VITAMIN B-6 DEFICIENCY : BIOCHEMICAL AND PHYSIOLOGICAL CHANGES WITH SPECIAL EMPHASIS ON ZINC STATUS

by

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Submitted in part fulfilment of the requirements for the degree of Doctor of Philosophy (Ph.D) in the Department of Human Physiology and Physiological Chemistry at the University of Durban-Westville.

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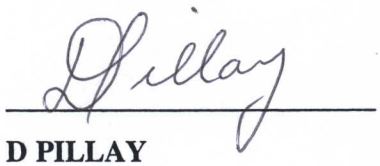
Date submitted : MAY 1999

DECLARATION

I, the undersigned, **DHANABAIKUM PILLAY**, declare that these studies represent original work by me and have not been submitted in any form to another university. Where use was made of the work of others, it has been duly acknowledged in the text.



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MAY 1999

ABSTRACT

Contradictory results regarding tissue and plasma zinc levels in vitamin B-6 deficient rats appear in the literature (52, 68, 72). These investigations have used either tissue or plasma zinc levels to assess zinc status during a vitamin B-6 deficiency. The conflicting reports, however, indicate the need for a more comprehensive approach in assessing the effect of vitamin B-6 deficiency on zinc status.

Zinc-metallothionein-1 (Zn-MT-1) is described as a rapidly turning over, metabolic buffer pool from which zinc is mobilised during a zinc deficiency. Erythrocyte Zn-MT-1 levels have been shown to be sensitive to dietary zinc intake and are reduced to nondetectabe levels in zinc deficient animals (31).

In the present study, the effect of vitamin B-6 deficiency on the zinc status of rats was investigated by determining erythrocyte zinc-metallothionein-1 levels and tissue-metallothionein levels together with plasma, tibia, kidney, testes and liver zinc concentrations. In addition, faecal and urinary zinc excretion as well as dietary zinc intake were monitored to establish zinc balance. To monitor other biochemical and physiological changes that may result from a vitamin B-6 deficiency, the food consumption, growth and physical condition of the experimental animals were recorded. In addition, blood pressure and the levels of pancreatic picolinic acid, serum erythropoietin, plasma insulin, blood glucose and plasma proteins were also measured.

In order to incorporate all these parameters, two sets of experiments were carried out. The first experiment had thirty-six male Wistar rats, divided into 4 groups of 9 rats each, viz. a control group (receiving 7 mg PN.HC1/kg diet), a vitamin B-6 deficient group (receiving 0 mg PN.HC1/kg diet), a vitamin B-6 deficient-picolinic acid (PA)-supplemented group (receiving 0 mg PN.HC1/kg diet supplemented with 0.2 g PA/kg diet) and a pair-fed group (receiving 7 mg PN.HC1/kg diet). The second experiment also consisted of four groups of nine rats each. The control, pair-fed and vitamin B-6 deficient groups received diets as in experiment 1. In addition, this experiment consisted of a partially deficient group receiving a diet containing 1 mg PN.HC1/kg diet.

Erythrocyte Zn-MT-1 levels were found to be significantly lower in the vitamin B-6 deficient and pair-fed groups compared with the control group ($p < 0.0004$). Since no significant difference in erythrocyte Zn-MT-1 levels existed between the pair-fed and deficient groups, the decreased levels were attributed to low caloric intake and a possible redistribution of erythrocyte zinc within the body.

A decrease in tissue and plasma zinc levels was observed in the vitamin B-6 deficient rats when compared with control and pair-fed rats. In addition, the vitamin B-6 deficient rats showed decreased kidney and pancreas metallothionein levels. A trend towards increased faecal and urinary zinc excretion was also noted in the vitamin B-6 deficient group compared to the pair-fed group. Since these two groups consumed the same amount of dietary zinc, these findings suggested a possible impairment of zinc absorption and a decrease in tissue zinc levels in the vitamin B-6 deficient group.

Vitamin B-6 deficiency was shown to reduce plasma protein levels (18). The decreased plasma albumin, globulin and total protein levels observed in this study support this finding. A protein deficiency would result in reduced zinc transport proteins and zinc-binding ligands, which in turn may impair zinc absorption and tissue zinc levels. Picolinic acid, a zinc binding ligand which requires vitamin B-6 for its synthesis was found to be significantly lower in the vitamin B-6 deficient group compared to the other groups. In addition, supplementation with picolinic acid was observed to increase tissue zinc levels in the tibia, testes and kidney of the vitamin B-6 deficient rats. These findings suggest that picolinic acid may play a role in the altered zinc status observed in the vitamin B-6 deficient group.

The results of the current study suggest that vitamin B-6 deficiency reduces body zinc status. The lower plasma protein levels observed in the vitamin B-6 deficient rats and the positive effect of picolinic acid supplementation on tissue zinc levels indicate that vitamin B-6 deficiency may exert its effect at the site of zinc absorption.

The low zinc levels in tissues such as bone, liver and erythrocytes of the vitamin B-6 deficient rats may imply that these tissues form part of a temporary storage pool for zinc and that under deficiency conditions, zinc may be mobilised to other priority tissues.

The above results indicate that the analysis of tissue, plasma, faecal, urinary and dietary zinc levels and plasma protein concentrations provides a reliable method for assessing zinc status in vitamin B-6 deficient rats, while erythrocyte Zn-MT-1 levels appear more suited to assessing zinc status brought about by a zinc deficient diet.

Vitamin B-6 deficiency was also observed to result in increased blood pressure, decreased plasma insulin levels and reduced serum erythropoietin levels, while the deficiency was found to produce no effect on blood glucose levels in rats.

Key words

Vitamin B-6
Zinc
Status
Metallothionein
Picolinic Acid

ACKNOWLEDGEMENTS

I wish to thank Professor P. Gathiram for his assistance and encouragement, and in particular, his many helpful comments and suggestions.

Many thanks are also due to the staff of the Department of Physiology and the BRC (U.D.W.) for their assistance and support during this study. I am also grateful to Mrs Florida Nel for her most efficient typing and to Suganya Chetty for her valuable assistance.

I must also express my gratitude to my parents for their support and encouragement and to Mani for her assistance and sense of humour when it was most needed, and in particular, I would like to say a very special thank you to Logesh for his (mostly) uncomplaining assistance and support above and beyond the call of duty.

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CHAPTER ONE

INTRODUCTION

Vitamin B-6 is an important member of the B-complex vitamins involved in a broad variety of biochemical reactions (160). A deficiency of vitamin B-6 would therefore be expected to have adverse consequences. Although the vitamin is widely distributed in most common foodstuffs (89), inadequate vitamin B-6 status has been reported in several studies (172). The vitamin B-6 status of these subjects was found to improve after receiving supplementation of the vitamin.

Another nutrient which has been widely researched is zinc. This trace element has long been recognised as essential for normal growth and physiological functions (136). Even though it is accepted that the body has an efficient mechanism for whole body zinc homeostasis, little is known about the mechanisms involved in tissue uptake of zinc. Even less is understood about the transport out of tissues into the plasma. Certain tissues, eg., the pancreas, secrete zinc as part of their normal physiological function. In other tissues, however, there is also a turnover of zinc which results in a loss of zinc from cells by mechanisms which are as yet unknown (75). Zinc homeostasis is maintained by manipulation of gastrointestinal absorption and gastrointestinal excretion of the element (75). The absorption appears to have an efficient homeostatic control (75). The control of its excretion has, however, not been fully established.

Various factors other than a low dietary zinc intake were shown to depress plasma zinc concentration. Low plasma zinc levels were reported during infection and other stresses (26). Interleukin-1 is believed to mediate the redistribution of plasma zinc to the liver in many stress conditions - the liver being the major organ for zinc metabolism (31). The net effect is the induction of metallothionein synthesis and a sequestration of some of the circulating zinc in the plasma (31). The zinc content in extracellular fluid pools and tissue also appears to be sensitive to changes in hormonal balance, particularly glucagon, insulin and glucocorticoids (31). Glucagon and glucocorticoids stimulate zinc uptake by the liver while infusion of insulin was shown to inhibit urinary excretion of zinc (31).

In addition, several dietary factors such as phytic acid, fibre and calcium may potentially inhibit the intestinal absorption of zinc (152).

Since both vitamin B-6 and zinc are known to participate in a wide variety of enzymatic systems, interactions between these two nutrients would be expected. Many symptoms of zinc deficiency and vitamin B-6 deficiency are known to be similar - these include decreased food consumption and growth, dermatological lesions and impaired immune function.

It has been proposed that vitamin B-6 deficiency may impair zinc absorption (46). A role for picolinic acid, a zinc-binding ligand, was suggested in zinc absorption (46). Picolinic acid, a tryptophan metabolite, requires vitamin B-6 for its synthesis. However, much controversy exists regarding the involvement of zinc-binding ligands, such as picolinic acid, in normal zinc absorption (70).

Various studies have shown that tissue levels of zinc or zinc uptake are directly related to vitamin B-6 intake in laboratory animals. Decreased zinc concentrations in plasma, liver, pancreas and muscle were reported in vitamin B-6 deficient rats (68). Other researchers, however, found that vitamin B-6 deficiency increased the zinc levels in various tissues (liver, pancreas, serum) (52). Investigators have also reported no change occurring in the zinc concentrations of the liver, pancreas, testes and lungs of vitamin B-6 deficient rats (63). These contradictory reports on the effects of vitamin B-6 deficiency on zinc levels suggest the need for an alternative approach in assessing body zinc status.

In vitamin B-6 studies, plasma zinc concentration is often used to monitor changes in body zinc levels (68). Plasma zinc levels are, however, known to be influenced by factors such as infection, disease, stress and steroid hormone administration (166).

More recently, metallothionein, an intracellular zinc-binding ligand, has been identified as the metabolic buffer pool for zinc (55). In addition, a key role for metallothionein in controlling zinc absorption has been proposed (146). Plasma and erythrocyte metallothionein levels were found to reflect dietary zinc intake (31). Furthermore, erythrocyte metallothionein is known to be less responsive to stress and infection than plasma metallothionein or plasma zinc (55). It has therefore been suggested that erythrocyte zinc-metallothionein-1 concentration may be a more reliable indicator of zinc deficiency or status (55).

The importance of analysing tissue and excretory zinc levels in assessing whole-body zinc cannot, however, be disregarded. Such analyses may provide useful information regarding zinc absorption, uptake and losses as well as zinc mobilisation from possible temporary zinc pools. Such information may be especially useful when an altered zinc status is caused by factors other than altered dietary zinc levels.

Although much work has been done to determine the association between vitamin B-6 and zinc in the body, many aspects of their relationship still remain unclear. It is known that a deficiency of vitamin B-6 alters the zinc levels in various tissues and body fluids and although some contradictory results have been found, it has been concluded that a deficiency of vitamin B-6 affects the body zinc status.

Previous studies involving vitamin B-6 deficiency and its effect on body zinc status have used either tissue or plasma zinc levels as an index (68, 52, 72). However, the current knowledge of zinc and its metabolism would bring into question the relevance of these findings regarding the status of zinc in the body. The use of tissue or plasma zinc levels as a criterion for determining changes in body zinc or zinc metabolism is increasingly criticised as inappropriate and inaccurate (149). The fact that a vitamin B-6 deficiency does in some way alter body zinc cannot be disputed. However, the most suitable method to assess the body zinc changes is of relevance. Furthermore, secondary factors which influence zinc absorption, utilisation and excretion also arise from vitamin B-6 deficiency, eg., decreased protein synthesis (149). These factors should also be monitored. In addition, the role, if any of picolinic acid in the resulting zinc changes has not been completely ascertained.

The primary aims of the present study are to provide more accurate information regarding zinc status during a vitamin B-6 deficiency and to determine the relevance of picolinic acid in vitamin B-6 deficiency and zinc status.

To achieve the above (within the confines of the available laboratory equipment and facilities) the following experimental procedures were chosen to assess zinc status :

1. Atomic absorption spectrophotometric measurement of zinc in various tissues and plasma.

2. A zinc balance study.

3. Erythrocyte-metallothionein-1 concentrations by radioimmunoassay.

4. Tissue metallothionein-1 concentration by the silver saturation method (179).

The vitamin B-6 status of all rats was determined at the end of the experimental period. In addition to growth and food consumption of the rats, other parameters on which vitamin B-6 deficiency may have an influence including blood pressure, serum erythropoietin, plasma insulin and plasma protein levels were also monitored in order to obtain a clearer insight into the general status of the rat during a vitamin B-6 deficiency.

To make an assessment of the involvement of picolinic acid in the above investigation, pancreatic picolinic acid concentrations were determined. In addition, picolinic acid was added to the diet of one group of vitamin B-6 deficient rats. This was to allow comparisons between vitamin B-6 deficient rats with and without picolinic acid supplementation, to enable the evaluation of whether picolinic acid alters zinc absorption or tissue zinc levels.

No studies have been reported which incorporated all the above parameters in investigating the zinc status during a vitamin B-6 deficiency. In addition, none of the work done on the involvement of picolinic acid in vitamin B-6 deficiency and zinc metabolism included measuring pancreatic PA levels in rats fed a vitamin B-6-free diet. It is hoped that the present study will be able to evaluate body zinc status during vitamin B-6 deficiency and lay to rest the argument on the probable involvement of picolinic acid in zinc absorption.

CHAPTER TWO

LITERATURE REVIEW

INTRODUCTION

Due to the nature of this study and the aim of the current investigations, the literature review which follows will concentrate on vitamin B-6 and zinc in general and the nature of their deficiency symptoms. The absorption of these two nutrients as well as their interaction with each other will also be discussed. An important aspect of the present investigation is the choice of method to determine possible deficiencies. The suitability of various methods that have been used will therefore also be reviewed.

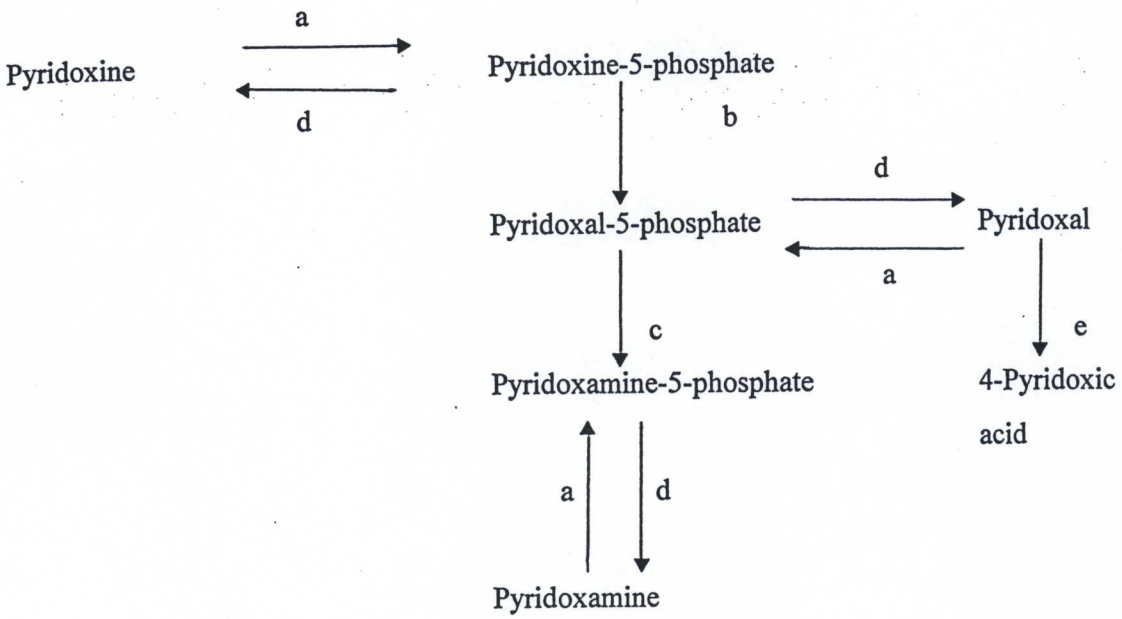
2.1 Vitamin B-6

2.1.1. Chemistry

Vitamin B-6 is the generic term for three closely related and interconvertible compounds, viz, pyridoxine, pyridoxal and pyridoxamine (6). These three forms have equal activity when administered parenterally to rats. The major excretory form of vitamin B-6 is 4-pyridoxic acid. The vitamin is metabolically active in humans as a coenzyme, mainly in the form of pyridoxal-5-phosphate and to a lesser extent as pyridoxamine-5-phosphate. The various forms of vitamin B-6 found in animal tissues are also interconvertible (40). These interconversions are summarized in Figure 1.

All forms of the vitamin are soluble in water, less soluble in alcohol and insoluble in ether. The various forms are also unstable in visible and ultraviolet light and are destroyed by heating (90).

The commonly available synthetic form of vitamin B-6 is pyridoxine hydrochloride (PN.HC1) which is odourless and easily crystallisable. Pyridoxine hydrochloride is the form of the vitamin typically used in nutrient supplements and animal diets.



The enzymes involved are :

a) Pyridoxal kinase

b) Pyridoxine-5-phosphate oxidase

c) Aminotransferase

d) Alkaline Phosphatase

e) Aldehyde oxidase

Figure 1: Interconversion of vitamin B-6 compounds in the liver (115).

2.1.2. Intestinal absorption of vitamin B-6

The hydrolysis of pyridoxal-5-phosphate (PLP) to pyridoxal (PL) and pyridoxamine-5-phosphate (PMP) to pyridoxine (PN) occurs in the intestinal lumen and is catalysed by intestinal phosphatases. Pyridoxal, pyridoxamine (PM) and pyridoxine are then absorbed (74). The intestinal absorption of PN has been investigated by several researchers in systems ranging from whole animals (12) to everted rat intestinal sacs (117). The general conclusions reached is that PN is absorbed by passive diffusion.

Absorption of the nonphosphorylated forms of vitamin B-6 was shown to be rapid (PL, 40%; PN, 23%; and PM, 18% in 10 minutes). It has been established that the transport of PN into mucosal tissue is proportional to the dose over 10 000 fold range of concentrations. This is a confirmation that PN transport occurs by passive diffusion (66).

Using a perfusion system, Mehansho *et al.* (1979), established that the absorption of PL is rapid and non-saturable (111). This observation was confirmed by Middleton (1984), who reported that PN, PL and PM cross the mucosal membrane by a non-saturable energy-independent process which appears to be passive diffusion (116).

2.1.3 Metabolism of vitamin B-6

While there is interconversion of the three forms of vitamin B-6 in the cells of the intestinal mucosa, the primary forms which leave these cells and are transported to the liver are, however, the forms which are initially absorbed (97). Uptake by liver cells occurs by facilitated diffusion (97). Although other tissues also contribute to vitamin B-6 metabolism, the liver is the primary organ for the interconversion and metabolism of the three forms of the vitamin (115). The metabolic steps involved are shown in Figure 1. The two metabolic steps which are common to all three forms are the phosphorylation step, in which a phosphate group is attached at 5-position in the presence of pyridoxal kinase, and the dephosphorylation step, in which the 5-phosphate group is removed, by the action of alkaline phosphatase (97). Pyridoxine-5-phosphate (PNP) and pyridoxamine-5-phosphate (PMP) are then converted to pyridoxal-5-phosphate (PLP) through the action of pyridoxine (pyridoxamine)-5-phosphate oxidase. This enzyme requires flavin mononucleotide (FMN) for its activation (104). Conversion of pyridoxal to 4-pyridoxic acid (4-PA) is catalysed by aldehyde oxidase. This reaction is irreversible and is one of the major routes for the metabolism of the excess vitamin B-6 consumed.

The PLP, formed in the liver but not utilized there, is released into the circulation (102). The liver is considered to be responsible for synthesis of the PLP found in the plasma (102). It has, however, been suggested that the muscle reservoir of PLP may serve as a source of PLP under conditions of caloric deficit (11). Pyridoxal-5-phosphate in the plasma is bound almost entirely to albumin (3). This binding protects the PLP from hydrolysis and permits delivery of PLP to other tissues. Although circulating PLP may not be the direct source of vitamin B-6 for many tissues, it accounts for 60-70% of the total vitamin B-6 present under conditions of normal vitamin B-6 intake (74). Pyridoxal is the next most abundant form and appears to be the circulating form used by most tissues (104).

Erythrocytes are active in vitamin B-6 metabolism and can rapidly convert plasma PN to PL. Since erythrocytes do not contain aldehyde oxidase and have only low aldehyde dehydrogenase activity, their conversion of PN to PL does not result in the wasteful formation of the dead-end catabolite, 4-pyridoxic acid (73). The PL concentration in erythrocytes is about 4 times greater than in the plasma (73).

It can therefore be seen that both the liver and erythrocytes play a critical role in processing pyridoxine before it can be utilized by certain organs and tissues which lack PMP oxidase activity. The liver metabolises the absorbed PN to PLP, PL and 4-PA which are then released into the circulation. Erythrocytes, however, convert PN to PL only.

2.1.4. Functions of vitamin B-6

Sauberlich (1985) reported that "over 100 enzymes are known to require vitamin B-6 as an activating co-enzyme". This illustrates the broad involvement of vitamin B-6 in the chemistry of the body (154). Pyridoxal-5-phosphate, the principal active form of vitamin B-6, readily forms a Schiff base with amino acids and other nitrogen-containing compounds and is known to participate in a wide range of enzymatic reactions, some of which are discussed below.

2.1.4.1. Transamination reactions

These reactions involve the interconversion of amino acids and their corresponding keto-acids. Pyridoxal-5-phosphate forms an essential part of the active site of transaminases. In the initial step of the reaction a PLP-enzyme complex is formed (90). The bound PLP reacts with an amino acid to yield the corresponding keto-acid and enzyme bound pyridoxamine phosphate. This reaction is reversible and the bound co-enzyme acts as an intermediate carrier of amino groups (40).

2.1.4.2 Desulfhydration and transulfuration reactions

Desulfhydration of cysteine and homocysteine requires the interactions of PLP. These amino acids are degraded to either pyruvate or 2-oxobutyrate + ammonia + hydrogen sulphide (40). The enzyme involved in the degradation of pyruvate is desulfurase, a PLP dependent enzyme. Pyridoxal-5-phosphate is required as a coenzyme in the metabolism of methionine from which cysteine is biosynthesised via the transulfuration reaction. The enzyme involved in this reaction is the PLP dependent transulfurase (40).

2.1.4.3 Nonoxidative decarboxylation reactions

These reactions also involve PLP as coenzyme. Decarboxylases are involved in the synthesis of gamma-aminobutyric acid (GABA), serotonin, histamine, epinephrine and norepinephrine.

The decarboxylation of glutamic acid to GABA in the brain requires the enzyme glutamate decarboxylase (PLP dependent). Gamma-aminobutyric acid is involved in the regulation of synaptic transmission in the nervous system (90). The degradation of GABA involves its transamination to succinic semialdehyde via the PLP dependent enzyme, GABA aminotransferase. The synthesis of serotonin involves the action of tryptophan decarboxylase on 5-hydroxytryptophan. Serotonin functions as a vasoconstrictor and as a neurotransmitter.

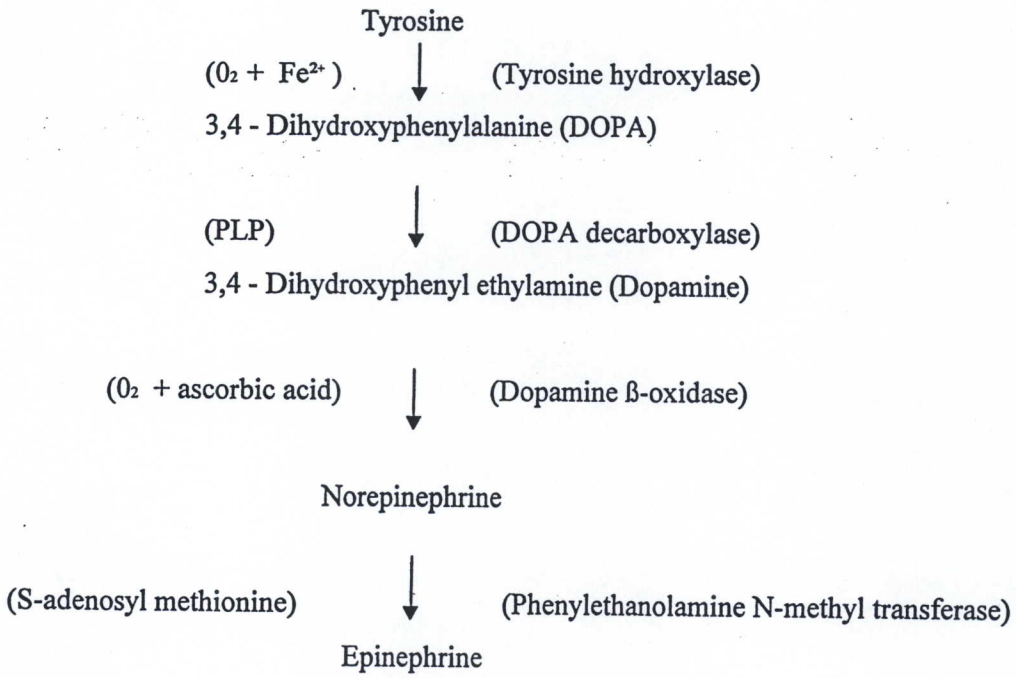


Figure 2: Role of vitamin B-6 in the synthesis of epinephrine and norepinephrine.

2.1.4.4 Tryptophan-niacin metabolism

The role of vitamin B-6 in the metabolism of tryptophan to niacin is one of the most extensively investigated vitamin B-6 - amino acid interrelationships (98). Only one step in the conversion of tryptophan to niacin requires PLP, viz. the kynureninase step. Kynureninase catalyses the conversion of 3-hydroxykynurenine to 3-hydroxyanthranilic acid. This conversion is illustrated in Figure 3.

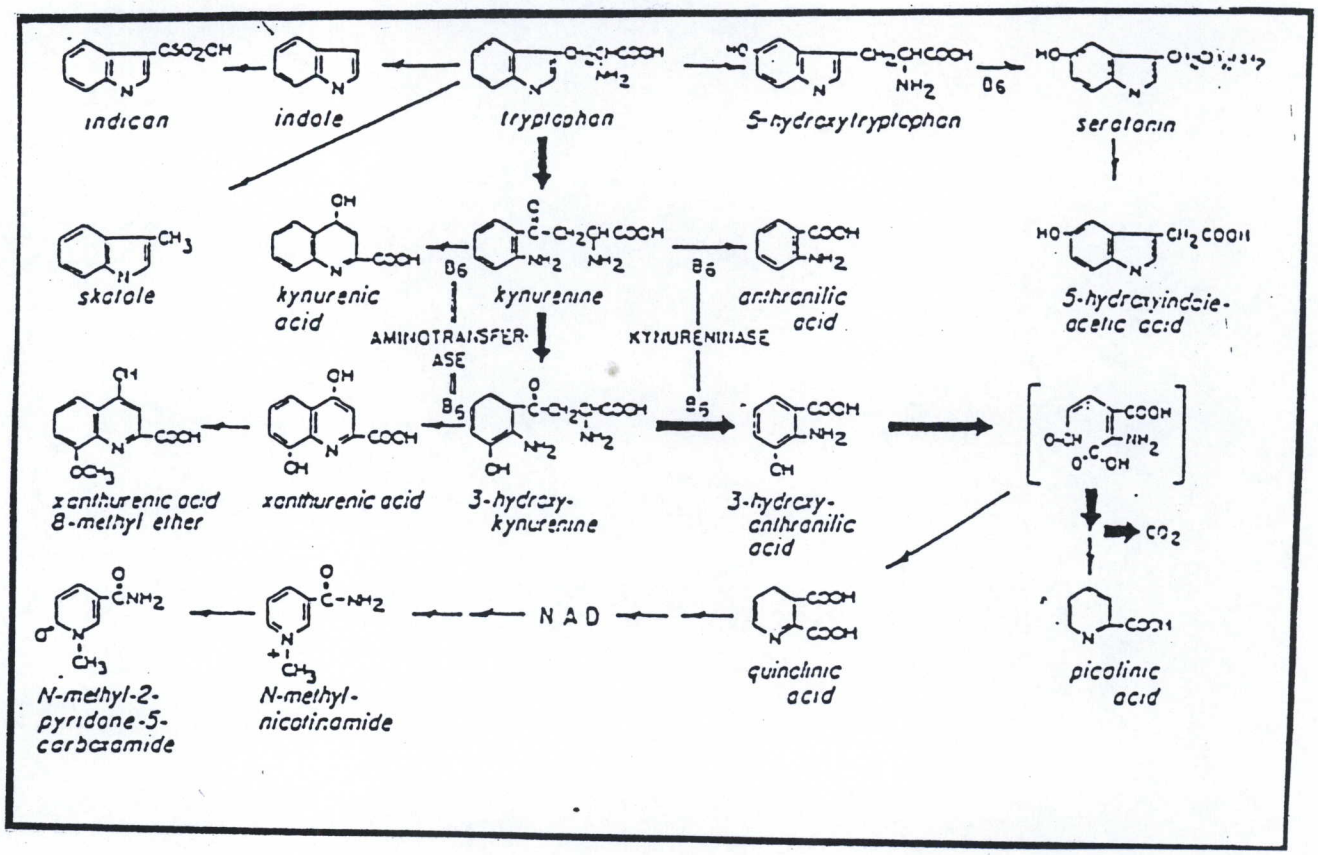


Figure 3: Tryptophan metabolism showing the sites of action of vitamin B-6. The large arrows indicate the pathway for degradation of the majority of ingested tryptophan (97).

2.1.4.5 Pyridoxal-5-phosphate and glycogen phosphorylase

Glycogen phosphorylase, a PLP dependent enzyme, initiates the catabolism of glycogen (90). It has been suggested that PLP is not a coenzyme for glycogen phosphorylase but is involved rather in altering the conformation of the enzyme (40). A deficiency of vitamin B-6 was shown to result in decreased activity of liver and muscle glycogen phosphorylase while an excess intake of vitamin B-6 resulted in increased total amount of glycogen phosphorylase and vitamin B-6 in the muscle (97). It was therefore concluded that muscle serves as a reservoir for vitamin B-6. However, the PLP in muscle, associated with glycogen phosphorylase, does not serve as a source of vitamin B-6 during a vitamin B-6 deficiency but does during a caloric deficit (97).

2.1.4.6 Steroid Function

Vitamin B-6 has recently been implicated in steroid function (142). Pyridoxal-5-phosphate has been utilized as an *in vitro* tool for the study of hormone receptors (142). At physiological concentrations of PLP, reversible reactions between target receptors and the respective hormones, oestrogen, androgen, progesterone and glucocorticoids occur (97). This interaction results in inhibition of the binding of the steroid-receptor complex to DNA. A deficiency of vitamin B-6 results in a greater retention of steroids in the nuclear fraction of target cells, thereby increasing the sensitivity of the end-target cell to the steroid (9).

2.1.4.7 Essential fatty acid metabolism

A role for vitamin B-6 in fatty acid metabolism has been recognised since the 1930's (110, 140). Vitamin B-6 deficiency was shown to reduce the amount of unsaturated essential fatty acids in tissues (110). Vitamin B-6 deficiency was reported to have an inhibitory effect on linoleic acid metabolism (153). Cunnane *et al.* (1985) observed that a vitamin B-6 deficiency in rats resulted in lower body weight, lower thymus and subcutaneous fat weights and higher liver weight (fatty liver) (36). On analysis of the fatty acid of phospholipids of plasma, liver, thymus and skin, they found the same defect in all of these tissues, viz., increased concentrations of linoleic acid and gamma-linolenic acid and decreased concentration of arachidonic acid. It was proposed that these effects were consistent with decreased desaturation of linoleic acid or decreased elongation of gamma-linolenic acid (Figure 4).

In addition, Cunnane *et al.* (1985) reported an increase in the total essential fatty acid content in liver triglyceride of vitamin B-6 deficient rats (36). They suggested that vitamin B-6 may also have a role in fatty acid mobilization from triglyceride to phospholipid. Since vitamin B-6 was found to result in abnormal metabolism of linoleic acid and gamma-linolenic acid, they concluded that vitamin B-6 is indispensable for efficient utilization of dietary essential fatty acids.

Since cholesterol metabolism is dependent on essential fatty acid availability, especially arachidonic acid, a deficiency of vitamin B-6 may also affect cholesterol metabolism.

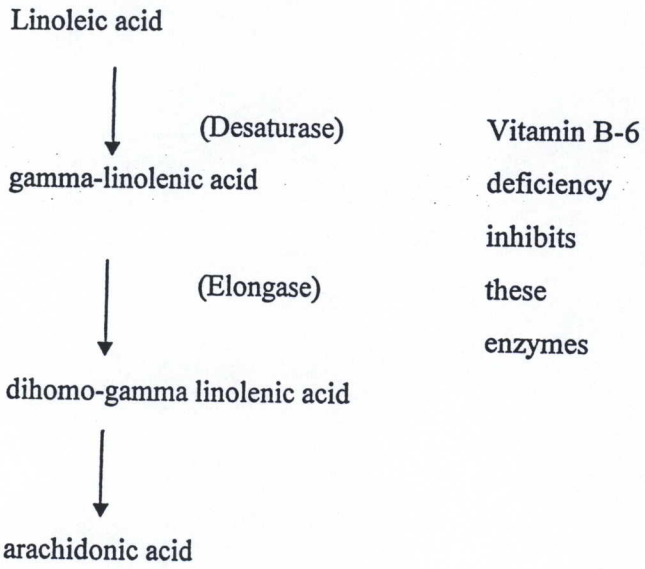


Figure 4: Inhibitory effect of vitamin B-6 deficiency on linoleic acid metabolism (36).

The above shows that vitamin B-6 is involved in a wide variety of biochemical reactions. The involvement of vitamin B-6 in protein, carbohydrate and lipid metabolism as well as in steroid hormone action, erythrocyte function, immune functions and the nervous system illustrates the diverse functions of this vitamin.

2.1.5 Vitamin B-6 deficiency and associated disease states

Vitamin B-6 is seen as one of the "problem" vitamins in nutritional surveys. Studies show that most children and adolescents do not have adequate amounts of vitamin B-6 in their diets to meet the Recommended Dietary Allowance (RDA). Similarly, breast-fed infants do not meet their RDA for vitamin B-6 without considerable maternal supplementation. Overt clinical vitamin B-6 deficiency is rare in developed countries. However, biochemical evidence of inadequate vitamin B-6 status has been revealed in approximately 25% of individuals in well-nourished populations (65).

