deficiencies.

2. The elevation of liver Fe content in group 3 is interesting. Considerable evidence seems to indicate the existence of an sideroblastic anemia in the deficient state.

Sideroblastic anemia is characterised as having varing numbers of hypochromic, microcytic red cells in the blood. Increased Fe stores in the reticulo-endothelial system and in parenchymatous organs such as the heart, pancreas and liver is found. Serum cholestrol is raised and Fe binding protein is saturated (Housten, 1985). Kinetic studies show increased Fe turnover but impaired Fe utilization.

While a pyridoxine free diet fed to dogs led to a hypochromic sideroblastic anemia (Prasad, 1978) no such reports have been made concerning rats. In man, pyridoxine responsive anemias were originally classified as sideroblatic. The response may be due to PN supplementation. PN supplementation prevented the inhibition of hemoglobin synthesis by PL-Fe complexes caused by increased Fe stores at the site of Hb synthesis (Bishop and Bethel, 1959). Not all cases cited are responsive to therapy.

However, Kirksey and Tabacchi, 1967 reported increased liver Fe stores. However, no mention was made a sideroblastic anemia. Although Hb content was not significantly altered; polycythemia, lower mean corpuscular Hb and mean corpuscular volume was found. Several instances have been reported of polycythemia vera associated with sideroblastic anemia (Prasad, 1978). Thus pyridoxine deficiency may have resulted in a sideroblastic anemia.

Thus it is possible that a dietary deficiency of pyridoxine would lead to a sideroblastic anemia due to decreased availability of PLP. The synthesis of hemoglobin is dependent on PLP. At a very early stage in the synthetic pathway PLP is needed for the formation of succinyl-CoA and -aminolevulinic acid. Hence hematopoiesis will be affected by the lack of PLP. If hemoglobin synthesis is impaired less Fe will be incorporated into protoporhyrin to form heme in the final biosynthetic step Fe metabolism will be affected resulting in increased Fe storage in certain tissues. The high proportion of Fe present in Hb indicates that any condition influencing the level of Hb in the blood will greatly affect the Fe status of the body.

3) Thus increased Fe stores is due to less Fe being incorporated into hemoglobin due to the lack of PLP. This increased Fe may have been augmented or caused by the decrease of Cu observed in the present study. The possibility is very likely as a considerable elevation in liver Fe was observed. Dietary deficiencies or excesses of some micronutrients create secondary disorders in tissue levels of other micronutrients (Sherman and Tissue, 1981). The interaction of Cu and Fe illustrates this point very well. Cu is transported to the liver were is is incorporated in ceruloplasmin. Ceruloplasmin links the Cu and Fe metabolism, an interaction causing the occurrence of anemia in the case of Cu deficiency. As a ferroxidase,

ceruloplasmin catalyses the conversion of Fe from the ferrous to ferric state before Fe is transported from the liver for hemoglobin synthesis. The postulated anemic state and increased liver Fe found in copper deficiency may be attributed to inadequate ceruloplasmin for the moblilization of Fe from the liver (fig.19) (Peereboom, 1985).

Thus in addition to the effect of PLP, decreased levels of Cu may impair moblization of Fe resulting in increased Fe stores.

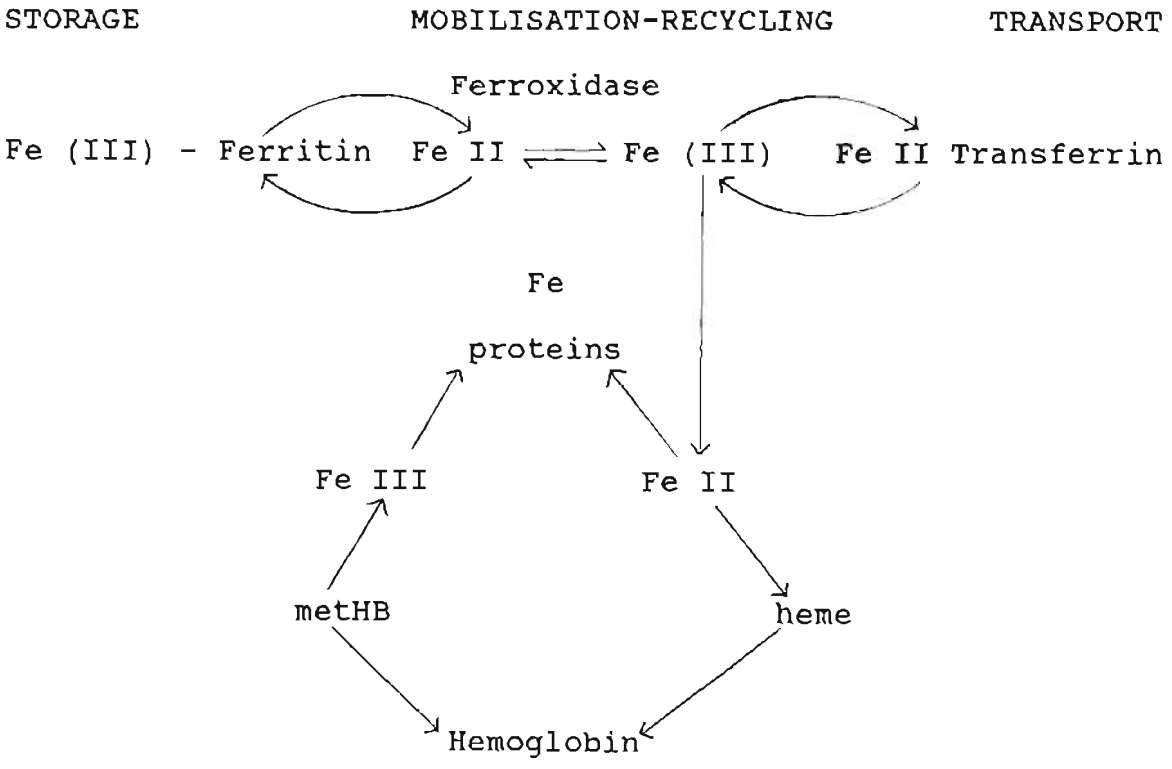


Fig. 19 : THE CENTRAL ROLE OF CERULOPLASMIN (Ferroxidase) IN LINKING Cu AND Fe METABOLISM (Peereboom, 1985)

5.6 ZINC CONTENT OF TISSUES

There were no significant differences of zinc levels of tissues between the groups studied.

