THE EFFECT OF VITAMIN B-6 DEFICIENCY ON THE BIOAVAILABILITY OF ZINC IN THE RAT

by

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CONTENTS

PAGE

LIST OF FIGURES

LIST OF TABLES

CHAPTER ONE

INTRODUCTION

A large portion of modern nutrition research is concerned with the absorption and utilization of nutrients. The term bioavailability is used to describe these properties of foods. Although the concept of bioavailability applies to all nutrients, investigators are at present showing particular interest in the bioavailability of trace elements since a number of factors affect their absorption and assimilation.

The trace element of interest in the present study is zinc (Zn). Zn is one of the most extensively studied trace elements and has been long recognized as an essential nutrient for normal growth and physiological functioning. In fact, Zn is known to be an essential co-factor in at least a hundred enzymes (Peereboom, 1985).

Another nutrient that has been very widely researched is vitamin B-6. The biologically active form of vitamin B-6, pyridoxal-5-phosphate, is known to participate in over a hundred different enzymatic reactions involved in

carbohydrate metabolism, lipid metabolism, immune function, amino acid metabolism, gluconeogenesis, steroid hormone action, and nervous system function.

Since both Zn 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 noted 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 (Brown, 1981).

The effect of vitamin B-6 deficiency on the Zn levels of various tissues has been researched. However, much controversy exists with regard to the various results obtained. Both increased and decreased levels of tissue Zn concentration have been reported in vitamin B-6 deficiency (Hsu, 1965; Gershoff, 1967). No studies pertaining to the effect of vitamin B-6 deficiency on the bioavailability of Zn could be found in the literature.

For these reasons, the present study was undertaken to determine the effect of vitamin B-6 deficiency on the Zn bioavailability in rats.

Various studies dealing with the effect of different dietary Zn levels on Zn bioavailability have been done. Slope-ratio techniques using femur and tibia Zn concentrations have shown conclusively that dietary Zn levels affects bone Zn concentrations and body weight (Momčilović et al, 1975; Stuart et al, 1986).

Tibia Zn concentrations and growth were chosen in the present study as parameters to determine the effect of a vitamin B-6 deficiency on the bioavailability of Zn in rats.

CHAPTER TWO

LITERATURE SURVEY

2.1. Vitamin B-6

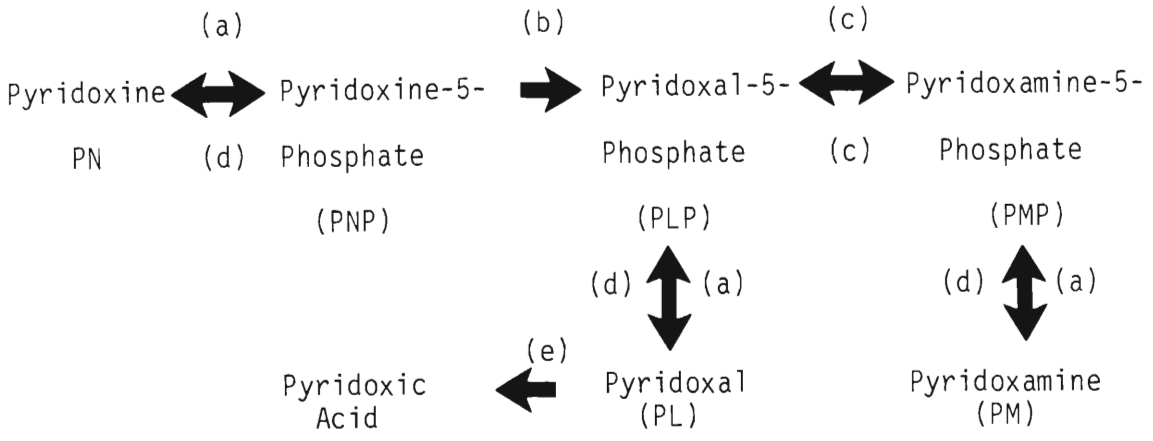
Vitamin B-6 (Pyridoxine) is the name which is used to describe the major forms of 3-hydroxy-5-hydroxymethyl-2-methyl pyridine namely pyridoxine (PN), pyridoxal (PL) and pyridoxamine (PM). Vitamin B-6 is classified as a water soluble vitamin and is generally found bound to proteins in biological materials.

2.1.1. Chemistry

The three naturally occurring forms of vitamin B-6 are pyridoxine (new terminology pyridoxol), pyridoxal and pyridoxamine (Driskell, 1984). The structure of these three vitamins forms, along with those of the coenzymes pyridoxal phosphate and pyridoxamine phosphate are shown in Figure 1. The various forms of the vitamin are water soluble and fairly stable in acids and dry heat but not in moist heat. They are unstable in visible and ultraviolent light, particularly when in neutral or alkaline solutions (Shideler, 1983).

The B-6 vitamin forms are interconvertible in animal tissues (Ink, 1984). These interconversions are summarized

in Figure 2.



The enzymes involved are:

(a) PL kinase

(b) PNP oxidase

(c) aminotransferase

(d) phosphatase, and

(e) PL oxidase; aldehyde dehydrogenase.

FIG. 2: Interconversion of vitamin B-6 compounds.

Pyridoxine hydrochloride (PN.HCl), the commonly available synthetic form of vitamin B-6, is easily crystallizable, odourless and has a melting point of 206°C. PN.HCl is the form of the vitamin typically used in nutrient supplements and animal diets. The hydrochloride is fairly unstable in light and moisture (Driskell, 1984).

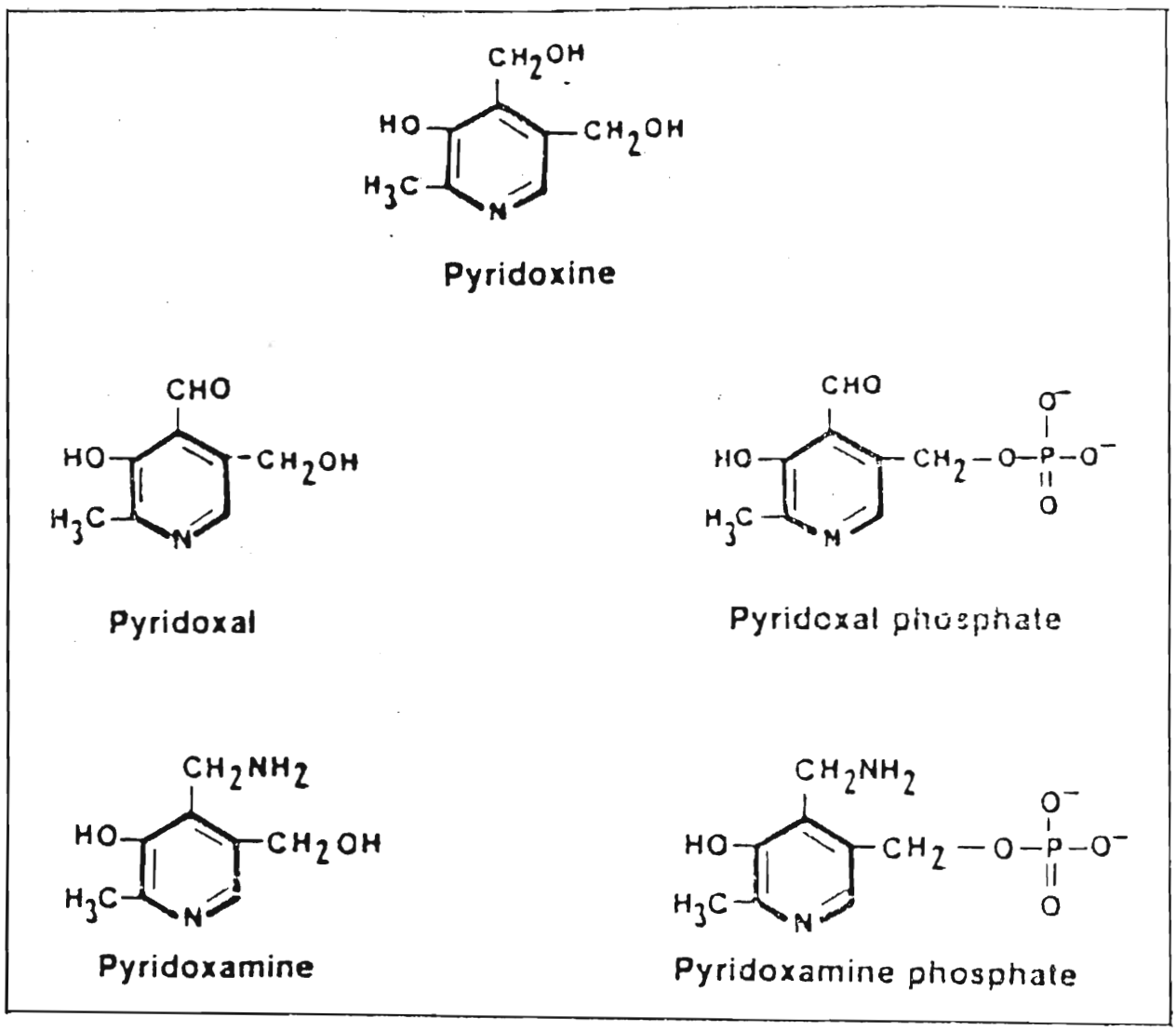


FIG. 1: Biochemical structures of the various forms of vitamin B-6.

2.1.2. Metabolism of vitamin B-6

Vitamin B-6 absorption occurs primarily in the jejunum but also in the ileum by passive diffusion (Rutishauser, 1982). Absorption in the colon is very slight. Before absorption occurs, PLP is hydrolysed to PL and PMP to PM. The hydrolysis occurs in the intestinal lumen and is catalysed by intestinal phosphatases. PL, PM and PN are then absorbed (Ink, 1984).

Although interconversion of the three forms of vitamin B-6 occurs in the intestinal cell, the primary forms which leave the intestinal cell and are transported to the liver are the forms which are initially absorbed. The liver is the primary organ for the interconversion and metabolism of the three forms of vitamin B-6. (Leklem, 1988). The metabolic steps involved are shown in Figure 2.

Pyridoxal is thought to be the circulating form used by most tissues. PL concentration in plasma is regulated by some binding by serum albumin but also by tighter binding to haemoglobin inside the erythrocyte (Henderson, 1985). PL uptake is by passive diffusion followed by phosphorylation. The liver is considered to be responsible for the synthesis of PLP found in plasma. PLP in circulation is bound primarily to albumin, a

mechanism which protects the PLP from hydrolysis and also permits delivery of PLP to other tissues. The PLP which is bound to glycogen phosphorylase of muscle represents the major body pool of vitamin B-6. The major vitamin B-6 excretory product is 4-pyridoxic acid which is formed from PL by the action of aldehyde oxidase or by the action of a NAD dependent aldehyde dehydrogenase (Ink, 1984).