2.1.5.1 Deficiency symptoms

Animals

In the rat and mouse a symmetrical scaling dermatitis resembling that of essential fatty acid deficiency is usually observed on the tail, paws, nose, chin and upper thorax during a vitamin B-6 deficient state (150). In addition, vitamin B-6 deficiency causes poor growth (eventually weight loss) and muscular weakness (170), retarded mental ability, anaemia, impaired immune responses, oedema (150) and hyperirritability (160, 180).

Liver sterol esters and phospholipids are altered in deficient rats (40). A change in plasma cholesterol levels also occurs. However, investigators do not agree on whether plasma cholesterol levels are increased, unchanged or decreased in vitamin B-6 deficient rats. Insulin insufficiency has also been reported (40). In addition, vitamin B-6 deficiency causes nerve degeneration resulting in convulsive seizures and neuronal dysfunction. Reproductive performance of both male and female rats is decreased in the deficiency state (150).

Similar symptoms occur in hamsters, dogs, chicks, swine, calves, turkeys, monkeys and rabbits (150).

Humans

Deficiency of vitamin B-6 in adult humans results in seborrhoeic dermatitis, hypochromic microcytic anaemia, nausea, vomiting, hyperirritability, depression, loss of weight and lesions of the mucous membranes (40, 150, 160).

In addition, vitamin B-6 deficiency results in abnormal tryptophan and methionine metabolism (133, 161). Elevated urinary excretion of both tryptophan and methionine metabolites has been reported to occur during vitamin B-6 deficiency (133, 161). Serum and erythrocyte aspartate aminotransferase and alanine aminotransferase activities are reduced (150) and increased serum and decreased urinary levels of free amino acids also occur (22).

In pregnant women, vitamin B-6 deficiency results in lethargy, fatigue, mental depression and impaired metabolism (160, 182). Vitamin B-6 deficiency in infants results in growth retardation (150), hyperirritability (183), gastro-intestinal distress (183), convulsive seizures (150) and hypochromic microcytic anaemia (90, 150).

The prototypical pyridoxine responsive anaemia was described as being hypochromic and microcytic with elevated serum iron (91). Most cases of anaemia have abnormalities in iron metabolism indicative of a genetically determined error of metabolism which increases the dependence of erythropoiesis on vitamin B-6. It was suggested that the block in haeme synthesis in vitamin B-6 deficiency occurs at an early stage in the synthetic pathway, viz., during the formation of succinyl-CoA and in the decarboxylation of α-amino-β-keto-adipic acid (Figure 5).

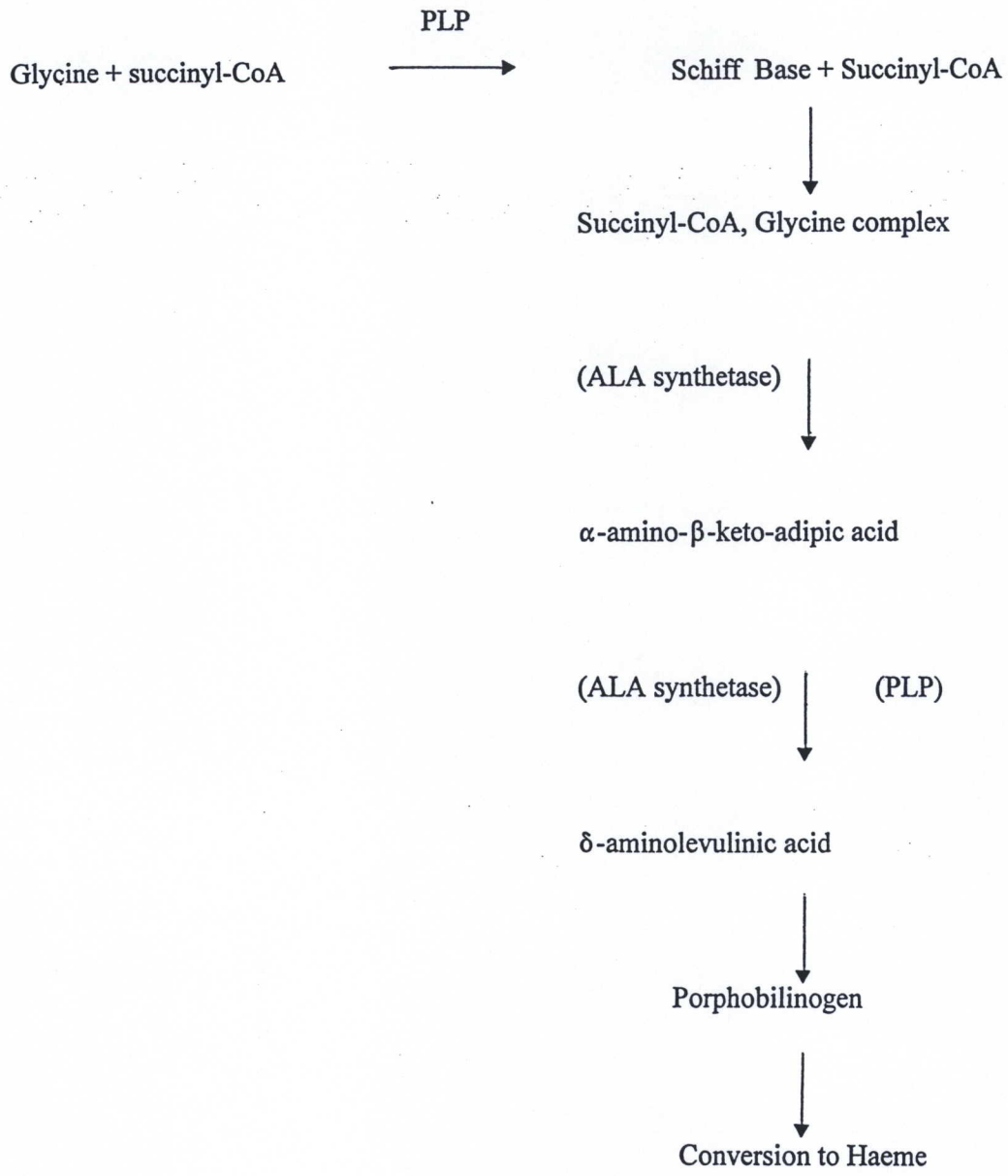


Figure 5: The role of vitamin B-6 in haeme synthesis (91).

Hypovitaminosis is not common in respect of vitamin B-6 since vitamin B-6 is found in abundance in a variety of foods. Deficiencies do, however, occur in patients taking drugs which are known to antagonize vitamin B-6. Some of the antagonists include penicillamine, hydralazine, L-dopa, cycloserine and deoxypyridoxine (160).

2.1.5.2 Impaired vitamin B-6 levels in disease states

Since vitamin B-6 plays a vital role in intermediate metabolism, certain clinical disorders, discussed below, could produce a deficiency of the vitamin.

2.1.5.2.1 Liver disease

A high incidence of vitamin B-6 deficiency has been observed in patients with chronic liver disease (89). This was also found to occur in patients with acute viral hepatitis and extra hepatic obstructions (120). Elevation of plasma PLP occurred in patients with chronic liver disease when supplemented with pyridoxine hydrochloride or PLP. Excretion of 4-pyridoxic acid was also elevated during supplementation (91). The reduced plasma PLP levels in chronic liver disease have been attributed to an increased clearance of PLP from plasma rather than to a reduced synthesis of PLP by the damaged liver (89). It was postulated that the increased PLP clearance may be due to enzymatic degradation in the liver or by dephosphorylation of PLP in the plasma resulting from increased levels of alkaline phosphatase (4).

2.1.5.2.2 Arteriosclerosis

Decreased plasma PLP levels were reported in patients with acute myocardial infarction (84), indicating that the overall vitamin B-6 status may be impaired in these patients. The vitamin B-6 dependent enzyme, cystathionine ß-synthase, is involved in the conversion of methionine to cystathionine and a reduced activity of this enzyme results in accumulation of homocysteine (108). Homocysteinaemia was shown to produce vascular lesions in animals and humans with arteriosclerosis (91). More recent investigations have shown that increased intake of vitamin B-6 is associated with decreased risk of myocardial infarction (20, 179). This association between vitamin B-6 and the risk of myocardial infarction was found to be independent of plasma homocysteine levels. It was concluded that protective mechanisms of vitamin B-6, other than its role in homocysteine metabolism, may be more important in the study of coronary disease. (179).

2.1.5.2.3 Renal disease

A vitamin B-6 deficiency has been reported in patients with chronic renal failure and in patients undergoing haemodialysis (85). This deficiency is due to an enhanced breakdown of PLP in plasma and can be corrected by supplementation with pyridoxine hydrochloride (85).

2.1.5.3 Vitamin B-6 status during pregnancy and lactation

Plasma PLP levels in pregnant and lactating rats were found to be greatly depressed when compared to the concentration in virgin female rats. (87) These levels were not elevated by adding a high excess of PN to the diet. It was suggested that pregnant and lactating rats have diminished abilities to absorb PN or to synthesize PLP from dietary pyridoxine. Another explanation was that PLP in plasma is enzymatically destroyed at a greater rate during pregnancy. (87).

Pregnant women have significantly lower plasma PLP levels than non-pregnant women (159). They also have elevated excretions of tryptophan metabolites (91). Oestrogen was reported to increase the activity of tryptophan-oxygenase, resulting in an increased turnover of L-tryptophan and thus increasing the need for vitamin B-6 (14).

Plasma PLP levels in pre-eclamptic women are close to the lower limit of the acceptable range observed in normal pregnant women. Pre-eclamptic placentae have sub-normal levels of pyridoxal kinase, pyridoxine phosphate oxidase and vitamin B-6 vitamers (91). This would suggest that vitamin B-6 metabolism in these placentae is abnormal.

The abnormal tryptophan metabolism found in pregnant women results in the excretion of 3-hydroxykynurenine, xanthurenic acid and kynurenic acid. Xanthurenic acid exerts a diabetogenic effect in animals by forming a stable complex with insulin and thus acts as an insulin antagonist (124). It has been postulated that the increased plasma levels of xanthurenic acid occurring during pregnancy may be responsible for gestational diabetes (91).

2.1.5.4 The effect of vitamin B-6 deficiency on blood pressure

Various studies have indicated that lowered vitamin B-6 status is associated with hypertension in rats (92, 134). Paulose *et al.* (1988) reported that pyridoxine deficiency in rats caused true arterial hypertension which could be completely reversed within 24 hours by pyridoxine treatment (134). It was concluded that since reversal occurred within such a short period of time, vessel wall damage could be excluded as the primary cause of the hypertension. A primary renal cause of hypertensions was also excluded in these animals. Since pyridoxine deficiency results in slow growth and suppressed appetite in rats, the possibility that malnutrition may contribute to the development of hypertension was investigated. It was found that rats subjected to generalised malnutrition had significantly lower blood

pressure compared with controls and pyridoxine-deficient rats (134). It was therefore concluded that the hypertension seen in pyridoxine-deficient rats was not a consequence of generalised malnutrition in these rats (134).

Paulose *et al.* (1988) also investigated the possibility that the reversible hypertension seen in the pyridoxine-deficient rats may be related to general sympathetic stimulation (134). They found that treatment of pyridoxine-deficient rats with a single dose of pyridoxine (10 mg/kg body weight) not only reversed the blood pressure to normal levels within 24 hours, but also restored hypothalamic serotonin and gamma-aminobutyric acid as well as plasma norepinephrine and epinephrine to normal levels. It was concluded that the results indicated an association between pyridoxine deficiency and sympathetic stimulation leading to hypertension (134).

Dietary calcium intake plays an important role in the regulation of blood pressure by regulating the concentration of cytoplasmic calcium. A high dietary calcium level reduces blood pressure and a low calcium intake has been shown to cause hypertension. It has been suggested that reduced dietary calcium depletes calcium from membrane storage sites, causing a less stable membrane of the vascular smooth muscle which results in enhanced calcium influx, increased tone and reactivity (92).

On investigating the relationship between calcium channel function and vitamin B-6 status, Lal and Dakshinamurti (1993) reported that when caudal artery segments from vitamin B-6-deficient hypertensive (B-6 DHT) rats were incubated, *in vitro,* with $^{45}Ca^{2+}$, calcium uptake was significantly increased compared with in tissues from control rats fed a vitamin B-6-sufficient diet (93). Their study revealed that the administration of calcium antagonists to conscious B-6 DHT rats was effective in lowering the systolic blood pressure (SBP). It was also noted that blood pressure was altered by subtle changes in vitamin B-6 intake and status and that this effect on SBP was limited to the functional state of the calcium channels. (93).

In a more recent study, Lal and Dakshinamurti (1995) reported that increasing the concentration of vitamin B-6 in diets containing normal calcium concentration resulted in reduced blood pressure in these rats (92). It was also noted that an increased concentration of vitamin B-6 in the diet attenuated the blood pressure-increasing effect of low dietary calcium. It was suggested that vitamin B-6 may correct the membrane abnormality leading to calcium influx by a mechanism similar to that of calcium antagonists. Lal and Dakshinamurti (1995) concluded that dietary vitamin B-6 deficiency and low dietary calcium seem to share the mechanisms responsible for increasing systolic blood pressure (92).

A recent study by Lal *et al.* (1996) has led to the suggestion that animal models of hypertension can be classified on the basis of their response to a vitamin B-6 supplement (94). These workers observed that the addition of a vitamin B-6 supplement to the diets of male Zucker obese rats made hypertensive, resulted in a complete attenuation of the hypertension and the removal of the supplement in these rats resulted in the return of the hypertensive state in two weeks. Similar changes in systolic blood pressure were noted in Zucker lean controls treated with vitamin B-6. The ingestion of sucrose by male Sprague-Dawley rats was found to cause modest elevation of systolic blood pressure which was attenuated by the addition of the vitamin B-6 supplement to their diets. However, there was no such response to the addition or removal of dietary vitamin B-6 supplement in the spontaneously hypertensive rats (SHRs). It was suggested that the etiology of hypertension in SHRs is quite distinct from that in Zucker obese rats and in rats ingesting sucrose (94).

In the present study, the blood pressure of rats fed a vitamin B-6 free diet, a partially deficient vitamin B-6 diet (1 mg PN.HCl/kg diet) and control diets was monitored.

2.1.6 Nutritional requirements

All species of animals studied to date require vitamin B-6. The rat is most often used as an animal model in research into human nutrition.

Variations in the composition of the diet, as well as genetic and environmental factors, add to the difficulty of determining the nutritional requirement for vitamin B-6 in animals, including humans. The quantity of protein in the diet affects the requirement for vitamin B-6 in humans and animals. As the protein level of the diet increases, so does the requirement for vitamin B-6 (58).

The relationship between vitamin B-6 requirement and protein intake is thought to result from the increased activities of pyridoxal-5-phosphate-dependent enzymes which catabolize excess amino acids when dietary protein intake is high (61).

It has also been established that women have lower plasma PLP concentrations than men with similar dietary vitamin B-6 to protein ratios (143). In addition, it has been found that compared with men, women subjects excreted a greater percentage of vitamin B-6 intake as 4-PA and excreted greater amounts of postload urinary tryptophan metabolites at three levels (0.5, 1.0 and 2.0 g protein/kg body weight) of protein intake. Women were also found to exhibit abnormal tryptophan metabolism on the high protein diet (2.0 g protein/kg body weight) and men did not. It was concluded that increased protein intake had a greater effect on the vitamin B-6 status of women than on the status of men (61).

It is therefore clear that in establishing dietary vitamin B-6 requirements, the dietary protein intake must be considered.

2.1.6.1 For animals

0Information concerning enzymatic activities, weight gain, reproductive performance and tissue PLP content has been used by researchers to establish the vitamin B-6 requirement of the laboratory rat. The Committee on Animal Nutrition of the National Research Council (NRC) in 1962 concluded that 12 μg of pyridoxine hydrochloride (PN.HC1) per day was adequate to meet the requirements of growing, pregnant or lactating rats. Beaton and Cheney (1965) reported that for maximal weight gain, the

requirement was between 40 and 80 μg PN.HCl per day (8). For maximal erythrocyte alanine aminotransferase activity and glutamic pyruvase enzyme activity, the requirements are 40 to 80 μg per day and 80 μg or more per day, respectively. It has also been suggested that rat diets should provide about 100 μg PN.HCl per day to ensure the adequacy of the intake.

The American Institute of Nutrition (AIN) Ad Hoc Committee on standards for nutritional studies recommended 7mg PN.HCl/kg diet as the requirement for optimal growth and development for rats and mice (141). The vitamin B-6 requirement for rats given by the NRC in 1978 for growth, gestation and lactation was 6 mg/kg diet (141). The NRC has also established the vitamin B-6 requirements, based upon growth data, of other animals including rabbits, pigs, dogs, fish, poultry, cats and hamsters.

2.1.6.2 **For humans**

The recommended dietary allowance (RDA) for adult males is 2.2 mg vitamin B-6 daily and for females older than 15 years, 2 mg/day (40, 150). As the recommended daily allowances are related to the protein intake, the recommended optimal ratio was ascertained to be 0.02 mg vitamin B-6 per gram protein (90, 61).

During pregnancy and lactation, the requirement for vitamin B-6 increases (103). The recommended allowance for pregnant women is 2.6 mg/day. It has also been suggested that during pregnancy vitamin B-6 intake should be increased to between 5.5 - 7.6 mg daily, the quantity needed to raise blood PLP values to those of normal non-pregnant women (157).

The requirement for vitamin B-6 increases in breast-fed infants, women on oral contraceptives, the elderly and in individuals using various types of drugs, including alcohol (53, 40, 74, 148, 78, 131).

2.17. Assessment of vitamin B-6 nutritional status

Clinical symptoms accompanying vitamin B-6 insufficiency (dermatitis, anaemia, mucous membrane lesions) are non-specific and therefore unreliable indicators of vitamin B-6 status.

Many procedures for evaluating vitamin B-6 status of humans and other animals have been proposed. However, investigators do not agree as to which parameter is most suitable for the determination of vitamin B-6 status. Commonly used biochemical indices have included direct measurements of the vitamer forms of vitamin B-6 and indirect functional tests measuring the activity of vitamin B-6-dependent enzymes. The various techniques used are discussed below.

2.1.7.1 Tryptophan load test

The increased urinary excretion of xanthurenic acid following tryptophan loading is the basis of a common test for vitamin B-6 deficiency. This test has been used in a wide variety of animals, including humans (118). Vitamin B-6 is an essential requirement for the conversion of tryptophan to nicotinic acid (niacin). Pyridoxal-5-phosphate functions as a coenzyme for kynureninase and kynurenine aminotransferase. Kynureninase is affected more by a vitamin B-6 deficiency than is the aminotransferase; therefore, xanthurenic acid is excreted in larger quantities than are the other tryptophan metabolites. Early and more severe stages of vitamin B-6 deficiency, however, cannot be accurately diagnosed using the tryptophan load test. Healthy pregnant women excrete higher than normal levels of xanthurenic acid. This is also the case in riboflavin-deficient individuals. This technique is, nevertheless, still widely used in population surveys.

2.1.7.2 Erythrocyte alanine aminotransferase

The measurement of alanine aminotransferase (ALT) activity in erythrocytes is another method used to determine vitamin B-6 nutritional status in animals. Alanine aminotransferase activity has been reported to decrease in erythrocytes, leucocytes and serum of vitamin B-6 deficient individuals. This method is considered to be as sensitive as and more advantageous than the tryptophan load test in the assessment of long-term vitamin B-6 status (87).

2.1.7.3 Urinary 4-pyridoxic acid excretion

The determination of 4-pyridoxic acid excretion is also used to monitor vitamin B-6 status. Fluorometric and HPLC techniques are used for its assay. Between 20% and 50% of dietary vitamin B-6 is excreted as pyridoxic acid. The measurement of pyridoxic acid excretion is, however, indicative of immediate dietary intakes of vitamin B-6 and not of body reserves. In addition, accurate 24 hour urine collections are difficult to obtain (87).

2.1.7.4 Plasma PLP concentration

Plasma PLP is the circulating store form of vitamin B-6. It therefore represents a direct and versatile indicator of vitamin B-6 status of animals. This method involves the direct measurement of the active coenzyme and is reflective of tissue levels (87).

In contrast to urinary vitamin B-6 excretion, fasting plasma PLP levels have been shown to correlate well with the nutritional status of an individual (105). Plasma PLP levels were found to rise with increasing pyridoxine intake. The PLP concentration in plasma correlates well with the PLP content of skeletal muscle which is the major storage pool of vitamin B-6 compounds in rats (105). Pyridoxal-5-phosphate analysis therefore provides an accurate and sensitive means of assessing vitamin B-6 status.

Ubbink *et al.* (1986) optimised a high performance liquid chromatography (HPLC) technique, which is rapid, specific, accurate and sensitive, for the determination of plasma PLP and PL (175). This method was chosen to assess the vitamin B-6 status of the rats in the present study.

2.1.8 Interaction of vitamin B-6 with trace elements

The metabolic participation of vitamin B-6 represents a versatility probably not matched by any other coenzyme. Pyridoxal-5-phosphate is required by over one hundred enzymes as an activating coenzyme. It is therefore not surprising that diverse interactions exist between vitamin B-6 and other nutrients which play important roles in nutrition.

For the purpose of this study, the interrelationship of vitamin B-6 with zinc in particular will be discussed in detail.

Trace elements act as catalysts in a wide range of enzyme systems. They carry out their functions through substrate activation or by specific binding to the enzyme itself (49). Since vitamin B-6 is also extensively involved as a catalyst or co-factor in various metabolic pathways, interactions between vitamin B-6 and trace elements in common metabolic pathways would be expected.

Numerous studies have been done to determine the effect of vitamin B-6 deficiency on various trace elements.

2.1.8.1 The effect of dietary vitamin B-6 deficiency on tissue zinc levels

Much controversy exists about vitamin B-6 deficiency and tissue zinc levels.

Hsu (1965) reported a significant decrease in zinc content of plasma, liver, pancreas and cardiac muscle of vitamin B-6 deficient rats when compared with control rats (68). No substantial differences in brain, kidney and spleen zinc concentrations were, however, observed between the deficient and control rats by this researcher.

Gershoff (1967) observed that vitamin B-6 deficiency in rats resulted in increased levels of zinc in the pancreas, serum and kidney (52). These results were not in agreement with those obtained by Hsu in 1965 (68).

The experimental protocol employed by Gershoff (1967) was, however, different from that used by Hsu (1965). Hsu's control rats were maintained on a diet containing 20 mg PN.HC1/kg for six weeks whereas the control rats in Gershoff's study were fed a diet containing only 4 mg PN.HC1/kg for twenty three days. These variations may have contributed to the different results observed.

Hsu (1965) injected radioactive zinc into vitamin B-6 deficient and control animals. He observed increased radioactive zinc concentration in plasma, liver, gastrointestinal tract, pancreas and testes of the vitamin B-6 deficient animals when compared with controls (68). He concluded that "since vitamin B-6 deficient rats exhibited a decrease in zinc content in certain tissues, an increased uptake of administered zinc would be expected". This observation was confirmed when zinc deficient rats injected with radioactive zinc were shown to retain more zinc in their tissues than those receiving zinc supplemented diets (68). The latter finding suggests an impaired zinc absorption at the level of the gastrointestinal tract rather than at the tissue uptake level in zinc deficient rats.

In the experiment performed by Gershoff (1967), half the rats in each group, i.e. the control and vitamin B-6 deficient groups, were fasted during the final 24 hours of the experimental period. Among the non-fasted (fed) animals it was noted that the vitamin B-6 deficient rats showed an increase in tissue zinc levels, particularly in the pancreas where the zinc levels were over a hundred percent greater than those of the non-fasted controls. It was also observed that when control rats were pair-fed or fasted for 24 hours, a rise in zinc levels in the pancreas, serum and liver occurred which was particularly significant in the fasted rats. The effect of fasting on tissue zinc levels was found to be significant in vitamin B-6 deficiency. Gershoff maintained that these observations would explain the results obtained by Hsu (1965) who pair-fed his rats and then fasted them overnight before determining the tissue zinc levels. It was concluded that Hsu's observation on the decreased tissue zinc levels of pyridoxine deficient rats was to a large extent a reflection of the high tissue levels of zinc in his fasted control rats (52).

Ikeda *et al.* (1979) reported no significant difference in the zinc content of the liver, spleen, pancreas, lung or testes between vitamin B-6 deficient and control rats (72). This is in agreement with the more recent findings of Mackraj *et al.* (1994) who also found no significant differences in the tissue zinc levels of vitamin B-6 deficient rats (107). Ikeda *et al.* (1979) however, did observe a significant increase in kidney zinc levels of the vitamin B-6 deficient rats (72). This is in keeping with the findings of Gershoff (1967) but does not agree with the results obtained by Hsu (1965).

The above observations indicate that a vitamin B-6 deficiency probably impacts on the zinc status of animals.

Possible factors that could explain the different results obtained are :

1. The composition of the diet. The PN.HC1 content of the control diet determines the vitamin B-6 status of control rats. The PN.HC1 requirement for rats and mice as set out by the Committee on Animal Nutrition, National Research Council (125) is 7 mg PN.HC1/kg diet. The vitamin B-6 levels in the control diets used by Hsu in 1965 (20 mg PN.HC1/kg) and Ikeda *et al.* in 1978 (12 mg PN.HC1/kg) were therefore in excess of the recommended levels, whereas the concentration in the control diet used by Gershoff in 1967 (4 mg PN.HC1/kg) was less than the required concentration for optimum vitamin B-6 status. This suggests the possibility that his control rats were vitamin B-6 deficient, which could have influenced his results.

2. The duration of the study. This may contribute to the severity of vitamin B-6 deficiency in the vitamin B-6 deficient rats and therefore also to tissue trace element levels.

3. Inter-individual and interspecies variation may also explain some of the different results obtained.

2.1.8.2 The effect of vitamin B-6 deficiency on zinc absorption/metabolism : influence of picolinic acid

Prasad *et al.* (1982) reported a significant increase in the absorption of zinc from the diet of vitamin B-6 deficient rats when compared with control and pair-fed rats. It was postulated that a lack of vitamin B-6 may lead to an impairment of nucleic acid synthesis. This would result in a subsequent inhibition of protein synthesis and cell division and repair. Since vitamin B-6, as a co-enzyme, catalyses a variety of enzymatic reactions involved with nitrogen metabolism, it has been postulated that in a deficiency state a marked alteration in the brush border membrane could arise, which may result in an increase in zinc uptake. This would explain the increased zinc content in various tissues of vitamin B-6 deficient rats as observed by Gershoff (1967). It may be argued, however, that such an alteration in the brush border should result in an elevation of all other metals as well. Prasad *et al.* (1982) also measured the uptake of calcium and cadmium and observed an increased uptake in vitamin B-6 deficient rats compared with control and pair-fed animals (138). The percentage increase, however, varied, suggesting a non-specific and general increase.

The lack of specificity was attributed to the fact that all three metals (Ca, Cd and Zn) have distinct modes of transport across the brush border (138). Cadmium follows a passive diffusion process while zinc absorption involves a passive diffusion and a carrier-mediated facilitative diffusion component (138). Calcium absorption, on the other hand, involves an active carrier-dependent mechanism and a second mechanism which shows linearity with the concentration of the metal ions (138).

In contrast to the above findings Evans and Johnson (1981) observed that vitamin B-6 deficiency resulted in a decreased dietary zinc absorption (46). This was attributed to a decreased production of picolinic acid (PA).

Using a technique known as modified gel filtration chromatography, Evans *et al.* (1979) had obtained evidence which suggested that the dominant zinc-binding ligand in the intestinal lumen and enterocytes is picolinic acid (pyridine-2-carboxylic acid), a product of tryptophan metabolism (44). Pyridoxal is required as a co-factor by the enzyme kynureninase in the pathway from tryptophan to picolinic acid (Figure 6). It was therefore suggested that if endogenous PA facilitates dietary zinc absorption, a deficiency of either vitamin B-6 or tryptophan would impair zinc absorption (44). Evans and Johnson (1980) subsequently reported that both zinc absorption and pancreatic PA concentrations were increased as the level of vitamin B-6 was increased (47). It was further noted that supplementation with PA ameliorated the impaired zinc absorption resulting from a tryptophan or vitamin B-6 deficiency. It was concluded that the absorption of zinc is affected by levels of both dietary tryptophan and vitamin B-6 and because PA is a metabolic product of tryptophan and depends on vitamin B-6 for its production, these results provide strong evidence that endogenous PA is essential for normal zinc absorption (42, 47).

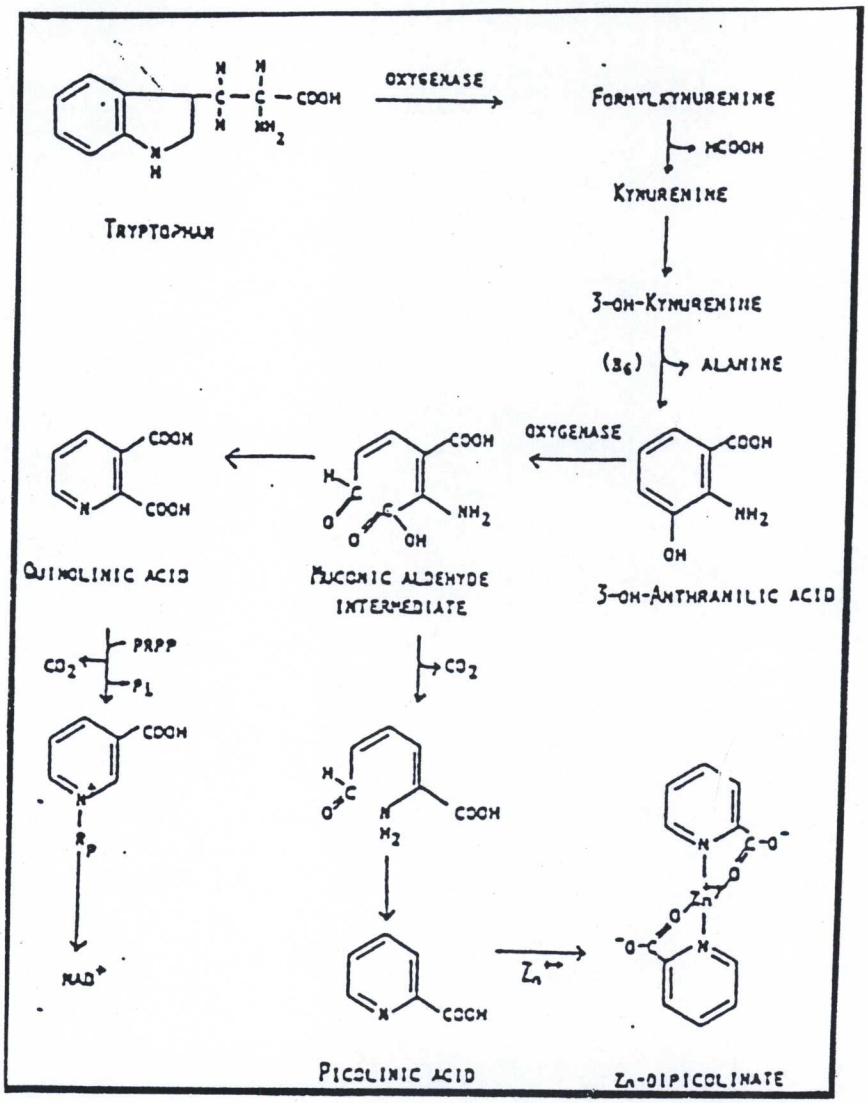


Figure 6: Metabolism of tryptophan to picolinic acid.

The observations of Evans and Johnson (1980) would lend support to the results obtained by Hsu (1965), who reported decreased zinc levels in several tissues of rats fed a diet deficient in vitamin B-6 (68).

The above conclusions reached by Evans and Johnson (1980) have, however, been criticised by Hurley and Lönnerdal (1980) (70), who maintained that the data presented by Evans and Johnson (1980) was inadequate to warrant their conclusion for the following reasons:

1. Picolinic acid is not a significant end product of tryptophan metabolism in the rat. Only 7.4% of 3-hydroxyanthranilic acid, an intermediate metabolite of tryptophan, is converted to PA by rat liver extracts and only 4% is converted to urinary PA, *in vitro*.

2. Evans and Johnson (1980) based their conclusion on the fact that PA is a good chelator of zinc. (47). Increasing the zinc absorption by addition of a chelating agent does not indicate that the chelating compound has a normal physiological role.

3. The analysis of pancreatic fluid and pancreatic tissue of rats showed no evidence of the presence of a low molecular weight zinc-binding ligand (70). Furthermore, the analysis of human milk did reveal the presence of a low molecular weight zinc-binding ligand which was identified as citrate and not PA.

However, further evidence for the involvement of PA in zinc absorption was provided by another study by Evans and Johnson in 1980 (43). It was reported that the zinc concentration of liver and kidneys from pups nursing dams fed zinc dipicolinate solution were significantly greater than the zinc concentrations of these tissues from pups nursing dams given a solution of zinc acetate. The conclusion drawn was that a greater quantity of dietary zinc is transferred from the intestine of the lactating female rat to the pups when zinc is fed in the form of zinc dipicolinate. It was also noted that when lactating rats were fed a diet supplemented with either nitrilotriacetic acid (NTA) or a zinc NTA complex, the zinc chelating ligand had no effect on the zinc concentration of organs from the nursing pups (43). These observations suggested that a specific type of ligand is required to facilitate zinc transport from the intestinal lumen into the body (43).

Researchers investigating the effect of dietary PA on the metabolism of zinc in the rat, found that rats receiving PA had a 10-fold increase in urinary excretion of total zinc (158). They concluded that PA has a considerable effect on zinc metabolism (158).

Krieger and Statter (1987), in their efforts to demonstrate the effect of tryptophan deficiency and PA on zinc metabolism (88), have produced results that may support the finding of Evans and Johnson (1980) (47). They noted that a deficiency of tryptophan results in low plasma and bone zinc concentrations in rats. It was concluded that the observations of Evans and Johnson (1980) could not be attributed to insufficient albumin synthesis since tryptophan is not a building block of albumin (88). In addition, 90% of tryptophan is non-covalently bound to albumin which seems to function as a carrier. Instead, the low zinc levels could be attributed to the fact that tryptophan supplies the zinc carrier, viz. PA, which is a strong bidentate ligand and important in zinc absorption (88).

Brown (1985), in his article dealing with possible roles for vitamin B-6 and trace metals in health and disease (19), also supported the observations of Evans and Johnson (1980) (47). His review favours the idea that PA enhances zinc uptake. He also supported the theory that the nutritional status of zinc may be related to vitamin B-6 status since several studies showed that zinc uptake or tissue zinc levels in laboratory animals are directly related to the levels of vitamin B-6 intake.