Thus far reports on the effect of vitamin B-6 deficiency on zinc uptake (Prasad, et al, 1982), tissue levels of zinc (Hsu, 1965; Gershoff, 1968) and zinc excretion (Channa, 1988) strongly point to the fact that the nutritional status of zinc may be related to vitamin B-6 status. In addition many symptoms of zinc deficiency and vitamin B-6 deficency are similar such as food intake, growth, dermatologic lesions and impaired immune function (Brown, 1981). Thus it is quite surprising that no significant change was noticed in zinc tissue levels if viewed in this light.

In addition to the above observations, the role of picolinic acid, a tryptophan metabolite, in zinc metabolism postulated by Evans, 1980 is debatable. A ligand, thought to originate from the pancreas was found in the intestinal wall and in the milk of certain species (Evans, 1980, Hurley and Lonnerdal, 1980). Its identity is still uncertain, although it is claimed to be protaglandin, citrate (Hurley and Lonnerdal, 1980) or picolinic acid (Evans, 1980). Picolinic acid enhances the absorption of zinc (Evans, 1980) but this property is shared by many other

chelating agents. It would be surprising if the effects of picolinic acid were unique when many other ligands are also present in the intestinal mucosa. Thus Evans' proposal that vitamin B-6 deficiency would result in decreased picolinic acid production leading to a malabsorption of zinc is debatable.

Regardless of whether picolinic acid plays a role in zinc uptake, other studies cited in the literature raise the intersting possibility of an interaction between Zn and vitamin B-6. The fact that this study failed to confirm this relationship is not surprising if one takes into account the fact that tissue levels were used to assess zinc status.

The use of tissue levels in defining Zn status could be misleading (Golden, 1989). He proposed the existence of 2 types of nutritional deficiencies based on the response of the tissue levels. While the type 1 nutrient deficiency leads to a reduction in tissue concentration; type II nutrient deficency does not. Zn falls into the latter category. So if a Zn deficiency does exist as Channa's results will tempt one to believe, it would be very difficult to diagnose by analysing trace element content of the tissues.

According to Golden, 1989 if the animal is at a stage of development where sensitivity to zinc is high it stops growing,

without a reduction in tissue concentration when given a diet that is low in zinc. He proposed that Zn is locked in tissues and is fundamental to the composition, integrity and day-to-day functioning of that tissue. Zinc would only be released if whole tissue is catabolized or small amounts are released if there is metabolic re-adjustment. These metabolic re-adjustments are proportionately small and totally unrelated to the zinc deficiency.

If whole tissue is being broken down and the excess nutrient liberated will have to be metabolized or excreted. This is one of the possible explanations for Channa's observation of increased zinc excretion.

The fact that zinc deficiency is characterized by a large fixed pool in the tissues makes analysis of zinc status using tissue content misleading. If a deficiency does exist analysis of the small, rapidly turning over free pool would be valid as this pool would be sensitive to zinc deficiency. Hence plasma or serum should be used. But use of plasma or serum to diagnose zinc deficiency is invalid as it does not reflect total body zinc and is subject to metabolic alteration. Anyway, according to the latest paper quoted on diagnosis of zinc deficiency, total body zinc bears a questionable relationship to zinc status as it is not a type I nutrient deficiency (Golden, 1989).

The latest research on the zinc diagnostic problem has recommended the assay of plasma zinc and red blood cell metallothionein-1 to diagnose zinc deficiency (Golden, 1989).

Thus in conclusion, the failure to show a reduction in tissue zinc levels in this study is due to the fact that a zinc deficiency is a type II nutrient deficiency. Also the failure to indicate significantly increased zinc levels in tissue supports results obtained in this laboratory by Channa.

Thus tissue zinc remains constant over a wide range of Zn intakes, indicating that there is efficent homeostatic control of zinc metabolism.

CHAPTER 6

CONCLUSION

The results of this study indicate that the lower levels of vitamin B-6 cited in the literature do not support maximal growth of the rat during restricted feeding. However, maximal growth is maintained at the lower levels of the vitamin when deficiencies of other nutrients do not exist.

This study shown that vitamin B-6 metabolism related to trace element status. It is clear that an alteration of Fe metabolism exists in vitamin B-6 deficiency. Elevated Fe stores in the liver and heart coud be due to the existence of a sideroblastic anemia during pyridoxine deficiency. The decreased muscle stores may have been due to a loss of myoglobin. The liver and muscle copper content was found to be lowered in pyridoxine deficiency. Thus its role in iron metabolism may have been impaired resulting in further elevation or iron stores. Various parameters have to be tested before the symptoms of the sideroblastic anemia can be confirmed.

Failure to show a link between zinc status and vitamin B-6 metabolism confirms the report by Golden, 1989 that the measurement of tissue levels is an invalid indicator of zinc

status. This is based on the assumption that zinc deficiency exists during a vitamin B-6 deficiency. As mentioned earlier considerable evidence points to this belief. It is therefore recommended that a valid assessment of zinc status such as measurement of plasma zinc or metallothionein -1 in red blood cells be carried out to shed more light on the issue.

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Summary

All previous studies on the relationship between vitamin B-6 and trace element metabolism point strongly to possible link between the two micronutrients. They function as catalysts in a wide variety of reactions and thus have common pathways. This study was designed to examine the effects of graded levels of vitamin B-6 on tissue trace element levels. In the first experiment 33 young male Wistar rats were divided into three groups of 11 rats each. Group 1 the vitamin B-6 adequate group received 3,5 mg/kg PN HCl. Group 2 a marginally deficient group received 1,75mg/kg of vitamin B-6 in the diet. Group 3 the deficient group received 0 mg/kg of vitamin B-6 in the diet. In a separate experiment 36 young male Wistar rats were divided into 3 groups and subjected to the same dietary treatments except that the group 2, was replaced by a pair-fed control group.

Plasma PL levels were analysed to determine the vitamin B-6 status of the animals. Tissue trace elements were assayed by atomic absorption spectrophotometry in tissue samples obtained from the study.

The growth rates and food consumption was significantly different in all groups except group 1 and 2 in experiment 1 indicating that these two factors are not altered during a

marginal deficiency. However, it was found that a lower intake of vitamin B-6 during restricted feeding does impair growth. Growth rate and food consumption of the deficient group was decreased when compared to the marginally deficient and control groups. The plasma PL levels were significantly different in all three groups in both series. A progressive decrease in PL from group 1 to group 3 was found.

No significant changes were found in zinc tissue levels. This contributed to the assessment of Zn in tissues as being invalid. Elevated liver and heart Fe levels found in Group 3 may be attributed to impaired mobilization, defective hematopiesis and the possible existence of an anemic state. Increased Cu excretion may have led to decreased in muscle and heart Cu levels observed. This study showed that Fe and Cu status were affected by the vitamin B-6 deficiency.