2.1.3. Biochemical functions of vitamin B-6

The principal active form of vitamin B-6 is PLP. PLP is attached to an ε—amino group of the apoenzyme via a Schiff base linkage and is known to participate in a wide range of enzymatic reactions including:

2.1.3.1. Transamination reactions

Transamination reactions are catalysed by amino-transferases. These enzymes are involved in the interconversions of a pair of amino acids into their corresponding ketoacids; generally these are α- amino and α-ketoacids. PLP is converted to pyridoxamine-5-phosphate in the process of acquiring the amino group, and is then converted back to PLP when the amino group is attached to a ketoacid, which then becomes an amino acid. Each aminotransferase is specific for the specified pair of amino and keto acids functioning as substrates, but is non-specific for the other pair. Most of the amino acids are substrates for transamination reactions (Driskell, 1984). A typical transamination reaction involving PLP as coenzyme is shown in Figure 3.

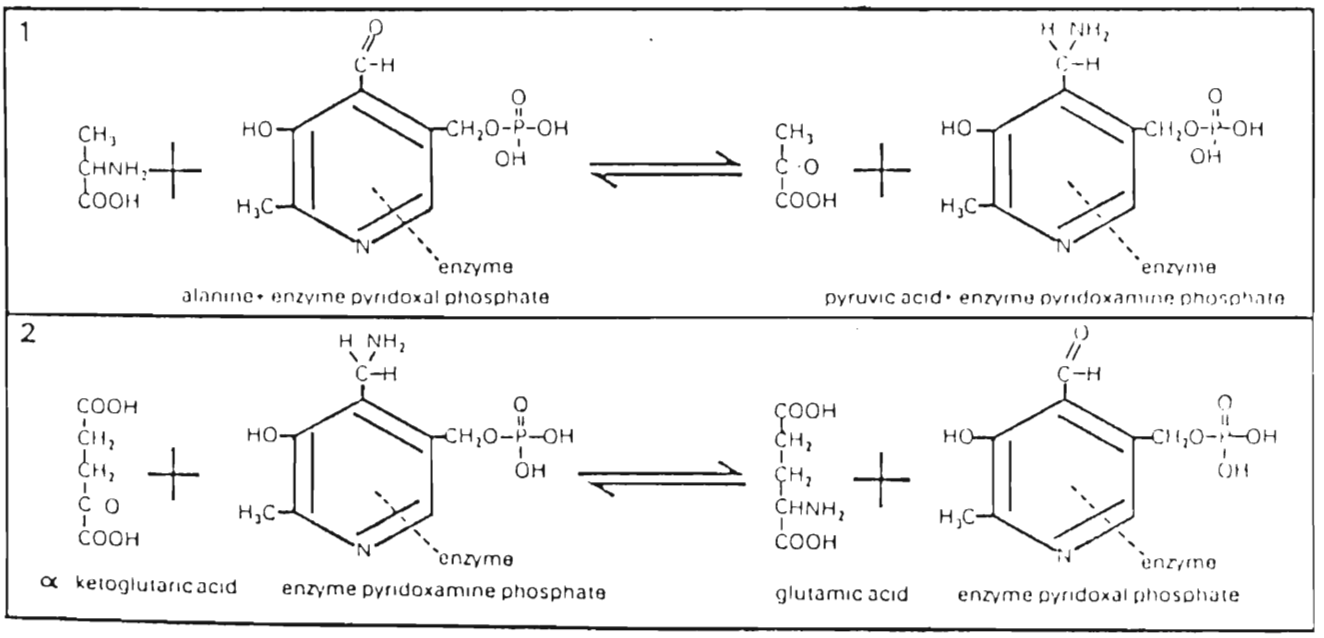


FIG. 3: Typical transamination reaction involving pyridoxal phosphate as coenzyme.

2.1.3.2. Vitamin B-6 and tryptophan - niacin metabolism

The involvement of vitamin B-6 in the conversion of tryptophan to niacin is one of the most extensively studied vitamin B-6 amino acid interrelationships. This in part is related to the use of the tryptophan load test to assess vitamin B-6 status (Leklem, 1988). There is only one step in the conversion of tryptophan to niacin which is a PLP requiring step viz. the kynureninase step. This enzyme catalyses the conversion of 3-hydroxykynurenine to 3-hydroxyanthranilic acid. This step is illustrated in Figure 4.

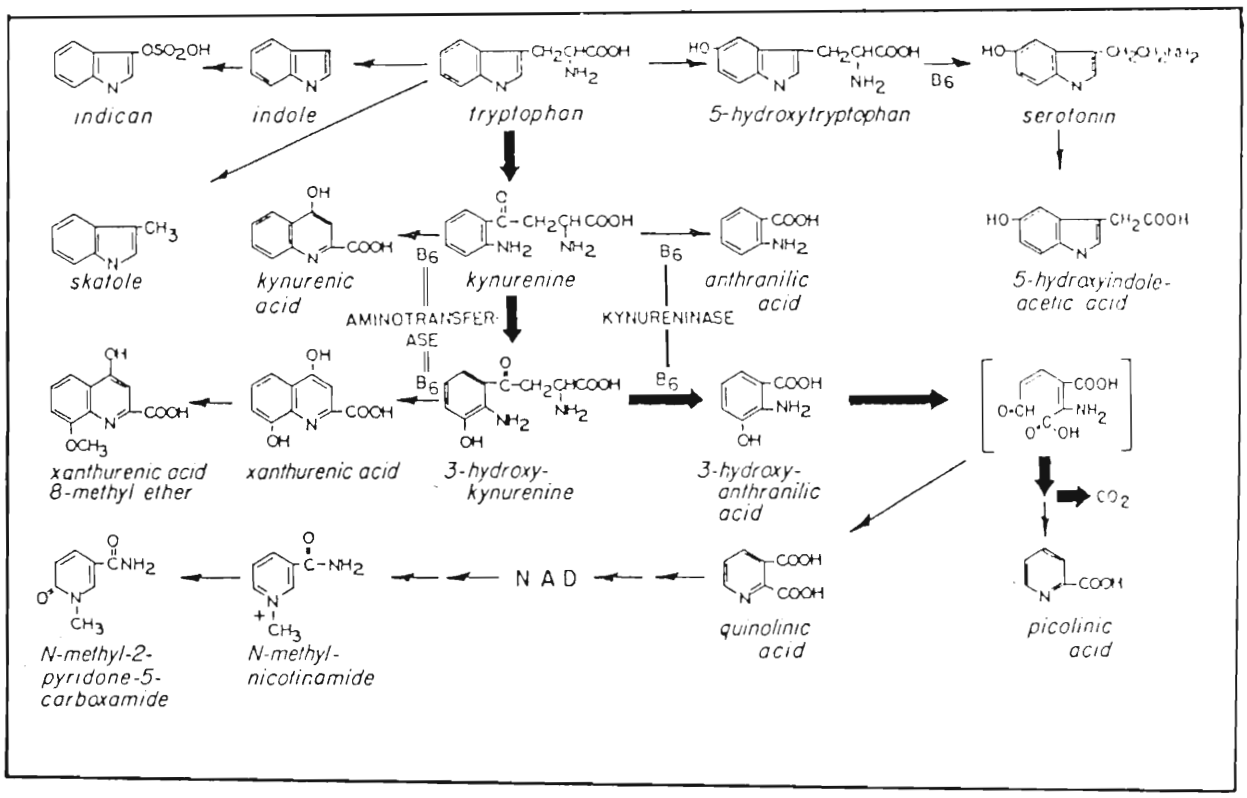


FIG. 4: Pathway of tryptophan metabolism showing major metabolites and sites of action of vitamin B-6 as pyridoxal phosphate. The large arrows indicate the pathway for degradation of the majority of ingested tryptophan.

2.1.3.3. Desulfhydration and transulfuration reactions

PLP is required as a coenzyme by enzymes involved in the metabolism of the nutritionally essential amino acid methionine, from which adequate amounts of cysteine is biosynthesised via the transulfuration reaction. The enzyme involved in this pathway is transulfurase (PLP dependent).

Desulfhydration of cysteine and homoserine requires the interaction of PLP. These amino acids are degraded to either pyruvate or 2-oxobutyrate + NH_3 + H_2S. The enzyme involved in the degradation to pyruvate is desulfurase a PLP dependent enzyme.

Desulfhydration and transulfuration reactions are illustrated in Figure 5.

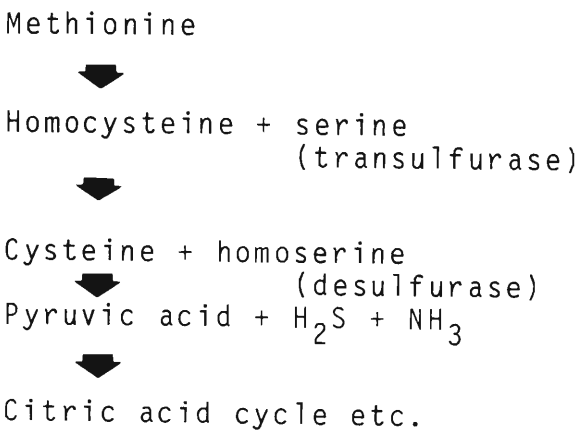


FIG. 5: Desulfhydrase and transulfhydrase reaction involving PLP.

2.1.3.4. Vitamin B-6 and nonoxidative decarboxylation reactions - GABA and serotonin synthesis

PLP is involved as coenzyme in nonoxidative decarboxylation reactions. Decarboxylases function in the systhesis of ɣ-aminobutyric acid (GABA) and serotonin.

PLP is required in the brain for the decarboxylation (glutamate decarboxylase) of glutamic acid to GABA, which is further metabolized to succinic acid. GABA is involved in the regulation of synaptic transmission in the nervous system. The degradation of GABA involves its transamination to succinic semialdehyde via the PLP dependent enzyme, GABA aminotransferase (Driskell, 1984).

Serotonin is synthesised by the action of tryptophan decarboxylase on 5-hydroxytryptophan as shown in Figure 6.

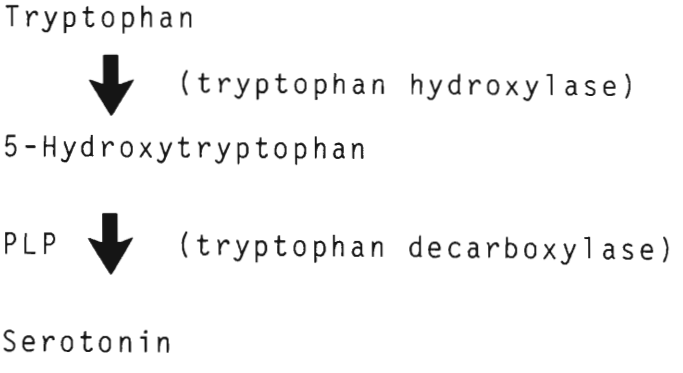


FIG. 6: Role of PLP in the synthesis of serotonin.