The above investigations provide enough evidence to suggest an association among vitamin B-6, PA and zinc levels.

However, there still seems to be disagreement regarding the involvement of PA in zinc absorption during a vitamin B-6 deficiency.

A study incorporating a vitamin B-6 deficient group (vitamin B-6 free diet), a vitamin B-6 deficient group supplemented with PA, a partially deficient group (low level of vitamin B-6 in diet), a pair-fed group and a control group may be better able to determine the involvement of PA in zinc absorption during a vitamin B-6 deficiency. Since no such studies have been reported it was decided to include all of the above groups in the current study. Furthermore, because pancreatic PA levels are believed to influence zinc absorption during vitamin B-6 deficiency the levels of this compound were determined in all groups.

2.1.8.3 The effect of vitamin B-6 deficiency on iron metabolism

Compared with normal control rats, vitamin B-6 deficient rats retain more iron in their tissues (72). The essentiality of vitamin B-6 in the biosynthesis of haemoglobin is well established. The increased tissue iron levels were therefore attributed to the fact that less iron was incorporated into haemoglobin during a vitamin B-6 deficiency (72).

It has also been reported that serum, liver and spleen iron levels were significantly increased in the vitamin B-6 deficient group compared with the control group (109). Hypochromic microcytic anaemia has been reported in experimental animals and humans during vitamin B-6 deficiency (109). It has been suggested that anaemia due to vitamin B-6 deficiency may be attributed to a defect of haeme biosynthesis with associated high serum iron levels (72).

Neal and Pearson (1962), working on the effect vitamin B-6 deficiency has on iron absorption, noted that iron intake of rats fed vitamin B-6 deficient diets depended on the amount of iron in the diet (125). It was observed that when the dietary iron level was 100 μg/day, the recommended level, the rats did not absorb increased amounts of iron. However, when the iron intake was increased to 1.0 mg/day, the vitamin B-6 deficient animals absorbed larger amounts of iron than the controls. They concluded that enhanced iron absorption in vitamin B-6 deficient rats does not occur under physiological conditions.

Kirksey and Tabacchi (1967), investigating the effects of pyridoxine deficiency on iron metabolism in the pregnant rat, reported impaired erythropoiesis including polycythemia accompanied by significantly lower mean corpuscular volume (MCV) and mean corpuscular haemoglobin (MCH) in the vitamin B-6 deficient rats when compared with controls (83). Also, both liver and spleen iron stores were elevated in the non-pregnant vitamin B-6 deficient rats. They also reported decreased duodenal iron concentrations, especially ferritin, in vitamin B-6 fed pregnant groups and a high level of iron in the duodenum of the vitamin B-6 deficient group. It was postulated that this high level of iron in the duodenum was part of a regulatory mechanism which blocked excessive absorption of iron. They also found that although the deficient rats had elevated levels of iron in all tissues, no major impairment in iron absorption was evident.

2.1.8.4 Effect of vitamin B-6 deficiency on calcium absorption

Prasad *et al.* (1982) reported a significant increase in the uptake of calcium in vitamin B-6 deficient rats when compared with control and pair-fed animals (138). The absorption of calcium is mediated by a biphasic mechanism comprising an active carrier-dependent component and a second passive diffusion component (51). It has been postulated that a lack of vitamin B-6 may result in an impairment of nucleic acid synthesis. This would lead to an inhibition of protein synthesis, cell division and repair, with a consequent alteration in the brush border membrane structure. This may explain the increase in calcium uptake observed in the vitamin B-6 deficient animals (138).

2.1.9 The effect of vitamin B-6 deficiency on plasma albumin and globulin levels

Pike and Brown (1959) observed that weekly changes occurred in the total plasma protein levels in vitamin B-6 deficient rats (137). The total plasma protein levels in the control rats, however, remained constant during the three week observation period. The vitamin B-6 deficient rats showed a significant increase in the concentration of total proteins in the plasma. It was postulated that the deficient animals were unable to draw upon plasma proteins for tissue utilization and, consequently, the concentration of total protein steadily increased (137).

On the other hand, in contrast to the above finding, Ross and Pike (1956) reported lower total protein concentration in the serum of vitamin B-6 depleted rats when compared with controls (149). They observed that the serum albumin levels varied little between depleted and non-depleted animals but the globulin concentrations of the vitamin B-6 deficient rats were lower than in the controls.

Further studies found either no change or very little change in plasma protein levels of vitamin B-6 deficient rats. Brown & Pike (1960) reported decreased haemocrit levels in vitamin B-6 deficient rats when compared with controls (18). The total plasma volume of vitamin B-6 deficient rats, however, was observed to have increased only slightly above the initial level. The total blood volume remained approximately the same throughout the experimental period. It was also noted that no difference in the concentration of serum proteins existed between the vitamin B-6 deficient and control rats. However, this observation was not in keeping with their earlier study, where a significant increase in total plasma protein was reported in vitamin B-6 deficient rats (137). The concentration of alpha-globulin was, however, observed to be higher in the vitamin B-6 deficient rats and concentrations of total circulating gamma-globulin were reduced in vitamin B-6 deficient animals. No clear explanation was provided for these conflicting results.

2.2 Zinc

2.2.1 General physiology of zinc

Although knowledge of the importance of zinc as an essential trace metal began over a century ago, many aspects of its metabolism still remain an enigma to scientists today.

The clinical use of zinc began in ancient Egypt where its oxide (or calamine) was employed for treatment of burns and wounds. The scientific basis for its effectiveness was, however, only discovered in the 1950's when studies on animals and wound healing in surgical patients demonstrated that zinc supplementation of the diet promoted healing. Even today the precise role of supplementary zinc therapy remains unclear (95).

Zinc is an essential trace element for both humans and animals and is necessary for the normal functioning of a large number of enzymes (77).

Zinc is present in all organs, tissues, fluids and secretions of the body. The greatest portion of body zinc is found in the skeletal muscle. Together with bone, these two tissues account for more than 80% of the total body zinc. Zinc is primarily an intracellular element. The concentration of zinc in extracellular fluids is relatively low compared with intracellular stores. Since the total amount of zinc present in the major tissues is much larger than that present in the plasma, relatively small changes in the zinc content of tissues such as liver can cause marked changes in levels of plasma zinc (75).

The distribution of zinc within tissues and the nature of its intracellular binding is still not clearly understood. It is believed that zinc is ubiquitously distributed within cells and that the major proportion of zinc within cells is protein-bound. There is evidence which indicates that different proteins have different affinities for zinc and that zinc may have a controlling role in certain enzymes such as fructose-1,6-diphosphatase, rather than being firmly bound and an integral part of the enzyme. Since zinc plays a controlling role within cells it would indicate the presence of a 'pool' from which zinc is readily available. The nature of this pool is as yet not clearly defined (75).

The relatively constant levels of zinc in tissues and body fluids in situations where the composition of the diet varies greatly indicates the presence of an efficient mechanism for whole body zinc homeostasis. Zinc homeostasis is thought to be maintained by manipulation of the gastrointestinal absorption and the gastrointestinal excretion of the element. Since little zinc is excreted in the urine, changes in urine zinc content contribute little to the maintenance of whole-body zinc homeostasis at normal dietary zinc intakes. A diet with a low zinc content results in an increase in the proportion of zinc absorbed from the diet. Decreases in the rate of excretion of zinc into the gastrointestinal tract are also known to occur in response to low dietary zinc. Conversely, an increased content of dietary zinc results in a reduced fractional absorption of zinc and an increased rate of gastrointestinal excretion of zinc (75).

2.2.2 Metabolic role of zinc

Zinc functions as an essential catalytic component in a great variety of enzymes. It also serves as a structural constituent of enzymic and non-enzymic proteins and of polynucleotides. Zinc appears to either confer structural and metabolic stability or govern the tertiary structure and therefore the biological function of a wide range of macromolecules (119). The role of zinc in alcohol dehydrogenase, for example, demonstrates its catalytic and structural roles. The zinc associated with the functional catalytically active centre can be differentiated from the zinc which is less firmly bound and involved in stabilizing the polymeric structure of the enzyme (119).

Over two hundred zinc dependent enzyme activities have been identified. A few of those relevant to humans and animals are listed in Table 1.

Zinc is probably the metal most widely distributed amongst metalloenzymes. Metal activated enzymes are divided into two classes, viz. metalloenzymes in which the metal is firmly bound, and metal-enzyme complexes that are easily dissociated and require the continuous presence of the activating metal in the medium (25).

Zinc is believed to have a fundamental role in protein metabolism. It has been compared with essential amino acids since both zinc deficiency and deficiencies of essential amino acids result in a rapid onset of anorexia, growth retardation, raised blood ammonia and urea concentrations, decreased protein synthesis and reduced DNA/RNA ratios (1).

The significant growth retardation resulting from zinc deficiency has led to much research in an attempt to determine whether the effect is due to inhibition of cell replication or of cell hypertrophy. Most investigators suggest that DNA synthesis and cell replications are impaired by zinc deficiency to a greater extent than protein synthesis and cell hypertrophy (25).

The activity of various enzymes involved in DNA synthesis, viz., thymidine kinase, DNA polymerase, thymidine synthetase and ribonucleotide reductase, has been found to be reduced by zinc deficiency (25). It has been suggested that in normal cells, a lack of zinc prevents individual cells from acquiring the groups of proteins that must be synthesised during each cell cycle before the commencement of DNA synthesis (25).

Much evidence exists to suggest that zinc has a direct role in gene expression. Zinc has been shown to be present in and to interact with the various chromatin components, viz., DNA, RNA, histone and non-histone proteins. More research is, however, needed to verify that nutritional modulation of *in vivo* zinc concentrations or specific zinc pools can directly influence the synthesis of nucleic-acid-synthesizing enzymes and affect the regulation of other genes (27). The involvement of zinc in DNA transcription, the translation processes and in the proper structure and functioning of the translated product suggests that zinc is essential for virtually all aspects of normal cell metabolism (27).

Zinc has a diverse role in the central nervous system. It has been proposed that zinc has a regulatory and structural role in nerve growth factor. Neuropsychiatric features, prominent in human zinc deficiency, suggest a role for zinc in neural functions such as taste, appetite control, olfactory function, vision and neuromuscular co-ordination (1). The distribution of zinc among all enzyme classes suggests a wide range of functions for zinc in brain and neurotransmitter homeostasis (1).

Early studies have indicated a relatively high concentration of zinc in bone compared with other tissues (67). Golub *et al.* (1996) reported that moderate reduction of dietary zinc in rhesus monkeys from the beginning of puberty through the postmenarcheal period resulted in retardation of skeletal growth, maturation and mineralization (56). This demonstrated that moderate zinc deficiency in the presence of an adequate intake of all other nutrients adversely affects skeletal growth and mineralisation during adolescence (56).

Zinc is highly concentrated in the hypertrophic zone of epiphyseal cartilage and is known to influence the activity of osteoblast alkaline phosphatase. However, further studies are necessary to understand the mechanism whereby zinc deprivation inhibits skeletal growth and mineralization *in vivo* (80). Zinc also serves as a potent inhibitor of bone resorption as well as a promoter of bone formation (80).

It has also been postulated that zinc plays an important role in fatty acid and carbohydrate metabolism (60). However, more research is needed in this field before its exact role can be established.

Table 1: **Mammalian zinc metalloenzymes.**

ENZYME	ROLE OF ZINC
Alcohol dehydrogenase	Catalytic and structural
Alkaline phosphatase	Catalytic and structural
Amino peptidase	Catalytic
Angiotensin converting enzyme	Catalytic
Collagenase	Catalytic
Carboxypeptidases	Catalytic
Carbonic anhydrase	Catalytic
Fructose 1,6-diphosphatase	Regulatory and structural
Glyceraldehyde-3-phosphate dehydrogenase	Catalytic

2.2.3 Zinc absorption

The exact site of zinc absorption along the mammalian gastrointestinal tract has not been determined. Antonson *et al.* (1979), in *in vivo* studies in the rat reported that the ileum contributes 60%, the jejunum 20% and the duodenum 19% to the overall absorption of zinc (14). This differs from a later study by Solomons (1982) who observed that in *in vitro* studies, the ileum had the greatest capacity for zinc absorption, whereas in *in vivo* studies, the duodenum was found to be the preferred site. Negligible absorption of zinc occurs in the stomach, caecum and colon (38).

Jackson *et al.* (1981) suggested that two mechanisms of zinc absorption exist. They observed that in normal rats, the absorption of zinc is proportional to the dietary intake of zinc and a relatively slow, obligatory, carrier-mediated mechanism predominates (77). The latter mechanism is independent of homeostatic control and involves binding of zinc to mucosal ligands from where it is released into the body. It was therefore suggested that under normal conditions, body zinc levels are controlled by regulation of the amount of zinc excreted (77). However, when the zinc level in the diet is low, the proportion of zinc absorbed increases. Jackson *et al.* (1981) suggested that this increased zinc absorption does not occur by increasing the mucosal binding of zinc but rather by a second carrier-mediated process for zinc absorption which becomes activated (77). Zinc is rapidly transported into the body without remaining in the mucosa for any substantial length of time. It was hypothesised that this second controllable mechanism would need to be situated near the pyloric end of the small intestine since all absorption by this process was observed to be complete within one hour of administration of ^{65}Zn (77).

Based on the evidence of Jackson *et al.* (1981) it may be possible that the site of maximal zinc absorption changes in accordance with the dietary zinc level (77). When dietary zinc levels are low, the duodenum (pyloric end of intestine) probably accounts for the major portion of the zinc absorbed since the second carrier-mediated mechanism for zinc absorption, as proposed by Jackson *et al. (1981)*, comes into effect. Under normal conditions (normal dietary zinc levels) there is no need for this second mechanism to be activated and, therefore, the duodenum may not be the site of maximal zinc absorption. The ileum may then account for maximal absorption of zinc (38, 165).

It has also been argued that although the ileum has the largest capacity for zinc absorption, the duodenum with its lower capacity for zinc absorption has the first opportunity to absorb zinc from digesta and thus much less is left for absorption by the ileum (96).

Research by Menard and Cousins (1983) indicated that the mechanism of zinc transport across the brush border of intestinal epithelial cells probably involves interaction between the metal in a chelated form and the membrane surface (111). It was proposed that a number of specific binding ligands might fulfill this role. The existence of a specific low molecular weight zinc-binding ligand was suggested by a number of investigators (59, 156). As stated previously, this ligand, believed to originate from the pancreas, was found in the intestinal wall and in the milk of certain species (45, 100). Evans *et al.* (1979) have attempted to characterize this zinc-binding ligand and have described it as picolinic acid (PA) (44). It was suggested that during the process of absorption, tryptophan is metabolised in the exocrine cells of the pancreas, producing PA. Picolinic acid is then secreted from the pancreas into the intestinal lumen where a zinc-dipicolinate complex is formed. This complex facilitates the transport of zinc across the brush border (42).

Other studies, however, cast doubt on claims that ligands secreted from the pancreas play a role in zinc absorption. Hurley and Lönnerdal (1980) have refuted the observations of Evans (1980) regarding the effect of picolinic acid on zinc absorption. These workers maintained that the ability of PA to enhance zinc absorption is due to the fact that it is a good chelating agent and chelating agents can improve the absorption of zinc in a variety of conditions. This, however, does not imply that PA has a normal physiological role in zinc absorption. Other compounds that have no normal physiological role in zinc absorption have also been shown to improve zinc absorption, eg. ethylenediaminetetraacetic acid (EDTA) (86).

Other researchers have reported that pancreatic fluid and tissue do not contain a low molecular weight zinc-binding ligand (99). Earlier work by Hahn and Evans (1973) proposed that the low molecular weight zinc compound in pancreatic tissue homogenate may in fact be due to proteolytic degradation of zinc metallo-proteins (59).

2.2.3.1 Role of intracellular zinc-binding ligands

The intracellular distribution of intestinal zinc has been extensively studied. The basic concepts involve hypothetical pools for zinc and the distribution of zinc to specific cellular sites for specific functions. The potential function of various intracellular zinc-binding ligands in controlling zinc absorption has been widely researched. A key role for metallothionein (MT) in this process was suggested by Richards and Cousins in 1977 (146). It was proposed that MT of the intestinal mucosa reflects dietary zinc status, and the amount of MT present in the mucosa is negatively correlated with zinc absorption.

Further research has indicated that synthesis of MT is induced by zinc administration (31). Little MT was found in intestines of zinc-deficient rats, whereas dietary repletion with zinc increased MT in a dose-dependent fashion. The inducible nature of intestinal MT led to the postulation that MT is an integral regulatory component for zinc absorption (31).

Studies by Richards and Cousins (1977) have shown that the major zinc-binding fraction in the cytosol of mucosal cells (enterocytes) is metallothionein (146). They suggested that zinc, taken up into these mucosal cells, may either be transported across the basolateral membrane and finally into the hepatic portal blood or become bound within the cells to MT, which prevents its transfer to the blood. The amount of metal-free thionein in the zinc-absorbing cells of the intestinal mucosa may, therefore, be the major determinant of the extent of zinc absorption (146).

The following model as depicted in Figure 7, was proposed by Cousins in 1982 (35) to explain the mechanism of zinc absorption at the level of the intestinal mucosal cell :

- A portion of the dietary zinc which enters the lumen of the small intestine is transported across the mucosal brush border membrane (shown as A in Figure 7).

- The newly acquired cytoplasmic zinc within the enterocytes equilibrates with a "zinc pool" and is either shunted into high molecular weight proteins and metallothionein or is transferred to the blood (shown as B in Figure 7). The intracellular binding phase is thought to depend on rates of protein synthesis and degradation and an availability of binding sites. Metallothionein may act as the expandable (inducible) intracellular zinc-binding compartment.

- In animals with adequate zinc status, a significant amount of zinc is transferred to the blood. An increase in dietary zinc may result in a concomitant increase in plasma zinc concentration and the synthesis of thionein polypeptide chains.

- A major portion of the zinc that enters the enterocytes from the plasma becomes bound to MT, a phenomenon which is directly related to zinc status (Figure 7).

- Appreciable amounts of zinc may also be secreted into the intestinal lumen. Therefore, "A" in Figure 7 is represented as a bidirectional transport.

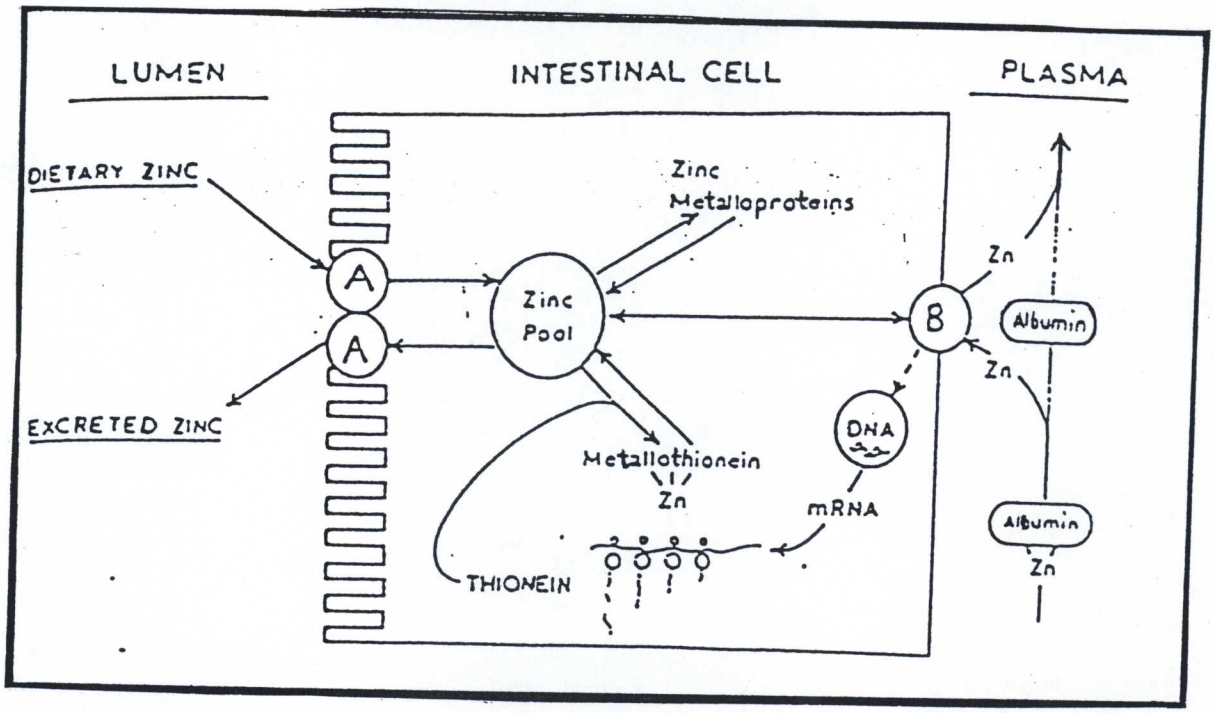


Figure 7 : Proposed mechanism for the regulation of intestinal zinc absorption. Broken lines indicate zinc concentrations which may signal the induction of intestinal metallothionein biosynthesis (165).

Investigations using isolated rat intestines indicate that zinc binds to albumin when it leaves the basolateral membrane and enters the portal blood (163). This is in agreement with earlier work by Smith *et al.* (1979) who showed that albumin is the principal portal transport protein for zinc (161). Therefore, factors which impair protein synthesis, such as occurs in vitamin B-6 deficiency, may be expected to reduce zinc absorption.

2.2.3.2 Regulation of zinc absorption - influence of metallothionein

Numerous experiments have been performed to determine the mechanisms involved in the regulation of zinc balance. Zinc is absorbed and excreted by the small intestine. However, the extent to which these processes contribute to overall zinc homeostasis is still unclear. Much interest has centred on the possible role of intestinal metallothionein in zinc absorption (169).

Metallothionein was initially believed to serve as a storage protein for zinc. Subsequent work, however, indicated that metallothionein may be involved in the metabolism of body zinc in ways other than storage (106).

In a study by Cousins (1979) it was concluded that metallothionein had a specific function in Zn metabolism and that the absorption of Zn, presented to the intestinal mucosa, is inversely related to the mucosal metallothionein content i.e. when body zinc stores are high, Zn taken up by the mucosa is preferentially sequested by the high thionein concentration. Thus only a small proportion is available for transfer across the basolateral membranes of the mucosal cells. However, when body zinc stores are low, little or no thionein is present in the absorptive cell and a higher proportion of the zinc entering the mucosal cells from the intestinal lumen is available for transcellular transfer (34).

Starcher *et al.* (1980), investigating the relationship between zinc absorption and intestinal metallothionein (169), produced results that were in conflict with those of Cousins (1979) (32). They found that zinc absorption is directly proportional to intestinal metallothionein levels, implying a significant role for metallothionein in zinc absorption. It was also shown that when metallothionein induction was blocked with actinomycin D, the absorption of ^{65}Zn (given orally) was decreased suggesting a positive relationship between zinc absorption and metallothionein synthesis. It was, however, conceded that other intestinal proteins or peptides that may be important in the absorption process may also be inhibited by actinomycin D (169).

A study by Coppen and Davies (1987) showed that the zinc bound to intestinal metallothionein was indeed related to the zinc status of the rats (29). It was found that when the dietary zinc supply was deficient, little or no Zn-thionein was detectable but as the zinc supply increased from adequate to excessive levels, there was a linear increase in Zn levels associated with metallothionein (29).

In addition, Coppen and Davies (1987) also noted that metallothonein may also play a role in modulating Zn excretion when dietary Zn contents exceed requirements for growth and that when dietary Zn is excessive, body Zn burden is modulated entirely by changes in Zn excretion (29). However, the exact role played by metallothionein in the control of Zn absorption or excretion could not be elucidated (29).

2.2.3.3 Exogenous factors affecting zinc absorption

The exogenous factors that may reduce the availability of zinc for intestinal absorption are discussed below :

2.2.3.3.1 Protein

Both the type and the dietary supply of protein appear to affect the extent of zinc absorption. Pedersen and Eggum (1983) showed that zinc absorption from diets containing 8%, 16% and 24% protein increased with increasing protein intake (135). In another study it was reported that the absorption of oral ^{65}Zn in rats fed a 5% protein diet was significantly less than in rats fed a 15% protein diet (178).

The apparent absorption of zinc in rats increases when the diet is supplemented by certain amino acids, eg. histidine and cysteine. However, it is not certain whether these changes indicate a higher absorption of zinc or whether it may be due to other changes in zinc metabolism caused by the altered amino acid intake (152). It has been shown that more important than the level of dietary protein, is its source (127). Various studies have reported that zinc in soybean protein is less available to animals than zinc in casein (127). The apparent absorption of zinc by rats fed casein was 84% compared with 44% by rats fed soybean protein (127). It was therefore concluded that zinc in plant proteins, such as soybean protein, is bound such that it is absorbed less efficiently than the zinc in animal proteins such as casein (127).

2.2.3.3.2 Phytic acid

Various studies have confirmed that the addition of phytic acid to the diet reduces the absorption of zinc (50). The structure and chemistry of phytic acid may explain the mechanism of this antagonistic effect. At pH values encountered in foods, phytic acid has a strong negative charge and thus a strong potential to bind positively charged molecules. It has been established that after copper, zinc forms the most stable complexes with phytic acid (152). It was proposed that the dietary basis for the zinc deficiency syndrome seen in the Middle East was the high phytate content of the diet.

2.2.3.3.3 Calcium

Several animal studies indicate that increases in dietary calcium levels decrease the intestinal absorption of zinc (168). The detrimental effect of high calcium on zinc bioavailability has been attributed to the presence of phytate in the diet. Zinc was found to co-precipitate with phytate and calcium at the alkaline pH in the lumen of the small intestine (168). It has been shown that the effect of calcium in decreasing zinc absorption in rats depended also on the phosphorous intake (168). Studies in human subjects, however, showed that increasing dietary calcium had no effect on zinc absorption or balance (168).

2.2.3.3.4 Fibre

The inhibition of zinc absorption by foods rich in dietary fibre has been shown by tolerance tests and zinc retention studies (151). Zinc was found to be less readily available for intestinal absorption when added to wholewheat bread than when added to white bread. This was attributed to the binding of zinc by fibrous components (lignin and hemicellulose) of the diet. However, fibre-rich foods are known to contain higher levels of potential zinc absorption antagonists like phytates. It has thus been suggested that fibre *per se* has very little or no effect on zinc availability.

Other exogenous components that may potentially affect zinc bioavailability include oxalates, iron, copper and cadmium (152, 164).

2.2.4 Systemic transport of zinc

Plasma zinc represents only 10-20% of the zinc in whole blood. The remainder is localized within the erythrocytes in which anhydrase is the major binding site (33). Approximately two-thirds of the zinc present in the plasma is bound to albumin and one-third to α_2-macroglobulin (26). It is believed that about twelve proteins in the serum bind zinc *in vitro*. The order of zinc binding was found to be albumin » α_2-macroglobulin › transferrin, with only minor binding by other proteins (33).

Albumin bound zinc is referred to as a loosely bound, exchangeable zinc pool, and the ability of albumin to give up bound zinc is thought to be an essential feature of the transfer of zinc between plasma and tissues (31).

2.2.5 **Cellular uptake from the systemic circulation**

The major tissues which contribute to the regulation of systemic zinc are shown in Figure 8. the kidney and liver are considered the most significant sites of zinc metabolism.

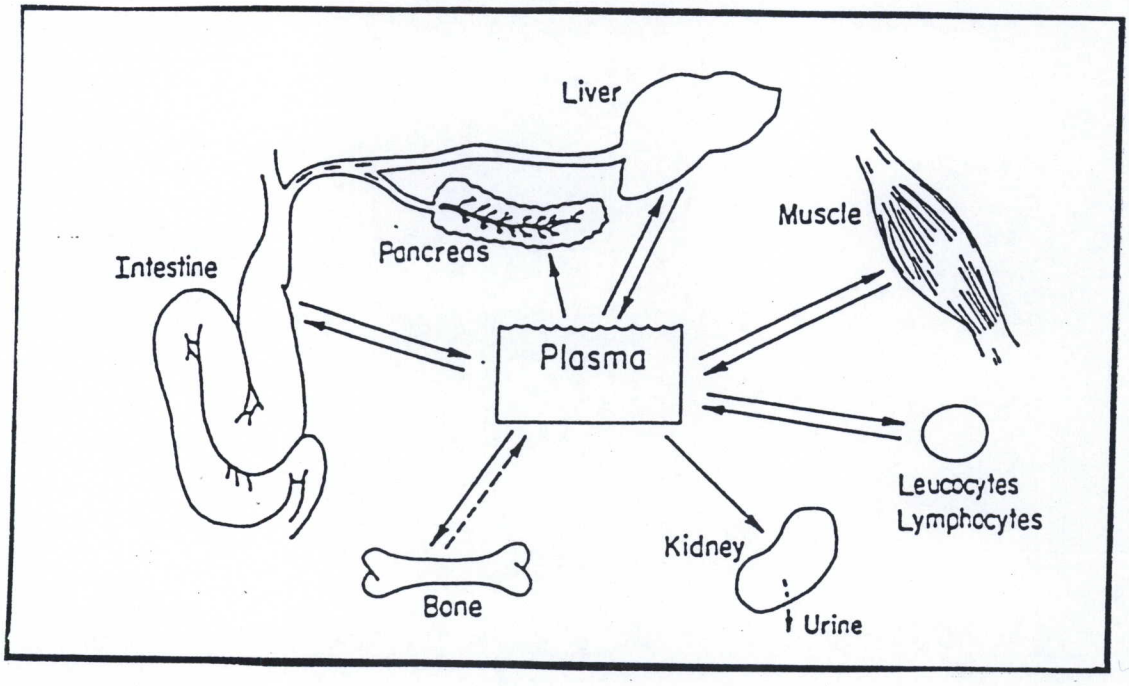


Figure 8 : The major tissues contributing to the regulation of systemic zinc (33).

2.2.5.1 Liver uptake

Most of the zinc from the intestine is transported to the hepatocytes bound to albumin (33). Major fluctuations in dietary zinc supply cause only minimal changes in the hepatic concentration of zinc (31).

Zinc uptake by hepatocytes occurs by two distinct phases. The first is a fast-uptake phase which represents initial binding to specific sites on the plasma membrane surface. This phase is believed to be a carrier-mediated phase. Since most of the zinc in the portal plasma is bound to albumin it has been suggested that there could be a zinc-albumin interaction with the plasma membrane at this point. The second phase of uptake was found to be a linear function in which zinc is accrued at a much slower rate (31).

A pharmacokinetic model of zinc metabolism has demonstrated that the first-order mass transfer coefficient and linear binding constant are highest for the liver. This would probably mean that hepatic mechanisms control plasma zinc levels (33).

2.2.5.2 Kidney uptake

After the liver, the kidney has been demonstrated as the most important site of metabolic significance for zinc. This would suggest that the kidney has a substantial influence on the plasma zinc compartment.

Renal insufficiency was shown to result in reduced serum zinc concentrations (33). This has been correlated with increased urinary zinc bound to ligands of smaller molecular weights than albumin. Infusion of amino acids into the systemic circulation, of both rats and humans, increased urinary zinc excretion (33). It was hypothesised that a shift of circulating zinc from the protein-bound to ultrafilterable pool could reduce plasma zinc concentrations. This hypothesis was based, in part, on the observation that the levels of plasma proteins are not altered despite significant hypozincaemia (33).

Renal zinc excretion was also demonstrated to be hormonally regulated (33). Insulin, when added to the vascular perfusate, inhibited the excretion of zinc in the perfused dog kidney while infusion of glucagon was shown to increase zinc excretion (33).

2.2.7 Clinical features of zinc deficiency

Zinc deficiency affects virtually every organ system, with the most negative effects occurring in those tissues where rapid cellular division occurs. Therefore, young rapidly developing and growing animals show the most severe effects of a direct or induced zinc deficiency (27).

The signs and symptoms of zinc deficiency are similar in different animals. Since zinc is an essential component in the diet of animals, animals that consume zinc-deficient diets will eventually develop a variety of pathologies. The most commonly observed symptoms include anorexia, growth retardation, dermatitis, alopecia, ocular lesions, testicular atrophy and impaired reproductive capacity (27, 171).

In rats, zinc deficiency is characterized by growth retardation, immature hair coats, scaly feet and fissures at the corners of the mouth (54, 171). Some of these features are also observed in vitamin B-6 deficiency.

In monkeys, Zn deprivation was shown to affect activity and attention before the onset of growth retardation (57).

Congenital malformations have been observed in rat foetuses of zinc-deficient females. These include misshapen heads, clubbed feet, short lower jaws and long bones and fused or missing digits (60). Other skeletal defects observed include fused ribs; curvature of the spinal column; incomplete ossification in the ribs, vertebrae and cranial bones; and missing vertebrae in the tail. Malformation of the brain, heart, lung and urogenital system were also observed (71).

In birds, the most common symptoms arising from zinc deficiency include abnormal feather growth, reduced egg production and hatchability (136). In ruminants, hair loss, parakerotosis, cracking and fissures around hooves, reduced testicular size, decreased libido and retarded sexual maturity are characteristic signs of zinc deficiency. In sheep, zinc deficiency results in decreased immune response, poor wound healing, lethargy and poor wool quality (136).

Zinc deficiency in humans has been identified in several areas of the world. Individuals experiencing rapid growth or those under stress may be especially vulnerable to marginal zinc deficiencies (136). Marginal zinc deficiency is fairly common in western societies and is believed to result from inadequate dietary intake, malabsorption and increased rates of loss from the body. Symptoms of marginal zinc deficiency include failure of appetite and growth, night blindness, mental lethargy and skin lesions.

Clinical features of severe zinc deficiency include dermatological features comprising an early skin rash around the nostrils, chin and corners of the mouth with hyperkeratotic lesions in prolonged zinc deficiency; growth retardation and failure to thrive; eye abnormalities and neuropsychiatric changes comprising a change in mood, loss of effect and emotional lability (2).

Other symptoms of severe zinc deficiency include liver cirrhosis, impaired wound healing, sickle-cell anaemia, impaired immune response, epilepsy, loss of taste and smell (136) and malformation and abnormalities of skeletal growth (80).

2.2.8 Assessment of body zinc status

Deficiency of micronutrients is being increasingly recognised as a contributory factor for the high incidence of infections (10). Suboptimal Zn nutriture has been reported among apparently healthy children living in North America, China, Malawi, Thailand, Guatemala and Africa (48).