References

American Institute of Nutrition AD HOC COMMITTEE, 1977. Report of the American Institute of Nutrition Ad Hoc Committee on Standards for Nutritional Studies. J. Nutr. 107 : 1340 - 1348.

Anderson, B.B., 1980. Red-cell metabolism of Vitamin B-6, p. 53 - 83. In: Tryfiates, G.P. (ed.), Vitamin B-6 metabolism and role in growth. Food and Nutr. Press, Westport CN.

Bacon, R.R. and Tavill, A.S., 1984. Role of the liver in normal Fe metabolism. Seminars in Liver Disease 4 (3) : 181 - 192.

Beaton, C.H. and Cheney, M.C., 1965. Vitamin B-6 requirement of the male Albino Rat. J. Nutr. 87 : 125 - 132.

Beisel, W.R., 1979. Metabolic balance studies - their continuing usefulness in nutritional research. Am. J. Clin. Nutr. 32 : 271 - 274.

Bishop, R.C., Bethel, F.H., 1959. Hereditary hypochromic anaemia with transfusion hemosiderosis treated with pyridoxine. New Eng. J. Med. 261 (10) : 486 - 489.

Brown, R.R., 1981. The tryptophan load test as an index of vitamin B-6 nutrition, p. 321 - 340. In: Leklem, J.E. and Reynolds, R.D. (eds.), Methods in vitamin B-6 nutrition: analysis and status Assessment, Plenum Press, New York.

Channa, M.L., 1988. MSc Thesis: The effect of Vitamin B-6 deficiency on copper, zinc and iron balance in the rat, University of Durban-Westville, Durban.

Clegg, M.S., Keen, C.L., Lonnerdal, B. and Hurley, L.S., 1981. Influence of ashing techniques on the analysis of trace elements in animal tissue. Biological Trace Element Research 3 : 107 - 115.

Dakshinamurti, K. and Chauhan, M.S., 1981. Chemical analysis of pyridoxine vitamers, p. 99 - 122. In: Leklem, J.E. and Reylonds, R.D. (eds.), Methods in vitamin B-6 nutrition: Analysis and status assessment. Plenum Press, New York.

Delves, H.T., 1982. Some clinical aspects of trace elements. Ann. Clin. Biochem. 19 : 302 - 306.

Delves, H.T., 1985. Assessment of trace element status. Clinics in endocrinology and metabolism 14 (3) : 725 - 761.

Driskell, J.A., 1984. Vitamin B-6, p. 379 - 400. In: Lawrence, J.M. (ed.), Handbook of Vitamins: Nutritional, biochemical and clinical aspects, Marcel Dekker, New York.

Driskell, J.A., Strickland, L.A., Poon, C.H. and Foshee, D.P., 1973. The vitamin B-6 requirement of the male rat as by behavioural patterns, brain pyridoxal phosphate and nucleic acid composition and erythrocyte alanine transferase activity. J. Nutr. 103 : 670 - 680.

Evans, G.W., 1980. Normal and abnormal Zn absorption in man and animals: The tryptophan connection. Nutr. Rev. 38 : 295 - 296.

Evans, G.W. and Johnson, E.C., 1980. Effect of Vitamin B-6 and Picolinic acid on Zn absorption in the rat. J. Nutr. 111 : 68 - 75.

French, S.W., 1966. Effect of chronic ethanol ingestion on liver enzyme changes induced by thiamine, riboflavin, pyridoxine or choline deficiency. J. Nutr. 88 : 291 - 302.

Gershoff, S.N., 1968. Effect of vitamin B-6 deficiency and restricted calorie intake on tissue zinc levels. Proc. Soc. Exp. Biol. Med. 127 : 1207 - 1210.

Golden, M.H.N., 1989. The diagnosis of Zinc deficiency, p. 323 - 332. In: Mills, C.F. (ed.), Zinc in Human Biology, Springer-Verlag, London.

Gray, L.F. and Daniel, L.J., 1973. Effect of copper status on the urinary excretion of tryptophan metabolites and amino acids by the rat. J. Nutr. 103 : 1764 - 1771.

Gregory, J.F. and Kirk, J.R., 1981. Determination of vitamin B-6 compounds by semi-automated continuous flow and chromatographic methods, p. 149 - 170. In: Leklem, J.E. and Reynolds, R.D. (eds.), Methods in Vitamin B-6 nutrition: Analysis and status assessment, Plenum Press, New York.

Henderson, L.M., 1985. Intestinal absorption of vitamin B-6 vitamers, p. 25 - 33. In: Reynolds, R.D. and Leklem, J.E. (eds.), Current topics in nutrition and disease, Vol. 13, Vitamin B-6: Its role in health and disease, Alan R. Liss, New York.

Houston, J.C., Joiner, C.L. and Trounce, T.R. (eds.), 1985. A Short Textbook of Medicine, 8th edition, Hodder and Stroughton, London.

Hsu, J.M., 1965. Zinc content of tissues of pyridoxine deficient rats. Proc. Soc. Exp. Biol. Med. 119 : 177 - 180.

Hurley, L.S. and Lonnerdal, B., 1980. Zinc absorption and tryptophan metabolism. Nutr. Rev. 38 : 295 - 296.

Ikeda, M., Hosotani, T., Ueda, T., Kotake, Y. and Sakakibara, B., 1979. Observations of the concentration of zinc and iron in tissues of vitamin B-6 germ-free rats. J. Nutr. Sci. Vitaminol. 25 : 151 - 158.

Ink, S.L. and Henderson, L.M., 1984. Vitamin B-6 metabolism. Ann. Rev. Nutr. 4 : 455 - 470.

Jackson, M.J., Jones, D.A. and Edwards, R.H.T., 1982. Tissue zinc levels as an index of body zinc status. Clinical Physiology 2 : 333 - 343.

Kirksey, A., Driskell, J.A. and Miller, I.E., 1969. Pyridoxine deficiency and iron metabolism in the pregnant rat: Fetal responses. J. Nutr. 99 : 9 - 15.

Kirksey, A. and Tabacchi, M.H., 1967. Pyridoxine deficiency and iron metabolism in the pregnant rat: Maternal responses. J. Nutr. 93 : 229 - 240.