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

2.1.3.5. Synthesis of epinephrine and norepinephrine

PLP - dependent enzymes are involved in the synthesis of epinephrine and norepinephrine from either phenylalanine or tyrosine (Sauberlich, 1985). Tyrosine is decarboxylated by the PLP - containing tyrosine decarboxylase to form 3,4-dihydrophenylalanine (DOPA). Under the influence of the same enzyme 3,4-dihydroxyphenyl ethylamine (dopamine) is formed. Dopamine is then converted to norepinephrine and epinephrine as shown in Figure 7.

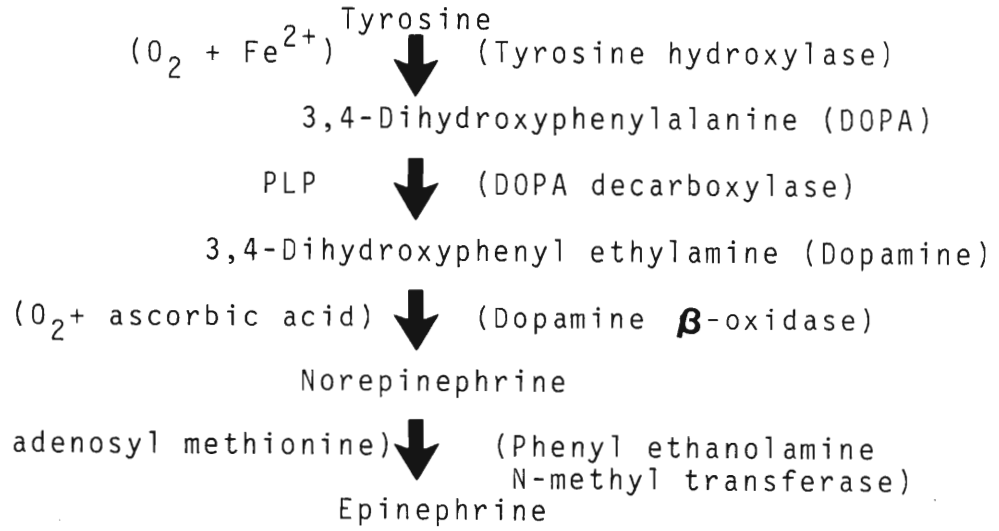


FIG. 7: Role of Vitamin B-6 in the synthesis of norepinephrine and epinephrine.

Both norepinephrine and epinephrine are involved in

carbohydrate metabolism and in other reactions in the body (Driskell, 1984).

2.1.3.6. Biosynthesis of porphyrins and heme

The first step in the biosynthesis of porphyrins is the condensation of glycine and succinyl CoA to form δ-amino-levulinate (ALA). This reaction is catalyzed by δ-aminolevulinate synthase, a PLP enzyme in the mitochondria. This enzyme converts glycine and succinate to α-amino-β-ketoadipic acid, which then loses CO_2 to form ALA, which in turn is converted to protoporphyrin and heme (Stryer, 1975) as illustrated in Figure 8.

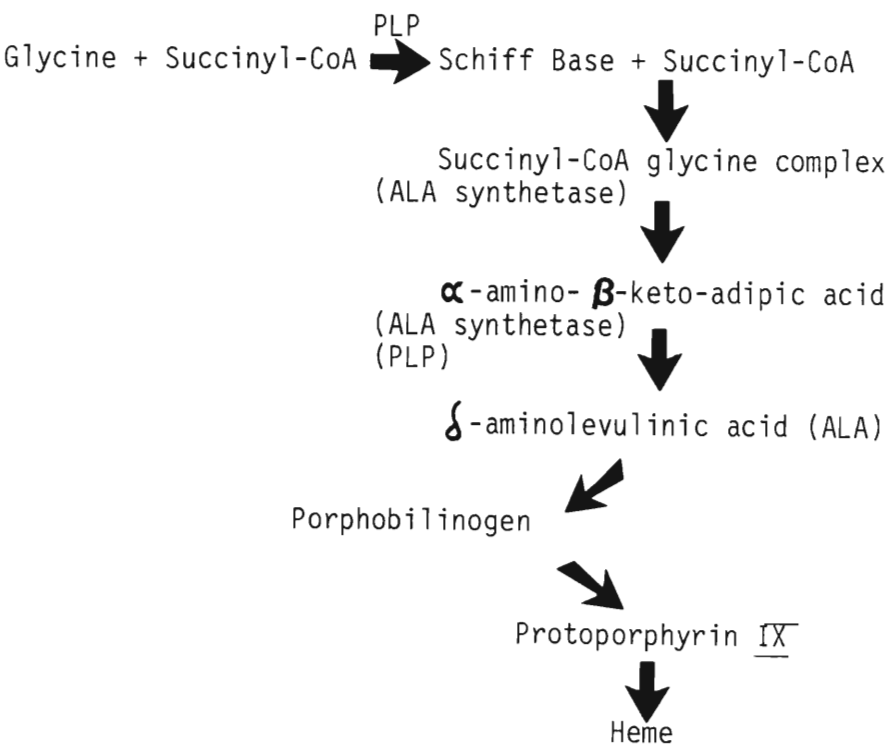


FIG. 8: The role of vitamin B-6 in heme synthesis.

2.1.3.7. Vitamin B-6 and glucose homeostasis

Glycogen phosphorylase, a PLP-dependent enzyme, is involved in glucose homoeostasis. A deficiency of vitamin B-6 in rats was shown to result in decreased activities of liver and muscle glycogen phosphorylase (Leklem, 1988). Glycogen phosphorylase initiates the catabolism of glycogen. PLP also acts as a coenzyme for the transaminase enzyme involved in gluconeogenesis.

2.1.3.8. Vitamin B-6 and lipid metabolism

Although it is known that vitamin B-6 is involved in lipid metabolism, its mechanism of action has not yet been established. The carcasses of animals deficient in the vitamin contain less lipid than those of controls; fatty liver, dermatitis, arteriosclerosis, and elevated plasma lipids and cholestrol levels are also symptomatic of vitamin B-6 deficiency. It is believed that vitamin B-6 affects lipid metabolism via its effects on hormones (Driskell, 1984).

2.1.3.9. Steroid function

PLP has recently been implicated in steroid function. At physiological concentrations, PLP was found to act reversibly with receptors for estrogen, androgen,

progesterone and glucocorticoids. The reaction involves the formation of a Schiff base with a lysine residue on the steroid receptor. It has been established that as a result of this interaction, there is inhibition of the binding of the steriod-receptor complex to DNA (Litwack, et al, 1985). Several studies, involving rats, have found that a deficiency of vitamin B-6 results in a greater retention of steroids in the nuclear fraction of target cells. It has been hypothesised that there would then be increased sensitivity of the end-target cell to the steroid. These studies suggest that there is a physiological relevance to the PLP-steroid receptor interaction (Leklem, 1988).

From all of the above, it is clear that vitamin B-6 is involved in a wide variety of biochemical reactions.

2.1.4. Manifestation of vitamin B-6 deficiency in animals

Vitamin B-6 has proved to be an essential growth factor for all species of animals studied to date. The exact manifestations of vitamin B-6 deficiency may vary from one species to another but the main effects are:-

- Loss of appetite, poor food utilization, poor weight gain or weight loss and muscular weakness (Rutishauser, 1982).

- Symmetrical scaling dermatitis (acrodynia) on the tail, paws, nose, chin and upper thorax.

- Hypochromic microcytic anaemia (Labadarios and Shephard, 1985).

- Nerve degeneration leading to convulsive seizures and neuronal dysfunction followed by death.

- Decreased reproductive performance of both males and females.

- A brown secretion around the eyes and a flow of tears with weakening of vision until complete blindness is reached.

Other symptoms observed include fatty liver, arterio-sclerotic lesions in rhesus monkeys, oedema, behavioural alterations, insulin insufficiency, altered plasma cholesterol levels and hypertension in the adult rat (Dakshinamurti and Paulose, 1988).

2.1.5. Assessment of vitamin B-6 nutritional status

Several biochemical procedures have been developed and utilized for the assessment of vitamin B-6 status of humans and of other animals. There is however much disagreement among investigators as to which parameter is the best criterion to assess vitamin B-6 status. The various techniques used include the following:

The measurement of xanthurenic acid excretion following a tryptophan load. Vitamin B-6 is required for the conversion of tryptophan to the vitamin niacin, as shown in Figure 4. Increased xanthurenic acid following a tryptophan load is excreted by subjects who are deficient in vitamin B-6. This method is the preferred technique for use in population surveys (Driskell, 1984).

Another method employed to determine vitamin B-6 status is the measurement of urinary pyridoxic acid levels. There are various techniques available to measure urinary pyridoxic acid. Usually fluorometric methods are used for this analysis. Pyridoxic acid excretion measurements are indicative of immediate dietary intakes of the vitamin and not of body reserves. Precise 24 hour urine collections are however difficult to obtain.

The measurement of alanine aminotransferase (ALAT) activity of blood was found to be more advantageous and as sensitive a criterion of vitamin B-6 nutrition in rats as the tryptophan load test. The activity of ALAT has been reported by several investigators to decrease in erythrocytes, leukocytes and serum of individuals depleted of vitamin B-6. It has, however, also been reported that the measurement of erythrocyte ALAT activity was more indicative of vitamin B-6 nutrition than the activity of this enzyme in whole blood, serum or plasma (Sauberlich et al, 1972).

In recent years, plasma PLP level has been used with increasing frequency as an indicator of vitamin B-6 status. This technique involves the direct measurement of the active coenzyme and is reflective of tissue levels. Lumeng et al, 1987 have reported that plasma PLP levels rise with increasing pyridoxine intake in the range between 0 to 100 µg per day. It was also established that the plasma PLP levels correlate well with the PLP content of skeletal muscle, the major storage pool of the vitamin B-6 compounds in rats. Based on their findings, Lumeng et al, 1978 concluded that plasma PLP measurement is the most direct and versatile indicator for evaluating vitamin B-6 nutritional status.

The measurement of plasma PL and PLP levels by high

performance liquid chromatography technique as devised by Ubbink et al, 1986 was employed in the present study to determine vitamin B-6 status in rats.