Evaluation of zinc nutriture has been difficult because of the lack of a marker of zinc status that is both sensitive and specific to changes in dietary zinc intake (173).

The need for a specific biochemical or functional test for zinc status is essential to the study of zinc nutrition in humans and animals. A large number of parameters such as zinc concentrations in plasma, serum, blood cells, femur, urine, hair, saliva and enzymatic activities have been used to assess zinc status (24, 30, 76, 166).

2.2.8.1 Plasma and serum zinc

The most widely used approach to assess zinc nutriture has been the determination of circulating plasma or serum levels of zinc. A number of investigators have shown a linear relationship between albumin concentration and zinc concentration in the blood; sixty to seventy percent of circulating zinc is loosely bound to albumin and 30 - 40% is tightly bound to α-2-macroglobulin. In normal subjects and hospitalized patients, albumin-bound zinc concentrations and total albumin concentration were highly correlated (166). It has been suggested that the total circulating zinc concentration reflects both serum albumin concentration and the affinity of albumin for zinc (166).

A decrease in plasma/serum zinc concentration has been presented as evidence of zinc deficiency (37).

The following two questions may, however, arise :

1. To what extent is a low serum/plasma zinc a reflection of impaired protein binding?

2. To what extent is the reduction due to total-body zinc deficiency?

The total concentration of zinc circulating in the plasma does not reflect total body zinc. The plasma zinc concentration changes in response to many conditions that do not involve zinc toxicity or deficiency (39). Plasma zinc levels also change in response to stress, infections, meals, short-term fasting and hormonal states (81). The fall in plasma Zn concentrations under these conditions (except fasting) may occur because of a redistribution of zinc to other tissues in response to a metabolic need (81). Circulating zinc may consequently be reduced for a short period of time. Plasma zinc can therefore, only be useful as a specific indicator of zinc status if the effects of zinc status and of these other metabolic conditions can be differentiated (81).

2.2.8.2 **Tissue zinc**

The direct measurement of the zinc content of tissues has also been used by several workers as an index of body zinc status (76, 121). Experimental zinc deficiency was shown to cause a decrease in the zinc content of various tissues but the results obtained have not always been in agreement.

Liver zinc levels were shown by Jackson *et al.* (1982) to decrease by almost 20% in zinc depleted rats compared with normal rats. This deficit was corrected in the repleted group (76). It was concluded that liver zinc levels may provide confirmation of existing zinc deficiency but, like plasma, liver zinc content is restored to normal by dietary repletion even though the total body zinc may still be low.

Hair represents a readily accessible tissue and has been extensively used as an indicator of zinc status. Hair is, however, much more susceptible to environmental contamination from exogenous sources than serum or most other tissues (166). In rats, hair zinc concentrations were found to correlate with the zinc content of bones and testes but not with those of kidney or liver (166). It was concluded that in rats, hair zinc reflects dietary intake rather than total body zinc (166). It has also been reported that hair zinc concentration is dependent on hair colour and sex (39). Children with acrodermatitis enteropathica were shown to have zinc levels related to age and body size (39). In addition, severe zinc deficiency was observed to retard hair growth to an extent that produced normal to high zinc levels (39). It is therefore clear that hair zinc is of little clinical value as an index of body zinc status.

Bone zinc levels were reported as being good indicators of total body zinc levels (76). Dangi and Kapoor (1983) observed an increased in femur zinc levels, which corresponded with increased dietary zinc (76). Bone zinc was shown to decrease to approximately half that of the normal value in zinc depleted rats (76). However, very little information regarding the part of the bone affected by zinc depletion is available. It has been hypothesised that only the growing areas of the bone are affected and, therefore, removal of only a small piece of bone for analysis (as during a bone biopsy) may not provide a reliable index of zinc deficiency (76).

Zinc concentration in muscle, testes and skin have also been used to assess the zinc status of zinc deficient animals. These tissues may be of some use in confirming an existing zinc deficiency but are of little value in assessing zinc status (39).

2.2.8.3 Balance studies

Balance studies provide accurate data on net intestinal absorption and body retention of ingested zinc. A negative zinc balance, suggesting a deficiency state, has been observed in conditions such as synthetic oral diets deficient in zinc and used in the treatment of inherited metabolic disorders, acrodermatitis enteropathica, pancreatic insufficiency and malabsorption and with many other dietary inadequacies and metabolic abnormalities (39). Balance studies are, however, considered to be labour intensive, requiring much skill, dedication and time (39).

2.2.8.4 Metallothionein

Golden (1987) suggested that the development of zinc deficiency and deficiencies of other nutrients like iron or calcium can be clearly differentiated (55). With the deficiency of most nutrients, the nutrient stores become mobilized and tissue concentrations are reduced with a resultant defect in one or more specific metabolic pathways. A zinc deficiency, however, results in a cessation of growth as a means of adapting to lower zinc intake and conserving tissue zinc. This occurs because no functional reserve or store of zinc appears to be available for use when zinc intake is reduced (55). With a more severe zinc deficiency, however, zinc balance becomes negative and there is a net loss of tissue zinc. The pool from which zinc is mobilized is a small, rapidly turning over, free pool. This metabolic buffer pool for zinc has been identified as zinc-metallothionein-I (55). It was suggested that metallothionein may be the key to making a diagnosis of zinc deficiency.

Metallothionein is a low molecular weight zinc-binding protein involved in various aspects of zinc metabolism and function. Mammalian tissues contain two major fractions of zinc-metallothionein, viz., metallothionein-I and metallothionein-II, which differ only by a single negative charge (139).

Metallothionein-II is specific for liver only. Under normal physiological conditions, metallothionein binds zinc and/or copper (139). It has been reported that tissue metallothionein concentrations are often proportional to zinc status (145). Metallothionein was reported as being reduced to non-detectable levels in the plasma and liver of rats made hypozincaemic (145). Moreover, a dose-dependent increase in metallothionein levels was observed in the pancreas, liver, kidney and small intestine after zinc sulphide administration (130). Dietary and liver zinc content are closely related (81) and it has been suggested that zinc may be the primary inducer of metallothionein synthesis in the liver (130, 139).

When the dietary zinc supply is increased sufficiently, metallothionein induction occurs primarily in the liver and intestine, followed by increased accumulation and/or redistribution of zinc in both tissues (31).

Experiments with rats have shown that hepatic and intestinal metallothionein levels which are normally low are decreased to trace levels by consuming a zinc deficient diet for only one day (31). Repletion with a high zinc diet increased metallothionein-bound zinc in both organs. Both hepatic and intestinal metallothionein levels increased in relation to dietary zinc levels. From these observations it was suggested that metallothionein is rapidly synthesised to bind extra zinc which may have a deleterious effect and that zinc binding to metallothionein is part of an intracellular function in which metallothionein serves as an intermediate or co-ordinating ligand to accommodate an influx of zinc into the intracellular pool. (31).

Bremner and Davies (1975) showed that restriction of food intake increased liver metallothionein-bound zinc in rats (15). It was reported that the magnitude of the effect of restriction of food intake on hepatic zinc distribution was dependent on the degree of starvation (15). This finding was corroborated by other researchers who noted that total starvation for 24 hours resulted in an accumulation of zinc-metallothionein (144). It was further noted that this response required RNA and protein synthesis, since actinomycin D was found to block the response (144). When protein synthesis was inhibited, serum zinc levels increased above normal, suggesting that metallothionein synthesis is necessary for hepatic uptake of endogenous zinc released into the blood as a result of tissue catabolism. It was also found that starvation did not cause a similar response in intestine or kidney (144). These observations

led to the conclusion that hepatic metallothionein synthesis, resulting from starvation, represents a conservation mechanism to decrease hepatic zinc loss. Feeding a zinc deficient diet prior to starvation did not diminish this effect indicating that readily mobilizable zinc stores had not been exhausted (144).

Metallothionein concentration in plasma is closely correlated with that in the liver (55). It has been observed that plasma metallothionein-I concentration increases in response to endotoxin and other stresses, while it decreases in a dose-dependent manner in response to a zinc-deficient diet (55). It was therefore suggested that measurement of metallothionein-I may serve as a mechanism to establish the cause of a low plasma zinc concentration (55). For example, if the low plasma zinc level was due to zinc deficiency, the zinc-metallothionein-I level would be decreased or absent, whereas if it was due to endotoxin or other stresses, its level would be increased (55). Therefore, the analysis of both plasma zinc and zinc-metallothionein-I should establish whether an individual was zinc deficient or not (55).

Metallothionein-I is also present in red blood cells in concentrations similar to those in plasma (55). Bremner *et al.* (1987) reported a major decrease in erythrocyte metallothionein-I level in marginal zinc deficiency (17). It was also established that unlike plasma metallothionein-I concentration, the RBC-metallothionein-I concentration was not affected by cold stress, endotoxin administration or restriction of food intake for 24 hours. It has been suggested that the assay of plasma zinc together with erythrocyte metallothionein-I may be used to make an unequivocal diagnosis of zinc deficiency (55).

Metallothionein can be detected in plasma and erythrocytes by a sensitive radioimmunoassay technique (112, 123).

From the above discussion, it can be seen that there is no consensus regarding the most appropriate method for the assessment of zinc status.

CHAPTER THREE

MATERIALS AND METHODS

3.1 EXPERIMENT 1

This experiment was designed to establish zinc status during a vitamin B-6 deficiency in experimental animals. To ascertain the zinc status, a zinc balance study was done where the faecal, urinary, dietary and tissue zinc levels were analysed. In addition, the plasma zinc and erythrocyte zinc-MT-1 levels were determined.

The vitamin B-6 status of all four groups of rats in this experiment was determined by measurement of plasma PLP.

To obtain a better understanding of the effects of vitamin B-6 deficiency on the animals, various other parameters were also monitored viz., insulin levels (RIA), blood glucose levels and plasma protein levels.

3.1.1 Subjects

All animals used in this experiment were male rats of the Wistar strain, bred at the Biomedical Resource Centre (BRC) at the University of Durban-Westville (UDW). The necessary ethical approval was obtained from the Ethics Committee of the UDW before the commencement of the study.

Thirty six male weanling rats (57.83 $\pm$ 8.08 g), age 3 weeks, were divided into four groups of 9 rats each viz., a control group receiving the normal AIN-76™ Purified Diet with 7 mg pyridoxine hydrochloride (PN.HC1) per kg diet; a deficient group receiving the same diet but with no pyridoxine hydrochloride (PN.HC1); a second deficient group (vitamin B-6 deficient-PA-supplemented) receiving the PN.HC1 deficient diet supplemented with 0.2 g picolinic acid/kg diet and a pair-fed group receiving the control diet.

The need for a pair-fed group was evident since a decrease in food consumption by the PN-deficient group was reported in various studies (23, 68).

3.1.2 Diet

Based on the recommendation of the American Institute of Nutrition (AIN) Ad Hoc Committee, 1977 on standards for nutritional studies, the AIN-76™ Purified Diet for rats and mice was chosen for use in the current project. The Committee reported that the AIN-76™ Purified Diet (for rats and mice) meets the known nutritional requirements of these species and supports growth, reproduction and lactation comparable to those from the best cereal based diets (141).

The composition of the AIN-76™ is shown in Table 2 and the vitamin and mineral mixtures used are shown in Tables 3 and 4.

Table 2 : AIN-76™ Purified Diet (for rats)

Ingredient	%
Casein	20.0
DL-Methionine	0.3
Cornstarch	15.0
Sucrose	50.0
Fibre	5.0
Corn oil	5.0
AIN Mineral mix	3.5
AIN Vitamin mix	1.0
Choline bitartrate	0.2
	100.0

Table 3 : AIN-76™ Vitamin Mixture

Vitamin	Per kg mixture
Thiamin.HC1	600 mg
Riboflavin	600 mg
Pyridoxine.HC1	700 mg
Nicotinic acid	3 g
D-Calcium pantothenate	1.6 g
Folic acid	200 mg
D-Biotin	20 mg
Cyanocobalamin (vitamin B-12)	1 mg
Retinyl palmitate (vitamin A)	+[1]
dl-α-Tocopheryl acetate (vitamin E)	+[2]
Cholecalciferol (vitamin D)	2.5 mg
Menaquinone (vitamin K)	5.0 mg
Sucrose, finely powdered to make	1000.0 g

+[1] As stabilised powder to provide 400 000 IU vitamin A activity

+[2] As stabilised powder to provide 5 000 IU vitamin E activity

Table 4 : AIN-76™ Mineral Mixture

Ingredient	g/kg mixture
Calcium phosphate, dibasic ($CaHPO_4$)	500.0
Sodium chloride (NaC1)	74.0
Potassium citrate, monohydrate ($K_3C_6H_5O_7.H_2O$)	220.0
Potassium sulphate (K_2SO_4)	52.0
Magnesium oxide (MgO)	24.0
Manganous carbonate (43-48% Mn)	3.5
Ferric citrate (16-17% Fe)	6.0
Zinc carbonate (70% ZnO)	1.6
Cupric carbonate (53-55% Cu)	0.3
Potassium iodate (KIO_3)	0.01
Sodium selenite ($Na_2SeO_3.5H_2O$)	0.01
Chromium potassium sulphate [$CrK(SO_4)_2.12H_2O$]	0.55
Sucrose, finely powdered to make	1000.0

Three different diets were prepared. The diet for the control group was prepared as in Table 2, i.e. comprising 7 mg PN.HCl/kg diet. The diet prepared for the vitamin B-6 deficient group was as per Table 2 but contained no PN.HCl and the diet for the vitamin B-6 deficient-PA-supplemented group also contained no vitamin B-6 but was supplemented with 0.2 g PA/kg diet. The pair-fed group consumed the control diet.

3.1.2.1 **Preparation of diets**

To avoid contamination, all equipment used in the diet preparation was first thoroughly washed with detergent (Contrad)(Merck), rinsed several times in deionised water and then air-dried. Plastic disposable gloves (Johnson and Johnson) were worn at all times during the preparation of the diets. Only plastic spatulas were used in dispensing the various nutrients.

All nutrients were weighed in plastic weighing boats using an electronic balance (Mettler BB 300). The vitamin and salt mixtures were prepared prior to the preparation of the complete diets. Constituents of a crystalline nature were pulverized in a porcelain mortar until a fine powdery consistency was achieved.

Two different vitamin mixtures were prepared - one with and one without vitamin B-6. In the preparation of salt and vitamin mixtures, the nutrients needed in small quantities were mixed first and then, by a number of triturated steps, all constituents were added and thoroughly homogenized in a large mortar. To prevent nutrients of a deliquescent nature from attracting moisture, they were first thoroughly mixed with anhydrous components of the diet and then stored in a desiccator prior to incorporation into the diets.

The vitamin and salt mixtures were first hand mixed and then further homogenised in a mechanical mixer (Forster Equipment Co., Eng.) for two hours. The salt and vitamin mixtures were then transferred to plastic bags, labelled and stored in air-tight and lightproof containers until required.

The final complete diets were prepared in batches of 2 kg each. The vitamin B-6 deficient-PA-supplemented diet was prepared by first thoroughly mixing the PA with a small amount of choline bitartrate (2 g of which was required to make a 1 kg of diet). The nutrients required in smaller quantities were mixed first before adding nutrients required in larger quantities. After

hand mixing in a mortar for approximately two hours, the mixture was transferred to a mechanical mixer for thorough homogenization for a further two hours. The completed diets were stored in plastic bags, in lightproof containers at approximately 3°C.

3.1.3 Induction of vitamin B-6 deficiency

All animals were housed individually in perspex metabolic cages (Techniplast) at the BRC, in an air-conditioned room with automatically controlled twelve hour light and dark cycles. The average temperature and humidity were 22°C and 55% respectively. The perspex food containers, attached to the cages, were structured such that only the head of the animal could be inserted into the container while feeding - minimizing food spillage and contamination. To prevent the risk of coprophagy, the metabolic cages were designed such that the faeces and urine were collected separately, in plastic containers situated below the floors of the cages. The food containers and cages were regularly washed with disinfectant and rinsed with deionised water.

Deionised water, made using an Elgastat B114 water deioniser (Elga Products, England), was supplied to the animals via water bottles attached to the cages. Both the water and the diets were supplied *ad libitum.*

Each group of rats was maintained on its particular diet for a period of six weeks. Food consumption was recorded daily and all animals were weighed once a week. The animals were examined daily for any physical signs of deficiency or disease. All faeces and urine excreted were collected daily, in plastic vials, for measurement of zinc content. Faeces samples were first dried in an oven (80°C) for 24 hours and then stored in a desiccator. Urine samples were stored at -23°C until analyzed. Urine and faecal samples for each week were pooled before analysis. Blood glucose concentrations for all rats were determined at the end of sixth week of the experiment.

3.1.4 Tissue sampling

At the end of the six-week experimental period, the food containers were removed from the metabolic cages and the rats were starved overnight but they had access to deionised water.

All surgical procedures were performed in the post-mortem laboratory at the BRC, University of Durban-Westville (UDW). To avoid contamination, all surgical instruments were sterilized prior to use. Rats were anaesthetized with halothane and blood samples removed by cardiac puncture using disposable plastic syringes and stainless steel needles. The blood samples were immediately transferred to labelled 5ml test tubes (free of zinc) containing EDTA as anticoagulant. The samples were centrifuged at 300 x g for 10 minutes and red blood cells and plasma separated. The red blood cell samples were rinsed with a saline solution and all samples were then stored at -23°C. The red blood cell samples were analyzed for zinc-metallothionein-1 concentration and the plasma samples for zinc, albumin, globulin, total protein and insulin levels.

The liver, kidneys, testes and tibias were carefully removed. All tissue samples were cleansed of adhering soft tissue. The soft tissue samples were first blotted to remove adhering blood (ashless filter paper) and then placed individually in labelled plastic vials. All vials were placed immediately in crushed ice before storage at -23°C until analyzed.

3.1.5 Reagents and laboratory equipment

To minimize contamination from water, reagents and laboratory equipment, all glassware and plasticware used in the experiment were first acid-washed by soaking in a 20% nitric acid solution for 48 hours. Glassware and plasticware were then rinsed several times with deionised water, thoroughly dried and stored in plastic bags until required.

3.1.6 Analytical procedures

3.1.6.1 The assessment of vitamin B-6 status

Plasma pyridoxal-5-phosphate (PLP) concentration has been recommended as a valid indicator, and is used with increasing frequency, to assess vitamin B-6 nutritional status (6). For the quantification of plasma PLP levels, the method devised by Ubbink *et al.* (1986) was employed (175). This technique is a rapid and sensitive high performance liquid chromatographic technique that makes use of an internal standard and is based on post-column semicarbazone formation of PLP, followed by fluorescence detection.

3.1.6.1.1 HPLC determination of plasma PLP levels

These analyses were made in the laboratory of Professor Ubbink, Department of Chemical Pathology, University of Pretoria, according to the method described below.

Reagents

The internal standard, 6-methyl-2-pyridine carboxaldehyde was obtained from Aldrich (Milwaukee, USA). Chromatography grade acetonitrile and all other reagents were purchased from Merck (Darmstadt, Germany). Pyridoxal-5-phosphate was also obtained from Merck.

PLP purification

The commercial PLP was purified by reverse-phase HPLC (Whatman Partisil 10 ODS-3 column; mobile phase: 10% glacial acetic acid in water) and then lyophilized. Reverse-phase ion-pair chromatography was used to check for impurities in purified PLP. Ultraviolet absorption spectra of the purified PLP was determined in a mixture containing 0.1 M hydrochloric acid, 0.1 M sodium hydroxide and 0.1 M sodium phosphate buffer (pH = 7.0).

Standards

Purified PLP was used to prepare working standards with concentrations of 15.08, 30.16, 45.24, 60.32 and 75.40 nM PLP. The internal standard was prepared by dissolving 6-methyl-2-pyridine carboxaldehyde in 25 ml of 0.1 M sodium dihydrogen phosphate; 20 ml of 0.5 M semicarbazide solution was then added and the mixture heated for 20 minutes at 40°C. After cooling, 0.1 M disodium hydrogen phosphate was added to adjust the pH to 7.0; the volume was finally adjusted to 500 ml using Na_2HPO_4-NaH_2PO_4 buffer (0.1 M; pH = 7.0).

Sample preparation

To 0.5 ml of plasma was added an equal volume of precipitation reagent. The mixture was shaken to ensure even protein precipitation. Prior to injection, the samples were centrifuged at 4000 rpm for 15 minutes and 200 μl of the supernatant was then transferred to individual microvials.

Instrumentation

A Beckman (Beckman Instruments, Berkeley, CA, USA) Model 112 solvent delivery module chromatograph was used. An LS 4 Perkin-Elmer fluorescence spectrometer (excitation wavelength: 367 nm; emission wavelength: 478 nm) was used for fluorescence detection.

Columns

A Whatman (Clifton, NJ, JSA) Partisphere C18 analytical column (110 x 4.7 mm I.D.; particle size 5 μm) was used. A Whatman reverse-phase guard cartridge was installed between the injector and the analytical column to protect the analytical column.

Chromatographic conditions

A solution of 0.05 M potassium dihydrogen phosphate (pH adjusted to 2.9 by use of concentrated orthophosphoric acid) containing 4% acetonitrile was used as mobile phase. Sodium hydroxide (2% w/v) was introduced for post-column alkalinization. The solvent delivery pump and the post-column reagent pump flow rates were 1 and 0.1 ml/min, respectively.

Assay procedure

The HPLC procedure was fully automated by using a Spectra-Physics SP8780 XR autosampler (San Jose, CA., USA) and a Varian 4270 integrator (Palo Alto, CA., USA). Prior to analysis of samples, the standards were used for calibration. Peak heights were used to quantify the fluorescence detector response. All samples were analyzed in duplicate.

3.1.6.2 The assessment of zinc status

3.1.6.2.1. Analysis of tissue, faecal, urine and dietary zinc contents

A flame atomic absorption spectrophotometric method was used for analysis.

Sample preparation

3.1.6.2.1.1 Tibia samples

Tibia samples were prepared as described by Clegg *et al.* (1981) (28). The samples were removed from the refrigerator and allowed to thaw at room temperature. The thawed samples were blotted dry on ashless paper. Tibias were then individually weighed (wet weight), using plastic weighing boats, on an electronic balance (Mettler BB 300) and the masses recorded. The samples were then placed in separate, labelled beakers, transferred to an oven and allowed to dry at 100°C for 24 hours. The latter was established as being the length of time required for the complete drying of tibia samples. The samples were then transferred to a desiccator and allowed to cool to room temperature. The dry weights of the tibias were then recorded.

3.1.6.2.1.2 **Kidneys, testes and liver samples**

The frozen tissue samples were brought to room temperature and then blotted (ashless paper) to remove excess moisture. Small sections (approximately 0.5 mg) of each sample were then accurately weighed (Mettler BB 300) before transfer to digestion flasks.

Wet ashing

To enable atomic absorption spectrophotometric analysis, the samples must be in a liquid form (28). The samples were then solubilized by the following method. Tissue samples (tibias, kidneys, testes and liver) were transferred to 50 ml Kjeldahl flasks, each containing 5 ml nitric acid and three glass beads. All samples were predigested for approximately 15 hours at room temperature. Samples were then heated and maintained at a constant temperature until the liquid in the digestion flask was transparent (approximately 4 hours). Samples were allowed to cool to room temperature and then transferred to 25 ml volumetric flasks. Samples were brought to volume with deionised water.

3.1.6.2.1.3 **Faecal and diet samples**

A modified wet ashing procedure of Clegg *et al.* (1981) was used to prepare diet and faecal samples for analysis. Dried powdered faecal samples weighing 0.3 g (or 1 g powdered dried diet samples) were transferred to acid washed Kjeldahl flasks containing 5 ml 65% nitric acid and 3 boiling beads. The samples were heated until a brown sediment formed on the sides of the flasks. Thereafter, 4 ml nitric acid and 2 ml 60% perchloric acid were added to each flask and heated further until the contents of the flasks turned clear (approximately 2 hours). The samples were allowed to cool and were diluted to 100 ml with 0.36% hydrochloric acid.

3.1.6.2.1.4 **Plasma samples**

Plasma samples were brought to room temperature. A volume of 0.5 ml of the plasma sample from each of the storage vials was transferred to polyethylene vials. The plasma samples were diluted fivefold by the addition of 2 ml double deionised water. The samples were carefully mixed before analysis.

Working standards were prepared using the zinc stock standard solution and a glycerol/water solution (5:95 by volume). To avoid analytical error, it was necessary that the working and reference standards and the plasma samples were of similar viscosities. The glycerol/water solution compensates for differences in viscosity between plasma samples (diluted fivefold) and zinc standards which are usually prepared using deionised water (28).

3.1.6.2.1.5 **Urine samples**

Urine samples were brought to room temperature and allowed to thaw. 10 ml of each sample was transferred to a centrifuge tube and centrifuged at 1500 rpm for 10 minutes The urine samples were diluted (1:3) with deionised water.

3.1.6.2.2 **Determination of zinc levels by flame atomic absorption spectrophotometry**

A Perkin-Elmer Model 2380 microprocessor controlled atomic absorption spectrophotometer (Perkin-Elmer Corporation, Norwalk, C.T., USA) was used.

Instrumentation procedures

A hollow cathode lamp specific for zinc was installed into the lamp compartment and the lamp control was adjusted until a lamp current of 15 mAmp (as specified on the lamp) was reached. A five minute lamp warm-up period followed.

The wavelength was set at 213.9 nm and the slit setting at 0.7 mm. The gain setting was used to optimize the wavelength and lamp current.

Prior to ignition, the air pressure was set to 280 kPa and the acetylene pressure to 140 kPa. A lean, blue, oxidizing air-acetylene flame was achieved.

Deionised water was aspirated for a few minutes to clear the burner slot of any contaminants. Working standards and samples were then analyzed.

Zinc working standards of 0.5 ppm, 1 ppm and 1.5 ppm were prepared to establish a linear standard curve. The calibration standards were analyzed after every 20 samples. Recalibration was performed if the known concentration varied by more than 5%.

Zinc concentrations of samples were calculated from values obtained directly off the FAAS.

Recovery Studies

Recovery studies were performed for each type of tissue analyzed. Samples from the control group were used for recovery studies. Before digestion, a known concentration of zinc stock standard (Spectrosol, BDH Chemicals, Poole, England) was added to the samples to enable the determination of percentage recoveries so as to standardize the digestion and atomic absorption procedures. Recoveries ranging from 96% to 99% were obtained.

3.1.6.2.2 **Determination of erythrocyte zinc-metallothionein-1 concentration**

The zinc-metallothionein-1 analysis was performed at the Rowett Research Institute, Scotland, UK, courtesy of Professor I. Bremner.

The radioimmunoassay procedure used was developed by Mehra and Bremner (1983) (112). The assay which is specific for metallothionein-1 was developed using high-titre antibodies from sheep that were immunized with a conjugate of metallothionein and rabbit immunoglobulin (112).

The procedure for the detection of antibodies and the radioimmunoassay, as described by Mehra and Bremner (1983), was as follows :

Antibodies were detected by their specific binding to ^{125}I-Zn-metallothionein-1. Titration curves were prepared as follows :

Doubling serial dilutions of test sera were prepared in gelatin buffer containing 0.2% heated normal sheep serum. These were incubated with 100 μl of ^{125}I-labelled protein in gelatin buffer containing 50 mM-2-mercaptoethanol for 18 hours at 4°C. Antigen-antibody complexes were then precipitated by the addition of 60 μl of an appropriate dilution of donkey anti-(sheep IgG) antiserum. Incubation was continued for a further 18 hours at 4°C. One millilitre of cold Tris/HC1 buffer (50 mM) was then added. The mixtures were centrifuged at 1500 g at 1°C for 1 hour. The supernatants were decanted and the precipitates counted for radioactivity in a gamma counter with NaI crystal. Percentage binding was then determined.

In the radioimmunoassay, dilutions of antisera, standards and unknowns were made in gelatin buffer. Dilutions of ^{125}I-labelled metallothionein-1 were made in gelatin buffer containing 50 mM-2-mercaptoethanol. Sufficient carrier serum from an untreated sheep was added with the antiserum to provide a final concentration of 0.1% serum. The unknown samples or solutions containing standard amounts of Zn-metallothionein-1 were preincubated with diluted antiserum in a final volume of 400 μl at 4°C for 24 to 30 hours.

Thereafter 100μl of a solution of ^{125}I-labelled metallothionein-1 containing 10 000 c.p.m. was added and further incubated at 4°C for 16 to 24 hours. Bound antibody was separated by the second antibody procedure as described above.

Sample preparation

Blood cells from 1 ml of blood were washed with saline, treated with 1 ml distilled water and lysed by repeated freeze thawing. Thereafter, 100 μl aliquots of the lysates were used for the metallothionein-1 assay, using the procedure as described above by Bremner *et al.* (1987) (17).

3.1.6.3. Determination of plasma albumin and globulin concentration

Plasma albumin and globulin levels were measured using the Kingsley method.

The reagents required for the analysis were prepared as follows :

1. Sodium sulphate (23%)

   250 g anhydrous sodium sulphate was dissolved in 600 ml distilled water by heating and stirring. The solution was then transferred to a one litre volumetric flask and brought to volume using distilled water. The flask was stoppered and maintained at a temperature of 37°C.

2. Biuret reagent

   92 ml of a saturated solution of NaOH (carbonate free) was added to a graduated cylinder (500 ml) and diluted to the 300 ml mark using distilled water. A volume of 100 ml of 1% crystalline copper sulphate was then added to the cylinder. After mixing, the solution was transferred to a bottle which was stoppered.

3. Standard protein

   5 ml of a stock plasma solution (Merck., Darmstadt, Germany) was diluted to 100 ml in a 100 ml volumetric flask with 15% NaC1 solution.

4. Diethyl ether (BDH Chemicals Ltd., Poole, Eng.)

Analytical procedure

The following procedure was carried out for each plasma sample from all four groups of rats.

A volume of 0.5 ml of plasma was transferred to a calibrated centrifuge tube and brought to 10 ml with 23% sodium sulphate solution. The contents of the tube were mixed by inversion and 2 ml of the uniform liquid was immediately transferred to a test tube labelled "Tube 1". This was used for total protein estimation. Three millilitres of ether was then added to the remaining solution in the centrifuge tube. The tube was stoppered and vigorously shaken and then

centrifuged at 3500 rpm for 5 minutes. After centrifugation, 2 ml of the clear fluid beneath the globulin precipitate was removed and transferred to a test tube labelled "Tube 2". This was used for albumin estimation. Two millilitres of standard protein was placed in a third tube and 2 ml distilled water in a fourth tube which served as the blank.

To each of the four tubes, 4 ml Biuret reagent was added, followed by 2.5 ml ether. The tubes were stoppered and vigorously shaken and then centrifuged for 5 minutes at 3 500 rpm. The solution beneath the ether layer was transferred to a photometer cuvette and the optical density was estimated using a wavelength of 520 nm. The spectrophotometer was set to zero with the blank. Total protein, albumin and globulin levels were then calculated.

3.1.6.4 Effect of vitamin B-6 deficiency on blood glucose levels

A Glucometer Elite™ (model 3904) blood glucose testing system was used to determine blood glucose levels in all four groups of rats.

The testing system comprises the Glucometer Elite™ meter, test strips, code strips, normal control and check strips.

The Glucometer Elite blood glucose testing process is based on an electrode sensor technology. Capillary action at the end of the test strip draws a small amount of blood into the reaction chamber and a reading is displayed in 60 seconds.

This system provides blood glucose results ranging from 2.2-27.8 mmol/l (40-500 mg/dl).

Method :

1. The tail of each rat was washed with soapy water rinsed thoroughly and blotted dry.

2. A test strip was inserted into the glucometer.

3. A sterile lancet was used to make a small incision at the tip of the tail to obtain a small drop of blood.

4. The test end of the test strip was held to the drop of blood until the meter signalled that the reaction chamber was filled with blood.

5. After 60 seconds, the blood glucose reading was obtained.

3.1.6.5 Determination of plasma insulin levels

A recently developed, commercially available radioimmunoassay kit was used for insulin analysis. The Amersham Biotrak™ rat insulin assay system provides a sensitive, reliable and precise quantitative determination of rat insulin.

The system uses a high specific activity [^{125}I] tracer together with a sensitive antiserum. Separation of the bound antibody from free fraction is achieved by simple magnetic separation.

3.1.6.5.1 General principles of the assay

Competition between unlabelled insulin and a fixed amount of ^{125}I-labelled human insulin for a limited number of binding sites is the basis of this assay procedure. With fixed quantities of antibody and radioactive ligand, the amount of radioactive ligand bound by the antibody will be inversely proportional to the concentration of added non-radioactive ligand.

The antibody bound insulin is then reacted with the second antibody reagent which contains the second antibody that is bound to magnetizable polymer particles. The antibody bound fraction is then separated by magnetic separation. Measurement of the radioactivity in the pellet enables the amount of labelled insulin in the bound fraction to be calculated. The concentration of unlabelled insulin in the sample is then determined by interpolation from a standard curve.

The assay pack contained the following assay components :

Assay buffer : Assay buffer concentrate 10ml. On dilution to 100 ml this gives 0.025 M phosphate buffer pH 7.5 containing 0.1% sodium azide.

Standard : Rat insulin, lyophilized. On reconstitution this bottle contains rat insulin at a concentration of 50 ng/ml.

Antiserum : Guinea pig anti-insulin serum, lyophilized.

Tracer : [^{125}I] human insulin 46 kBq, 1.25 μCi, lyophilized.

Amertex-M second antibody reagent : Sheep anti-guinea pig serum coated onto magnetizable polymer particles containing sodium azide.

3.1.6.5.2 **Radioimmunoassay procedure**

Reagent preparation

All reagents were allowed to equilibrate to room temperature.

Assay buffer : The bottle containing the assay buffer concentrate was warmed to 40°C until the gel-like material melted. The buffer concentrate was then transferred to a 100 ml measuring cylinder, diluted to 100 ml with deionised water and mixed. The assay buffer was used to reconstitute all other components.

Antiserum : The antiserum was reconstituted with 12.5 ml assay buffer.

Tracer : The tracer was reconstituted with 12.5 ml assay buffer.

Preparation of working standards

Standard : 2.0 ml assay buffer was added to the standard bottle and the stopper replaced. The contents were mixed by inversion and swirling.