Kok, F.J., Schrijver, J., Hofman, A., Witteman, J.C.M., Kruyssen, D.A.C.M., Remme, W.J., Valkenburg, H.A., 1989, Low vitamin B-6 status in patients with acute mycodardial infarction. AM.J. Cardiol. 63 : 513 - 516.

Labadarios, D. and Shephard, G.S., 1985. Vitamin B-6 : Use and deficiencies in clinical practice. Journal of Dietetics and Home Economics. 13, (1) : 22 - 24.

Labadarios, D. and Shephard, G.S., 1984. Vitamin B-6: metabolism in health. Journal of Dietetics and Home Economics 12, (3) : 90 - 95.

Leklem, J.E. and Reynolds, R.D., 1981. Recommendations for status assessment of vitamin B-6, p. 389 - 392. In: Leklem, J.E. and Reynolds, R.D. (eds.), Methods in vitamin B-6 nutrition - analysis and status assessment, Plenum Press, New York.

Leklem, J.E., 1988. Vitamin B-6 metabolism and function in humans, p. 3 - 28. In: Leklem, J.E. and Reynolds, R.D. (eds.), Current topics in nutrition and disease, Vol. 19. Clinical and physiological applications of vitamin B-6, Alan R. Liss, New York.

Levene, C.I., Murray, J.C., 1977. The aetological role of maternal vitamin B-6 deficiency in the development of atherosclerosis. Lancet : 628 - 629.

Litwack, G., Miller-Diener, A., Disorbo, D.M. and Schmidt, J.J., 1985. Vitamin B-6 and the glucocorticoid receptor, p. 177 - 191. In: Reynolds, R.D. and Leklem, J.E. (eds.), Current topics in nutrition and disease, Vol. 13, Vitamin B-6 - Its role in health and disease. Alan R. Liss. New York.

Lumeng, L., Ryan, M. and Li, T-K., 1978. Validation of the diagnostic value of plasma pyridoxal 5'-phosphate measurements in vitamin B-6 nutrition of the rat. J. Nutr. 108 : 545 - 553.

Lumeng, J., Li, T-K, and Lui, A., 1985. The interorgan transport and metabolism of vitamin B-6, p. 35 - 54. In: Reynolds, R.D. and Leklem, J.E. (eds.), Current topics in nutrition and disease, Vol. 13., Vitamin B-6: its role in health and disease, Alan R. Liss, New York.

Masse, P., Vuilleumier, J.P. and Weiser, H., 1988. Is pyridoxine an essential nutrient for bone? Int. J. Vit. Nutr. Res. 58 : 295 - 299.

Mercer, P.L., Gustafson, J.M., Higbee, P.T., Geno, C.E., Schweisthal, M.R. and Cole, T.B., 1984. Control of physiological response in the rat by dietary nutrient concentration. J. Nutr. 114 : 144 - 152.

Merrill, A.H. and Henderson, J.M.., 1987. Diseases associated with defects in vitamin B-6 metabolism or utilization. Ann. Rev. Nutr. 7 : 137 - 156.

Metcalfe, E. (ed.) 1987. Atomic Absorption and Emission Spectroscopy: Analytical Chemistry by Open Learning. John Wiley and Sons, Chichester.

Neal, R.A. and Pearson, W.W., 1962. Effect of pyridoxine deficiency on iron absorption in the rat. J. Nutr. 78 : 215 - 218.

Olson, R.E., 1987. Dietary protein and vitamin B-6 requirements. Nutr. Rev. 45 (1) : 23 - 25.

Peereboom, J.W.C., 1985. General aspects of trace elements and health. The Science of the Total Environment 42 : 1 - 27.

Perkin Elmer Corporation, 1976a. Manual for atomic absorption spectrophotometer - Model 2380.

Perkin Elmer Corporation, 1976b. Analytical methods for atomic absorption spectrophotometry.

Polansky, M., 1981. Microbiological assay of vitamin B-6 in foods. In: Leklem J.E. and Reynolds, R.D. (eds.), Methods of vitamin B-6 nutrition: analysis and status assessment, Plenum Press, New York.

Prasad, A.S., 1978. Trace elements and iron in human metabolism. Plenum Publishing Corp., New York.

Prasad, R., Lyall, V. and Nath, R., 1982. Effect of vitamin B-6 and B-1 deficiencies on the intestinal uptake of calcium, zinc and cadmium. Ann. Nutr. Metab. 26 : 324 - 330.

Reynolds, R.D., Natta, C.L., 1985. p. 301 - 316. Vitamin B-6 and sickle cell anemia : p. 301 - 316, In: Current topics in nutrition and disease. Vol. 13, Vitamin B-6: Its role in health and disease. Alan R. Liss, New York.

Rinehart, J.F. and Greenburg, L.D., 1949. Artherosclerotic lesions in pyridoxine deficient monkey. Am. J. Pathol. 25 : 481 - 491.

Rosenberg, I.H., 1989. Role of intestinal microflora in vitamin B-6 metabolism. Nutr. Rev. 47 (9) : 277 - 279.

Rutishauser, I.H.E., 1982. Vitamin B-6. Journal of Food and Nutrition 39, (4) : 158 - 167.

Sauberlich, H.E., 1985. Interaction of vitamin B-6 with other nutrients, p. 193 - 217. In: Reynolds, R.D. and Leklem, J.E. (eds.), Current topics in nutrition and disease, Vol. 13, Vitamin B-6: Its role in health and disease. Alan R. Liss, New York.

Serfontein, W.J., Ubbink, J.B., De Villiers, L.S., Rapley, C.H. and Becker P.J., 1985. Plasma pyridoxal 5-phosphate level as a risk index for coronary artery disease. Atherosclerosis. 55 : 357 - 361.

Sherman, A.R. and Tissue, N.T., 1981. Tissue iron, copper and zinc levels in offspring of iron-sufficient and iron-deficient rats. J. Nutr. 111 : 266 - 275.

Snell, E.E., 1981. Vitamin B-6 analysis: Some historical aspects, p. 1 - 19. In: Leklem, J.E. and Reynolds, R.D. (eds.), Methods in vitamin B-6 nutrition: analysis and status assessment, Plenum Press, New York.

Starcher, B.C., 1969. The effects of pyridoxine deficiency on aortic elastin biosynthesis, Proc. Soc. Exp. Biol. Med. 132 : 379 - 382.

Sturman, J.A., 1981. Vitamin B-6 and sulphur amino acid metabolism, p. 341 - 371. In: Leklem, J.E. and Reynolds, R.D. (eds.), Methods in vitamin B-6 nutrition: analysis and status assessment, Plenum Press, New York.