2.1.6. Vitamin B-6 and its interrelationship with trace elements

Trace elements are those elements which are essential to human and animal biological function. Most trace metals have been shown to have substantial chemical reactivity. Many trace metals are cofactors in enzymatic catalysis of a great many reactions involving over half the body's enzymes. Metals carry out these functions through substrate activation or by specific binding to the enzyme itself (Fitzgerald and Tierney, 1984).

As already mentioned, the biologically active form of vitamin B-6, PLP, is known to participate as an activating coenzyme for over 100 enzymes (Sauberlich, 1985). The diversity of pyridoxal-5-phosphate is illustrated by its involvement in a wide range of functions including gluconeogenesis, amino acid metabolism, immune function, carbohydrate metabolism, lipid metabolism, nervous system function and steroid hormone action. With such a broad involvement in the biological functioning of the body, a lack of sufficient vitamin B-6 would be expected to have adverse consequences.

Since both vitamin B-6 and trace elements are known to participate in a great many systems, interactions

between certain trace elements and vitamin B-6 would be expected. Various studies have been undertaken to determine the effect of vitamin B-6 deficiency on various trace elements. A few of these findings include:

- A reported elevation of iron (Fe) stores in the liver and kidney resulting from PN deficiency (Ikeda et al, 1979). The increased Fe levels were attributed to the fact that less Fe is incorporated into haemoglobin in PN deficiency since PLP is involved in haemoglobin synthesis.

- Dietary deprivation of pyridoxine was found to result in an increased fecal excretion of ^{54}Mn. However, ^{54}Mn levels were increased in the brain and testicles while a reduction of injected ^{54}Mn was observed in the heart and liver (Hsu, 1965).

- A deficiency of vitamin B-6 in rats resulted in a non-specific increase in the in vitro intestinal uptake of calcium and cadmium indicating increased tissue levels of these elements in a vitamin B-6 deficiency state (Prasad et al, 1982).

- Changes in tissue concentrations of zinc (Zn) have been reported in vitamin B-6 deficient animals. Hsu, 1965 reported decreased concentrations of Zn in

plasma, liver, pancreas and heart in pyridoxine deficient rats. However, Gershoff, 1967 found increased levels of Zn in the pancreas, serum and liver of vitamin B-6 deficient rats.

These various findings indicate a metabolic link between PN and various trace elements. Because of the contradictory findings with regard to vitamin B-6 deficiency and tissue Zn levels, the present study was undertaken to determine the effect of vitamin B-6 deficiency on Zn bioavailability.

Although no studies have been found in the literature concerning vitamin B-6 deficiency and Zn bioavailability, the various findings on the effect of PN deficiency on tissue Zn levels would suggest a strong likelihood that Zn bioavailability would be affected by a vitamin B-6 deficiency.

2.2. Zinc

2.2.1. Early discovery

Zn does not occur naturally in a free, uncombined state. It was discovered by accident in the fourth century A.D. that brass was produced when a certain earth (zinc bearing) was heated with copper. Metallic zinc was first produced during the 13th century in India by reducing calamine (zinc oxide plus a small percentage of ferric oxide) with organic substances such as wool. Ebener of Nürnberg is reported to have recognized Zn as a discrete element in 1509 and in 1746 Marggraf rediscovered the metal in Europe (Halsted et al, 1974).

2.2.2. Biological functions of zinc

Zinc has a great diversity of functions - greater than for any other organic nutrient (Mills, 1989). Having been first shown to be required for normal growth in rats and mice in the 1930s, there is now no doubt that Zn is essential for normal life in both humans and animals (Jackson, 1989).

Zinc plays both a unique and extensive role in the biochemistry of enzymes and other biological molecules. This is due to certain advantageous chemical properties of metal ions. Zn has a highly concentrated charge,

Zn^{2+}. It is also a small ion with a radius of 0.65A. Zn has a high affinity for electrons and is thus a very effective attacking group. It does not show variable valence (oxidation state change), and for this reason is preferred over other metal ions in certain circumstances since redox activity introduces the risk of free radical reactions (Williams, 1989).

Over two hundred Zn dependent enzyme activities have been identified and such enzymes occur in all phyla. Some of those that are relevant to humans and animals are listed in Table 1. Various studies have shown that Zn is probably the metal most widely distributed amongst metalloenzymes. Metal activated enzymes are subdivided into two classes: metalloenzymes in which the metal is firmly bound, and metal-enzyme complexes that are readily dosspcoated and require the continuous presence of the activating ion in the medium (Aggett, 1985).

Enzyme	Role of Zinc
Alcohol dehydrogenase	Catalytic and Structural
Superoxide dismutase	Structural
Alkaline phosphatase	Catalytic and Structural
Fructose 1,6-diphosphatase	Regulatory and Structural
Amino peptidases	Catalytic
Angiotensin converting enzyme	Catalytic
Collagenase	Catalytic
Carboxypeptidases	Catalytic
Carbonic anhydrase	Catalytic
Glyceraldehyde-3-phosphate dehydrogenase	Catalytic

Table 1: Some mammalian zinc metalloenzymes.

A generalized theory of metalloenzyme activity in which catalysis depends on the existence of an entatic state has been suggested. It is postulated that binding of the metal to the enzyme protein results in a distorted and partial co-ordination sphere around the metal ion and that the energy of distortion is released or transferred to the substrate. This theory fits in with recent observations on the structure of four Zn enzymes - carbonic anhydrase, liver alcohol dehydrogenase, thermolysin, and carboxypeptidase. These enzymes have been shown to possess similar active sites centred around a Zn^{2+} ion (Chesters, 1982).

Recent research has shown that Zn plays an important role as a structural constituent of enzymic and non-enzymic proteins and of polynucleotides. Zn is believed to govern the biological functions of a wide range of macromolecules by conferring on them both structural and metabolic stability. The function of Zn in alcohol dehydrogenase for example, demonstrates its catabolic as well as organizational roles - where it is possible to differentiate between the Zn associated with the functional catalytically active centre and the Zn which is less firmly bound and involved in stabilizing the polymeric structure of the enzyme (Mills, 1989).

It is postulated that Zn plays a fundamental role in protein metabolism. Zn has, in fact, been compared to an essential amino acid since in both Zn deficiency and deficiencies of amino acids, there is a rapid onset of anorexia, growth retardation, sensitivity to nitrogen intake, raised blood ammonia and urea concentrations, decreased synthesis of proteins and reduced DNA/RNA ratios (Aggett, 1985).

Recent studies strongly suggest that Zn plays a role in gene expression. Zn has been shown to be present and to interact with the various chromatin components, namely DNA, RNA, histone and non-histone proteins. The involvement of Zn in the synthesis of DNA, DNA transcription, translation of mRNA into protein and in the proper structure and functioning of the translated product suggests that Zn is necessary for virtually all aspects of normal cell metabolism (Clegg et al, 1989).

Zinc is believed to play a diverse role in the central nervous system. It has been proposed that Zn has a regulatory and structural role in nerve growth factor. Neuropsychiatric features are prominent in human Zn deficiency. These indicate a role for Zn in neural functions such as taste, appetite control, olfactory function, vision, and neuromuscular co-ordination (Aggett, 1985). Also, since zinc is distributed among

all enzyme classes, it has a wide range of functions in brain and neurotransmitter homeostasis (Prohaska, 1987).

Early studies have indicated a relatively high concentration of zinc in the bone as compared to other tissues (Hove et al, 1938). The discovery that "Zn is found in close proximity to the sites of calcification and that Zn is detected exactly in the layer of preosseous tissue whose calcification is imminent" suggests an important biochemical role for Zn in bone formation (Calhoun et al, 1974). It has been theorised that Zn affects the regulatory mechanism of ossification and that an adequate concentration of Zn is required at the site of the bone formation as a requisite for complete calcification. The various findings of Calhoun et al, 1974 have led them to conclude that Zn has an active role in bone metabolism. The exact mechanism however remains unknown.

It has also been postulated that Zn plays an important role in fatty acid and carbohydrate metabolism. However, more research is needed in this field before its exact role can be elucidated (Halstead et al, 1974).

2.2.3. Metabolism of zinc

2.2.3.1. Zinc absorptions

Cellular uptake from the intestinal lumen

The basic aspects of Zn absorption metabolism are shown in Figure 9. Studies to determine the site of intestinal absorption in rats indicate that the duodenum contributes 60%, the ileum 30%, and the jejunum 10% to the overall absorption of Zn. Negligible absorption of Zn occurs in the stomach, caecum and colon (Davies, 1980).

Numerous experiments have elucidated the biphasic nature of Zn uptake by the intestine. Davies, 1980 and Smith and Cousins, 1980 observed two phases of Zn absorption as a function of the luminal Zn concentration. A fast mucosal uptake phase across the brush border membrane is followed by a slower phase which involves transport of zinc across the basolateral membrane. The fast phase of uptake, with increasing luminal Zn concentrations, is postulated to represent saturability of binding sites on the brush border membrane. At high Zn concentrations, the membranes may become leaky and allow Zn to enter the cell and bind non-specifically to cell proteins and other ligands.