Working standards were prepared as follows :

1. 9 polypropylene tubes were labelled 0.01, 0.02, 0.039, 0.078, 0.158, 0.312, 0.625, 1.25 and 2.5 respectively.
2. 500 μl of assay buffer was pipetted into each tube.
3. 500 μl of stock standard (50 ng/ml) was pipetted into the 2.5 tube and thoroughly vortex mixed.
4. 500 μl was transferred from the 2.5 tube to the 1.25 tube and thoroughly vortex mixed.
5. This serial dilution was successively repeated with the remaining tubes.
6. 100 μl aliquots from each serial dilution gave rise to 9 standard levels of rat insulin ranging from 0.0-1 to 2.5 ng/tube.

Assay protocol

1. Polypropylene tubes were labelled in duplicate for total counts (TC), non-specific binding (NSB), zero standard (Bo), standards and samples.

2. 200 μl assay buffer was pipetted into the NSB tubes.

3. 100 μl assay buffer was pipetted into the Bo tubes.

4. Starting with the most dilute, 100 μl of each standard was pipetted into the appropriately labelled tubes.

5. 100 μl of each unknown sample was pipetted into appropriately labelled tubes.

6. 100 μl of antiserum was pipetted into all tubes except NSB and TC.

7. 100 μl of tracer was pipetted into all tubes. The TC tubes were stoppered and put aside for counting.

8. All tubes were thoroughly vortex mixed. The tubes were covered and incubated for 4 hours at room temperature.

9. The bottle containing the Amertex-M second antibody reagent was gently swirled to ensure a homogenous suspension. To each tube except the TC was added 250μl of the second antibody reagent. All tubes were vortex mixed and incubated for 10 minutes at room temperature.

10. The antibody-bound fraction was separated by magnetic separation. The rack was attached to the Amertex-M separator base such that all tubes were in contact with the base plate. After 15 minutes, separation was achieved and the supernatant discarded, without removing the rack from the separator base. The separator was kept inverted and the tube placed on a pad of absorbent tissue and allowed to drain for 5 minutes.

11. On completion of magnetic separation, the rims of the inverted tubes were blotted on the tissue pad to remove any adhering liquid.

12. The radioactivity present in each tube was determined by counting for at least 60 seconds in a gamma scintillation counter.

13. Plasma insulin concentrations of samples were then determined by interpolation from a standard curve.

3.2 EXPERIMENT 2

INTRODUCTION

It was decided that in order to obtain a clearer insight into the relationship between picolinic acid levels and tissue zinc status and to determine the effect of a vitamin B-6 deficiency on picolinic acid levels a second experiment would be performed. In addition to measuring picolinic acid levels, the zinc status of various tissues was determined by measuring their zinc-metallothionein content.

Erythropoietin levels were determined by radioimmunoassay. The vitamins B-6 status of all rats was measured as in experiment 1.

Blood pressure measurements using the tail cuff method, were performed once a week from the first week to the end of the experimental period.

3.2.1 Experimental animals

Only male Wistar rats were used in this experiment. The necessary ethical approval was obtained prior to the commencement of the experiment.

Thirty six male weanling rats were divided into four groups of 9 rats each viz., a control group receiving the normal AIN-76™ Purified Diet (as in experiment 1) with 7 mg PN.HC1/kg diet; a partially deficient group receiving the same diet with 1 mg PN.HC1/kg diet; a deficient group receiving the diet with no PN.HC1 and a pair-fed group receiving the control diet.

The preparation of the diet and the housing, feeding and monitoring of the weight and food consumption of the animals were as in experiment 1.

However, the duration of this experiment was extended by 2 weeks i.e. 8 weeks compared to the 6 week duration of experiment 1.

Tissue sampling : Tissue isolation and storage procedures were as for experiment 1.

3.2.2 Analytical procedures

The assessment of the vitamin B-6 status of the rats was done as in experiment 1 (courtesy of Prof. Ubbink University of Pretoria, SA).

3.2.2.1 Analysis of tissue zinc- metallothionein content

The silver saturation method as developed by Scheuhammer and Cherian (1991) was used to measure tissue metallothionein (155).

Slight modifications to the method were made to best suit the tissues and laboratory equipment used.

The principle of this method relies on the high Ag^+ binding capacity and heat stability of MT compared to other cytosolic ligands. Excess Ag^+ is added to tissue supernatant. This displaces all other metals from MT. In addition Ag^+ binds to MT and other cytosolic proteins and ligands. Haemoglobin is then added to the sample which removes silver from all ligands except MT. The sample is then heated which results in precipitation of haemoglobin-bound Ag^+. The addition of haemoglobin and the heating steps are repeated resulting in supernatant containing Ag^+ exclusively bound to MT. The concentration of MT can be determined by the amount of silver in the final supernatant (155).

3.2.2.1.1 Reagents and sample preparation

The following solutions were prepared :

Sucrose	-	(0.25M)
Glycine	-	(0.5M). The pH was adjusted to 8.5 with 4N NaOH
$AgNO^3$	-	(9.27mM; 1000 μg Ag^+/ml). Working solutions (20 μg Ag^+/ml glycine buffer) were made weekly

Red Blood cell

haemolysate - 20 ml of rat blood was added to 40 ml of heparinized isotonic KCl solution (1.15% w/v) and centrifuged at 1800 rpm for 5 min. The pellet was resuspended in 60 ml of 1.15% KCl solution and centrifuged at 1800 rpm for a further 5 minutes. The washing and centrifugation steps were repeated once. The RBC pellet was lysed in 25 ml of 30 mM Tris base buffer, pH 8.0 at room temperature. The lysate was then centrifuged at 11 500 rpm for 10 minutes and the supernatant was divided into aliquots (2 ml) and stored at -20°C.

Sample preparations - Liver, kidney and pancreas tissues were analysed. Briefly, 0,5 g of the tissue (minced) was placed into a centrifuge tube. Thereafter, 2 ml of the sucrose solution was added and the sample was homogenized at high speed for 90 seconds. The homogenate was then centrifuged in a refrigerated centrifuge at 18 000 rpm, for 25 minutes. The supernatant was stored in sealed plastic test tubes (-20°C) until required.

3.2.2.1.2 **Procedure**

1. 200 ml of tissue supernatant was added into a glass test tube (all analyses were performed in duplicate).

2. 0.6 ml of glycine buffer was added to the sample.

3. 0.5 ml of the 20 μg Ag^{+}/ml solution was then added and the solution was incubated at room temperature for 5 minutes.

4. 100 μl haemolysate was added and the solution was mixed thoroughly.

5. The sample was then heated in a boiling water bath for 2 minutes. (After the heating step it was ensured that no red colour was present in the solution. A red colour would indicate incomplete removal of haemoglobin).

6. The sample was then centrifuged for 5 minutes at 2 800 rpm.

7. Another 100 ml haemolysate was then added and the heating and centrifugation steps repeated. The final volume of the sample was 1.5 ml.

8. The resulting supernatant was then transferred to plastic Eppendorf micro-test tubes and centrifuged for 5 minutes at 15 500 rpm.

9. The silver concentration of the final supernatant was measured by atomic absorption spectrophotometry using an air-acetyle flame.

10. A standard curve for silver was generated using known amounts of silver in glycine buffer.

The amount of MT in the sample was calculated using the following equation.

$$\mu g \text{ MT/g tissue} = \frac{(C_{Ag} - C_{BKG}) \times 3.55 \times V_T \times SDF}{SV}$$

C_{Ag} = concentration of silver in final supernatant
C_{BKG} = background reading in supernatant of blank
V_T = total volume in assay sample
SDF = sample dilution factor
SV = sample volume used in the assay

Note : (a) 1 μg Ag represents 3.55 μg MT

3.2.2.2 Determination of Picolinic acid concentration

The concentration of pancreatic picolinic acid was determined by minor modification of the method used by Evans and Johnson (46).

3.2.2.2.1 Procedure

1. The pancreatic tissue was homogenised in 1,5 ml of deionised water.

2. 1 ml of 5M H_2SO_4 was then added to the homogenate which was thoroughly mixed and centrifuged at 15 000 rpm.

3. The supernatant was then adjusted to pH 7 using $Ba(0H)_2$ and centrifuged at 15 000 rpm.

4. The clear supernatant was removed and applied to a 1 x 5 cm column of DOWEX 1 in the formate form.

5. The column was then rinsed with 5 ml deionised water and a linear gradient mixer was then attached to the column. The mixer contained 50 ml deionised water in the mixing chamber and 50 ml 0.1 M formic acid in the other chamber.

6. Fractions of 2 ml were collected.

7. Those fractions that showed an ultraviolet-absorbing substance (254 nm) and corresponded with the elution volume of picolinic acid were pooled and the volume determined.

8. The absorbence at 265 nm of a 1 ml aliquot of the pooled fraction was determined with a spectrophotometer. The concentration of picolinic acid was calculated from the molar extinction coefficient of picolinic acid at 265 nm.

Recovery Studies

Recovery studies were performed using pancreatic tissue samples from the control group. A known concentration of picolinic acid (99%) was added to the samples prior to application to the column to determine the percentage recoveries and to standardise the analytical procedure. Recoveries ranging from 95% to 98% were obtained.

3.2.2.3 Determination of Serum Erythropoietin levels by radioimmunoassay

The EPORIA Erythropoietin Double Antibody RIA Kit (Ramco Laboratories, Inc., Texas) was used for erythropoietin analysis.

3.2.2.3.1 Principle of the test

The basic principle of radioimmunoassay applies i.e. there is competition between a radioactive and a non-radioactive antigen for a fixed number of antibody binding sites. The amount of (^{125}I) - labelled erythropoietin bound to the antibody is inversely proportional to the amount of erythropoietin (EPO) present in the test sample. The free and bound antigen are separated by using an accelerated double antibody system followed by centrifugation. The precipitate of the antibody - bound tracer complex remains in the tube and can be measured in a gamma counter after the supernatant is decanted.

The assay kit contained the following reagents :

1. Erythropoietin Standards : One vial containing 5 ml of OmU/ml human EPO and six vials, 1 ml each, containing approximate concentrations of 5, 10, 25, 50, 100 and 200 mU/ml human erythropoietin in protein-based buffer with sodium azide as preservative.

2. Erythropoietin (^{125}I) Reagent : One vial, 11 ml, containing 185 kBq of [^{125}I] - labelled EPO in protein-based buffer with sodium azide as a preservative.

3. Erythropoietin Antiserum : One vial, containing rabbit anti-human EPO serum in protein-based buffer with sodium azide as a preservative.

4. Erythropoietin Precipitating Reagent : One bottle, 110 ml, containing goat anti-rabbit gamma globulin serum in a buffer with polyethylene glycol as a precipitating acid and sodium azide as a preservative.

5. Erythropoietin Controls : Two vials, 1 ml each, containing low and high levels of human EPO in protein based buffer with sodium azide as preservative.

3.2.2.3.2 **Assay Procedure**

All reagents were allowed to reach room temperature and thoroughly mixed before use. Standards, controls and unknowns were assayed in duplicate.

1. Test tubes were labelled and arranged in duplicate for total counts, non-specific binding (NSB), standards, controls and unknown samples.

2. 100 μl of the standards, controls and unknowns were added to appropriate tubes. To the NSB tubes, 200 μl of the OmU/ml standard was added.

3. 100 μl of EPO antiserum was added to all tubes except the NSB and total count tubes.

4. All tubes were then vortexed, covered and incubated at room temperature for 4 hours.

5. 100 μl of EPO (I^{-125}) reagent was added to all tubes.

6. All tubes were vortexed, covered and incubated at room temperature for 16 hours.

7. 1 ml of precipitating reagent was added to all tubes except total counts and then all tubes were vortexed.

8. All tubes were incubated at room temperature for 30 minutes.

9. All tubes, except the total counts, were then centrifuged for 20 minutes at 3000 rpm.

10. All tubes, except total counts, were then decanted by simultaneous inversion with a sponge rack into a radioactive waste receptacle. The tubes were allowed to drain on absorbent paper for 30 seconds and then gently blotted to remove any droplets adhering to the rims before returning them to the upright position.

11. All tubes were counted in a gamma-counter for one minute to determine the radioactivity present.

12. Erythropoietin concentrations of samples were then determined from a standard curve of the % B/B_0 (percentage bound of the standards) against the EPO concentrations on semi-log graph paper.

3.2.2.4 Determination of blood pressure of rats

The IITC Model 31 computerised blood pressure monitor was used to determine the blood pressure of the rats in the present study. It works with an IITC hardware BP system to determine blood pressure and heart rate. The system uses an automatic scanner and pump in addition to sensing cuff and amplifier to measure and count the heart beat pulses in the animals tail. The results are displayed as data plots and summary data on a computer screen (176).

The IITC Model 31 employs a photoelectric sensor in the tail sensing cuff for pulse detection, thereby permitting measurements at the lowest ambient temperature of any such device available. This low ambient reduces heat stress on the animal. Therefore the Model 31 yields results that are close to the values obtained by cannulation (176).

Both heat stress and temperatures below 25°C modifies the blood pressure of rats. This effect can be prevented by warming the animal slightly with a table lamp. The holder also needs to be warmed since the animal is very sensitive to any difference in temperature (176).

Completely untrained animals are difficult to test because they move a lot and cause a noisy base line. The rats in this study were therefore trained for one hour for a few days before being tested.

The following procedure was followed to acclimatize the rats and record the blood pressure measurements :

1. The rat was placed in its holder a few minutes before beginning the test, to allow it to acclimatize.

2. The tail gate was removed from the empty holder.

3. The head gate was positioned to accommodate the length of the animal.

4. The holder was preheated by placing it under a table lamp for a few minutes.

5. The rat was placed in front of the open end of the tube and entered the holder. The tail gate was then slipped over the tail.

6. The animal was warmed with a lamp while in the holder.

7. The tail cuff was then slipped over the tail and attached.

Set up, calibration and measurement procedures for the IITC Model 31 computerised blood pressure monitor were then followed according to the instruction manual and the blood pressure measurement for each rat was recorded.

3.3 Statistical analysis

Individual analysis of variance (ANOVA) was performed on growth, food consumption and faecal and urinary zinc excretion for each week (for differences between the groups at each week). Duncans multiple range tests were used for multiple comparisons. These tests were also used to compare blood pressure levels, tissue zinc, plasma protein, plasma PLP, plasma insulin, plasma zinc, erythrocyte zinc-metallothionein, tissue metallothionein, pancreatic picolinic acid and serum erythropoietin concentrations among the four groups of rats.

All statistical analysis were done in collaboration with Ms E Gouws (Institute of Biostatistics of the Medical Research Council of South Africa).

All data are presented as means $\pm$ SD.

CHAPTER FOUR

RESULTS

4.1 EXPERIMENT 1

4.1.2 The effect of vitamin B-6 deficiency on food consumption

The mean weekly food consumption for all four groups, over the six week period, is shown in Table 5. The food consumption in the deficient groups was observed to increase in the first two weeks of the experiment. Thereafter a gradual decline in food intake was apparent in these two groups (Figure 9). The control group, on the other hand, showed an increase in food intake in the first four weeks of the experiment and this was followed by a gradual decline in food consumption in the final two weeks of the experiment. The food consumption of the vitamin B-6 deficient and the vitamin B-6 deficient-PA-supplemented groups was significantly lower ($p < 0.0001$, Figure 9) than the control group from the second week to the sixth week. The food consumption of the vitamin B-6 deficient group decreased significantly from 73.44 ± 13.38g at the end of the second week to 51.55 ± 11.72 g at the end of the experimental period and in the vitamin B-6 deficient-PA-supplemented group from 73.33 ± 12.31 g to 59.77 ± 15.20 g. There was no significant difference in food consumption between the vitamin B-6 deficient and the vitamin B-6 deficient-PA-supplemented groups.

In all the tables which follow, "deficient (PA)" represents the vitamin B-6 deficient-PA-supplemented group and "deficient" represents the vitamin B-6 deficient group.

Table 5 : The effect of vitamin B-6 deficiency on weekly food consumption (g).

(Mean ± S.D.)

GROUP	n	WEEK 1	WEEK 2	WEEK 3	WEEK 4	WEEK 5	WEEK 6
Control	9	68.44[a] ±9.90	92.22[a] ±7.17	109.44[a] ± 10.71	129.12[a] ± 10.66	119.11[a] ± 11.40	114.0[a] ± 13.04
Deficient (PA)	9	65.55[a] ± 9.48	73.33[b] ± 12.31	63.66[b] ± 16.25	67.66[b] ± 14.57	60.33[b] ± 11.36	59.77[a] ± 15.20
Deficient	9	62.22[a] ± 10.05	73.44[b] ± 13.38	66.44[b] ± 13.54	67.66[b] ± 14.51	53.77[b] ± 9.37	51.55[a] ± 11.72

Mean values for each week not followed by the same superscript letter denote a significant difference ($p < 0.05$).

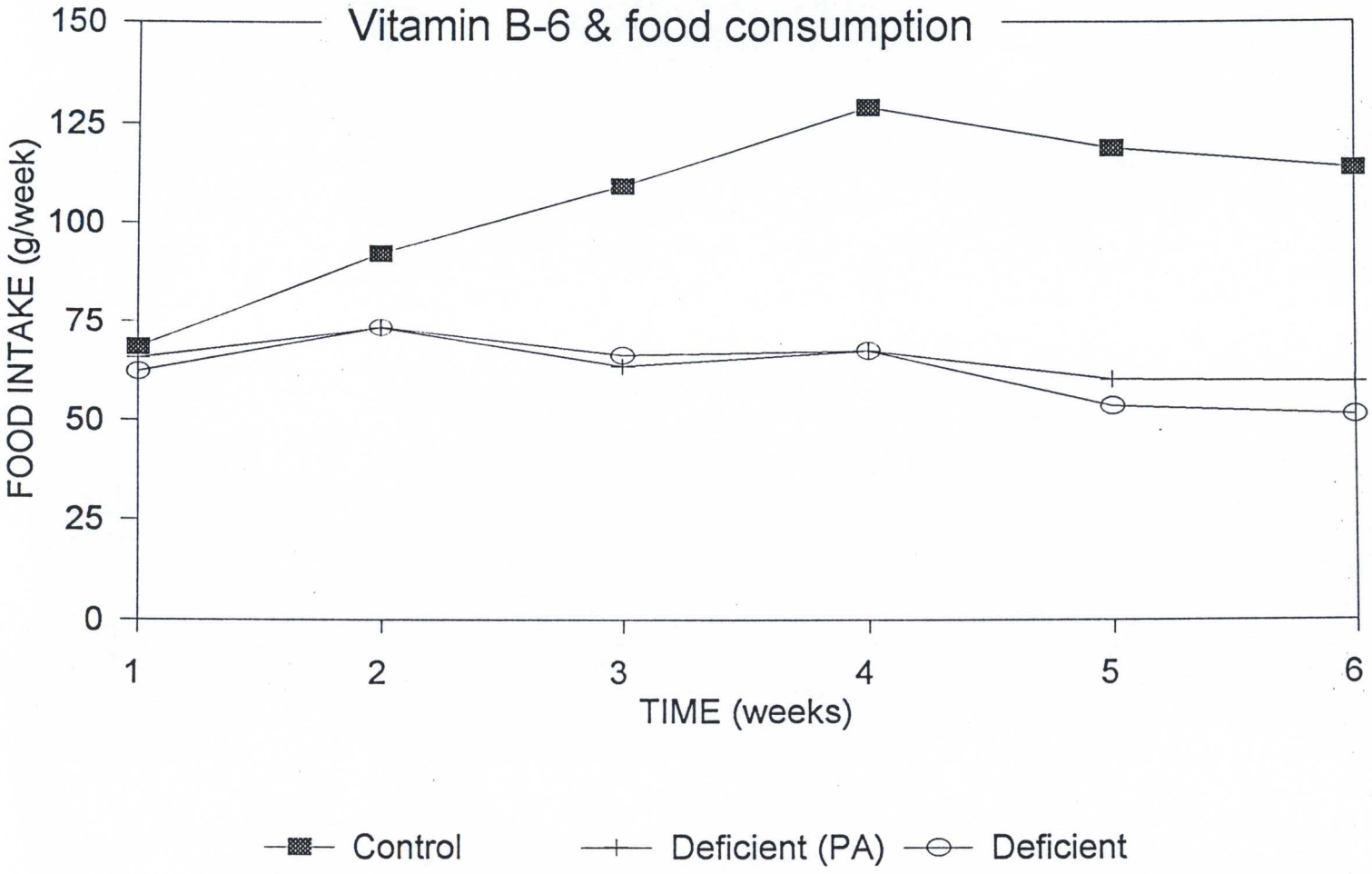


Figure 9 : Vitamin B-6 and food consumption

4.1.2 The effect of vitamin B-6 deficiency on growth

Changes in mean body mass per week, for the four groups are shown in Table 6. There was no significant difference in the mean weights among the four groups at the beginning of the experiment. In keeping with the food intake pattern, none of the experimental groups showed significant differences in their mass when compared with the control group at the end of the first week. However, by the end of the second week the mean body mass of the vitamin B-6 deficient (115.77 $\pm$ 20.96 g) and the vitamin B-6 deficient-PA-supplemented (114.55 $\pm$ 160.04 g) groups were significantly lower than the control (135.88 $\pm$ 12.50 g) and pair-fed (134.88 $\pm$ 11.81 g) groups ($p < 0.001$). There was no significant difference in body mass between the vitamin B-6 deficient and the vitamin B-6 deficient-PA-supplemented groups or between the control and pair-fed groups. This pattern continued until the end of the third week. By the fourth week the mean mass of the pair-fed group (168.22 $\pm$ 10.28 g) was significantly lower than the control group (209.00 $\pm$ 10.88 g), while the vitamin B-6 deficient (128.44 $\pm$ 25.24 g) and the vitamin B-6 deficient-PA-supplemented (128.22 $\pm$ 24.19 g) groups showed significantly lower body weights compared with both the control and pair-fed groups ($p < 0.001$). This trend continued to the end of the six-week experimental period.

Table 6 : The effect of vitamin B-6 deficiency on growth. (Mean ± S.D., body mass in grams).

GROUP	DAY 1	WEEK 1	WEEK 2	WEEK 3	WEEK 4	WEEK 5	WEEK 6
Control	55.33[a] ± 7.08	90.77[a] ± 9.53	135.88[a] ± 12.50	169.33[a] ± 10.97	209.00[a] ± 10.88	232.22[a] ± 13.93	252.00[a] ± 15.41
Pair-fed	57,33[a] ± 8.35	93.66[a] ± 13.17	134.88[a] ± 11.81	151.11[a] ± 10.70	168.22[b] ± 10.28	181.33[b] ± 10.19	191.66[b] ± 11.82
Deficient (PA)	60.66[a] ± 9.48	92.11[a] ± 11.82	114.55[b] ± 16.04	121.22[b] ± 19.16	128.22[c] ± 24.19	132.88[c] ± 26.28	132.66[c] ± 27.59
Deficient	57.77[a] ± 8.04	88.44[a] ± 12.98	115.77[b] ± 20.96	122.22[b] ± 20.77	128.44[c] ± 25.24	130.33[c] ± 29.26	129.44[c] ± 30.06

Means values not followed by the same superscript letter denote a significant difference ($p < 0.05$).

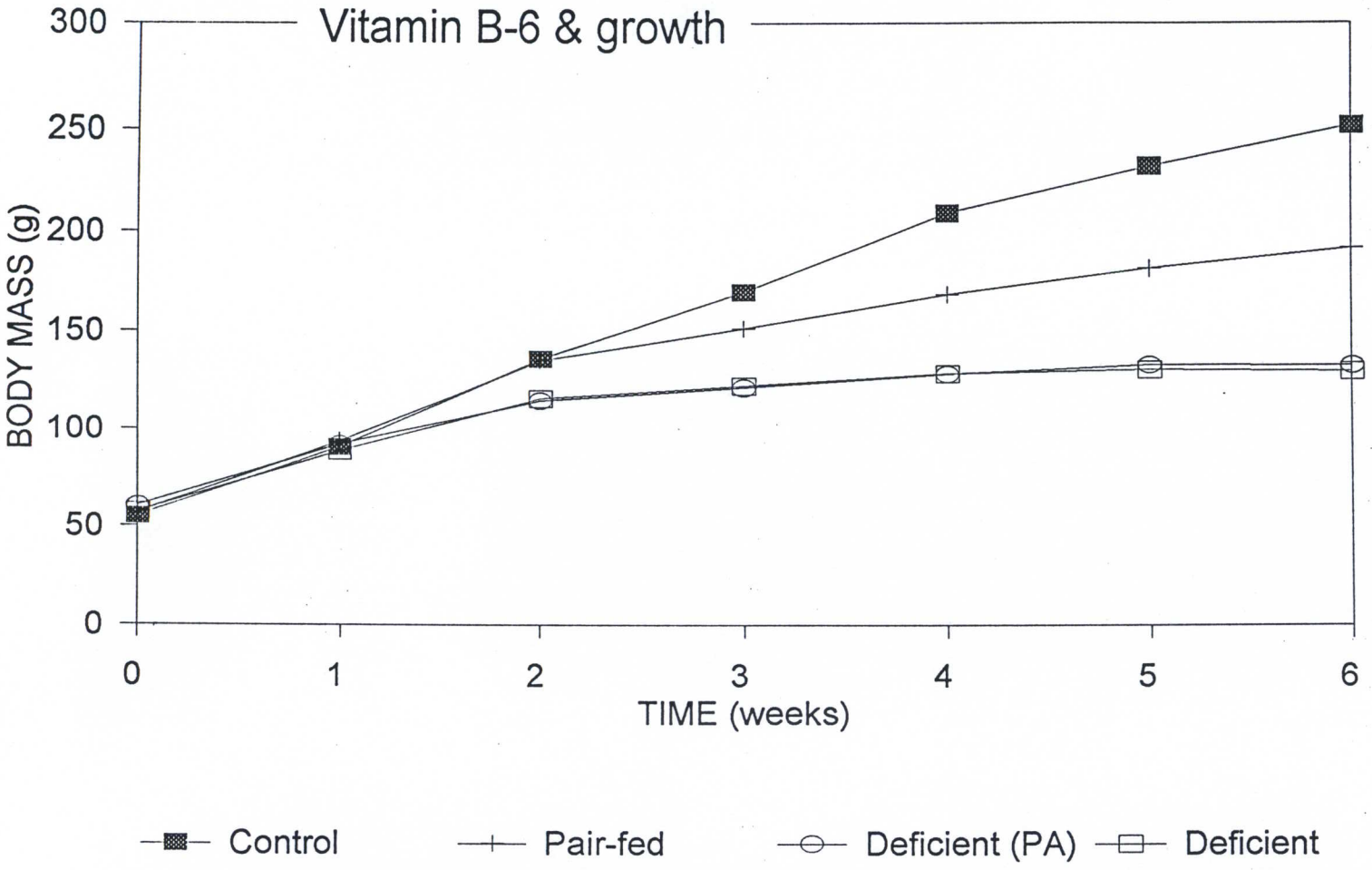


Figure 10 : Vitamin B-6 and growth

4.1.3 The effect of vitamin B-6 deficiency on plasma and tissue zinc concentrations

4.1.3.1 Zinc concentration in plasma

As shown in Table 7, the vitamin B-6 deficient (1.35 $\pm$ 0.08 μg/ml) group had a significantly lower plasma zinc concentration compared with pair-fed (2.03 $\pm$ 0.07 μg/ml) and control (1.99 $\pm$ 0.06 μg/ml) groups ($p < 0.0001$). No significant difference in plasma zinc levels was apparent between the control and pair-fed rats or between the vitamin B-6 deficient and the vitamin B-6 deficient-PA-supplemented groups.

4.1.3.2 Zinc concentration in the testes

There was no significant difference in zinc levels of the testes between the control (46.11 $\pm$ 2.20 μg/g) and vitamin B-6 deficient-PA-supplemented (45.66 $\pm$ 5.74 μg/g) groups (Table 7). Both these groups had significantly higher zinc concentrations compared with the vitamin B-6 deficient group (38.77 $\pm$ 4.79 μg/g) ($p < 0.001$). In addition, the pair-fed group (52.11 $\pm$ 2.42 μg/g) showed a significantly higher zinc level compared with the other three groups ($p < 0.0001$).

4.1.3.3 Zinc concentration in the liver

The vitamin B-6 deficient (41.00 ± 3.31 μg/g) and the vitamin B-6 deficient-PA-supplemented (45.66 ± 6.80 μg/g) groups had significantly lower liver zinc concentrations compared with both the control (57.00 ± 8.91 μg/g) and pair-fed (61.66 ± 7.48 μg/g) groups ($p < 0.0001$, Table 7). There was no significant difference in liver zinc levels between the control and pair-fed groups or between the two vitamin B-6 deficient groups

4.1.3.4 Zinc concentration in the kidney

The control group (64.11 ± 8.20) had significantly higher kidney zinc concentration compared with the other three groups ($p < 0.0001$, Table 7). The pair-fed (50.11 ± 5.27 μg/g) and vitamin B-6 deficient-PA-supplemented (47.66 ± 3.83 μg/g) groups showed significantly higher zinc levels compared with the vitamin B-6 deficient (38.44 ± 3.43 μg/g) group. There was however, no significant difference in kidney zinc levels between the pair-fed and vitamin B-6 deficient-PA-supplemented groups.

4.1.3.5 Zinc concentrations in the tibia

Tibia zinc levels were significantly lower in the vitamin B-6 deficient group (435.22 ± 47.47 μg/g) compared with the control (485.66 ± 27.19 μg/g), pair-fed (494.77 ± 46.97 μg/g) and vitamin B-6 deficient-PA-supplemented (474.55 ± 30.17 μg/g) groups ($p > 0.015$, Table 7). No significant difference in tibia zinc concentration was observed among the latter three groups.

Summary of tissue and plasma zinc concentrations

It can be observed from the above results that the zinc levels in the liver, kidney and plasma were significantly lower in the vitamin B-6 deficient and vitamin B-6 deficient-PA-supplemented groups compared to the control and pair-fed groups ($p < 0.0001$). Supplementation with PA during vitamin B-6 deficiency increased zinc concentrations in the tibia, testes and kidneys.

Table 7. The effect of vitamin B-6 deficiency on plasma (μg/ml) and tissue zinc (μg/g) levels (means $\pm$SD)

GROUP	n	Tibia	Testes	Liver	Kidney	Plasma
Control	9	485.66^{a} $\pm$ 27.19	46.11^{a} $\pm$ 2.20	57.00^{a} $\pm$ 8.91	64.11^{a} $\pm$ 8.20	1.99^{a} $\pm$ 0.06
Pair-fed	9	494.77^{a} $\pm$ 46.97	52.11^{b} $\pm$ 2.42	61.66^{a} $\pm$ 7.48	50.11^{b} $\pm$ 5.27	2.03^{a} $\pm$ 0.07
Deficient (PA)	9	474.55^{a} $\pm$ 30.17	45.66^{a} $\pm$ 5.74	45.66^{b} $\pm$ 6.80	47.77^{b} $\pm$ 3.83	1.37^{b} $\pm$0.08
Deficient	9	435.22^{b} $\pm$ 47.47	38.77^{c} $\pm$ 4.79	41.00^{b} $\pm$ 3.31	38.44^{c} $\pm$ 3.43	1.35^{b} $\pm$ 0.08

Mean values for each group not followed by the same superscript letter denote a significant difference ($p < 0.05$).

4.1.4 The effect of vitamin B-6 deficiency on plasma pyridoxal-5-phosphate and zinc-metallothionein-1 levels

4.1.4.1 Plasma PLP

The vitamin B-6 deficient (13.95 ± 4.08 nmol/1) and the vitamin B-6 deficient-PA-supplemented (16.60 ± 5.60 nmol/1) groups had significantly lower plasma PLP levels compared with the control (373.33 ± 166.39 nmol/1) and pair-fed (421 ± 150.64 nmol/1) groups ($p < 0.0001$, Table 8, Figure 11). However, no significant difference in plasma PLP concentrations were observed between the control and pair-fed groups or between the two vitamin B-6 deficient groups.

4.1.4.2 Zinc-metallothionein-1

Zinc-metallothionein-1 levels were expressed as per ml of erythrocytes and per mg of haemoglobin (Table 8). Whether expressed as per ml erythrocytes or per mg haemoglobin the control group had increased erythrocyte Zn-MT-1 levels compared to the other three groups ($p < 0.0004$).

No significant difference in erythrocyte Zn-MT-1 levels existed among the pair-fed, vitamin B-6 deficient and vitamin B-6 deficient-PA-supplemented groups.

Table 8 : The effect of vitamin B-6 deficiency on plasma PLP and erythrocyte Zn-MT-1 concentrations (means ± SD).

GROUP	n	PLASMA PLP (nmol/1)	RBC Zn-MT-1 (ng/ml)	Hb Zn-MT-1 (ng/mgHb)
Control	9	373.33[a] ± 166.39	53.79[a] ± 15.47	0.57[a] ± 0.20
Pair-fed	9	421.00[a] ± 150.64	29.49[b] ± 6.37	0.25[b] ± 0.06
Deficient (PA)	9	16.60[b] ± 5.69	33.60[b] ± 14.66	0.38[b] ± 0.19
Deficient	9	13.95[b] ± 4.08	30.09[b] ± 9.51	0.33[b] ± 0.13

Mean values not followed by the same superscript letter are significantly different ($p < 0.05$).