Tryfiates, G.P., 1986. Pyridoxal phosphate and metabolism, p. 423 - 447. In: Dolphin, D., Poulson, R. and Avramovic, O. (eds.), Vitamin B-6 pyridoxal phosphate, Part A, John Wiley and Sons, New York.

Ubbink, J.B., Serfontein, W.J. and De Villiers, L.S. 1985. Stability of pyridoxal-5-phosphate semicarbazone: applications in plasma vitamin B-6 analysis and population surveys of vitamin B-6 nutritional status. Journal of Chromatography 342: 277 - 284

Ubbink, J.B., Serfontein, W.J. and De Villiers, C.S. 1986. Analytical recovery of protein-bound pyridoxal-5-phosphate in plasma analysis. Journal of Chromatography 375 : 399 - 404.

Van den Berg, H., Bogaards, J.J.P., Sinkeldam, E.J., and Schreurs, W.H.P., 1982. Effect of different levels of vitamin

B-6 in the diet of rats on the content of pyridoxine -5'-phosphate and pyridoxal-5'-phosphate in the liver. Internat. J. Vit. Nutr. Res. 52:407 - 416.

Versieck, J., 1985. Trace elements in human body fluids and tissues. Crc. Crit. Revs. Clin. Lab. Sci. 22 (2) : 97 - 183.

Underwood, E.J., 1971. Trace Elements in Human and Animal Nutrition. Academic Press, New York.

Williams, A.K., 1974. Vitamin B-6 : Gas liquid chromatography of pyridoxol, pyridoxal and pyridoxamine. J. Agric. Food Chem. 22, 107 - 109.

Williams, B.C. and Wilson, K. (eds.), 1984. A biologist's guide to principles and techniques of practical biochemistry, p. 72 - 126. Edward Arnold, London.

Willet, C.W., 1985. Does a low vitamin B-6, increase the risk of coronary heart disease? p. 337 - 346. In: Current topics in nutrition and disease, Vol. 13, Vitamin B-6: Its role in health and disease. Alan R. Liss., New York.

Winge, D.R., 1984. Normal physiology of copper metabolism. Seminars in Liver Disease. 4(3) : 239 - 251.

APPENDIX 1 : **FOOD CONSUMPTION : MEAN DAILY INDIVIDUAL FOOD CONSUMPTION (g) OVER 8 WEEKS** : Experiment 1

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 1 CONTINUED

Two-tailed P-values for Table 2 - Effect of vitamin B-6 deficiency on daily food consumption : Experiment 1

GRP	WEEK 1	WEEK 2	WEEK 3	WEEK 4	WEEK 5	WEEK 6	WEEK 7	WEEK 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 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	12,6	14,2	14,6	18,0	19,6	17,8	18,2	17.5
	2	15,3	14,0	14,4	16,3	15,5	15,6	16,5	16.7
	3	13,9	14,4	15,8	17,4	16,8	16,5	18,0	16.6
	4	14,5	16,2	18,6	20,0	19,5	18,7	18,1	20.6
	5	15,8	16,5	18,5	19,2	18,9	16,2	16,3	16,5
	6	15,3	17,6	20,2	22,6	20,8	19,4	19,5	19,6
	7	14,3	16,4	17,0	18,7	18,4	15,1	17,4	18,9
	8	15,1	16,2	17,0	18,3	17,4	16,6	17,6	16,7
	9	12,8	15,7	17,4	18,3	18,2	17,2	15,1	19,4
	10	14,8	13,9	14,5	15,1	13,1	13,7	15,2	12,9
	11	12,7	13,7	16,1	19,2	18,3	16,4	18,0	18,1
	12	14,1	12,9	14,4	15,5	15,5	16,9	15,5	17,3
3	1	10,1	9,5	8,6	9,3	6,5	6,3	8,5	7,8
	2	10,9	10,7	9,5	9,8	8,4	8,2	7,1	6,8
	3	11,4	8,5	8,4	7,6	8,7	8,5	8,7	9,7
	4	13,2	11,5	9,2	10,2	10,0	7,7	6,7	6,3
	5	12,8	10,8	9,4	10,4	9,5	9,5	9,0	9,5
	6	10,6	10,0	10,2	8,9	9,7	9,4	9,4	8,1
	7	12,6	12,5	11,6	8,5	8,3	10,7	10,7	10,6
	8	9,5	8,8	7,8	8,1	9,8	11,0	10,4	8,6
	9	10,7	10,1	9,5	8,1	8,0	6,4	7,7	6,6
	10	11,5	10,9	9,6	8,1	8,5	8,7	9,8	8
	11	11,1	11,3	8,7	8,1	8,7	8,4	6,8	7,4
	12	12,5	11,3	10,4	9,6	10,2	10,1	10,2	11,52

APPENDIX 2 CONTINUED

Two-tailed P-values for table 3 - Effect of Vitamin B-6 deficiency on daily food consumption : Experiment 1

GROUP	WEEK 1	WEEK 2	WEEK 3	WEEK 4	WEEK 5	WEEK 6	WEEK 7	WEEK 8
1 vs 3	0,0001	0,0000	0,0000	0,0000	0,0000	0,0000	0,0000	0,0000

A significant difference exists between groups if the P value is <0,05

APPENDIX 3 : **GROWTH : WEEKLY INDIVIDUAL ANIMAL MASS (g)** :
Experiment 1

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 3 : CONTINUED

Two-tailed P-values for Table 4 - Effect of vitamin B-6 deficiency on percentage body mass gained : Experiment 1

GROUP	WEEK 1	WEEK 2	WEEK 3	WEEK 4	WEEK 5	WEEK 6	WEEK 7	WEEK 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 4 : **GROWTH: WEEKLY INDIVIDUAL ANIMAL MASS (g)**
Experiment 2