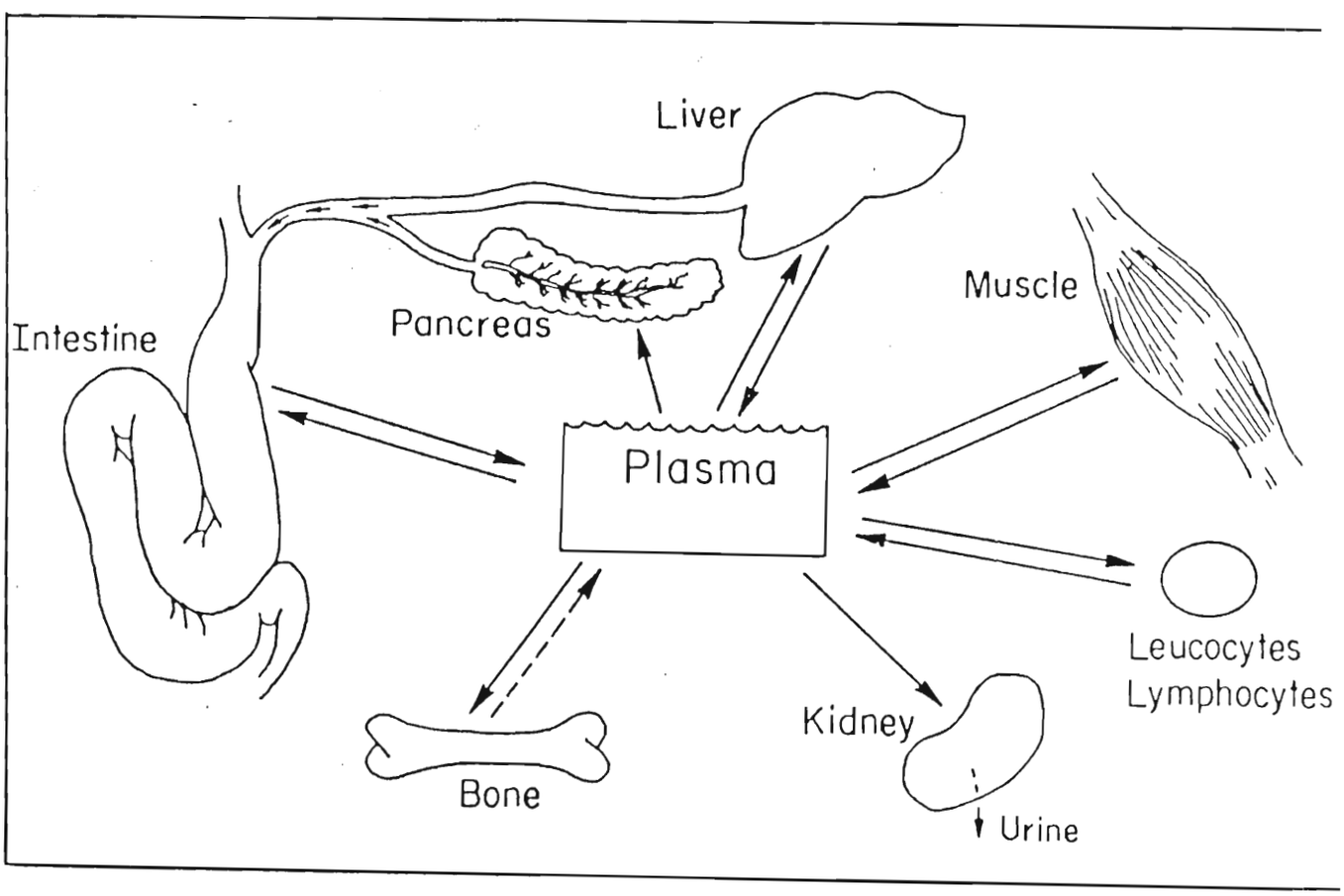


FIG. 9: Basic aspects of mammalian zinc metabolism. Absorption of dietary Zn occurs from the small intestine. Absorbed Zn is transported in portal plasma bound to albumin. Muscle and bone represent the largest Zn pools.

Jackson et al, 1981 have also suggested that there are two mechanisms of Zn absorption. One of these may be induced by low dietary Zn levels. This hypothesis is supported by the finding that Zn absorption may have a more pronounced mediated component in response to Zn depletion.

Menard and Cousins, 1983 using brush border membrane vesicles (BBMV) in their investigations regarding Zn transport, concluded that at low Zn concentrations (0.2mM) uptake is saturable and occurs by a carrier-mediated process which is not energy dependent and that at high Zn concentrations, uptake is linear indicating a passive diffusion process.

Research carried out by Menard and Cousins, 1983 suggest that the mechanism of Zn transport across the brush border of intestinal epithelial cells probably involves interaction between the metal in a chelated form and the membrane surface. It was proposed that a number of specific binding ligands might fulfil this role.

The existence of a specific low molecular weight Zn-binding ligand was suggested by a number of researchers (Hahn and Evans, 1973 and Schricker and Forbes, 1978). This ligand, thought to originate from the pancreas, was found in the intestinal wall and in

the milk of certain species (Evans et al, 1975 and Lonnedal et al, 1980). Evans and co-workers have attempted to characterize this Zn-binding ligand of pancreatic origin and have described it as picolinic acid (PA). They maintain that during the process of absorption, PA is secreted from the pancreas into the intestinal lumen where a Zn-dipicolinate complex is formed. This complex facilitates the transport of zinc across the intestinal cells (Evans and Johnson, 1981).

Other studies however throw doubt on claims that ligands secreted from the pancreas play a role in Zn absorption (Davies, 1980). Cousins, 1979 has emphasized the difficulty of preventing the appearance of artefacts when attempts are made to isolate Zn-binding ligands from the intestinal mucosa.

Metallothionein - The intracellular Zn-binding ligand

The intracellular distribution of intestinal Zn has been extensively researched. The basic concepts involve hypothetical pools for Zn and the distribution of the metal to specific cellular sites for definitive functions eg. Zn to membranes for stabilization and to metalloenzymes and intracellular binding sites as part of regulatory mechanisms.

The potential function of various intracellular Zn-binding ligands in controlling zinc absorption has been widely studied. A key role for metallothionein (MT) in this process was suggested by Richards and Cousins, 1977. Further research has also indicated that this intestinal protein is inducible by Zn administration. Inducibility was confirmed by feeding and injection experiments. Little MT was found in intestines of Zn-depleted rats, whereas dietary repletion with Zn 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 Zn absorption (Cousins, 1985).

Mucosal MT is believed to reflect dietary Zn status and the amount of MT present in the mucosa is negatively correlated to Zn absorption, thereby exerting homeostatic control of Zn metabolism (Lönnerdal, 1989). Studies by Richards and Cousins, 1977 have shown that the major Zn-binding fraction in the cytosol of mucosal cells is metallothionein. They suggest that Zn taken up into the intestinal cells may either be transported across the serosal membrane into the hepatic portal blood or, become bound within the cells to MT which prevents its transfer to the plasma. Thus, the amount of metal-free thionein in the Zn-absorbing cells of the mucosa may be the major determinant of the extent of Zn

absorption.

The following model (Figure 10), postulated by Cousins, 1979 attempts to explain the mechanism of Zn absorption at the level of the intestinal mucosal cell.

- A portion of the dietary Zn which enters the lumen of the small intestine is transported across the mucosal brush border membrane (shown as A in Figure 10).
- Within the intestinal cell, newly acquired cytoplasmic Zn equilibrates with a "Zn pool" and is either shunted into high molecular weight proteins and MT or is transferred to the plasma (shown as B in Figure 10).
- A major portion of Zn that enters the intestinal mucosa from the plasma becomes bound to MT, a phenomenon that is directly related to Zn status (shown as C in Figure 10).
- Because appreciable amounts of Zn may also be secreted into the intestinal lumen, "A" in Figure 10 is represented as bidirectional transport.

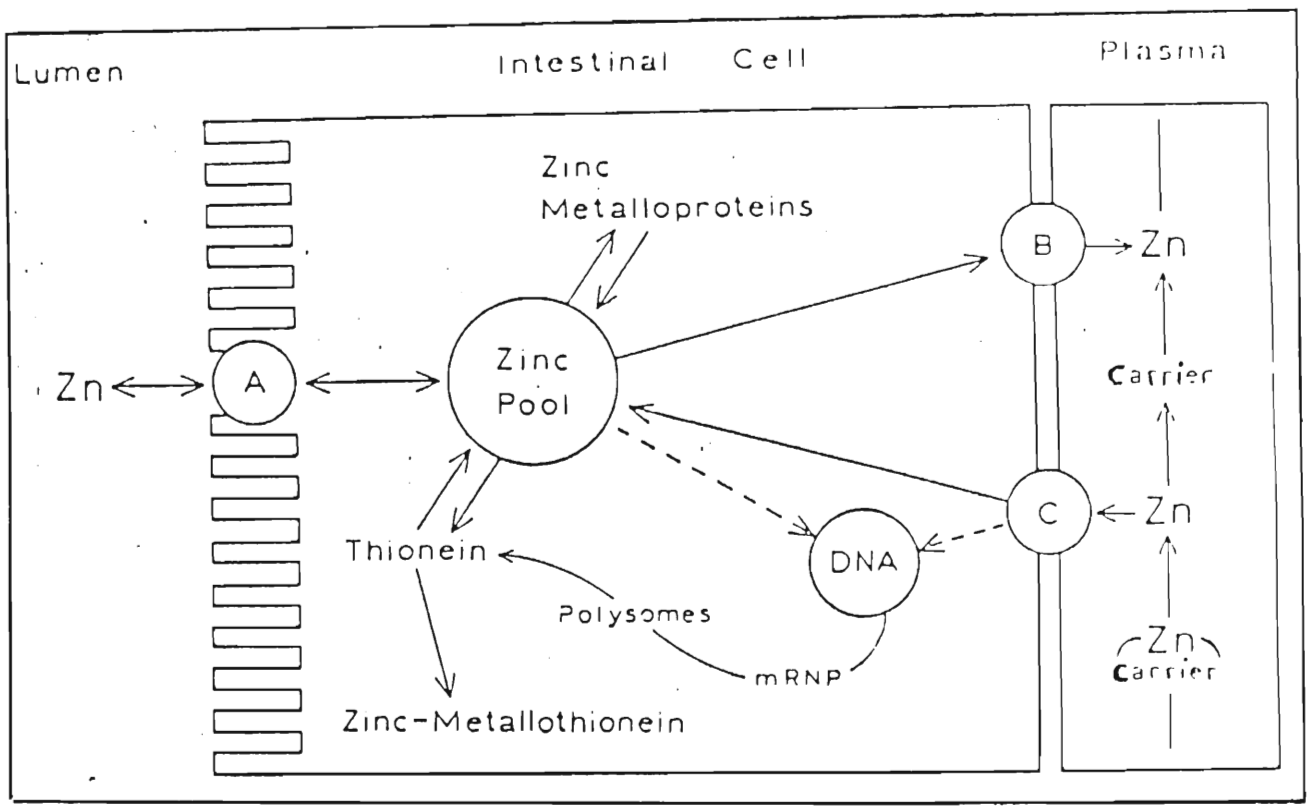


FIG. 10: Proposed mechanism for the regulation of intestinal zinc absorption. Dash lines indicated Zn concentrations which may signal the induction of intestinal metallothionein biosynthesis.

Transfer of Zn to portal circulation

Studies with isolated rat intestine (Smith and Cousins, 1980) indicate that Zn is bound to albumin as it leaves the serosal surface of the gut wall. This finding is in agreement with earlier work by Smith et al, 1979 who obtained direct evidence that albumin is the principal portal transport protein for Zn as it leaves the intestine.

2.2.3.2. Plasma transport of zinc

Only 10-20% of the Zn in whole blood is found in plasma. The remainder is localized within the erythrocytes in which carbonic anhydrase is the major binding site. Approximately two-thirds of the Zn present in plasma is bound to albumin and one-third to α_2-macroglobulin (Chesters and Will, 1981). It is believed that about twelve proteins in human serum bind Zn in vitro. The order of Zn binding was found to be albumin $\gg$ α_2-macroglobulin $>$ transferrin, with only minor binding by other proteins (Cousins, 1989).