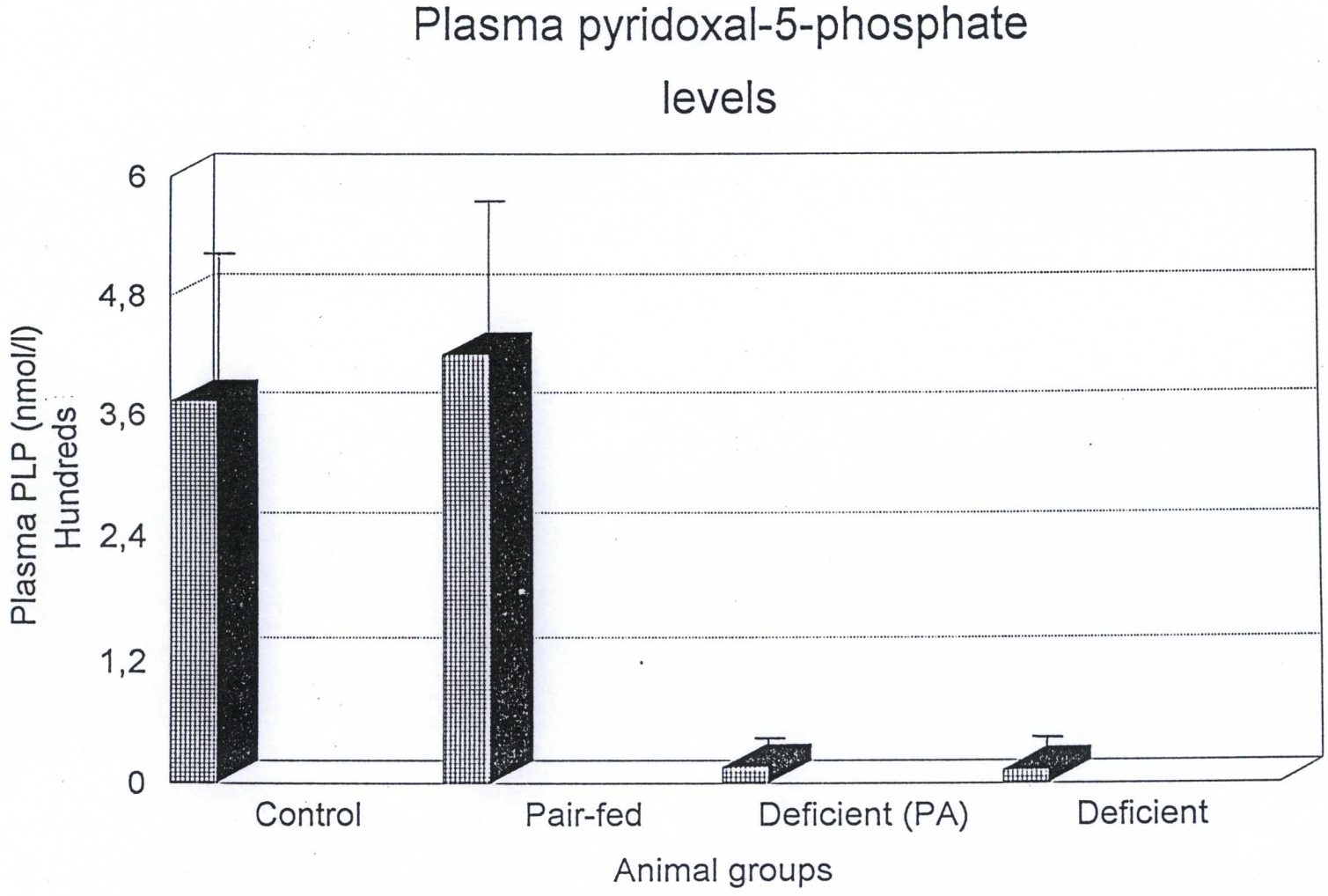


Figure 11 : Plasma pyridoxal-5-phosphate levels.

4.1.5 The effect of vitamin B-6 deficiency on haemoglobin levels

The mean haemoglobin concentrations of the four groups are given in Table 9. The vitamin B-6 deficient (8.71 ± 0.81 g/dl) and vitamin B-6 deficient-PA-supplemented (8.33 ± 1.07 g/dl) groups had significantly lower ($p < 0.0001$) haemoglobin levels compared with the control (9.8 ± 1.49 g/dl) and pair-fed (10.29 ± 1.35 g/dl) groups. No significant difference existed between the control and pair-fed groups or between the vitamin B-6 deficient and vitamin B-6 deficient-PA-supplemented groups.

Table 9 : The effect of vitamin B-6 deficiency on haemoglobin concentration (means ± SD).

GROUP	n	HAEMOGLOBIN (g/dl)
Control	9	9.80 ± 1.49^{a}
Pair-fed	9	10.29 ± 1.35^{a}
Deficient (PA)	9	8.33 ± 1.07^{b}
Deficient	9	8.71 ± 0.81^{b}

Mean values not followed by the same superscript letter are significantly different ($p < 0.05$).

4.1.6 The effect of vitamin B-6 deficiency on plasma insulin and blood glucose levels

The mean insulin concentrations of the four groups are shown in Table 10. Plasma insulin levels in the vitamin B-6 deficient (0.81 $\pm$ 0.27 ng/ml) and the vitamin B-6 deficient-PA-supplemented (0.65 $\pm$ 0.15 ng/ml) groups were significantly lower than in the control (1.91 $\pm$ 0.58 ng/ml) and pair-fed (2.62 $\pm$ 0.5 ng/ml) groups ($p < 0.0001$). The control group showed significantly lower plasma insulin concentrations compared with the pair-fed group ($p < 0.0001$). No significant difference in insulin levels existed between the vitamin B-6 deficient and the vitamin B-6 deficient-PA-supplemented groups.

However, no significant difference in blood glucose concentration existed among the four groups (Table 10).

Table 10 : The effect of vitamin B-6 deficiency on plasma insulin and blood glucose concentrations (means ± SD).

GROUP	INSULIN (ng/ml)	BLOOD GLUCOSE mmol/1
Control	1.91 ± 0.58^{a}	5.81 ± 1.09^{a}
Pair-fed	2.62 ± 0.50^{b}	5.97 ± 0.60^{a}
Deficient (PA)	0.65 ± 0.15^{c}	5.87 ± 0.24^{a}
Deficient	0.81 ± 0.27^{c}	5.79 ± 0.70^{a}

Mean values for each group not followed by the same superscript letter are significantly different ($p < 0.05$)

4.1.7 Total plasma protein, albumin and globulin concentrations

The mean values of plasma albumin, globulin and total protein are shown in Table 11. Plasma albumin, globulin and total protein levels were observed to be significantly lower in the vitamin B-6 deficient and vitamin B-6 deficient-PA-supplemented groups than in the control and pair-fed groups ($p < 0.001$). No significant difference in plasma albumin, globulin or total protein concentrations existed between the vitamin B-6 deficient and the vitamin B-6 deficient-PA-supplemented groups or between the pair-fed, and control groups.

Table 11 : Plasma albumin, globulin and total protein concentrations (g/dl). The values represent means ± SD.

GROUP	Total protein	Albumin	Globulin
Control	6.81± 0.07[a]	3.83± 0.11[a]	2.98± 0.16[a]
Pair-fed	6.53± 0.09[a]	3.71± 0.15[a]	2.81± 0.15[a]
Deficient (PA)	4.95± 0.15[b]	2.85± 0.17[b]	2.10± 0.05[b]
Deficient	4.88± 0.19[b]	2.78± 0.18[b]	2.08± 0.05[b]

Mean values not followed by the same superscript letter are significantly different ($p < 0.05$).

4.1.8 The effect of vitamin B-6 deficiency on dietary zinc intake, faecal and urinary zinc excretion and zinc balance (μg/week)

The mean dietary zinc intake, faecal and urinary zinc excretion and zinc balance per week are shown in Tables 12(a) to 12(f) and each of these is discussed below.

4.1.8.1. Dietary zinc intake

The control group showed a significantly higher dietary zinc intake compared with the pair-fed, vitamin B-6 deficient and vitamin B-6 deficient-PA-supplemented groups from the second week to the end of the experimental period ($p < 0.0001$). There was no significant difference in dietary zinc intake among the latter three groups.

4.1.8.2 Faecal zinc excretion

No significant difference in faecal zinc excretion (expressed as a percentage of their dietary zinc intake) was observed among the four groups.

4.1.8.3 Urinary zinc excretion

Whilst there were no significant differences in the dietary zinc levels and levels of zinc excretion in the faeces among the pair-fed and the two deficient groups, the urinary zinc excretion was significantly greater in the deficient groups as compared to the pair-fed groups ($p < 0.001$). The urinary zinc excretory levels in the vitamin B-6 deficient and the vitamin B-6 deficient-PA-supplemented groups were significantly greater ($p < 0.0001$) than the control group from the third to the fifth weeks of the experiment.

4.1.8.4 Zinc Balance

All four groups showed a positive zinc balance throughout the experimental period. However, the control group had a significantly higher zinc balance compared with the pair-fed, vitamin B-6 deficient and the vitamin B-6 deficient-PA-supplemented groups ($p < 0.001$). No significant difference in zinc balance existed among the latter three groups.

The dietary intake of zinc by the sixth week of the experiment of the vitamin B-6 deficient group was the same as the pair-fed group but the faecal and urinary zinc excretion was greater in the former group, though the changes did not reach significance. This would mean that zinc absorption was lower in the deficient group. PA supplementation increased the total food intake and hence the zinc intake in the vitamin B-6 deficient PA-supplemented group when compared to the vitamin B-6 deficient group in the final two weeks of the experiment. However, since this difference did not reach significance there was no difference in zinc balance between the deficient groups.

Table 12(a): The effect of vitamin B-6 deficiency on zinc intake, faecal and urinary zinc excretion and zinc balance (μg/week). Values are expressed as means $\pm$ SD.

GROUP	WEEK 1			
	INTAKE	FAECAL	URINE	BALANCE
Control	2053.33^{a} $\pm$ 297.02	$1299,00^{b}$ $\pm$ 218.19	60.66^{b} $\pm$ 13.02	693.66^{a} $\pm$ 308.55
Pair-fed	1866.67^{a} $\pm$ 301.78	1282.11^{b} $\pm$ 142.73	66.88^{ab} $\pm$ 9.72	517.66^{a} $\pm$ 363.36
Deficient (PA)	1970.00^{a} $\pm$ 284.60	1535.22^{a} $\pm$ 355.47	72.55^{a} $\pm$ 8.06	362.22^{a} $\pm$ 328.22
Deficient	1866.67^{a} $\pm$ 301.78	1171.78^{b} $\pm$ 164.59	60.66^{b} $\pm$ 7.76	634.22^{a} $\pm$ 333.23

Mean values not followed by the same superscript letter are significantly different ($p < 0.05$)

Table 12(b): The effect of vitamin B-6 deficiency on zinc intake, faecal and urinary zinc excretion and zinc balance (μg/week). Values are expressed as means $\pm$ SD.

GROUP	WEEK 2			
	INTAKE	FAECAL	URINE	BALANCE
Control	2766.67[a] $\pm$ 215.17	1149.11[ab] $\pm$ 166.53	54.77[b] $\pm$ 7.01	1562.78[a] $\pm$ 251.42
Pair-fed	2170.00[b] $\pm$ 412.15	1049.78[b] $\pm$ 301.39	53.22[b] $\pm$ 15.56	1067.0[b] $\pm$ 584.86
Deficient (PA)	2200.00[b] $\pm$ 369.56	1313.11[a] $\pm$ 180.23	91.33[a] $\pm$ 11.82	795.55[b] $\pm$ 336.03
Deficient	2170.00[b] $\pm$ 412.15	1138.22[ab] $\pm$ 217.88	90.66[a] $\pm$ 15.04	941.11[b] $\pm$ 483.11

Mean values not followed by the same superscript letter are significantly different ($p < 0.05$)

Table 12(c): The effect of vitamin B-6 deficiency on zinc intake, faecal and urinary zinc excretion and zinc balance (μg/week). Values are expressed as means $\pm$ SD.

GROUP	WEEK 3			
	INTAKE	FAECAL	URINE	BALANCE
Control	3179.67^{a} $\pm$ 542.30	2270.11^{a} $\pm$ 845.31	129.55^{ab} $\pm$ 49.77	780.00^{a} $\pm$ 626.75
Pair-fed	1993.33^{b} $\pm$ 406.41	1608.44^{b} $\pm$ 250.02	109.55^{b} $\pm$ 18.00	275.33^{a} $\pm$ 521.00
Deficient (PA)	1920.00^{b} $\pm$ 476.94	1417.11^{b} $\pm$ 193.43	143.55^{a} $\pm$ 27.49	359.33^{a} $\pm$ 622.32
Deficient	1993.33^{b} $\pm$ 406.41	1418.11^{b} $\pm$ 242.71	131.11^{ab} $\pm$ 23.20	444.11^{a} $\pm$ 346.72

Mean values not followed by the same superscript letter are significantly different ($p < 0.05$)

Table 12(d) : The effect of vitamin B-6 deficiency on zinc intake, faecal and urinary zinc excretion and zinc balance (μg/week). Values are expressed as means $\pm$ SD.

GROUP	WEEK 4			
	INTAKE	FAECAL	URINE	BALANCE
Control	3873.75[a] $\pm$ 320.08	2295.13[a] $\pm$ 219.55	115,12[b] $\pm$ 10.77	1463.50[a] $\pm$ 401.97
Pair-fed	2030.00[b] $\pm$ 435.51	1308.67[b] $\pm$ 101.74	107.00[b] $\pm$ 20.91	613.33[b] $\pm$ 476.89
Deficient (PA)	2030.00[b] $\pm$ 437.32	1268.22[b] $\pm$ 180.08	137.66[ab] $\pm$ 25.66	624.11[b] $\pm$ 571.73
Deficient	2030.00[b] $\pm$ 435.51	1317.11[b] $\pm$ 221.53	125.22[a] $\pm$ 13.70	587.66[b] $\pm$ 401.80

Mean values not followed by the same superscript letter are significantly different ($p < 0.05$)

Table 12(e): The effect of vitamin B-6 deficiency on zinc intake, faecal and urinary zinc excretion and zinc balance (μg/week). Values are expressed as means $\pm$ SD.

GROUP	WEEK 5			
	INTAKE	FAECAL	URINE	BALANCE
Control	3573.33^{a} $\pm$ 342.19	2554.11^{a} $\pm$ 173.11	125.22^{b} $\pm$ 11.49	$894,00^{a}$ $\pm$ 279.26
Pair-fed	1613.33^{b} $\pm$ 281.33	1273.44^{b} $\pm$ 176.75	110.22^{c} $\pm$ 9.28	229.66^{b} $\pm$ 363.50
Deficient (PA)	1810.00^{b} $\pm$ 341.04	1315.78^{b} $\pm$ 185.67	133.77^{ab} $\pm$ 8.02	360.44^{b} $\pm$ 416.05
Deficient	1613.33^{b} $\pm$ 281.33	1307.33^{b} $\pm$ 184.00	142.33^{a} $\pm$23.33	163.66^{b} $\pm$ 277.51

Mean values not followed by the same superscript letter are significantly different ($p < 0.05$)

Table 12(f): The effect of vitamin B-6 deficiency on zinc intake, faecal and urinary zinc excretion and zinc balance (μg/week). Values are expressed as means $\pm$ SD.

GROUP	WEEK 6			
	INTAKE	FAECAL	URINE	BALANCE
Control	3460.00[a] $\pm$ 371.38	2659.22[a] $\pm$ 185.82	134.77[a] $\pm$ 7.31	666.00[a] $\pm$ 483.71
Pair-fed	1546.67[b] $\pm$ 351.81	1204.67[b] $\pm$ 199.05	102.77[b] $\pm$12.22	239.22[b] $\pm$ 435.70
Deficient (PA)	1787.78[b] $\pm$ 445.97	1381.78[b] $\pm$ 181.36	148.22[a] $\pm$ 17.26	257.77[b] $\pm$ 380.11
Deficient	1546.67[b] $\pm$ 351.81	1287.33[b] $\pm$ 285.75	145.77[a] $\pm$ 22.69	113.55[b] $\pm$ 383.33

Mean values not followed by the same superscript letter are significantly different ($p < 0.05$).

4.2 EXPERIMENT 2

For convenience of expression the vitamin B-6 deficient group (receiving 7 mg PN.HC1/kg diet) will be referred to as the "deficient group" and the group receiving the 1 mg PN.HC1/kg diet will be referred to as the "partially deficient group".

4.2.1 The effect of vitamin B-6 deficiency on food consumption

The mean food consumption of all four groups is shown in Table 13 and Figure 12. The food pattern trend for the first six weeks of this experiment correlates with that observed in experiment 1 for the vitamin B-6 deficient group. The control group in experiment 2, however, did not show a gradual decline in food consumption after the fourth week as was observed in experiment 1. Instead the food consumption in the control group continued to increase until the fifth week and this was followed by no noticeable change in food consumption for the following three weeks. The partially deficient group showed significantly higher food consumption compared to the deficient group from the third week onwards ($p < 0.001$).

Table 13 : The effect of vitamin B-6 deficiency on weekly food consumption (Means ± SD).

GROUP	WEEK 1	WEEK 2	WEEK 3	WEEK 4	WEEK 5	WEEK 6	WEEK 7	WEEK 8
Control	70.31±9.70[a]	89.37±8.41[a]	118.77±10.72[a]	134.15±10.51[a]	140.31±12.40[a]	142.12±13.78[a]	139.00±14.01[a]	140.87±15.71[a]
Partially Deficient	68.45±10.43[a]	87.31±13.47[a]	95.44±17.74[b]	120.33±15.51[b]	120.12±15.44[b]	118.31±12.51[b]	115.40±13.37[b]	116.79±14.37[b]
Deficient	68.14±10.04[a]	85.41±12.37[a]	90.44±13.41[b]	86.43±15.72[c]	75.55±10.31[c]	74.60±8.47[c]	70.12±10.34[c]	68.94±9.81[c]

Mean values not followed by the same superscript letter denotes a significant difference ($p < 0.05$)

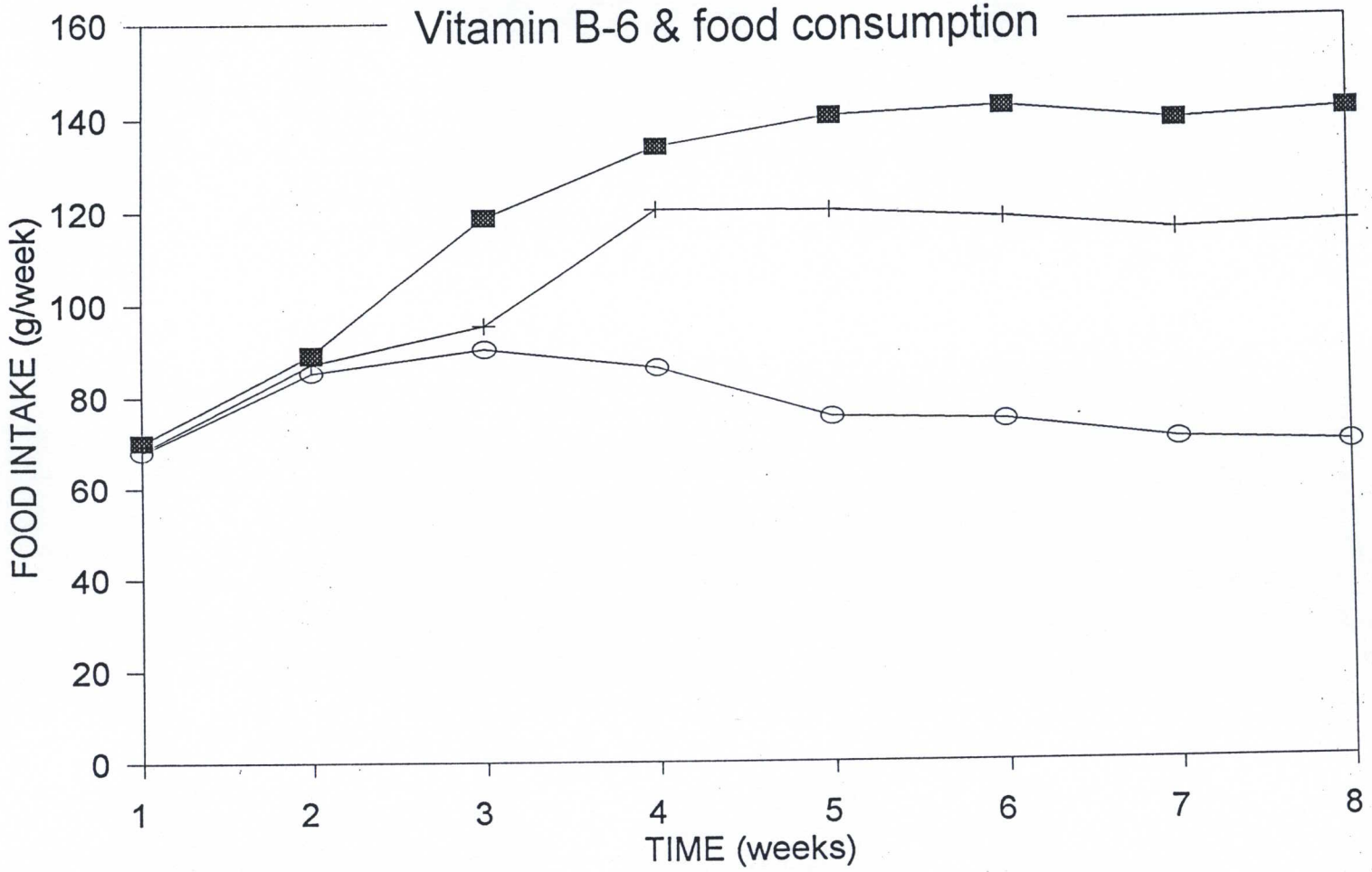


Figure 12 : Vitamin B-6 and food consumption.

4.2.2 The effect of vitamin B-6 deficiency on growth

The overall growth pattern for the control, pair-fed and vitamin B-6 deficient groups were more or less similar in experiments 1 and 2 (Figures 10 and 13 respectively). The higher final body weights reached by these three groups in experiment 2 were due to the extended experimental period. The partially deficient group showed a significantly higher mean body mass compared to the pair-fed and deficient groups from the third week onwards ($p < 0.001$) (Table 14).

Table 14 : The effect of vitamin B-6 deficiency on growth (g) (Means ± SD)

GROUP	DAY 1	WEEK 1	WEEK 2	WEEK 3	WEEK 4	WEEK 5	WEEK 6	WEEK 7	WEEK 8
Control	53.41±8.07[a]	82.64±10.31[a]	124.83±15.31[a]	164.87±13.77[a]	205.31±16.39[a]	234.07±16.39[a]	255.74±16.87[a]	268.59±17.31[a]	275.41±16.54[a]
Pair-fed	50.20±8.74[a]	78.98±13.74[a]	115.00±11.78[b]	125.51±10.81[b]	136.72±13.39[b]	156.61±17.47[b]	162.31±13.31[b]	168.41±14.87[b]	171.37±14.24[b]
Partially Deficient	50.90±7.21[a]	78.93±14.36[a]	114.92±13.31[b]	148.41±18.34[c]	160.90±18.01[c]	172.72±20.73[c]	180.21±25.73[c]	188.25±29.31[c]	192.43±30.41[c]
Deficient	50.13±8.34[a]	78.84±12.79[a]	100.70±20.34[c]	124±17.47[b]	138.63±20.78[b]	140.31±25.21[d]	141.27±30.74[d]	139.83±31.44[d]	137.34±30.27[d]

Mean values not followed by the same superscript letter denotes a significant difference ($p < 0.05$)

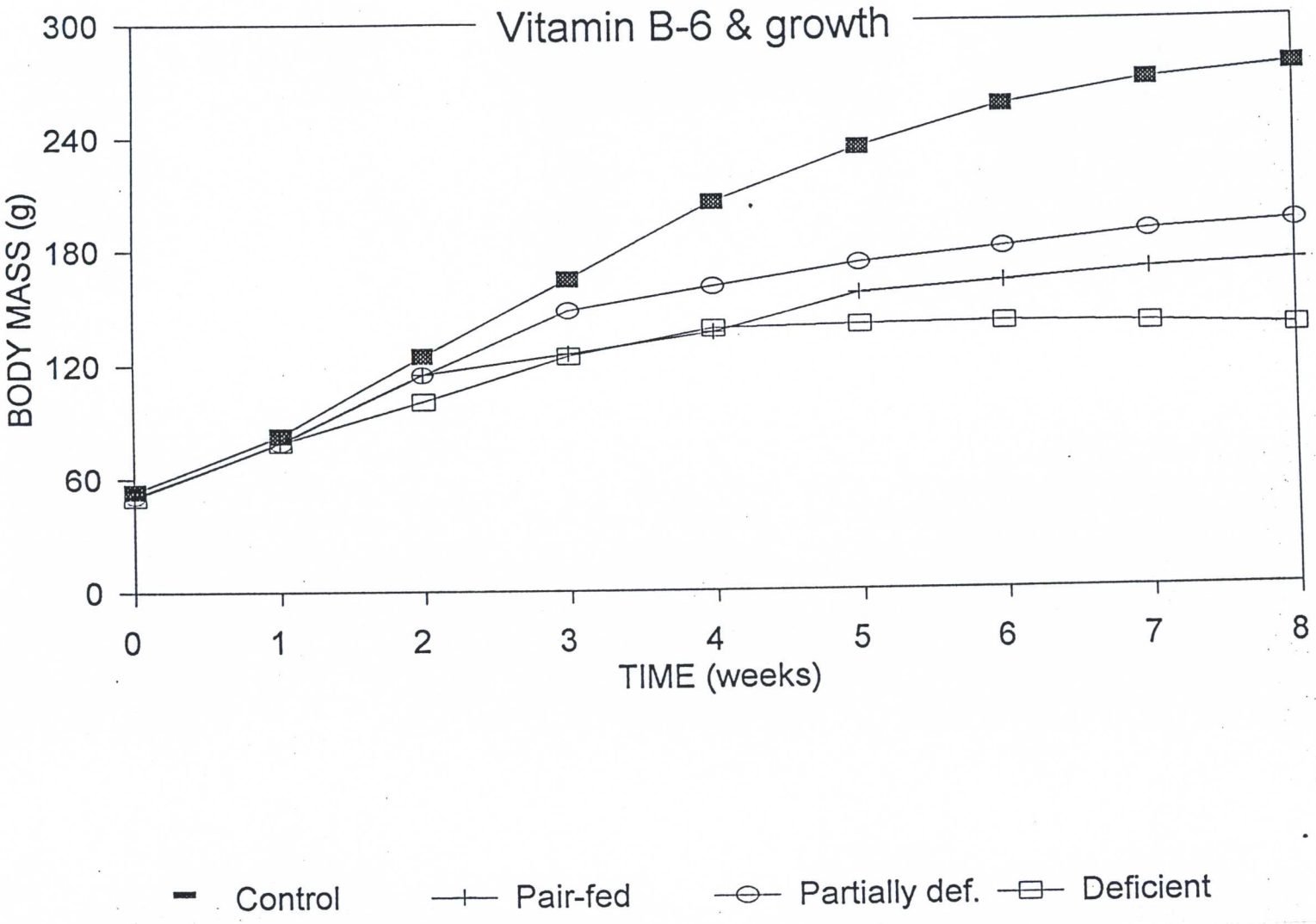


Figure 13 : Vitamin B-6 and growth.

4.2.3 The effect of vitamin B-6 deficiency on tissue zinc-metallothionein levels

4.2.3.1 Kidney zinc - MT levels

The control group (35 ± 7 μg/g) had significantly higher kidney metallothionein level compared with the pair-fed (29 ± 5 μg/g), the partially deficient (28 ± 4 μg/g) and the deficient (10 ± 3 μg/g) groups ($p < 0.001$), Table15). There was no significant difference in kidney MT levels between the pair-fed and the partially deficient groups; while the deficient group had significantly lower kidney MT levels compared to the other three groups.

4.2.3.2 Pancreas zinc - MT levels

The MT levels were found to be significantly lower in the vitamin B-6 deficient group (16 ± 2 μg/g) compared with the control (28 ± 5 μg/g), pair-fed (21 ± 3 μg/g) and the partially deficient (23 ± 3 μg/g) groups ($p < 0.001$, Table 15). However, no significant difference in pancreatic MT levels was noticed between the pair-fed and partially deficient groups.

4.2.3.3 Liver zinc - MT levels

Unlike the kidney and the pancreas there was no significant difference in liver MT levels among the control (31 ± 6 μg/g), pair-fed (32 ± 6 μg/g), partially deficient (30 ± 5 μg/g) and deficient (33 ± 4 μg/g) groups (Table15).

Summary of tissue metallothionein levels

The kidney and pancreas MT levels were increased in the control group compared to the other three groups while the deficient group had significantly lower MT levels than the other groups. This trend was not observed in the liver where no difference in MT levels was apparent among the four groups of rats.

Table 15 : The effect of vitamin B-6 deficiency on tissue metallothionein levels (means ± SD)

GROUP	n	KIDNEY MT $\mu g/g$	LIVER MT $\mu g/g$	PANCREAS MT $\mu g/g$
Control	9	35 ± 7^{a}	31 ± 6^{a}	28 ± 5^{a}
Pair-fed	9	29 ± 5^{b}	32 ± 6^{a}	21 ± 3^{b}
Partially Def.	9	28 ± 4^{b}	30 ± 4^{a}	23 ± 3^{b}
Deficient	9	20 ± 3^{c}	33 ± 5^{a}	16 ± 2^{c}

Mean values for each group not followed by the same superscript letter denote a significant difference ($p < 0.05$).

4.2.4 The effect of vitamin B-6 deficiency on plasma pyridoxal 5-phosphate and picolinic acid levels

4.2.4.1 Plasma PLP

The vitamin B-6 deficient (37 ± 8 nmol/l) and the partially deficient 304 ± 117 nmol/l) groups had significantly lower plasma PLP levels compared with the control (567 ± 147 nmol/l) and pair-fed (609 ± 157 nmol/l) groups ($p < 0.0001$), Table 16). In addition the vitamin B-6 deficient group showed significantly lower plasma PLP levels compared to the partially deficient group ($p < 0.001$). The difference in plasma PLP levels between the deficient and the pair-fed and control groups in this experiment correlates with the pattern observed for these groups in experiment 1. The higher values for the control and pair-fed groups in experiment 2 compared to experiment 1 are probably due to the two week longer duration of the second experiment.

4.2.4.2 Pancreatic picolinic acid

The pancreatic PA level was found to be significantly lower ($p < 0.0001$) in the deficient group (0.94 ± 0.33 μmoles/g) compared to the control (3.04 ± 0.61 μmoles/g), the pair-fed (2.90 ± 0.45 μmoles/g) and the partially deficient (2.08 ± 0.47 μmoles/g) groups. Furthermore, the PA concentration was observed to be lower in the partially deficient group than in the pair-fed and control groups ($p < 0.001$). There was no significant difference in pancreatic PA levels between the control and pair-fed groups (Table 16).

Table 16: The effect of vitamin B-6 deficiency on plasma PLP and picolinic acid levels (means ± SD)

GROUP	n	PLASMA PLP (nmol/l)	PICOLINIC ACID (μmoles/g)
Control	9	567 ± 147[a]	3.04 ± 0.61[a]
Pair-fed	9	609 ± 157[a]	2.90 ± 0.45[a]
Partially Deficient	9	304 ± 117[b]	2.08 ± 0.47[b]
Deficient	9	37 ± 8[c]	0.94 ± 0.33[c]

Mean values not followed by the same superscript letter are significantly different ($p < 0.05$).

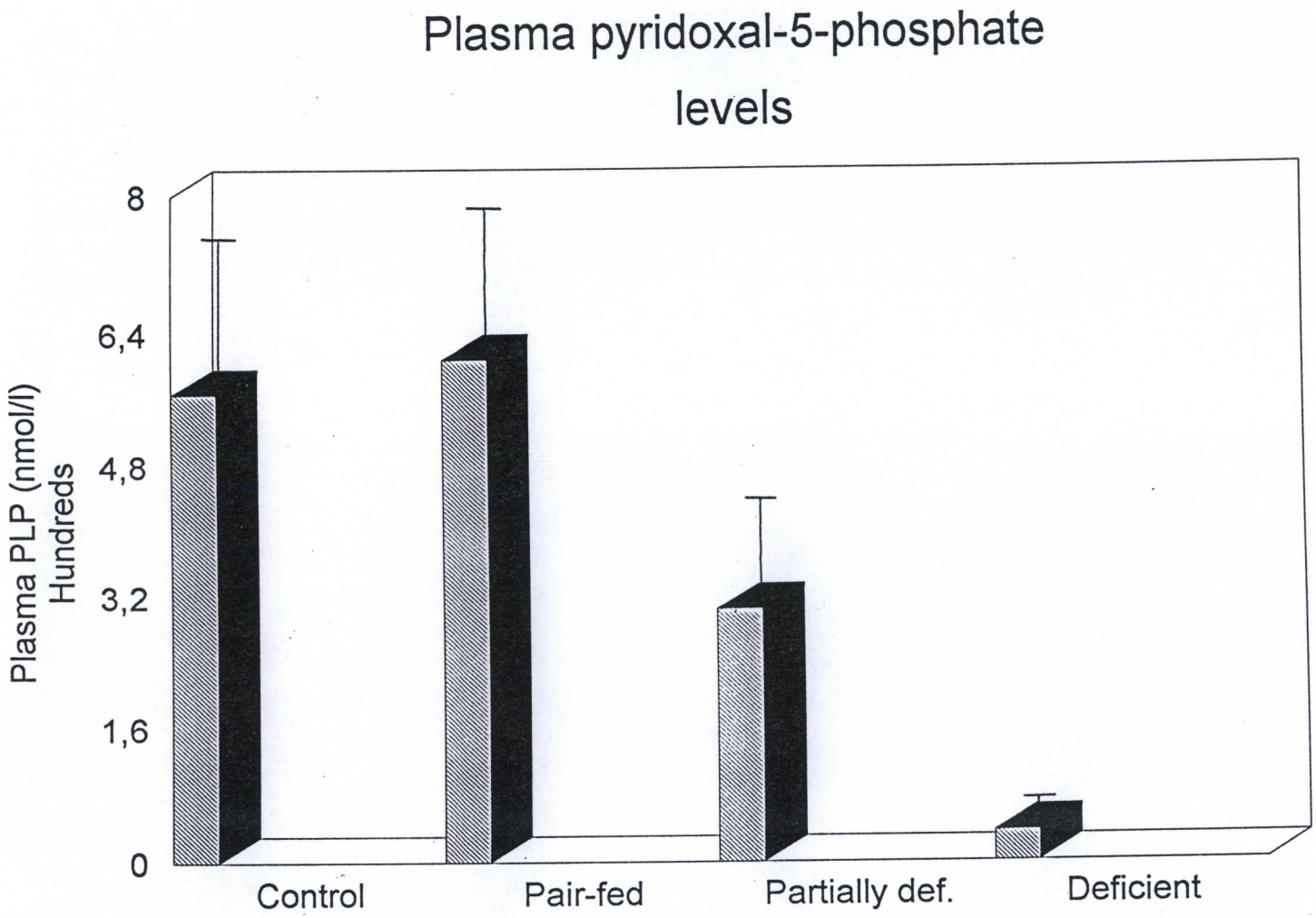


Figure 14 : Plasma pyridoxal-5-phosphate levels.