GRP	NO.	DAY 0	WK 1	WK 2	WK 3	WK 4	WK 5	WK 6	WK 7	WK 8
1	1	70	120	160	195	245	285	310	340	363
	2	95	155	200	230	270	303	327	346	362
	3	80	125	160	205	255	290	323	352	373
	4	90	155	200	240	293	321	343	355	374
	5	85	150	200	240	281	314	321	349	345
	6	80	155	200	250	304	339	367	388	409
	7	85	150	200	235	280	305	312	327	340
	8	90	145	200	235	272	302	325	345	357
	9	70	120	160	195	243	282	311	330	351
	10	70	120	160	185	217	236	264	281	283
	11	70	125	160	200	258	297	327	357	382
	12	80	125	160	185	227	256	289	320	342
2	1	80	129	142	168	174	178	174	188	197
	2	80	126	146	166	177	186	184	198	208
	3	85	135	149	178	193	206	199	213	221
	4	75	127	149	176	188	198	192	214	228
	5	80	123	150	179	192	201	196	205	209
	6	85	136	155	184	191	194	188	203	211
	7	80	124	140	169	176	185	183	197	206
	8	90	133	157	188	203	212	203	212	224
	9	80	133	155	182	207	179	207	223	235
	10	75	125	149	180	193	201	196	205	215
	11	70	124	152	175	187	182	193	209	214
	12	85	120	150	173	187	189	195	208	213
3	1	70	112	130	139	146	134	132	135	142
	2	70	120	145	152	170	145	152	155	151
	3	75	118	129	141	144	186	191	200	219
	4	85	145	165	164	173	167	172	173	180
	5	90	145	165	178	191	200	208	212	216
	6	80	119	145	162	174	149	148	166	172
	7	75	140	165	176	168	158	177	181	178
	8	90	129	145	150	159	179	192	190	189
	9	75	120	145	150	154	156	159	157	152
	10	75	130	155	161	168	180	182	192	195
	11	70	120	140	136	147	153	161	156	159
	12	95	140	155	161	172	182	186	186	192

PENDIX 4 CONTINUED

Two-tailed P-values for table 5 - Effect of vitamin B-6 deficiency on percentage body weight gained : Experiment 2

GROUP	WEEK 1	WEEK 2	WEEK 3	WEEK 4	WEEK 5	WEEK 6	WEEK 7	WEEK 8
1 vs 2	0,4333	0,0000	0,0001	0,0000	0,0000	0,0000	0,0000	0,0000
1 vs 3	0,2022	0,0015	0,0000	0,0000	0,0000	0,0000	0,0000	0,0000
2 vs 3	0,6434	0,9077	0,0015	0,0008	0,0047	0,0282	0,0018	0,0018

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

APPENDIX 5 : **TISSUE LEVELS OF Cu IN GROUPS FED VARYING LEVELS OF VITAMIN B-6 IN DIET**

1. **KIDNEY**

ANIMAL NO	GRP 1 3,5mg/kg PN	GRP 2 1.75mgkg/ PN	GRP 3 0mg/kg PN	GRP 1 3,5mg/kg PN	GRP 2 Pair-fed control	GRP 3 0mg/kg PN
1	4,83	4,69	5,97	3,96	3,38	3,99
2	6,21	6,92	4,50	4,50	4,98	3,95
3	6,45		4,50	4,84	5,38	3,94
4	6,23	4,35	5,18	4,44	5,50	4,43
5	5,91	4,24		5,98	5,50	3,90
6	6,50	5,34	6,86	4,38	3,84	3,82
7		4,43	5,32	3,85	4,35	3,95
8	6,18	6,93	5,93	4,28	4,31	3,87
9	5,93	5,01	6,96	5,38	3,91	3,91
10	4,99	4,46	6,29	4,39	5,36	3,84
11	5,00	4,91		3,41	5,47	4,32
12				3,43	3,35	3,95

TISSUE LEVELS OF Cu IN GROUPS FED VARYING LEVELS OF VITAMIN B-6 IN DIET

2. **LIVER**

ANIMAL NO	GRP 1 3,5mg/kg PN	GRP 2 1,75mg/kg PN	GRP 3 0mg/kg PN	GRP 1 3,5mg/kg PN	GRP 2 Pair-fed Control	GRP 3 0mg/kg PN
1	3.89	4,83	2,49	4,38	4,42	5,31
2	4,50	5,37	3,50	3,49	4,76	5,01
3	3,94	4,50	3,85	4,50	4,48	5,15
4	5,01	2,41	3,01	4,09	3,93	5,29
5	2,45	2,94	2,94	3,35	3,98	2,40
6	4,44	3,50	2,96	4,00	3,35	3,91
7	4,44	4,02	2,94	3,95	4,35	3,35
8	3,46	2,02	3,05	3,35	4,90	2,44
9	4,69	3,50	3,43	3,35	3,88	3,09
10	5,50	2,99	3,98	3,98	5,43	2,42
11	4,79	3,43	3,82	4,45	3,42	3,41
12				4,46	4,32	2,92

APPENDIX 5 : TISSUE LEVELS OF Cu IN GROUPS FED VARYING LEVELS OF VITAMIN B-6 IN DIET

3. HEART

ANIMAL NO	GRP 1 3,5mg/kg PN	GRP 2 1.75mg/kg PN	GRP 3 0mg/kg PN	GRP 1 3,5mg/kg PN	GRP 2 Pair-fed control	GRP 3 0mg/kg PN
1	3,17	4.38	4,34	2,96	4,91	3,56
2	2,97	3,01	4,96	4,00	5,10	5,59
3	5,19	3,00	4,72	3,01	5,25	2,95
4	6,00	4,01	5,53	3,44	3,97	3,80
5	2,35	2,35	6,50	4,34	3,58	2,96
6	2,45	5,5	6,34		5,01	3,35
7	3,50	3,81	2,92	3,42	4,40	4,41
8	2,38	3,01	5,79	3,85	4,35	3,38
9	3,37	3,88	8,26	2,93	3,87	3,63
10	3,98	1,45	2,98	3,91	3,91	3,39
11	2,88		2,25	2,88	3,92	3,72
12				2,88	4,46	3,95

TISSUE LEVELS OF Cu IN GROUPS FED VARYING LEVELS OF VITAMIN B-6 IN DIET

4. MUSCLE

ANIMAL NO	GRP 1 3,5mg/kg PN	GRP 2 1.75mg/kg PN	GRP 3 0mg/kg PN	GRP 1 3,5mg/kg PN	GRP 2 Pair-fed control	GRP 3 0mg/kg PN
1	0,49	1,47	0,96	1,00	1,00	1,00
2	0,96	1,00	0,50	1,95	1,46	0,98
3	1,50	0,96	1,46	1,48	1,00	0,99
4	0,50	0,96	0,50	1,48	0,99	0,97
5	1,47	1,49	0,49	1,96	0,97	0,48
6	1,94	0,98	1,46	0,96	0,96	1,00
7	1,06	1,50	0,49	1,94	1,00	0,98
8	0,96	1,46	0,99	2,43	1,00	0,49
9	0,96	0,97	1,00	0,99	0,99	0,50
10	0,99	1,49	1,44	1,45	0,98	0,49
11	1,00	1,50	1,02	1,48	0,98	0,98
12				1,47	1,44	0,49