The albumin bound Zn is frequently referred to as a loosely bound, exchangeable Zn pool and the ability of albumin to give up bound Zn is thought to be an essential feature of the transfer of Zn between plasma

and tissues (Cousins, 1985).

2.2.3.3. Cellular uptake from the systemic circulation

The major tissues which contribute to the regulation of systemic Zn are illustrated in Figure 9. Since the liver and kidney are considered the most significant sites of Zn metabolism, Zn uptake by the liver and kidney will be discussed in the present study.

Hepatic uptake

Zn is transported to the hepatocytes from the intestine bound to albumin (Cousins, 1985). Hepatic uptake of Zn occurs rapidly after absorption. Marked fluctuations in the dietary zinc supply cause only minimal changes in the Zn content of the liver, demonstrating that uptake and efflux are closely regulated (Cousins, 1989).

In early experiments to characterize Zn uptake by the liver, it was concluded that binding or uptake followed first-order kinetics and a passive mechanism of Zn accumulation was proposed. However, more recent experiments have shown that Zn accumulation by liver cells is temperature dependent and energy dependence and saturable kinetics was suggested. It has subsequently been demonstrated that Zn uptake by liver cells occurs

by two distinct phases - the first, a fast-uptake phase, represents initial binding to specific sites on the plasma membrane surface. In the second phase of accumulation, Zn is accrued at a much slower rate (Cousins, 1985).

A pharmacokinetic model of ^{65}Zn metabolism has demonstrated that the first order mass transfer coefficient and linear binding constant are highest for the liver. This strongly suggests that control of plasma Zn levels involves hepatic mechanisms (Cousins, 1989).

Kidney Uptake

The kidney has been shown to play an important role in Zn metabolism. Kinetic data have demonstrated that the first-order mass transfer coefficient and binding constants for Zn place the kidney in line after the liver as a site of metabolic significance. The kidney is therefore believed to have a substantial influence on the plasma zinc compartment.

Renal insufficiency has been shown to result in reduced serum Zn concentrations. This has been correlated to increased urinary Zn bound to ligands of smaller molecular weight than albumin.

Hormonal regulation of renal Zn excretion has been demonstrated. Insulin has been found to inhibit the excretion of Zn in perfused dog kidney when added to the vascular perfusate, while infusion of glucagon was shown to increase Zn excretion in the same model.

The effect of renal MT on renal handling of Zn has not yet been determined. It has been found, however, that endotoxin and interleukin-1 administration increases MT mRNA levels in the kidney. These mRNA levels were found to increase in the kidney and intestine under practical feeding conditions, suggesting that these organs may influence Zn metabolism through MT related shifts in cellular uptake.

2.2.3.4. Body zinc store

Zinc is present in all organs, tissues and secretions of the body. Because of the large bulk of skeletal muscle, this tissue contains the greatest portion of body Zn. Together with bone, these two tissues account for more than 80% of the total body Zn. Zinc is primarily an intracellular ion and intracellular Zn accounts for over 95% of the total body Zn (Jackson, 1989). Extracellular fluids have a relatively low Zn concentration, with plasma representing approximately 0.1% of total body Zn in humans.

Several researchers have accepted that an effective body Zn store does not exist and, therefore in all species studied, a marked decrease in dietary Zn is followed quickly by harmful signs of Zn deficiency eg. skin lesions, growth failure and reduced food intake (Golden, 1989). It is postulated that since there is no store from which to increase Zn supply, the body decreases its demand for Zn by reducing food intake and therefore growth rate.

Other researchers have, however, argued in favour of a functional, though limited, Zn store. Various experiments, using weanling rats, have demonstrated that pre-treatment with excess dietary Zn results in increased bone zinc concentrations, while Zn-deficient diets given to the same experimental animals resulted in decreased bone zinc concentrations. Brown et al, 1979 showed that in weanling rats given a Zn-deficient diet without pre-treatment, there was a significant fall in femur Zn concentration despite continued femur growth. They postulated that Zn was released from bone during periods of dietary zinc deficiency.

2.2.3.5. Zn excretion

Zinc is excreted almost entirely via the faeces. Animal

experiments have demonstrated that faecal excretion is chiefly by way of the pancreatic juice regardless of the route of Zn intake. Negligible amounts are excreted by the liver into bile (Halsted et al, 1974). Skin, hair and nails, being high in Zn, are also important routes of Zn excretion. Although little Zn is excreted in the urine, urinary Zn content does appear to respond to large changes in body status such that overt depletion of Zn results in a reduction in urinary Zn content (Jackson, 1989).

2.2.3.6. Whole-body Zn homeostasis

The relatively constant levels of Zn in tissues and body fluids in situations where the composition of the diet varies suggests that there is an efficient mechanism for whole-body homeostasis. Zn homeostasis appears to be maintained by manipulation of both gastrointestinal absorption and gastrointestinal excretion of the element. Since little Zn is excreted in the urine, changes in urine Zn content can contribute little to the maintenance of whole-body homeostasis at normal dietary Zn intakes (Jackson, 1989). Low Zn diets result in an increase in the proportion of Zn absorbed from the diet, but decreases in the rate of excretion of Zn into the gastrointestinal tract are also known to occur in response to a reduction in dietary Zn (Baer and King,

1984). Conversely, a reduced fractional absorption of Zn and an increase in the rate of gastrointestinal excretion of Zn were found to occur in response to an elevation in dietary Zn. Thus, it is apparent that the body is able to respond to relatively large variations in dietary Zn so as to maintain a relatively constant body Zn content (Jackson et al, 1984).

2.2.4. Manifestation of Zn deficiency in humans and animals

In animals

The signs and symptoms of Zn deficiency are markedly similar in different animals. Zinc is an important nutrient and a Zn deficiency in animals may result in diverse symptoms, the most commonly observed of which include dermatitis, emaciation, alopecia, ocular lesions, testicular atrophy, retarded growth and anorexia.

In ruminants, parakeratosis, hair loss, cracking and fissures around hooves, reduced testicular size, decreased libido, and retarded sexual maturity are characteristic signs of Zn deficiency. The most common symptoms observed in birds include decreased and abnormal feather growth, reduced egg production and

hatchability. In sheep, Zn deficiency results in lethargy, increased tendency to bleed, decreased immune response, bad wound healing and poor quality of wool (Peereboom, 1985). Zn deficiency in rats is characterized by growth retardation, immature hair coats, scaly feet and fissures at the corners of the mouth (Swenerton and Hurley, 1967 and Giugliano and Millward, 1984).

Congenital malformations have been observed in rat fetuses delivered from Zn-deficient females. These malformations include misshapen heads, clubbed feet, fused or missing digits, and short lower jaws and long bones (Halsted et al, 1974). Other skeletal defects observed were fusion of ribs, curvature of the spinal column, missing vertebrae in the tail and incomplete or retarded ossification in ribs, vertebrae and cranial bones. Malformation of internal organs including the brain, heart, lung and urogenital system also occurs (Hurley, 1981).

In humans

In humans, the Zn store in bone and muscle is not readily available if the oral Zn intake is not sufficient. Zn deficiency may therefore easily develop. Individuals with rapid growth or people under stress may be

especially vulnerable to marginal Zn deficiencies (Peereboom, 1985). Marginal Zn deficiency is fairly common in Western, industrialized societies and is thought to result from inadequate dietary intake (or availability), malabsorption and increased rates of loss from the body. Symptoms of marginal Zn deficiency include failure of appetite and growth, mental lethargy, night blindness, decreased taste (hypgeusia) and skin lesions.

Food components such as phytic acid and fibre were found to decrease the absorption of Zn. It has also been observed that in diets containing a high content of cereals, the bioavailability of Zn is poor (Peereboom, 1985). In the Middle East, some of the growth retardation, skin lesions and impaired sexual development of adolescent malnourished boys was found to be caused by Zn deficiency. Also acrodermatitis enteropathica, with severe skin lesions, hair loss, and diarrhoea, was diagnosed as resulting from an inherited defect of Zn absorption.

Clinical features of severe zinc deficiency include:

- Neuropsychiatric changes, comprising a change in mood, loss of affect and emotional lability. As the Zn deficiency progresses, anorexia and dysfunction of

smell and taste develop. The subjects become irritable, depressed and easy to anger. Signs of cerebellar disturbances may occur, characterized by a fine tremor and slurred speech.

- Eye abnormalities, including a mild dry conjunctivitis, increased keratinization of the eyelids, corneal oedema, and bilateral cataracts.
- Dermatological features, comprising an early skin rash involving the nostrils, corner of mouth, and chin; hyperkeratotic lesions due to prolonged Zn deficiency; symmetrical facial dermatitis; a papular acneiform rash with haemorrhagic brown discoloration may develop parakeratotic skin lesions of feet, ankles and knees.
- Growth retardation and reproductive abnormalities, including failure to thrive, impaired weight gain, hypogonadism, and impaired testicular development (Aggett, 1989).
- Other symptoms of severe Zn deficiency include impaired immune response, liver cirrhosis, impaired wound healing, sickle-cell anaemia, epilepsy and loss of taste and smell (Peereboom, 1985).

2.3. The effect of vitamin B-6 deficiency on tissue Zn levels

Although experimental work investigating the influence of vitamin B-6 deficiency on Zn metabolism has been undertaken by various researchers, much controversy exists as to the various results obtained.

Hsu, 1965 found that the zinc content of plasma, liver, pancreas and heart of PN-deficient rats was significantly lower than that of PN-treated animals. However, no substantial differences in Zn concentrations of the brain, kidney and spleen were observed between the deficient and control animals.

Gershoff, 1967 using a different experimental protocol to determine the tissue Zn content of PN-deficient rats, obtained results which were in disagreement with those obtained by Hsu (1965). Gershoff found that PN deficiency resulted in increased levels of Zn in the pancreas, serum and kidney of rats.

Hsu's (1965) results showed that in addition to the decreased Zn levels observed in plasma, liver, pancreas and heart tissues of the vitamin B-6 deficient rats, there was an increase in injected radioactive zinc concentration. The uptake of Zn-65 by the plasma and

liver of PN-deficient rats was found to be significantly higher than that of their respective controls. This observation was in agreement with a previous study (Hammam et al, 1965) which demonstrated that Zn-deficient rats retained more injected radioactive Zn-65 in their tissues than those receiving Zn supplemented diets. It was therefore maintained that since PN-deficient rats showed a decrease in Zn concentration in certain tissues, an increased uptake of administered Zn-65 would be expected.