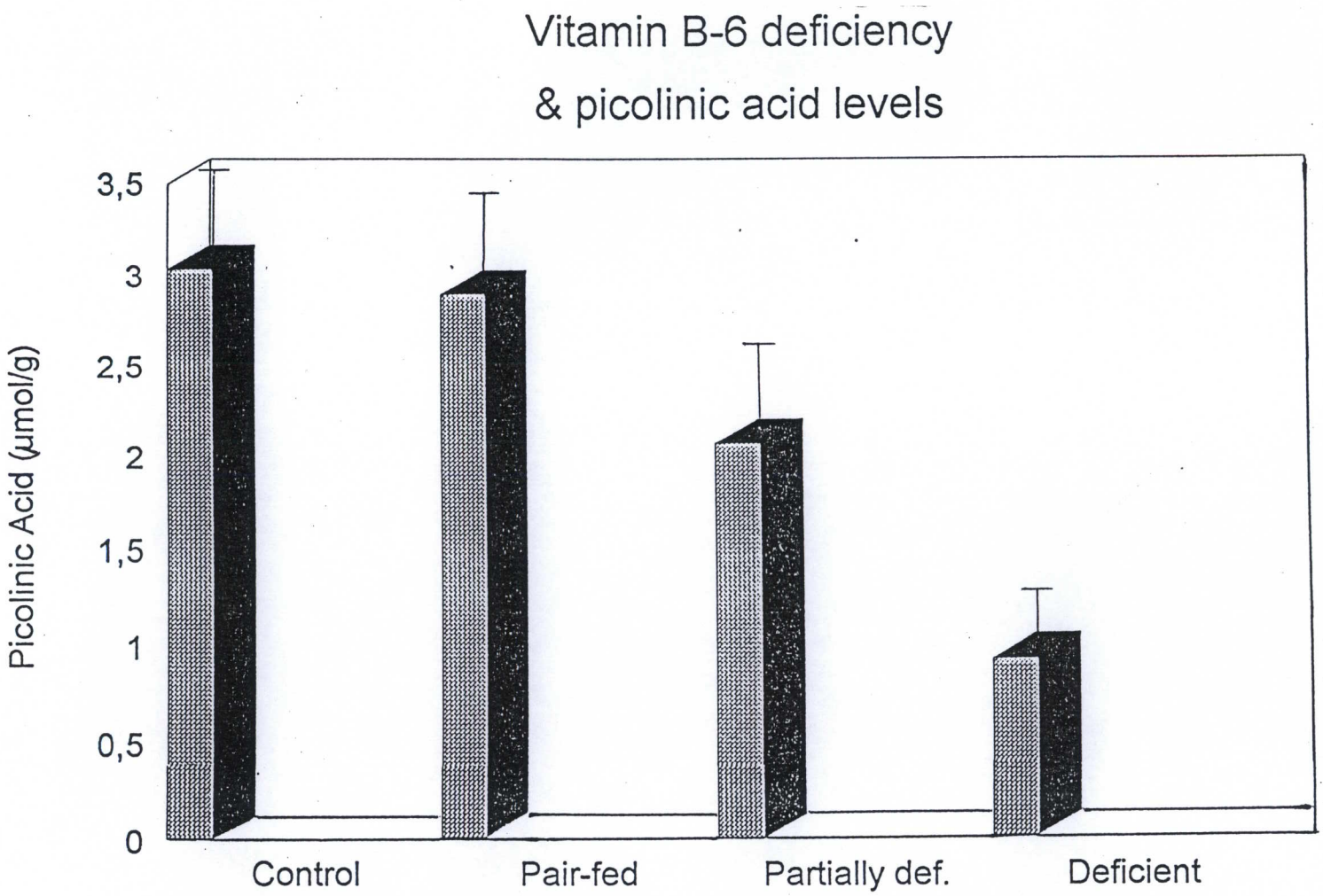


Figure 15 : Vitamin B-6 deficiency and picolinic acid levels.

4.2.5 The effect of vitamin B-6 deficiency on plasma protein, haemoglobin and serum erythropoietin levels

4.2.5.1 Total plasma proteins

The total protein levels were significantly lower in the vitamin B-6 deficient (4.32 $\pm$ 0.11 g/dl) and partially deficient (5.77 $\pm$ 0.13 g/dl) groups when compared with control (6.90 $\pm$ 0.16 g/dl) and pair-fed (6.64 $\pm$ 0.07 g/dl) groups ($p < 0.001$). In addition the deficient group had significantly lower total protein levels than the partially deficient group ($p < 0.001$). There was no significant difference in total protein levels between the control and pair-fed groups. The total plasma protein levels in this experiment are similar to the values observed in experiment 1 for the control, pair-fed and vitamin B-6 deficient groups.

4.2.5.2 Haemoglobin

The vitamin B-6 deficient group (8.37 $\pm$ 1.01 g/dl) had significantly lower haemoglobin values compared to the control (10.21 $\pm$ 1.40 g/dl), pair-fed (10.27 $\pm$ 1.36 g/dl) and the partially deficient (9.87 $\pm$ 1.27 g/dl) groups ($p < 0.0001$). There was no significant difference in haemoglobin levels between the control, pair-fed and partially deficient groups. The haemoglobin levels in the control, pair-fed and deficient groups of experiment 1 correlate with the values for these groups in experiment 2.

4.2.5.3 **Serum Erythropoeitin**

Serum erythropoietin levels were found to be significantly lower in the deficient group (34.35 ± 4.29) compared to the control (38.41 ± 3.22 μU/ml), pair-fed (38.17 ± 4.11 μU/ml) and partially deficient (37.96 ± 5.68 μU/ml) groups ($p < 0.001$). There was no significant difference in erythropoeitin levels among the control, pair-fed and partially deficient groups.

Table 17 : The effect of vitamin B-6 deficiency on plasma total protein, haemoglobin and serum erythropoietin levels (means ± SD)

GROUP	n	TOTAL PLASMA PROTEIN (g/dl)	HAEMOGLOBIN (g/dl)	SERUM ERYTHROPOIETIN μU/ml)
Control	9	6.90 ± 0.16^a	10.21 ± 1.40^a	38.41 ± 3.22^a
Pair-fed	9	6.64 ± 0.07^a	10.27 ± 1.36^a	38.17 ± 4.11^a
Partially Deficient	9	5.77 ± 0.13^b	9.87 ± 1.27^a	37.96 ± 5.68^a
Deficient	9	4.32 ± 0.11^c	8.37 ± 1.01^b	34.35 ± 4.29^b

Mean values not followed by the same superscript letter are significantly different ($p < 0.05$).

4.2.6 The effect of vitamin B-6 deficiency on blood pressure

The mean systolic blood pressure of each group over the 8 week period is shown in Table 18. The systolic blood pressure of the vitamin B-6 deficient group was significantly higher than the other three groups from the fourth week to the end of the experimental period ($p < 0.001$). The partially deficient group showed a significantly higher blood pressure compared to the control and pair-fed groups from the sixth to the eighth week ($p < 0.001$). There was no significant difference in mean systolic blood pressure between the control and pair-fed groups.

Table 18 : The effect of vitamin B-6 deficiency on the systolic arterial blood pressure (mmHg) of rats (means ± SD)

GROUP	n	D 1	W 1	W 2	W 3	W 4	W 5	W 6	W 7	W 8
Control	9	118 ± 4[a]	115 ± 5[a]	118 ± 6[a]	115 ± 4[a]	116 ± 4[a]	115 ± 5[a]	118 ± 4[a]	120 ± 3[a]	120 ± 4[a]
Pair-Fed	9	115 ± 6[a]	117 ± 5[a]	116 ± 4[a]	118 ± 5[a]	116 ± 6[a]	117 ± 4[a]	119 ± 5[a]	118 ± 4[a]	119 ± 6[a]
Partially Deficient	9	116 ± 5[a]	118 ± 3[a]	118 ± 4[a]	119 ± 5[a]	117 ± 4[a]	119 ± 5[a]	125 ± 4[b]	127 ± 5[b]	127 ± 5[b]
Deficient	9	115 ± 3[a]	118 ± 5[a]	120 ± 4[a]	119 ± 4[a]	126 ± 5[b]	133 ± 6[b]	140 ± 7[c]	144 ± 4[c]	147 ± 5[c]

Mean values not followed by the same superscript letter denote a significant difference ($p < 0.05$)

CHAPTER FIVE

DISCUSSION

5.1 Physical appearance

The first noticeable physical symptom in the vitamin B-6 deficient groups was poor growth which became apparent from the second week onwards. By the fourth week, a symmetrical scaling dermatitis (acrodynia) on the nose, upper thorax, tail and paws of the vitamin B-6 deficient groups was observed. Growth retardation and dermatitis are also commonly observed symptoms in zinc deficient rats (27). Similar lesions have been observed by others and were described as resembling that of essential fatty acid deficiency (150). In fact, a deficiency of vitamin B-6 was shown to reduce the amount of unsaturated essential fatty acids in tissues (110) and early studies demonstrated that supplementation with oils containing essential fatty acid had a sparing effect on the severity of the deficiency (140). However, the exact interrelationship between the essential fatty acids and the vitamin B-6 deficiency is as yet unknown.

The vitamin B-6 deficient groups also showed areas (upper thorax and forelimbs) of alopecia and an overall unkempt appearance of their fur when compared to control and pair-fed rats. These symptoms have also been reported in zinc deficient rats (54). By the fifth week, most vitamin B-6 deficient rats had skin lesions on their snouts and paws. These symptoms were similar to those reported by Driskell *et al.* in 1984 (40) and Prasad *et al.* in 1982 (138). These physical symptoms appeared more severe in the vitamin B-6 deficient group in experiment 2 of the current study. This is probably because these animals were exposed to the vitamin B-6 deficiency for two weeks longer than the vitamin B-6 deficient group in experiment 1. Also apparent among the vitamin B-6 deficient group was a general lethargy when compared to control rats. This symptom was also reported in human subjects who were vitamin B-6 deficient (101). Since vitamin B-6 plays an essential role in amino acid and carbohydrate metabolism and since many enzymes are vitamin B-6 dependent, the above symptoms would be expected during a vitamin B-6 deficiency.

5.2 Food consumption and growth

A decrease in food consumption is a much published symptom arising from vitamin B-6 deficiency (23). The vitamin B-6 deficient and the vitamin B-6 deficient-PA-supplemented groups in the present study (experiment 1) consumed an average of 8.93 $\pm$ 1.17 g and 9.28 $\pm$ 0.77 g of food per day, respectively, while the control animals consumed an average of 15.03$\pm$ 3.1 g per day (Table 5, Figure 9). This may in itself account for the significant difference in body mass observed between the control and vitamin B-6 deficient groups ($p < 0.001$) from the second week to the end of the experimental period (Table 6). However, the pair-fed rats which also consumed an average of 8.93 g of food per day had significantly higher body weights when compared to both the vitamin B-6 deficient groups from the second week onwards ($p < 0.001$). These observations correlate with the food consumption and growth patterns seen in the control (17.39 $\pm$ 3.91 g per day), pair-fed (11.06 $\pm$ 1.45 g per day) and deficient (11.06 $\pm$ 1.45 g per day) groups of experiment 2 (Table 13, Figure 12). The partially deficient group consumed significantly more food per day than the deficient group from the fourth week to the end of the experimental period. The body mass of the partially deficient group (114 $\pm$ 13 g) was, however, significantly higher than the deficient group (100 $\pm$ 20 g) from the second week and not the fourth week as would be expected from the food consumption patterns. The low body weights of the vitamin B-6 deficient groups may be attributed to both the low caloric intake and the lack of vitamin B-6 in their diets. This would suggest that factors other than decreased caloric intake also contributes to the decreased growth rate of the vitamin B-6 deficient groups. This finding is in agreement with previous suggestions that vitamin B-6 intake also plays a critical role in body weight/growth. A significant difference in body mass was also noted between the control and pair-fed groups from the fourth week onwards in experiment 1 ($p < 0.01$) (Table 14). The control group consumed 105 μg PN.HC1/day compared to 62 μg PN.HC1 consumed by the pair-fed group. A survey of the literature reveals that the vitamin B-6 requirement for rats as reported by individual investigators ranges from 22 μg/day to 80 μg/day. Lumeng *et al.* in 1978 (105) found that maximal growth occurred in weanling rats receiving 29 μg/day of PN.HC1, while Van den Berg (1982) reported that 24 μg/day of pyridoxine is sufficient to produce maximal growth in rats (177). Other researchers have reported higher levels of vitamin B-6 (75 μg/day) as being necessary for optimal growth in rats (114). These reports suggest that the pair-fed group in the present study consumed the required amount of vitamin B-6 for maximal growth. Therefore, the significantly lower body weights of the pair-fed rats when compared to controls may be due entirely to their low caloric intake.

A decrease in the transport and cellular uptake of amino acids was reported in dietary vitamin B-6 deficient rats (120). Heindel and Riggs (1978) provided evidence to suggest that vitamin B-6 itself plays no direct role in amino acid transport (64). Instead they suggested that vitamin B-6 produces its effect by its role in the synthesis of growth hormone. It has been established that the physiologically active level of growth hormone is reduced in dietary vitamin B-6 deficiency (64). The decrease in amino acid transport was therefore associated with a reduction in the growth hormone content of the pituitary gland. Administration of growth hormone was found to restore the *in vivo* amino acid transport to normal levels faster than injected vitamin B-6. The reduced growth hormone production may also play a role in the decreased growth rate of the vitamin B-6 deficient rats.

The other endocrine factor which may contribute to the transport defect of amino acids and growth in vitamin B-6 deficiency is insulin (69). Researchers have suggested that the low body weights resulting from vitamin B-6 deficiency may be linked to the decreased insulin levels observed in vitamin B-6 deficient rats (69), as was also the case in the current study where the plasma insulin concentration (Table 10) in the vitamin B-6 deficient rats (0.81 ± 0.27 ng/ml) were significantly lower than the control (1.91 ± 0.58 ng/ml) and pair-fed (2.62 ± 0.50 ng/ml) groups ($p < 0.0001$). However, the reduced insulin level in the vitamin B-6 deficient group was not accompanied by an altered plasma glucose level. A dietary deficiency of vitamin B-6 is accompanied by decreased serum and pancreatic insulin content (69). Furthermore, insulin insufficiency is associated with decreased appetite leading to decreased food intake and growth failure (7). The decreased appetite was found to be partially overcome by administration of insulin which in turn resulted in improved growth (7). Therefore administration of insulin has a somewhat similar effect as administration of growth hormone in vitamin B-6 deficient rats.

In view of the above, it may be suggested that the low insulin levels in the vitamin B-6 deficient group may have also contributed to the decreased food intake and the resultant reduced growth.

5.3 Plasma pyridoxal-5-phosphate (PLP)

Plasma PLP concentration is one of the indicators of vitamin B-6 status (175). In both experiments 1 and 2, vitamin B-6 deficiency caused a highly significant ($p < 0.0001$) reduction in plasma PLP levels. In experiment 1 the plasma PLP concentrations in control and pair-fed groups were reduced from 373.33 $\pm$ 166.39 nmol/l and 421 $\pm$ 150.64 nmol/l, respectively, to 13.95 $\pm$ 4.08 nmol/l in the vitamin B-6 deficient and 16.6 $\pm$ 5.69 nmol/l in the vitamin B-6 deficient-PA-supplemented groups (Table 8). In experiment 2, plasma PLP concentration in the deficient group was 37 $\pm$ 8 nmol/l compared to 567 $\pm$ 147 nmol/l in the control and 609 $\pm$ 157 nmol/l in the pair-fed groups ($p < 0.0001$) (Table 16). The PLP levels in the control and pair-fed groups were higher in experiment 2 compared to the findings in experiment 1. This may be attributed to the two week longer duration of experiment 2. The PLP level in the partially deficient group (284 $\pm$ 117 nmol/l) was also significantly lower than the control and pair-fed groups but significantly higher than the deficient group (Table 16). These findings confirmed the vitamin B-6 deficient status of the experimental groups.

The changes in plasma PLP levels of the vitamin B-6 deficient group, observed in this study, were in keeping with those reported in the literature (23) while PLP levels of the control and pair-fed groups were higher than some reported values (189.58 nmol/l for control rats) (23) but in agreement with others (331.7 nmol/l and 337 nmol/l for control and pair-fed rats, respectively (184). These variations may be due to the different levels of PN.HCl in the diet between the current experiment (105 μg/day) and the reported levels (45 μg/day) (23).

No significant difference in plasma PLP concentration was noted between the control and pair-fed groups. This would suggest that the pair-fed group consumed enough vitamin B-6 (62 μg/day) to maintain a normal vitamin B-6 status, and therefore researchers who have suggested 75 to 100 μg/day of vitamin B-6 as the required amount to maintain vitamin B-6 status and optimal growth in rats may have overestimated the required level (114).

5.4 Vitamin B-6 deficiency and blood pressure

The results of the current study (experiment 2) reveal that pyridoxine deficiency leads to the development of hypertension after four weeks on the diet. The hypertensive state in the vitamin B-6 deficient group persisted until the end of the experimental period (Table 18) with the deficient group showing a significantly higher systolic blood pressure of 147 $\pm$ 5 mmHg compared to 120 $\pm$ 4 mmHg in the control and 119 $\pm$ 6 mmHg and 127 $\pm$ 5 mmHg in the pair-fed and partially deficient groups, respectively. This observation was in keeping with the findings of Paulose *et al.* (1988), who found that the vitamin B-6 deficient group had a significantly higher blood pressure compared to the control group from the fifth week (134). After eight weeks these researchers reported a systolic blood pressure of 143 $\pm$ 6 mmHg in the deficient group and 123 $\pm$ 3 mmHg in the control group. In the present study even the partially deficient group developed a significantly higher systolic blood pressure during the final two weeks of the experiment compared to the control group ($p < 0.001$). This shows that the increase in arterial blood pressure is related to the degree of deficiency as well.

The reduced food consumption and slow growth pattern in vitamin B-6 deficiency may be eliminated as possible contributory factors to the development of hypertension since the pair-fed group which was subjected to reduced caloric intake did not develop increased systolic blood pressure. This observation would be in keeping with the findings by Paulose *et al.* (1988), who reported that rats subjected to generalised malnutrition showed significantly lower blood pressures compared to pyridoxine-deficient rats (134). It was further noted that treatment of pyridoxine-deficient rats with pyridoxine not only reversed blood pressure levels to normal within 24 hours but, in addition, restored hypothalamic serotonin and gamma-aminobutyric acid as well as plasma epinephrine and norepinephrine levels to normal. This may indicate that the hypertension which develops during pyridoxine deficiency is associated with an increase in sympathetic excitation (134).

It has also been reported that low calcium intake is associated with hypertension (92). Vitamin B-6 deficiency has been shown to cause a significant reduction in the uptake of calcium by the enterocytes (92). The reduced dietary calcium may deplete calcium from membrane storage sites, resulting in a less stable membrane of the vascular smooth muscle (92). This in turn could result in increased calcium influx, increased tone and reactivity in the arteriolar smooth muscle, leading to elevated peripheral resistance resulting in hypertension (92).

Although none of the literature reviewed related zinc deficiency to the development of hypertension, it is known that both zinc and vitamin B-6 share many deficiency symptoms. Zinc deficiency results in, for example, altered levels of norepinephrine in the brain (129) and it has been reported that calcium metabolism is abnormal during a zinc deficiency (79). Calcium and norepinephrine are only two examples of substances whose altered levels are associated with the development of hypertension in rats that are deficient in vitamin B-6. It is, however, beyond the scope of the present study to determine the association, if any, between the development of hypertension in vitamin B-6 deficient rats and the zinc status of the animals. It would provide for interesting future research, however, to monitor the extent to which altered zinc levels affect various neuroactive compounds like serotonin, the catecholamines including norepinephrine and whether this may relate to the development of hypertension in pyridoxine deficiency.

5.5 Vitamin B-6 deficiency and zinc status

Zinc is an essential trace element for normal life in both humans and animals having first been shown to be required for normal growth in rats and mice in the 1930's (174). It has since been reported that animals which consume zinc-deficient diets eventually develop a variety of pathologies including anorexia, growth retardation, abnormal immune function, sparse hair growth, impaired connective tissue metabolism, dermatological lesions, impaired reproductive capacity and behavioural defects (27, 54, 171).

Even though the body has an efficient mechanism for maintaining whole body zinc homeostasis, many examples of acquired deficiencies appear in the literature. A low dietary intake of zinc is the most frequent cause of zinc deficiency. Zinc deficiency may, however, also occur when its dietary level is considered adequate. This may be due to other dietary components, and their levels, which may influence either the absorption, utilisation or excretion of zinc (75). One such dietary component, which has been widely researched, is vitamin B-6. Various studies, to determine the effect of vitamin B-6 deficiency on body zinc levels, have been undertaken. These studies, however, have produced conflicting results. Hsu (1965) found reduced zinc levels in various tissues of vitamin B-6 deficient rats (68). Gershoff (1967), on the other hand, reported increased zinc concentrations in tissues of vitamin B-6 deficient rats (52). Ikeda *et al.* (1979) (72) and Mackraj *et al.* (1994) (107) on the other hand, found no significant changes in tissue zinc concentration arising from vitamin B-6 deficiency.

These conflicting results may be partly due to variations in the duration of the experimental period (23 days for Gershoff's study compared with six weeks for Hsu's study). This variation may have resulted in experimental groups with different vitamin B-6 status, which in turn would have given rise to differences in the zinc levels in the tissues. The duration of the current study was six weeks for experiment 1 and eight weeks for experiment 2 and, in accordance with Hsu's (1965) findings, the vitamin B-6 deficiency reduces plasma and pancreatic zinc levels.

In establishing zinc requirements, it is important to consider the concentration and source of dietary zinc since these influence whole-body zinc utilisation. In humans, an intake of approximately 10-12 mg/day from animal protein-based meals is considered sufficient to maintain zinc equilibrium in adults (82). The American Institute of Nutrition has recommended a concentration of 30 mg/kg diet as suitable to provide for the zinc requirements of rats and mice (141).

In this study (experiment 1), the concentrations of zinc in the liver, kidney, testes, tibia and plasma of the vitamin B-6 deficient rats were found to be significantly lower than that in the control and pair-fed groups ($p<0.0001$, Table 7).

Under normal conditions, the amount of zinc absorbed is proportional to the dietary intake of zinc. When the dietary zinc intake is low, the proportion of zinc absorbed increases (77). Zinc homeostasis is maintained by regulation of gastrointestinal absorption and excretion of zinc. In experiment 1 of the present study, the vitamin B-6 deficient and pair-fed groups consumed significantly less diet and consequently significantly less zinc ($p < 0.0001$) than the control rats (Table 12). Increased absorption and decreased excretion of zinc would therefore have been expected in the pair-fed and vitamin B-6 deficient groups. However, it was noted that the faecal and urinary zinc excretion (as a percentage of their intake) in the vitamin B-6 deficient rats tended to be higher than that in the pair-fed and control groups in the final two weeks of the experiment. Since both the vitamin B-6 deficient and pair-fed groups consumed the same amount of diet, the increased faecal zinc excretion by the vitamin B-6 deficient group implies impaired zinc absorption in this group. Furthermore, the vitamin B-6 status in the pair-fed group was similar to that of the control group in both experiments 1 and 2 of this study.

It has been reported that vitamin B-6 deficiency causes an impairment of dietary zinc absorption (42). This was attributed to decreased production of a zinc binding ligand, viz., picolinic acid (PA), which requires pyridoxal as an essential co-factor for its synthesis (42). It was proposed that PA facilitates the passage of zinc across the enterocytes. The pancreatic PA levels observed in experiment 2 of the current study would concur with the above report. The deficient group in the present study had significantly lower pancreatic PA levels (0.94 ± 0.33 μmoles/g) compared to the control (3.04 ± 0.61 μmoles/g) and pair-fed (2.90 ± 0.45 μmoles/g) groups. Picolinic acid production and PA supplementation will be discussed later in this chapter.

The effect of vitamin B-6 deficiency on zinc absorption was also ascribed to reduced protein synthesis in vitamin B-6 deficiency (69). Significantly lower plasma albumin (2.78 ± 0.18 g/dl), globulin (2.08 ± 0.05 g/dl) and total protein (4.88 ± 0.19 g/dl) levels were observed in the vitamin B-6 deficient rats when compared with control (3.83 ± 0.11 g/dl, 2.98 ± 0.16 g/dl and 6.81 ± 0.07 g/dl, respectively) and pair-fed (3.71 ± 0.15 g/dl, 2.81 ± 0.15 g/dl and 6.53 ± 0.09 g/dl, respectively) groups in experiment 1. This trend was also noted for total protein levels in experiment 2, where the deficient group (4.32 ± 0.11 g/dl) showed significantly lower levels than the control (6.90 ± 0.10 g/dl), pair-fed (6.64 ± 0.07 g/dl) and partially deficient (5.77 ±0.13 g/dl) groups. The present finding is in agreement with other studies where reduced serum total protein levels were reported in vitamin B-6 deficient rats when compared with controls (149). The partially deficient group in the current study also had significantly lower total protein levels than the control and pair-fed groups. Vitamin B-6 deficiency was shown to decrease amino acid transport and cellular uptake (69). This together with the reduced growth observed in the vitamin B-6 deficient group confirms the effect of vitamin B-6 deficiency on plasma protein synthesis.

Reduced plasma protein synthesis may contribute to the apparent reduction in zinc absorption in the vitamin B-6 deficient group and may explain the higher faecal zinc excretion by this group compared with the pair-fed group in the latter stages of the experiment. Intestinal absorption of zinc involves equilibration within a "zinc pool" (32). A major portion of the zinc that enters the enterocytes becomes bound to metallothionein, an intracellular zinc-binding protein (32). This binding phase depends on the rate of protein synthesis and degradation and availability of binding sites (32). Therefore reduced metallothionein concentration may result in zinc being secreted back into the intestinal lumen (32). In this study (experiment 1), as shown in Table 8, a significantly lower erythrocyte Zn-MT-1 level was observed in the vitamin B-6 deficient group (30.09 ± 9.51 mg/ml) compared to the control group (53.79 ± 15.47 mg/ml) ($p<0.0004$). In addition, significantly lower kidney and pancreatic MT levels ($p < 0.0001$) were observed in the vitamin B-6 deficient group (20 ± 3 μg/g and 16 ± 2 μg/g, respectively) compared to the control (35 ± 7 μg/g and 28 ± 5 μg/g, respectively) and pair-fed (29 ± 5 μg/g and 21 ± 3 μg/g, respectively) groups (Table 15). From the above it may be assumed that enterocyte metallothionein levels would also be reduced. This coupled with the reduced PA concentration (experiment 2, Table 16) found in the deficient group (0.94 ± 0.33 μmoles/g) compared to the control group (3.04 ± 0.61 μmoles/g) may contribute to reduced zinc absorption and towards

increased faecal zinc losses observed in the vitamin B-6 deficient group. This in turn would be expected to result in lowered plasma zinc and erythrocyte Zn-MT-1 levels in vitamin B-6 deficiency and hence in less zinc being available for uptake by tissues. Furthermore, the observed reduction in tissue zinc levels in vitamin B-6 deficiency indicates that zinc has been mobilised from these tissues to tissues of greater need.

A decrease in urinary zinc may be expected when zinc absorption is low or during the onset of a zinc deficiency. However, it has been reported that urinary zinc concentration increases markedly during starvation (167). Renal conservation of zinc was reported to occur during the initial stages of zinc deficiency in rats, but after 30 days, the excretion of zinc was observed to increase (181). In the initial stages of the current experiment, no significant difference in urinary zinc output existed between the vitamin B-6 deficient (60.66 $\pm$ 7.76 μg/week) and the pair-fed (66.88 $\pm$ 9.72 μg/week) and control (60.66 $\pm$ 13.02 μg/week) groups as shown in Table 12a. However, by the end of the second week, the urinary zinc excretion of the vitamin B-6 deficient group (90.66 $\pm$ 15.04 μg/week) was higher than that of the pair-fed (53.22 $\pm$ 15.66 μg/week) and control (54.77 $\pm$ 7.01 μg/week) groups (Table 12b). This trend continued until the end of the experimental period. The increased urinary zinc excretion by the vitamin B-6 deficient rats corresponded with the period when a significant difference in body weight/growth existed between the vitamin B-6 deficient and pair-fed groups. The increased urinary zinc excretion may therefore be attributed to muscle catabolism resulting from reduced protein synthesis during vitamin B-6 deficiency (75).

An active or facilitated uptake of zinc by all tissues has been proposed (75). Little, however, is known about the uptake mechanism. The decreased zinc levels in the testes, liver, tibia and kidney of the vitamin B-6 deficient group observed in the present study (Table 7), may be also linked to the decreased albumin levels found in the vitamin B-6 deficient rats when compared to control and pair-fed groups. Since albumin functions as the primary carrier of zinc in plasma (33), the plasma albumin concentration probably exerts some influence on the zinc absorption from the small intestine (75). In the liver, for example, the majority of zinc presented to the hepatocytes is bound to albumin (33) and consequently reduced levels of albumin may imply reduced uptake of zinc by the liver. It was further reported that reduced albumin levels would slow down zinc uptake (33). Therefore the inhibition of plasma protein synthesis may have a role in retarding hepatic uptake of zinc.

Marked alterations in dietary zinc supply were reported to produce only small changes in the zinc concentration in the liver, demonstrating that uptake and efflux are closely regulated (33). The significantly lower liver zinc concentration in the vitamin B-6 deficient group (41.00 $\pm$ 3.31 μg/g) compared to control (57.00 $\pm$ 8.91 μg/g) and pair-fed (61.66 $\pm$ 7.48 μg/g) groups ($p < 0.0001$) (Table 7) may therefore indicate an impairment in this regulatory mechanism during vitamin B-6 deficiency. Most of the zinc in hepatocytes is exchangeable with plasma and the reduced levels of zinc in hepatocytes during hypozincaemia was attributed not only to altered zinc uptake rate, but also to altered zinc efflux (33).

Several studies have shown that the zinc concentration in bones is markedly influenced by the zinc level in the diet (21). It was argued that bone may serve as a functional, though limited, zinc store. Other researchers (62) showed that by pre-treating young quails with excess dietary zinc, their bone zinc levels increased. An ensuing period of dietary zinc deficiency was found to result in decreased bone zinc concentration. Calhoun *et al.* (1978) obtained similar results using weanling rats (21). They concluded that zinc was released from bone during periods of dietary zinc deficiency.

The significantly lower ($p < 0.01$) tibia zinc concentration in the vitamin B-6 deficient group (435.22 $\pm$ 47.47 μg/g) compared with pair-fed (494.77 $\pm$ 46.97 μg/g) and control (485.66 $\pm$ 27.19 μg/g) groups (Table 7) lends support to the suggestion that zinc is mobilized from bone under conditions where zinc absorption and tissue uptake of zinc may be affected. Other studies have shown that although zinc concentrations in bone, liver and plasma decrease in zinc deficient animals, muscle zinc concentration is conserved (76) even though muscle comprises 50-60% of the whole-body zinc. It was proposed that some tissues lose zinc in order to support other tissues (81). This hypothesis categorises tissues as either low or high priority tissues. Bone and to a lesser extent plasma, liver and testes are low priority tissues. Zinc is mobilized from these tissues to support high priority tissues such as muscle and skin (81). Therefore, in addition to the apparent impairment of intestinal zinc absorption and tissue zinc uptake as a consequence of reduced synthesis of zinc carriers and/or zinc-binding ligands, redistribution of zinc from bone, liver, testes, kidney and plasma may also contribute to the decreased zinc levels observed in these tissues of the vitamin B-6 deficient rats. However, the exact mechanism affecting zinc mobilization is not known.

In the current study (experiment 1), all four groups were in positive zinc balance, the control group, however, had a significantly higher zinc balance (666.00 ± 483.71 μg) at the end of the experimental period compared with the pair-fed (239.22 ± 435.70 μg), vitamin B-6 deficient (113.55 ± 383.33 μg) and vitamin B-6 deficient-PA-supplemented (257.77 ± 380.11 μg) groups (Table 12(f)). No significant difference existed among the latter three groups. The pair-fed group, however, showed a trend towards a higher zinc balance compared with the vitamin B-6 deficient group. This would imply that, apart from the low caloric intake, a lack of dietary vitamin B-6 may have contributed to the low zinc balance of the vitamin B-6 deficient group. A trend towards a higher zinc balance was also apparent in the vitamin B-6 deficient-PA-supplemented group which may imply a role for picolinic acid in zinc absorption.

The low zinc balance in the vitamin B-6 deficient group compared with the pair-fed group in the final week of the experimental period also lends support to the suggestion that vitamin B-6 deficiency impairs zinc absorption. This would explain the lower zinc content in the tissues and plasma of the vitamin B-6 deficient group compared with the pair-fed group (Table 7).

5.6 Picolinic acid production and the effect of picolinic acid supplementation on zinc absorption and tissue zinc levels during vitamin B-6 deficiency.

Picolinic acid (PA) is a product of tryptophan metabolism. Pyridoxal is required as a cofactor by the enzyme kynureninase in the pathway from tryptophan to picolinic acid (Figure 3). Studies have shown that the addition of PA to normal rat diets promoted growth and increased absorption and retention of dietary zinc (42). It has been proposed that the pancreas produces a zinc binding ligand, viz. picolinic acid which is then secreted into the lumen of the intestine where it forms a complex with zinc. This complex facilitates the passage of zinc through the luminal membrane of the absorptive cells (42). Therefore, if picolinic acid facilitates dietary zinc absorption, a deficiency of vitamin B-6 should impair zinc absorption.

Studies have further demonstrated that ligation of the pancreatic ducts markedly decreases the absorption of zinc (45). It was suggested that the production of PA from tryptophan and the secretion of this ligand into the intestinal lumen may be the rate limiting step in the absorption of dietary zinc (45).

Other researchers have, however, argued against a role for picolinic acid in zinc absorption (70). It was proposed that PA is only a minor metabolite of tryptophan. It was further suggested that PA is a good chelator of zinc and that increasing the zinc absorption by the addition of a chelating agent does not indicate that the chelating compound has a normal physiological role in absorption.

However, further studies have shown that when diets were supplemented with nitrilotriacetic acid (NTA) or a zinc-NTA complex, the zinc chelating ligand had no effect on the zinc concentration of organs from nursing pups, whereas zinc dipocolinate was found to increase the zinc concentration in the liver and kidneys of nursing pups (43). It was concluded that a specific type of ligand (viz. PA) is required to facilitate zinc transport from the intestinal lumen into the body (43).