APPENDIX 5

Two-tail P values for table 8. Cu content of the liver, kidney, heart and muscle

Experiment 1

GROUP	LIVER	KIDNEY	HEART	MUSCLE
1 vs 2	0,1147	0,7032	0,7512	0,2749
1 vs 3	0,0043	0,8602	0,0613	0,5089
2 vs 3	0,4690	0,5320	0,0760	0,0480

Experiment 2

GROUP	LIVER	KIDNEY	HEART	MUSCLE
1 vs 2	0,2975	0,7073	0,0012	0,0023
1 vs 3	0,3698	0,0732	0,3719	0,0003
2 vs 3	0,1748	0,1185	0,0079	0,0177

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

APPENDIX 6 : **TISSUE LEVELS OF Fe IN GROUPS FED VARYING LEVELS OF VITAMIN B-6 IN DIET**

1. **LIVER**

ANIMAL NO	GRP 1 3,5mg/kg PN	GRP 2 1.75mg/kg PN	GRP 3 0mg/kg PN	GRP 1 3,5mg/kg PN	GRP 2 Pair-fed control	GRP 3 0mg/kg PN
1	153,7	141,41	229,08	215,95	171,90	295,05
2	160,5	138,18	250,03	120,52	166,35	301,25
3	165,85	105,68	427,88	189,30	162,85	335,88
4	175,80	118,24	255,00	204,06	149,80	312,50
5	175,80	204,01	265,29	140,06	138,17	347,12
6	189,52	156,67	291,58	208,00	162,05	196,07
7	153,47	145,58	324,35	159,58	163,29	290,11
8	152,67	159,06	174,72	166,19	168,63	262,18
9	175,80	163,03	289,46	196,98	180,19	377,42
10	142,5	206,18	347,91	209,66	140,32	351,55
11	198,1	160,99	300,57	135,38	217,71	306,91
12				189,30	186,18	323,10

: **TISSUE LEVELS OF Fe IN GROUPS FED VARYING LEVELS OF VITAMIN B-6 IN DIET**

2. **KIDNEY**

ANIMAL NO	GRP 1 3,5mg/kg PN	GRP 2 1.75mg/kg PN	GRP 3 0mg/kg PN	GRP 1 3,5mg/kg PN	GRP 2 Pair-fed control	GRP 3 0mg/kg PN
1	76,21	63,92	65,58	51,49		60,38
2	62,14	84,62	62,00	51,80	81,18	62,25
3	65,97	76,40	83,50	61,53	83,66	53,80
4	58,91	61,41	83,96	59,66	129,24	66,70
5	68,85	70,62	74,14	65,74	133,89	47,67
6	59,00	57,35	69,61	46,21	66,70	67,40
7	75,30	68,90	67,87	48,71	71,57	69,17
8	74,62	83,17	65,02	54,66	66,09	58,51
9	71,70	68,53	65,61	57,32	50,30	56,64
10	83,33	65,97	62,71	60,29	77,00	75,34
11	63,37	77,11	62,48	52,53	88,43	75,34
12				56,26	88,43	55,83

APPENDIX 6 : **TISSUE LEVELS OF Fe IN GROUPS FED VARYING LEVELS OF VITAMIN B-6 IN DIET**

3. **HEART**

ANIMAL NO	GRP 1 3,5mg/kg PN	GRP 2 1,75mg/ kg PN	GRP 3 0mg/kg PN	GRP 1 3,5mg/kg PN	GRP 2 Pair-fed control	GRP 3 0,mg/kg PN
1	84,47	85,60		72,90	67,33	70,77
2	87,25	93,27	95,98	90,34	64,70	72,71
3	87,26	87,5	99,71	64,55	68,19	76,86
4	99,50	75,53	104,38	87,43	104,17	74,13
5	82,06	80,04	110,12	80,92	98,16	78,90
6	75,49	87,00	101,73	72,61	89,64	89,05
7	86,00	75,43	87,72	77,79	88,88	76,10
8	80,31	81,74	96,06	69,85	89,94	79,30
9	81,89	80,10	92,51	71,78	125,73	82,93
10	90,14	83,17	92,94	89,53	90,51	77,52
11	89,42	81,56	99,53	51,47	76,96	77,50
12				49,52	77,72	79,98

: **TISSUE LEVELS OF Fe IN GROUPS FED VARYING LEVELS OF VITAMIN B-6 IN DIET**

4. **MUSCLE**

ANIMAL NO	GRP 1 3.5mg/kg PN	GRP 2 1.75PNmg/ kg	GRP 3 0mg/kg PN	GRP 1 3,5mg/kg PN	GRP 2 Pair-fed control	GRP 3 0,mg/kg PN
1	21,56	20,14	7,71	14,00	13,01	9,50
2	19,15	13,5	9,90	15,14	15,56	9,31
3	19,96	10,58	10,23	15,29	17,00	9,48
4	18,00	21,86	9,50	13,43	16,30	8,69
5	16,66	18,13	6,35	13,26	17,86	11,00
6	18,93	18,62	10,25	13,46	13,46	11,50
7	14,85	16,00	10,38	14,65	14,5	12,69
8	16,35	19,42	11,36	15,05	16,5	11,30
9	14,93	16,02	10,50	12,82	11,34	12,38
10	15,93	22,32	10,50	19,75	14,74	11,70
11	15,47	15,00	9,50	15,25	12,23	12,52
12				14,22	13,46	13,62

APPENDIX 6

Two-tail P values for Table 9
Concentration of Fe in Liver, Kidney, Heart and Muscle

Experiment 1

GROUP	LIVER	KIDNEY	HEART	MUSCLE
1 vs 2	0,1999	0,5993	0,1783	0,7676
1 vs 3	0,0001	0,9738	0,0007	0,0001
2 vs 3	0,0001	0,5767	0,0003	0,0001

Experiment 2

GROUP	LIVER	KIDNEY	HEART	MUSCLE
1 vs 2	0,3555	0,0006	0,0735	0,8172
1 vs 3	0,0000	0,0376	0,3556	0,001*
2 vs 3	0,0001	0,0074	0,1842	0,0007