In the experiment performed by Gershoff, 1967, half the rats in each group ie. the control and PN-deficient groups, were fasted during the final 24 hours of the experimental period. It was observed that among the fed animals, the PN-deficient rats showed an increased level of tissue Zn, particularly in the pancreata where the PN-deficient rats contained over a hundred per cent more Zn than those of the controls. Gershoff also noted that when control rats were pair-fed or fasted for 24 hours, there was a rise in the tissue Zn levels which was particularly significant in the fasted rats. The effect of fasting on Zn levels of vitamin B-6 deficient rats was not found to be significant. Gershoff maintained that these findings would explain the results obtained by Hsu, 1965 who pair-fed his rats and then fasted them overnight before determining tissue Zn content. Gershoff

thus concluded that Hsu's observation that PN deficiency in rats decreased tissue Zn concentrations was to a large extent a reflection of the high tissue levels of Zn in his fasted control rats.

Ikeda et al, 1979 reported that there was no significant difference in the Zn content of the liver, pancreas, spleen, lung or testes between PN-deficient and control rats. A significant increase in kidney Zn concentration was however observed in the deficient group. The results of Ikeda et al, are therefore in agreement with those of Gershoff, 1967 with respect to the increased Zn levels observed in the kidneys of PN-deficient rats. It will be recalled that Gershoff reported a significant difference in the Zn levels of various tissues of PN-deficient animals. This difference was particularly significant in the pancreata where the vitamin B-6 deficient rats contained over 100% more Zn than those of the control animals. The study of Ikeda et al however showed no significant difference in Zn levels of the pancreata between control and deficient groups.

In their paper, Ikeda et al reported that rats fed a vitamin B-6 diet with deoxypyridoxine injections showed increased urinary excretion of Zn combined with xanthurenic acid when compared to rats which were only fed a vitamin B-6 deficient diet. They therefore

concluded that a more severe vitamin B-6 deficiency (as caused by deoxypyridoxine injections) may give rise to a decrease in tissue Zn concentrations. If this hypothesis were shown to be true, it would verify the results obtained by Hsu, 1965 who reported decreased tissue Zn concentrations in vitamin B-6 deficient rats.

2.4. Zinc absorption as affected by a deficiency of vitamin B-6

Prasad et al, 1982 reported a significant increase in the uptake of Zn from the intestine of vitamin B-6 deficient rats when compared to control and pair-fed animals. To explain their results, Prasad et al, postulated that the lack of pyridoxine may lead to an impairment of nucleic acid synthesis. A decrease in nucleic acid synthesis would result in a subsequent inhibition of protein synthesis and cell division and repair. Since vitamin B-6, as the co-enzyme, catalyses a variety of enzymatic reactions involved with nitrogen metabolism, it was concluded that a significant alteration could be expected in the brush border membrane which may result in an increase in Zn uptake. These findings would explain earlier observations by Gershoff, 1967 who reported an increased Zn content in various tissues of PN-deficient rats.

However, various observations have been reported (Evans et al, 1979) which contradict the findings of Gershoff 1967. Instead these observations provide an explanation for decreased tissue concentrations resulting from a vitamin B-6 deficiency as reported by Hsu, 1965.

Evans and Johnson, 1981 observed that a vitamin B-6

deficiency resulted in an impairment of dietary Zn absorption. This result was attributed to a decreased production of picolinic acid (PA).

Using a technique known as modified gel filtration chromatography, Evans et al, 1979 obtained evidence which suggested that the dominant Zn-binding ligand in the rat intestine is picolinic acid (pyridine-2-carboxylic acid), a product of tryptophan metabolism. One of the enzymes, kynureninase, in the pathway from tryptophan to picolinic acid requires pyridoxal as a cofactor (see Figure 11). It was therefore maintained that if endogenous picolinic acid facilitates dietary Zn absorption, a deficiency of either vitamin B-6 or tryptophan should impair Zn absorption. Evans and Johnson (1981) subsequently also noted that both Zn absorption and pancreatic picolinic acid concentrations were increased as the levels of vitamin B-6 was increased. It was further observed that supplemented PA ameliorates the impaired Zn absorption caused by a tryptophan or PN deficiency. From these observations, Evans, 1980, concluded that Zn absorption is affected by levels of both dietary tryptophan and PN and because PA is a metabolic product of tryptophan and depends upon PN for its production, these results provide strong evidence that endogenous PA is essential for normal Zn absorption.

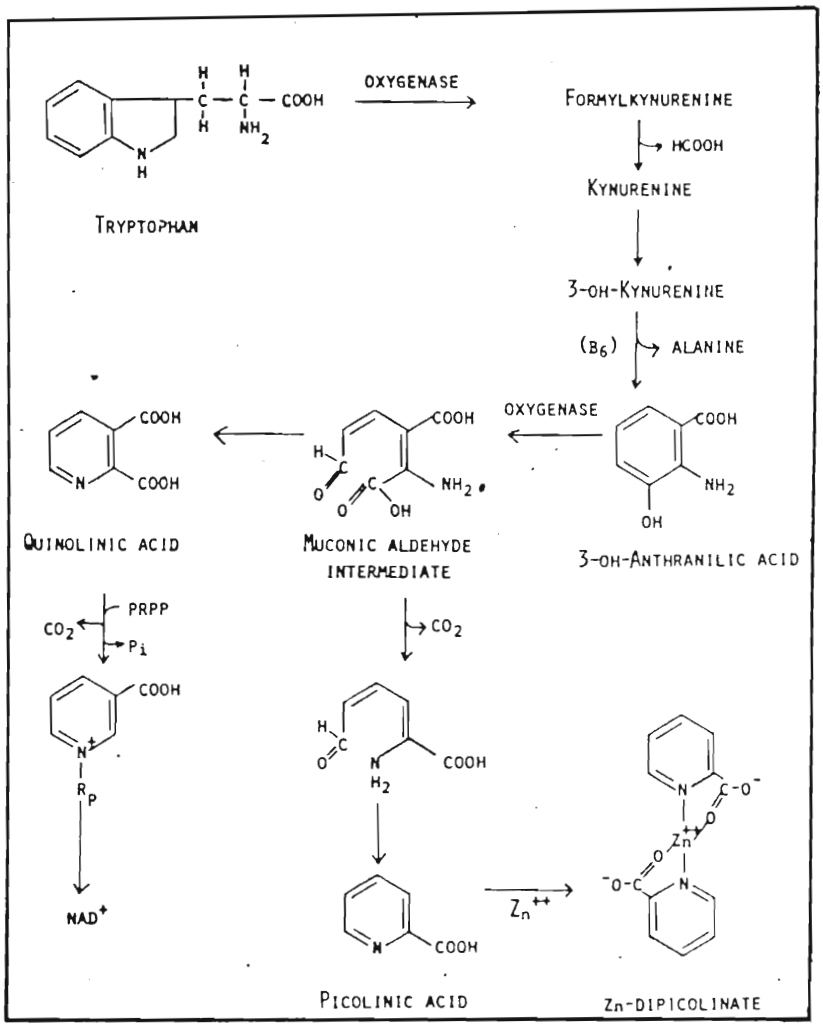


FIG. 11: Metabolism of tryptophan to picolinic acid.

The above observations of Evans and Johnson would also explain the results obtained by Hsu, 1965 who found decreased concentrations of Zn in several tissues from rats fed a diet deficient in vitamin B-6.

The conclusion reached by Evans, 1980 has however, been criticized by Hurley and Lönnerdal, 1980 who suggest that the data presented by Evans and Johnson, 1980 was not sufficient to warrant the conclusion they reached. Hurley and Lönnerdal, 1980 maintain:

1) Picolinic acid is not a significant end product of tryptophan metabolism in the rat and, in fact, 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 picolinate in vitro.
2) Evans and Johnson based a major part of their conclusion on the fact that PA is a good chelator of Zn. However, increasing the Zn absorption by addition of a chelating agent does not indicate that the chelating compound has a normal physiological role and,
3) In their laboratory, Hurley and Lönnerdal, 1980 noted that the analysis of pancreatic fluid and pancreatic tissue of rats showed no indication of the presence of a low molecular weight Zn-binding

ligand whereas the analysis of human milk did reveal the presence of a low molecular weight Zn-binding ligand which was identified as citrate and not PA.

Subsequent research has however produced results that favour the findings of Evans and Johnson, 1980. Krieger and Statter, 1987 in their efforts to demonstrate the effect of tryptophan deficiency and PA on Zn metabolism, have shown that a tryptophan deficiency leads to low plasma Zn levels and also low bone Zn concentrations in rats. They concluded that their findings could not be attributed to insufficient albumin synthesis because tryptophan is not a building block of albumin and because 90% of tryptophan is non-covalently bound to albumin which seems to function as a carrier. Instead, the low Zn levels are attributed to the fact that tryptophan supplies the Zn carrier, namely PA, which is a strong bidentate ligand and important in Zn absorption.

Brown, 1985 in his article dealing with the possible roles for vitamin B-6 and trace metals in health and disease agrees with the findings of Evans and Johnson, 1980. His report favours the idea that PA enhances Zn uptake and that the nutritional status of Zn may be related to vitamin B-6 status since several studies show that Zn uptake or tissue Zn levels in laboratory animals

are directly related to vitamin B-6 intake.

2.5. Zinc bioavailability

2.5.1. Definition

There are major discrepancies in the literature as regards the definition of bioavailability. Some researchers have equated bioavailability with absorption. The term absorption would however lack meaning unless it is qualified as either true or apparent absorption. True absorption is defined as the proportion of a nutrient in food which moves across the mucosa and into the body, while apparent absorption is the difference between the nutrient content of the food and faeces without reference to the immediate origin of the nutrient in the faeces (O'Dell, 1984).

In recent studies, bioavailability has been defined as the proportion of a nutrient in food which is absorbed and utilized (Schaafma et al, 1988). This definition is gaining wide acceptance and is used with increasing frequency by investigators researching trace element bioavailability.

2.5.2. Exogenous factors that affect Zn bioavailability

Exogenous factors that may suppress the availability of Zn for intestinal absorption include:

Protein: Studies show that a high protein diet significantly increases the apparent absorption of Zn and its deposition in bone as compared to a low protein diet (O'Dell, 1984). However, research has shown that more important than the level of dietary protein, is its source. Various studies have reported that Zn in soybean protein is less available to animals than that in casein. The apparent absorption of Zn by rats fed casein was 84% compared to 44% by those fed soybean protein. Soybean protein caused a lower percentage absorption of Zn from the intestine. From these findings it was concluded that Zn in plant proteins such as isolated soybean protein is bound so that it is absorbed less efficiently than the Zn in animal proteins such as casein (O'Dell, 1969).