The vitamin B-6 deficient-PA-supplemented group in this study (Table 7) was found to have significantly higher concentrations of zinc in the testes (45.66 ± 5.74 μg/g, $p < 0.0001$), kidney (47.77 ± 3.83 μg/g, $p < 0.0001$) and tibia (474.55 ± 30.17 μg/g, $p < 0.015$) compared with the non-supplemented vitamin B-6 deficient group (38.77 ± 4.79 μg/g, 38.44 ± 3.43 μg/g and 435.22 ± 47.47 μg/g respectively). Liver zinc levels also tended to be higher in the vitamin B-6 deficient-PA-supplemented group (45.66 ± 6.80 μg/g) when compared with the vitamin B-6 deficient group (41.00 ± 3.31 μg/g). Although the tibia zinc concentration of the vitamin B-6 deficient-PA-supplemented group showed no significant difference when compared with that in the control and pair-fed groups, the overall results observed may demonstrate that supplementation with picolinic acid ameliorates the impaired zinc uptake caused by vitamin B-6 deficiency and may therefore support the postulation that picolinic acid plays a role in cellular uptake of zinc.

However, since a response to supplementation cannot be taken as unequivocal evidence of an original deficiency (55), the pancreatic PA levels were measured in experiment 2 of the current study to ascertain whether the pancreatic production of PA was altered during a vitamin B-6 deficiency. Pancreatic picolinic acid levels were found to be significantly lower in the vitamin B-6 deficient group compared to the other three groups (Table 16). In addition the PA level of the partially deficient group (2.08 ± 0.47 μmoles/g) was also significantly lower than PA levels in the control (3.04 ±0.61 μmoles/g) and pair-fed (2.90 ± 0.45 μmoles/g) groups but significantly higher than the deficient group (0.94 ± 0.33 μmoles/g). These levels correlate well with the PLP levels of the four groups (Table 16), demonstrating the effect of altered vitamin B-6 levels in pancreatic production of PA. The pancreatic PA levels of the control, pair-fed and deficient groups also correlate with the zinc levels in the tibia, liver, kidney and plasma (Table 7), of control, pair-fed and vitamin B-6 deficient rats, suggesting a link between PA production and tissue and plasma zinc levels. The present observations would concur with those of Evans and Johnson (1981) who reported that both zinc absorption and pancreatic picolinic acid concentration were increased as the level of vitamin B-6 in the diet increased (46). The current findings were also in agreement with studies which showed that supplementation with picolinic acid increased zinc concentrations and growth rates in rats fed a vitamin B-6 deficient diet (47). In their study, however, Evans and Johnson (1981) did not investigate the

effect of a vitamin B-6-free diet on picolinic acid production, nor was the effect of a vitamin B-6-free diet on tissue zinc levels determined (46). The current study has revealed that rats receiving a vitamin B-6-free diet not only have significantly lower picolinic acid levels than rats receiving normal or partially deficient vitamin B-6 diets, but that these levels correlate with the tissue and plasma zinc levels in these rats. Although the extent of the involvement of picolinic acid in zinc absorption cannot be elucidated, these results would favour an association between vitamin B-6 deficiency, picolinic acid production and the degree of zinc absorption and tissue zinc levels. However, various secondary factors arise during a vitamin B-6 deficiency which may also affect tissue zinc content. For example, vitamin B-6 deficiency has been found to increase iron levels in serum, liver and spleen (109).

5.7 Assessment of zinc status in vitamin B-6 deficiency

Zinc homeostasis is maintained mainly by manipulation of gastrointestinal absorption and gastrointestinal excretion of the trace element. The efficiency of this mechanism for regulating whole body homeostasis is made apparent by the relatively constant levels of zinc in the tissues and body fluids when the level of the dietary zinc is markedly varied (75). Even in severe experimental zinc deficiency, there is almost no reduction in tissue zinc levels (55). The only tissue which shows a significant reduction in zinc concentration during a severe zinc deficiency is bone (55). However, it has been suggested that the zinc sequestered in bone is neither readily available nor exchangeable and a zinc deficiency may therefore be present without any change in bone zinc and vice versa (55). Measuring tissue zinc alone was therefore, not considered to be of much use in determining zinc status during an experimental zinc deficiency (55).

In vitamin B-6 deficiency, altered tissue zinc levels have been observed. Since both increased (52) and decreased (68) zinc levels have been reported in vitamin B-6 deficient rats much controversy exists regarding the validity of tissue zinc levels as a means of assessing body zinc status. In addition to this diagnostic problem is the fact that both vitamin B-6 and zinc deficiencies present with similar physical symptoms; the most obvious being decreased food intake, growth failure and dermatological lesions. However, in the current study a significant decrease in tissue zinc levels was observed not only in the tibia but also in the kidney, liver and testes of vitamin B-6 deficient rats. It is therefore the contention, of the present investigation, that although the measurement of tissue zinc levels alone may not be fully adequate in determining body zinc status, it does still provide valuable information when analysing zinc status.

Plasma and serum zinc have most frequently been used to diagnose zinc deficiency. Since hypoalbuminaemia is frequently accompanied by hypozincaemia, it has been suggested that plasma zinc concentration becomes reduced when albumin concentration is low because of a reduced number of zinc-binding sites in plasma (55). However, plasma or serum zinc levels have also been strongly criticised as being unsuitable to assess zinc status (166). It has been observed that under normal conditions, approximately one of every 50 albumin molecules is

associated with a zinc atom. Therefore, even under conditions where albumin levels are severely reduced, there would always be an excess of sites available for zinc binding. In addition, plasma zinc levels are reduced during infection, endotoxaemia and steroid hormone administration (55). Since these changes are attributed to metabolic redistribution of zinc within the free pool and not to a change in zinc status, plasma zinc is regarded as an unreliable indicator of zinc deficiency (55).

Zinc deficiency results in a cessation of growth, which is believed to be a means of adapting to lower zinc intakes and conserving tissue zinc (55). This occurs because no functional reserve or store of zinc is available for use when dietary zinc intake is decreased (55). However, in the event of the zinc balance becoming negative, the pool from which zinc is mobilized has been described as a small rapidly turning over, free, metabolic buffer pool, viz., zinc metallothionein-1 (55).

Both plasma and erythrocyte metallothionein levels are sensitive to dietary zinc intake and were to be reduced to nondetectable levels in zinc-deficient animals (31).

Measuring erythrocyte Zn-MT-1 levels is, as far as it is known, being used in this study for the first time to assess the effect of vitamin B-6 deficiency on zinc status.

In the present study (experiment 1) the erythrocyte Zn-MT-1 levels of the vitamin B-6 deficient (30.09 ± 14.66 mg/ml red blood cells) and the pair-fed (29.49 ± 6.37 mg/ml red blood cells) groups were significantly reduced when compared with the control group (53.79 ± 15.47 mg/ml red blood cells, Table 8). No significant difference in erythrocyte Zn-MT-1 levels existed among the pair-fed, vitamin B-6 deficient and vitamin B-6 deficient-PA-supplemented groups. A dual role for MT has been proposed wherein MT serves an uptake and storage function in liver cells and a sequestration function to control zinc absorption in enterocytes (145). The exact mechanism involved however, is still to be elucidated. Synthesis of MT involves accumulation of zinc and amino acids into the MT protein during the synthetic phase. The reduced amino acid transport and decreased protein synthesis which arise during vitamin B-6 deficiency will therefore affect metallothionein synthesis. This may imply an impairment in its storage and absorptive roles. An impaired binding of zinc to MT within the intestinal absorptive

cell, as discussed earlier, may result in appreciable amounts of zinc being secreted back into the intestinal lumen and lost in the faeces (Figure 7). This, together with the reduction in the zinc-binding ligands e.g. picolinic acid, would result in reduced zinc absorption in vitamin B-6 deficiency. However, erythrocyte Zn-MT-1 levels were also reduced in the pair-fed group and, in fact, there was no significant difference in erythrocyte Zn-MT-1 levels between the vitamin B-6 deficient and pair-fed groups. The pair-fed group, on the other hand, showed no reduction in plasma protein levels which indicates normal amino acids transport and protein synthesis in this group. To maintain zinc homeostasis when dietary zinc intake is low, as with the pair-fed group, zinc absorption is increased with a consequent increase in tissue zinc uptake. Zinc is bound to MT such that it readily disassociates from MT (165). Although the pair-fed group consumed significantly less zinc than the control group ($p < 0.0001$), the tissue zinc levels (tibia and liver) were not significantly different from the control levels and were even found to be significantly higher in the testes (52.11 ± 2.42 μg/g) compared to the control group (46.11 ± 2.2 μg/g). It is proposed here that under conditions of reduced dietary zinc intake, the zinc homeostatic mechanism is regulated such that not only is zinc absorption increased, but also the zinc uptake by tissues. This may suggest that even zinc bound to temporary storage pools like erythrocyte metallothionein may be released for tissue uptake. Zinc associated with the red blood cells accounts for at least 75% of that found in blood (33). It is therefore not inconceivable that under conditions of reduced dietary zinc intake, a portion of the zinc bound to MT may be released for uptake by certain priority tissues. This could account for the reduced erythrocyte Zn-MT-1 levels in the pair-fed group and there being no significant difference in plasma zinc concentrations between the pair-fed and control groups. In the vitamin B-6 deficient and vitamin B-6 deficient-PA-supplemented groups, on the other hand, the low erythrocyte Zn-MT-1 levels were also accompanied by reduced plasma and tissue zinc levels.

The problem with reconciling the normal plasma zinc levels observed in the pair-fed group with the reduced erythrocyte Zn-MT-1 levels in this group illustrates the importance of analysing plasma, tissue, faecal and urinary zinc content in assessing the zinc status of rats. This is of particular importance when zinc deficiency is brought about by factors other than reduced dietary zinc intake, for example, vitamin B-6 deficiency. Although it has been reported that the

use of plasma zinc and erythrocyte Zn-MT-1 levels may be used to make an unequivocal diagnosis of zinc deficiency (55), subsequent studies suggest that MT in the blood cells are associated mainly with the lighter cell fractions which are rich in reticulocytes (147). It has been reported that in zinc-injected rats, the additional cellular MT accumulated primarily in the reticulocytes and other young cells while concentrations in the mature erythrocytes were not greatly affected (147). In addition, blood cell MT levels were also observed to be influenced by severe iron deficiency, starvation and protein deficiency (147). It has therefore been suggested that care should be taken when interpreting blood cell MT levels and that MT concentrations would more appropriately be expressed as a function of reticulocyte numbers (147).

In view of the above, it can be seen that many complications may arise when interpreting erythrocyte Zn-MT-1 data especially as in the case of the present study where the alteration in zinc status arises from a vitamin B-6 deficiency. Therefore, analysis of tissue and plasma zinc levels as well as performing a balance study appear to be indispensable when trying to establish zinc status during a vitamin B-6 deficiency. Using these parameters not only can the cause of low zinc levels be established but the route of zinc loss may also be determined.

Studies have shown that MT concentrations in various tissues are influenced by zinc deficiency (185). The concentration of MT in plasma and liver are frequently related, suggesting that the liver is the main source of plasma MT (185). It has been reported that most types of stress results in increased MT production, especially in the liver, and increased hepatic uptake of zinc and a reduction of plasma zinc concentration (185).

The analysis of tissue metallothionein levels in experiment 2 of the current study revealed that kidney and pancreatic MT levels were significantly lower in the vitamin B-6 deficient group compared to the other three groups (Table15). The control group in turn had significantly higher MT levels than the pair-fed and partially deficient group. The kidney MT levels in the control (35 ± 7 μg/g), pair-fed (29 ± 5 μg/g) and vitamin B-6 deficient (20 ± 3 μg/g) groups correlate significantly with the kidney zinc concentrations observed in the control (64.11 ± 8.20 μg/g), pair-fed (50.11 ± 5.27 μg/g) and vitamin B-6 deficient (47.66 ± 5.27 μg/g) groups of experiment 1 ($p < 0.0001$). Studies have shown that a decreased food intake, low plasma and tissue iron levels or protein deficiency are associated with the decreased kidney MT levels (147).

The results of the current study would concur with this finding. It may therefore be surmised that the lower kidney MT levels observed in the vitamin B-6 deficient group (experiment 2) may be attributed to low food intake and decreased protein synthesis while the lower kidney MT level in the pair-fed group (compared to the control group) may be attributed to low food intake only, since no apparent difference in plasma protein levels existed between these two groups (Table 17). As far as it is known this is the first study to employ tissue MT levels when assessing zinc status during a vitamin B-6 deficiency.

However, the liver MT levels of the four groups of rats (Table 15) do not correlate with the findings in kidney and pancreatic MT levels. There was no significant difference in liver MT levels among the four groups of rats. This finding is in conflict with liver zinc levels observed in experiment 1 of the present study, where the vitamin B-6 deficient group had significantly lower zinc levels compared to pair-fed and control groups ($p < 0.0001$, Table 7). The reason for this discrepancy is unclear. However, factors contributing to these differences could be that these analyses were performed in two different experiments and conditions may have varied. Furthermore, it is known that stress factors may result in increased liver MT synthesis (16). It may therefore be argued that stress factors may have caused an increase in liver MT production but because of the reduced Zn levels in plasma and erythrocyte-MT, the increase in liver MT concentration was not accompanied by a concomitant increase in zinc levels. Since liver MT levels are greatly influenced by stress factors (many of which arise during a vitamin B-6 deficiency and a zinc deficiency), monitoring MT levels in this tissue may not be appropriate when assessing zinc status.

Although tissue MT levels alone cannot be used to measure the actual zinc status of an animal, much information can be gained regarding the cause of an increase or decrease in MT levels in specific tissues. This may be of some value when trying to ascertain the cause of an altered level of zinc in specific tissues or plasma eg. reduced kidney MT levels may indicate reduced protein synthesis, iron deficiency or zinc deficiency.

Morrison *et al.* (1988) have reported that fractionalisation of blood on a discontinuous Percoll gradient has shown that metallothionein occurs mainly in the lightest and youngest red cells ie., the reticulocytes (122). These researchers have also observed that treatments which induce erythropoiesis also increase the amount of metallothionein present in circulating blood cells

(122). These observations would therefore suggest that metallothionein levels are affected by erythropoietic activity and the numbers of reticulocytes (147). Plasma erythropoietin levels were found to increase in iron deficient rats (147). On the other hand protein deficiency and restriction of food intake have been observed to inhibit erythropoietin production and reticulocytosis. Protein deficiency and reduced food intake are well established symptoms of vitamin B-6 deficiency. Although an increase in tissue iron levels has been reported in vitamin B-6 deficient rats (72) researchers in this laboratory, studying the effect of vitamin B-6 deficiency on iron status, found no changes in iron absorption in vitamin B-6 deficient rats, which were found to be in positive iron balance (23). In the current study (experiment 2), serum erythropoietin levels were observed to be significantly lower ($p < 0.0001$) in the vitamin B-6 deficient group (34.35 ± 4.29 μU/ml) compared to the control (38.41 ± 3.22 μU/ml), pair-fed (38.17 ± 4.11 μU/ml) and the partially deficient (37.96 ± 5.68 μU/ml) groups. However, since there was no significant difference in erythropoietin levels among the latter three groups, it may be deduced that the lower erythropoietin levels in the vitamin B-6 deficient group are not due to the lower food intake in this group. Instead the protein deficiency observed in the vitamin B-6 deficient group may account for reduced erythropoietin levels in this group (Table 17). It therefore does not seem likely that the changes in erythropoietic activity during vitamin B-6 deficiency of the current study were substantial enough to play a major role in the altered red blood cell or tissue metallothionein levels observed in the deficient group.

The primary aim of the current study was to assess zinc status during vitamin B-6 deficiency in rats and to determine whether picolinic acid plays a role in zinc absorption. Previous studies, assessing the effect of vitamin B-6 deficiency on body zinc used tissue or plasma zinc levels as parameters (68, 52, 107). The lack of a definite conclusion, regarding zinc status in these studies indicated a need for a more extensive study which encompasses additional parameters to investigate the above relationship.

This study has conclusively shown that vitamin B-6 deficiency alters body zinc status and that erythrocyte Zn-MT-1 levels on its own cannot be used to assess body zinc status in this type of investigation. Furthermore, in order to study zinc status, a zinc balance study together with the analysis of plasma and tissue zinc levels is essential. These analyses together with erythrocyte and tissue MT levels have provided information which shows that not only is zinc status altered in vitamin B-6 deficient rats but has also produced possible explanations for the changes in tissue zinc levels observed in the deficient rats.

The observations, in the present investigation, regarding picolinic acid and its involvement in zinc absorption would favour a role for PA in zinc absorption and would therefore be in agreement with reports by various other researchers (42, 43, 45, 46). However, because of the various secondary factors arising from a vitamin B-6 deficiency which may also affect zinc status, the extent of the influence PA has on zinc status cannot be ascertained. The current study has, nevertheless, established that PA production is significantly reduced during vitamin B-6 deficiency and that these results correlate with tissue and plasma zinc levels during a vitamin B-6 deficiency. In addition, supplementation with PA increases zinc concentrations in the tibia, testes and kidney of vitamin B-6 deficient rats (Table 7).

CHAPTER SIX

CONCLUSION

From the findings of this study it may be concluded that :

1. A deficiency of vitamin B-6 in the diet results in an altered zinc status. Vitamin B-6 deficiency results in a lower zinc balance, significantly decreased tissue and plasma zinc levels, reduced erythrocyte Zn-MT-1 concentrations and significantly lower kidney and pancreatic MT levels.

2. Picolinic acid plays a role in zinc status during vitamin B-6 deficiency. Pancreatic picolinic acid production in the vitamin B-6 deficient group was significantly lower than in the pair-fed and control groups. This corresponded to the significantly lower tissue and plasma zinc levels in the vitamin B-6 deficient group. In addition, supplementation with PA increases tissue zinc levels. A role for picolinic acid in zinc absorption cannot therefore be ruled out.

3. The reduced zinc levels in tissues such as bone, liver and erythrocytes of the vitamin B-6 deficient group may suggest that these tissues form part of the temporary storage pool for zinc and that under deficiency conditions zinc may be mobilised to other priority tissues.

4. The reduced growth rate during a vitamin B-6 deficiency may be due to reduced plasma protein and insulin levels.

5. Vitamin B-6 deficiency contributes to the development of hypertension. The degree of raised arterial pressure is dependent on the concentration of vitamin B-6 in the diet.

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APPENDIX

The following article was sent for publication after the initial experiments (i.e. erythrocyte-Zn-MT-1 analysis) referred to in the text. Its conclusions reflect the findings in that study.

Internat. J. Vit. Nutr. Res. *67*
(1997) 22–26
Received for publication
December 6, 1995

Vitamin B_6
Zinc
Metallothionein
Status

Zinc Status in Vitamin B_6 Deficiency

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Summary: Three groups of young male Wistar rats were maintained on diets consisting of 7 mg pyridoxine hydrochloride/kg diet (control and pair-fed groups) and 0 mg pyridoxine hydrochloride/kg diet (deficient group) for six weeks. The zinc status of all rats was assessed by measuring their erythrocyte zinc-metallothionein-1 (Zn-Mt-1) and plasma zinc levels. A significant difference ($p < 0.001$) in plasma zinc levels was observed between the deficient group and the control and pair-fed groups (1.35 µg/ml ±0.08, 1.99 µg/ml ±0.06 and 2.03 µg/ml ±0.07 respectively). Erythrocyte Zn-Mt-1 levels were significantly lower in vitamin B_6 deficient rats when compared to control animals. No significant difference in Zn-Mt-1 levels existed between vitamin B_6 deficient and pair-fed groups suggesting that the reduced Zn-Mt-1 levels in vitamin B_6 deficient rats may be due entirely to their decreased food intake (8.9 g/day compared to 15 g/day of control rats).

Introduction

Since both zinc and vitamin B_6 are known to participate in a great variety of enzymatic systems, interactions between these two nutrients would be expected. It has, in fact, been observed that many symptoms of zinc deficiency and vitamin B_6 deficiency are similar – these include impairment of food consumption and growth, dermatological lesions and impaired immune function [1].

Conflicting reports on the influence of dietary levels of vitamin B_6 and tissue and plasma zinc levels have appeared in the literature. Thus far the concentration in various body fluids and tissues have been used to assess zinc status. Tissue levels of zinc were shown to be directly related to vitamin B_6 intake in laboratory animals [2–4]. Hsu [3] reported decreased zinc concentrations in plasma, liver, pancreas and heart of vitamin B_6 deficient rats; Gershoff [5] found that the zinc content of the pancreas, serum and kidneys was increased. On the other hand, Ikeda *et al* [6] found no significant difference in the zinc content of the liver, pancreas, spleen, lung and testes of vitamin B_6 deficient rats except for a significant increase in kidney zinc concentration. These conflicting results raise the possibility that the assessment of tissue zinc concentrations in vitamin B_6 deficient rats may be unreliable in assessing zinc status.

In vitamin B_6 studies, some investigators used plasma zinc concentration to monitor changes in body zinc [3]. Plasma zinc concentration is however also known to be influenced by factors such as infection, disease and steroid hormone administration [7].

The problem in diagnosing zinc deficiency or the zinc status of vitamin B_6 deficiency, therefore, seems to be one of finding a suitable indicator or method to determine the changes occurring in body zinc concentrations in vitamin B_6 deficiency.

More recently, zinc-metallothionein-1 (Zn-

Abbreviations used: Zn-Mt-1, zinc-metallothionein-1; PN. HCl, pyridoxine hydrochloride; PLP, pyridoxal-5-phosphate.

Mt-1) has been identified as the metabolic buffer pool for zinc and it has been suggested that the measure of Zn-Mt-1 concentration is a more reliable indicator of zinc status [8, 9]. Moreover, erythrocyte metallothionein appears less responsive to stress and infection than plasma metallothionein or plasma zinc in experimental animals [10].

We have chosen to use erythrocyte Zn-Mt-1 concentrations together with plasma zinc levels to assess the zinc status of vitamin B_6 deficient rats in this study.

Materials and Methods

Experimental animals: All animals used in this experiment were male rats of the Wistar strain bred at the Biomedical Resource Centre at the University of Durban-Westville. The necessary ethical approval was obtained before the commencement of the study.

Twenty seven weanling rats (57.8 ± 2.3 g), age 3 weeks, were divided into three groups of 9 rats each viz. a control group receiving the normal AIN-76™ purified diet with 7 mg pyridoxine hydrochloride (PN.HCl) per kg diet [11], a deficient group receiving the same diet but with no PN.HCl and a pair-fed group receiving the control diet.

The rats were housed individually in perspex metabolic cages in an air-conditioned room with average humidity and temperature of 55% and 22°C respectively and automatically controlled 12 hour light and dark cycles. Deionised water and the diets were supplied *ad libitum.* Food consumption was recorded daily and all animals were weighed once a week. Each group of rats was maintained on its particular diet for a period of six weeks. At the end of this period the rats were starved overnight (with access to deionised water) and then anaesthetized with halothane. Blood samples were obtained by cardiac puncture and transferred to plastic tubes free of zinc (using EDTA as anticoagulant). The samples were centrifuged and red blood cells and plasma separated. The red blood cell samples were rinsed with a saline solution and all samples were then stored at –23°C until assayed.

Analytical methods

Plasma pyridoxal-5-phosphate: The plasma pyridoxal-5-phosphate (PLP) concentration was analyzed in collaboration with Professor Ubbink (University of Pretoria) using the HPLC method of Ubbink *et al* [12, 13]. This method uses an internal standard and is based on post-column semicarbazone formation of PLP followed by fluorescence detection.

Plasma zinc: Plasma zinc levels were determined by flame atomic absorption spectrophotometry (Model 2380, Perkin-Elmer Corp., Norwalk, C.T., USA).

To compensate for differences in viscosity between plasma (diluted fivehold) and zinc standards, the zinc standards were prepared using a zinc stock standard solution (B. D. H. Chemicals, Poole, Eng.) and a solution of glycerol and deionised water (5 : 95 by volume). Plasma samples were diluted with deionised water prior to aspiration directly into the atomic absorption spectrophotometer [14].

Erythrocyte zinc-metallothionein-1: A radioimmunoassay technique was used to assay red blood cell Zn-Mt-1 concentrations [15]. Red blood cells from 1 ml of blood were separated after centrifugation and washed with saline, treated with 1 ml distilled water and lysed by repeated freeze thawing. Thereafter 100 µl aliquots of the lysates were assayed. The assay was specific for metallothionein-1 and no significant cross-reaction with rat metallothionein-II occurred [10].

Results

Effects of pyridoxine deficiency on food consumption and growth: The pyridoxine deficient group showed a distinct deficiency syndrome

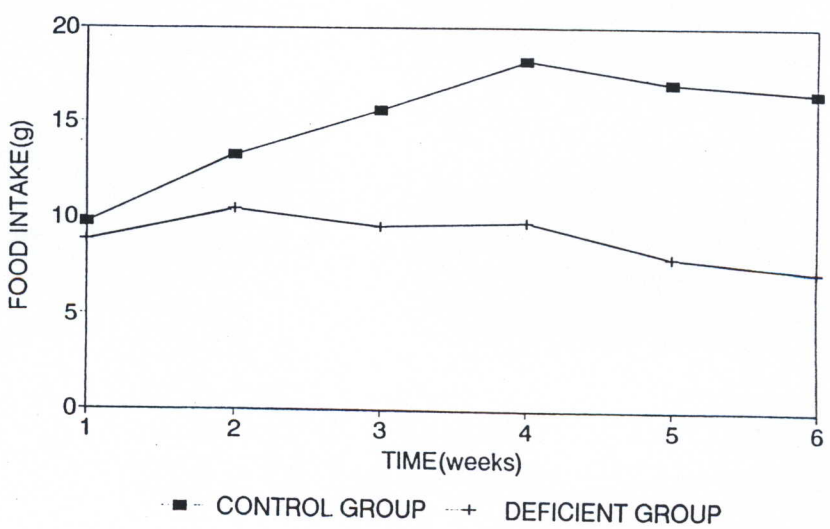

Figure 1: Food consumption of control and deficient rats during the six week experimental period. There was a significant difference in food consumption between the two groups from the second week onwards ($p < 0.05$).

characterized by alopecia and an area of acrodynia around the snout, paws and eyes. Notwithstanding these skin changes, there was no sign of infection. The food consumption of the deficient animals declined significantly from the second week onwards (Tab. I). The deficient rats each consumed an average of 8.9 g of food per day as compared to 15 g in the control group.

Weight gain in the deficient rats was markedly reduced ($p<0.001$) as compared to that in the control and pair-fed groups (Fig. 2). A significant difference in weight gain was also apparent between the control and pair-fed groups ($p<0.001$). The average initial weights were 55.3 ± 7.08 g in the control group, 57.3 ± 8.35 g in the pair-fed group and 57.8 ± 8.03 g in the pyridoxine deficient group. The final weights were 252 ± 15.41 g, 189.4 ± 12.74 g and 129.44 ± 30.06 g for the control, pair-fed and deficient groups respectively.

Plasma pyridoxal-5-phosphate: Plasma PLP levels of the vitamin B_6 deficient group were significantly lower than those of the control ($p<0.001$) and pair-fed groups ($p<0.001$). There was no significant difference in plasma PLP concentrations between the control and pair-fed groups (Tab. I).

Plasma zinc: The vitamin B_6 deficient rats showed significantly lower plasma zinc concentrations compared to control ($p<0.001$) and pair-fed ($p<0.001$) animals. There was no significant difference in plasma zinc levels betwen the control and pair-fed groups (Tab. I).

Erythrocyte zinc-metallothionein-1: Red blood cell Zn-Mt-1 concentrations were significantly lower in the vitamin B_6 deficient group compared to the control group ($p<0.001$). There was also a significant difference in Zn-Mt-1 levels between the control and pair-fed groups ($p<0.001$). Surprisingly, there was no significant difference in erythrocyte Zn-Mt-1 concentrations between pair-fed and vitamin B_6 deficient groups (Tab. I).

Statistical Analysis: Individual analysis of variants (ANOVAs) was performed on the growth and food consumption for each week. Duncan's multiple-range tests were used to determine differences in growth, food consumption, plasma PLP and Zn-Mt-1 levels between the three groups. A significant difference exists between groups if the p-value is <0.05.

Discussion

Several studies have reported that tissue zinc levels or zinc uptake are directly related to vitamin B_6 intake in rats [2, 3, 5]. This would suggest that the nutritional status of zinc may be related to vitamin B_6 status. Previous research, done in this laboratory, on vitamin B_6 and zinc bioavailability showed that the tibia zinc concentration of vitamin B_6 deficient rats (141.12 ± 6.02 µg/g) was significantly lower ($p<0.0001$) than control (161.34 ± 7.78 µg/g) and pair-fed control rats (159.11 ± 9.07 µg/g) [16].

Zn-Mt-1 was recently identified as the meta-

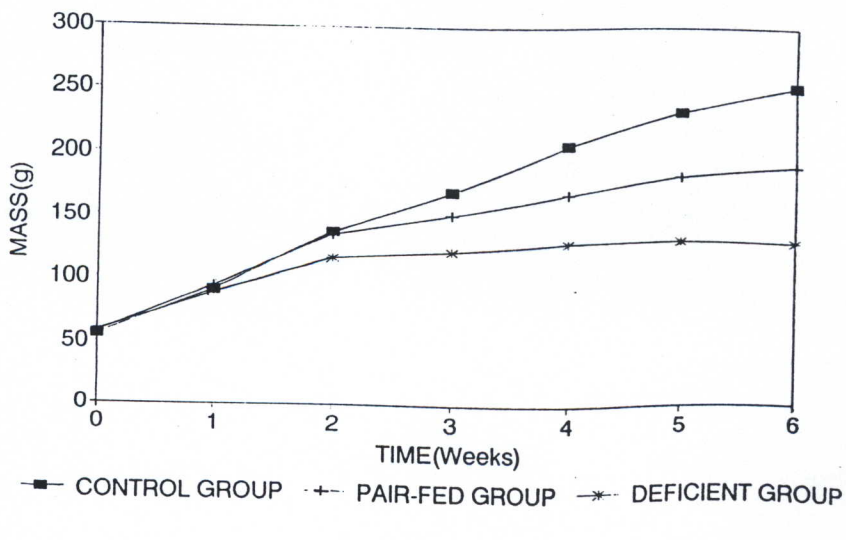

Figure 2: Mean body-mass of control, vitamin B_6 deficient and pair-fed rats during the 6 week experimental period. A significant difference in body-mass existed between all 3 groups from the third week onwards ($p<0.05$).

Table I: Effect of vitamin B_6 deficiency on plasma PLP, plasma Zn and erythrocyte Zn-Mt-1 levels

Group	Plasma PLP (n mol/l)	Plasma Zn (µg/ml)	RBC Zn-Mt-1 (µg/ml)
Control	373 ±166[a]	1.99±0.06[a]	53.79±5.16[a]
Pair-fed	421 ±150[a]	2.03±0.07[a]	29.49±2.13[b]
Deficient	13.9±4.2[b]	1.35±0.08[b]	30.09±3.17[b]

All values are expressed as means ± SD.
Values in each vertical column having different superscripts are significantly different ($P<0.05$).

bolic buffer pool for zinc [17]. Subsequent studies indicated that if there is zinc bound to MT-1 it would mean that there is sufficient zinc available to satisfy metabolic demands and therefore it may be unlikely that a zinc deficiency exists [17]. In this study we found that rats fed a vitamin B_6 deficient diet for six weeks had significantly reduced erythrocyte Zn-Mt-1 levels when compared to control rats ($p<0.001$). However, when compared to pair-fed control rats, the vitamin B_6 deficient rats did not show a significant difference in erythrocyte Zn-Mt-1 levels. These findings suggest that the reduced Zn-Mt-1 levels in the vitamin B_6 deficient group may be attributed to their decreased food intake (8.9 g/day compared to 15 g/day by the control group). A loss of appetite and the consequent decreased food intake are among the first symptoms of vitamin B_6 deficiency [18]. Contrary to the observations of other researchers, this study may imply that a vitamin B_6 deficiency does not alter zinc *per se*. The evidence presented in this study would suggest rather that the changes in tissue zinc levels observed by other researchers [3, 5, 16] may be a reflection of a metabolic redistribution of zinc within the free pool rather than a change in zinc status.

It is proposed that the redistribution of zinc may occur as a consequence of the reduced dietary zinc intake resulting from the reduced food consumption by the vitamin B_6 deficient rats. It has been established that zinc concentration in bone is markedly influenced by the dietary zinc intake and that zinc is released from bone during periods of dietary zinc deficiency [19]. It was argued that bone may serve as a functional, though limited, zinc store [19].

It has been suggested that some tissues lose zinc in order to support other tissues and that tissues may be categorised as either low or high priority tissues [20]. Bone and to a lesser extent plasma and liver are low priority tissues from which zinc is mobilized to support high priority tissues such as muscle and skin [20]. Studies have shown that although zinc concentration in bone, plasma and liver decreases in zinc deficient animals, muscle concentration is conserved [21] even though muscle comprises 50–60% of whole-body zinc. The significantly lower tibia zinc level of vitamin B_6 deficient rats compared with control rats observed in this laboratory [16] would lend support to the above hypothesis.

The significant difference in plasma zinc levels between pair-fed and vitamin B_6 deficient groups may, however, indicate that some factor other than decreased food consumption plays a role in reducing plasma zinc levels. It has been established that vitamin B_6 deficiency results in altered levels of certain hormones (e. g. insulin [22], growth hormone [23], glucocorticoids [24]) which play a role in zinc metabolism [25]. Steroid administration was found to decrease plasma zinc levels [7]. This change was attributed to metabolic redistribution within the zinc pool and not to a change in zinc status [17]. Vitamin B_6 deficiency has also been observed to alter various plasma factors which may affect zinc absorption. An increased iron level in various tissues including serum has been reported in vitamin B_6 deficient rats [6]. Also the intestinal uptake of calcium in rats was found to increase as a result of vitamin B_6 deficiency [26]. High levels of both iron and calcium are thought to impair the intestinal absorption of zinc [27, 28]. In addition, vitamin B_6 deficiency is reported to decrease plasma protein levels [29]. The latter could also be responsible for alterations in plasma zinc levels since approximately two thirds of the zinc present in plasma is bound to albumin.

Since metallothionein-1 is used to sequester free zinc in response to factors which cause a redistribution in plasma zinc concentration [17], it may be safe to conclude that erythrocyte Zn-Mt-1 levels provide an accurate reflection of the zinc status during vitamin B_6 deficiency. The results obtained in this study would indicate that the changes in Zn-Mt-1 concentrations are due to a decreased food consumption and would thus imply that vitamin B_6 deficiency does not *per se* alter the zinc status of rats.

Acknowledgements: We wish to thank Prof. I. Bremner (Rowett Research Institute, Aberdeen, Scotland) for the zinc-metallothionein-1 analysis.

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