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

APPENDIX 7 : **TISSUE LEVELS OF Zn IN GROUPS FED VARYING LEVELS OF VITAMIN B-6 IN THE DIET**

1. LIVER

ANIMAL NO	GRP 1 3,5mg/kg PN	GRP 2 1,75mg/kg PN	GRP 3 0mg/kg PN	GRP 1 3,5mg/kg PN	GRP 2 Pair-fed Control	GRP 3 0mg/kg PN
1	33,56	32,82	36,35	27,72	32,42	31,98
2	33,00	35,16	35,00	32,51	31,43	34,95
3	31,50	32,78	27,88	33,37	35,86	32,92
4	33,58	31,37	34,00	29,50	33,89	32,42
5	31,80	31,80	35,78	30,59	33,30	28,37
6	28,57	30,62	30,83	28,00	33,94	30,27
7	33,17	31,12	32,44	29,64	30,46	30,17
8	34,59	37,45	38,64	31,13	33,33	31,68
9	32,35	32,16	29,44	30,16	32,46	30,10
10	35,00	32,10	31,31	33,86	33,60	28,01
11	31,73	33,33	28,62	30,63	29,35	30,27
12				31,19	29,27	30,27

APPENDIX 7 CONTINUED

TISSUE LEVELS IN GROUPS FED VARYING LEVELS OF VITAMIN B-6 IN DIET

2. KIDNEY

ANIMAL NO	GRP 1 3,5mg/kg PN	GRP 2 1,75mg/kg PN	GRP 3 0mg/kg PN	GRP 1 3,5mg/kg PN	GRP 2 Pair-fed Control	GRP 3 0mg/kg PN
1	36,41	47,80	32,64	25,25	19,83	25,95
2	34,42	33,56	32,50	24,95	22,41	26,19
3	33,94	32,88	32,50	24,71	19,57	18,70
4	32,57	43,52	31,97	24,65	25,15	33,96
5	28,22	32,01	31,37	29,07	22,73	17,51
6	32,00	29,90	30,39	29,67	21,59	23,90
7	30,52	49,21	28,05	17,82	25,15	24,21
8	29,94	29,21	31,50	19,47	25,38	22,73
9	32,55	29,92	29,41	29,87	21,53	35,16
10	27,51	30,26	34,82	24,23	22,42	33,59
11	27,45	27,51	31,49	18,97	21,83	29,75
12				21,53	24,87	24,70

APPENDIX 7 CONTINUED

TISSUE LEVELS OF Zn IN GROUPS FED VARYING LEVELS OF VITAMIN B-6 IN DIET

3. **HEART**

ANIMAL NO	GRP 1 3,5mg/kg PN	GRP 2 1,75mg/kg PN	GRP 3 0mg/kg PN	GRP 1 3,5mg/kg PN	GRP 2 Pair-fed Control	GRP 3 0mg/kg PN
1	12,91	15,56	28,54	16,63	19,65	19,34
2	18,27	19,23	29,09	17,21	16,63	17,89
3	13,21	13,00	23,59	18,30	17,65	18,70
4	19,01	17,69	27,98	27,01	18,35	17,92
5	23,58	23,07	25,94	15,59	18,92	18,73
6	25,49	15,00	28,75	17,61	18,93	20,09
7	21,00	20,07	20,83	15,41	19,04	19,60
8	12,83	18,93	11,45	15,63	20,79	18,15
9	24,56	17,72	20,81	15,17	19,83	21,31
10	27,89	30,88	45,70	21,57	19,57	18,24
11	22,60			17,79	19,61	18,77
12					18,90	

APPENDIX 7 CONTINUED
TISSUE LEVELS OF Zn IN GROUPS FED VARYING LEVELS OF VITAMIN B-6 DIET

4. MUSCLE

ANIMAL NO	GRP 1 3,5mg/kg PN	GRP 2 1.75mg/kg PN	GRP 3 0mg/kg PN	GRP 1 3,5mg/kg PN	GRP 2 Pair-fed control	GRP 3 0mg/kg PN
1	8,82	12,17	12,04	9,00	9,63	7,50
2	12,43	13,00	12,37	9,28	7,29	
3	11,47	13,94	12,10	8,86	12,00	9,48
4	9,50	9,42	10,00	10,99	8,89	7,72
5	9,30	12,74		9,82	6,75	9,17
6	7,28	11,19	14,64	5,76	7,21	12,00
7	7,92	12,00	7,90	9,72	8,00	13,67
8	11,06	11,12	10,86	10,00	11,00	10,86
9	7,92	12,39	8,00	6,00	8,21	7,90
10	6,97	11,91	13,14	14,93	9,82	9,26
11	5,84	8,50	11,02	9,84	7,33	7,71
12				9,80	8,65	9,24

APPENDIX 7

Two-tail P values for table 10
Zn content in liver, kidney, heart and muscle

Experiment 1

GROUP	LIVER	KIDNEY	HEART	MUSCLE
1 vs 2	0,6457	0,5544	0,5262	0,0581
1 vs 3	0,9215	0,8182	0,0486	0,0411
2 vs 3	0,8696	0,6694	0,0233	0,1809

Experiment 2

GROUP	LIVER	KIDNEY	HEART	MUSCLE
1 vs 2	0,0567	0,7507	0,289	0,2365
1 vs 3	0,7726	0,4884	0,3091	0,5796
2 vs 3	0,0603	0,1408	0,8174	0,3400

A significant difference exists between groups if the P value is less than 0,0167 ($\underline{0,05}$)
3

APPENDIX 8

1) PERCENTAGE OF Cu RECOVERED AFTER DIGESTION

ANIMAL	LIVER	KIDNEY	HEART	MUSCLE
1	100	113	95	96
2	100	100	93	94
3	100	108	93	92
4	96	100	96	103
5	94	90	92	93
6	106	110	100	100
7	94	90	107	94
8	96	96	98	94
9	91	98	92	97
10	103	111	103	96
11	107	106	97	95
12	101	102	90	106

2) PERCENTAGE OF Fe RECOVERED AFTER DIGESTION

ANIMAL	LIVER	KIDNEY	HEART	MUSCLE
1	99	90	98	91
2	111	100	96	110
3	92	93	99	97
4	100	108	97	106
5	98	99	100	91
6	93	96	103	110
7	112	104	106	113
8	100	100	91	91
9	98	103	95	95
10	111	93	101	107
11	110	110	108	111
12	97	90	91	105

RECOVERY TEST

3) PERCENTAGE OF Zn RECOVERED AFTER DIGESTION

ANIMAL	LIVER	KIDNEY	HEART	MUSCLE
1	91	113	89	90
2	93	91	91	97
3	90	97	112	100
4	103	109	112	96
5	90	110	100	96
6	94	102	108	90
7	112	106	106	93
8	100	90	91	100
9	96	89	89	92
10	91	100	96	93
11	92	93	90	93
12	96	94	90	93