Phytate: The inhibitory effect of phytic acid on the absorption of Zn in experimental animals was first suggested in the early 1960s (Solomons, 1982). It was also speculated that the dietary basis for the Zn deficiency syndrome seen in the Middle East was the high

phytate content of the diet. Phytate-rich bread and purified phytate were found to reduce the apparent absorption of dietary Zn. These findings led O'Dell, 1969 to conclude that phytate binds zinc and when in sufficiently high concentrations in the diet, decreases the availability of Zn.

Fibre: The effect of dietary fibre on Zn bioavailability has been extensively studied (O'Dell, 1984). The inhibition of Zn absorption by foods rich in dietary fibre has been shown by tolerance tests and ^{65}Zn retention studies (Sanstead, 1982). ^{65}Zn was found to be less readily available for intestinal absorption when added to wholewheat bread than when added to white bread. The poor availability of Zn from such diets was attributed to the binding of Zn by fibrous components (eg. lignin and hemicellulose) of the diet.

Calcium: Dietary calcium (Ca) levels have long been associated with Zn bioavailability. It has however been determined that the detrimental effect of high Ca is dependent on the presence of phytate in the diet (O'Dell, 1984). Zn has been found to co-precipitate with phytate and Ca at the alkaline pH that is present in the lumen of the small intestine.

Other exogenous components that have been shown to

affect Zn bioavailability include oxalate, iron, copper and cadmium.

2.5.3. Endogenous factors affecting Zn bioavailability

Picolinic acid (PA), a product of pyridoxine-dependent tryptophan metabolism, is secreted into the intestinal lumen by the pancreas. PA has been shown to facilitate Zn absorption (Evans, 1980). PA forms dipicolinate complexes with many divalent cations. A deficiency of picolinic acid in rats fed diets low in tryptophan or pyridoxine was shown to impair the intestinal absorption of Zn. Supplementation of the low tryptophan or PN-deficient diets with PA was found to improve Zn absorption (Evans and Johnson, 1980; Evans and Johnson, 1981).

Evans, 1980 also attributed a failure of picolinic acid synthesis to be the cause of Zn malabsorption in infants with acrodermatitis enteropathica. These findings would suggest that PA plays an important role in the bio-availability of Zn.

2.5.4. Zinc bioavailability as affected by dietary Zn levels

Several studies have reported that dietary Zn levels affect both tissue Zn levels and body weight. Momčilović et al, 1975, using total femur Zn as the parameter of choice for Zn bioassay in rats, reported that total femur Zn and femur Zn concentrations increased with increasing levels of zinc in the diet. Using the slope-ratio technique to determine Zn bioavailability, Momčilović et al, 1975 found that dietary Zn not only affects femur Zn levels, but body weight as well. The body weight of rats was observed to increase with increasing levels of dietary zinc. This latter finding was in agreement with previous studies which also reported that dietary Zn affects body weight (Oberleas and Prasad, 1969; Williams and Mills, 1970).

Forbes and Parker, 1977 also studying the biological availability of Zn, reported that weight gain and total femur Zn increased with increasing dietary Zn concentration. In subsequent studies, Stuart et al, 1986 found that total tibia Zn and tibia Zn concentrations were higher in rats fed zinc-adequate diets than in rats fed marginally Zn-deficient diets.

2.5.6. Experimental approaches in the assessment of zinc bioavailability

Several techniques have been used to assess Zn bioavailability in man and animals.

The true absorption of Zn is a frequently used technique in studying Zn bioavailability (Hallmans et al, 1987; Lönnerdal et al, 1984). However, it has been argued that while true absorption constitutes a major component of bioavailability, it does not measure utilization or assimilation (O'Dell, 1984).

Metabolic balance studies are often used to determine Zn bioavailability, especially in human studies. In this approach, the amount of Zn in the stools is subtracted from the Zn in the diet, and an apparent absorption (not true absorption) is calculated. The balance method has however been found to be time consuming, tedious, exacting and expensive (Solomons, 1982).

Radioisotopic techniques have also been used in Zn bioavailability studies. Zn has several radioisotopes. ^{65}Zn has been the most widely used radioisotope but it has the disadvantage of having a radioactive half-life of 245 days and a biological half-life of over 500 days (Solomons, 1982).

Growth rate may also be employed as a means of determining Zn bioavailability when immature animals are used (O'Dell et al, 1972). However, the growth rate technique cannot be used in human studies since it is not possible in adults and cannot be ethically employed with infants (O'Dell, 1984).

Tissue concentration of Zn is often employed to determine Zn bioavailability (Stuart et al, 1986; Forbes and Parker, 1977; Momčilović et al, 1975). The slope ratio method is frequently applied in these studies and the tibia or femur is often the tissue of choice in studies involving rats.

Dangi and Kapoor, 1983 found that the mean femur Zn concentrations increased significantly with increased levels of dietary Zn and vice versa. In addition, Golden, 1989 in his publication reported that bone was the only tissue with an unequivocal reduction in Zn during a zinc deficiency. Giugliano and Millward, 1984 also found that bone Zn concentrations were reduced during a Zn deficiency in rats.

Calhoun et al, 1978 reported a significantly lower bone Zn level in Zn deficient rats as compared to control rats fed adequate zinc. They concluded that Zn is not

"locked" in the bone in young animals but that bone in young, growing animals serves as a Zn store and that Zn is mobilized from bone during Zn deficiency in young animals.

In view of the various positive reports pertaining to bone Zn concentrations as a method to measure Zn bioavailability, tibia Zn concentration and the growth of animals have been chosen, in the present study, as parameters for the assessment of Zn bioavailability as affected by vitamin B-6 deficiency in the rat.

CHAPTER THREE

MATERIALS AND METHODS

3.1. Structure of the study

- Diet preparation for control and experimental animals.
- The induction of pyridoxine deficiency in the experimental group.
- Pair-feeding of one group of animals with the deficient group.
- Monitoring of the food consumption daily and the body mass weekly of all rats.
- Collection of blood samples and bone (tibia) tissue at the end of the experimental period (eight weeks).
- Biochemical determination of plasma pyridoxal and pyridoxal-5-phosphate levels to establish the vitamin B-6 status of all three groups.
- The drying and digestion of bones by a wet ashing technique.
- Determination of tibia Zn levels by atomic absorption spectrophotometry.
- Statistical analysis of all experimental data obtained.

3.2. Diet

3.2.1. Composition of the diet

A diet (personal communication) comprising the following nutrients was selected for use in the present study:

	(g)
Sunflower oil	80
Cod liver oil	20
Germ oil	6
Sucrose	100
Agar	20
Salt mix	44
Vitamin mix	20
Dextrin white	510
Casein	200

Salt Mix

	(g)
$CaCO_3$	610.00
KH_2PO_4	696.00
$CaHPO_4.2H_2O$	688.00
Na_2HPO_4	142.00
$MgSO_4.7H_2O$	738.00
NaCl	233.00
Ca-lactate . $5H_2O$	154.00
KIO_3	2.14

$MnSO_4 \cdot H_2O$	0.56
$CuSO_4 \cdot 5H_2O$	0.56
$ZnCl_2$	0.52

Vitamin Mix	(g)
Vitamin K (Kapilin)	0.50
Thiamine	0.112
Riboflavin	0.250
Ca-d-Pantothenate	0.600
Vitamin B12 (Cytacon)	0.0015
Nicotinic acid	0.500
Folic acid	0.100
Biotin	0.020
Pyridoxine . HCl	0.121
Inositol	50.000
Choline	50.000
Vitamin C	5.000
Dextrin	893.00

The concentrations of the various components of the salt and vitamin mixtures were calculated and compared to the Hawk and Oser salt mix and the ICN vitamin diet fortification mixture (French, 1966). The concentrations of the various nutrients in the present diet were found to correlate closely with both the ICN vitamin diet fortification mixture and the Hawk and Oser salt mix. The vitamin mix in the present diet was also found to

closely resemble the water soluble vitamin mix recommended for rats (Kandutsch and Baumann, 1953)). Particular attention was paid to the pyridoxine content in the various diets.

The PN concentration in the present diet (2.42 mg PN.HCl/kg diet) was found to closely approximate that in the water soluble vitamin mix recommended for rats (2.50 mg PN/kg diet) and was also in keeping with the nutritional requirements for the rat as recommended by Cuthbertson, 1957, where the pyridoxine requirement for the rat is given as 2 mg PN/kg diet.

Also, Channa, 1988 using diets comprising 3.5 mg PN.HCl/kg and 1.75 mg PN.HCl/kg on two separate groups of rats found that although the 3.5 mg PN.HCl/kg diet enabled optimal growth of rats, the growth trends between the rats consuming the two different diets were not significantly different. This would suggest that 1.75 mg PN.HCl/kg of diet was sufficient to sustain normal growth in rats and that 3.5 mg PN.HCl/kg of diet may actually be in excess of the requirements for optimal growth of rats.

In view of the above, 2.42 mg PN.HCl/kg of diet was chosen for use in the present study as an appropriate concentration of PN for optimal growth of rats.

3.2.2. Diet preparation

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

Plastic weighing boats were used in the weighing of all nutrients which was done on an electronic balance (Mettler BB 300). The vitamin and salt mixtures were prepared prior to the preparation of the complete diet itself. All constituents of a crystalline nature were pulverized in a porcelain mortar until a fine powdery consistency was achieved. A porcelain pebble-mill was also employed to pulverize the crystal constituents that were needed in larger quantities eg. sucrose.

Two vitamin mixtures were prepared - one with and one without pyridoxine. In the preparation of the salt and vitamin mixtures, the compounds needed in small quantities were mixed first and then, through a number of triturated steps, all constituents were added and thoroughly homogenised in a large mortar. To prevent

nutrients of a deliquescent nature from attracting moisture, they were first mixed thoroughly with anhydrous components of the diet and then stored in a dessicator prior to incorporation into the diet.

After hand mixing, the vitamin and salt mixtures were further homogenised in a mechanical mixer (Forster Equipment Co, Eng.) for two hours. The two different vitamin mixtures and the salt mix were then transferred to plastic bags, properly labelled, and stored in air- and light-tight containers until required.

The final complete diets, one with and one without vitamin B-6, were prepared in batches of 4 kg each. This was done twice a week for the duration of the experiment. Here again, the nutrients required in smaller quantities were mixed before adding nutrients required in larger quantities. The dry ingredients were mixed first and only then were the oil components of the diet added. After hand mixing in a mortar using a pestle for approximately two hours, the mixture was transferred to a mechanical mixer for thorough homogenization for a further two hours. The completed diets were then dispensed into plastic bags, labelled and stored in a light-tight container at approximately 3°C.

3.3. Induction of vitamin B-6 deficiency

Thirty-three young male Wistar rats, supplied by the Biomedical Resource Centre (BRC), University of Durban-Westville (UDW), were used in this study. The rats with masses ranging from 60 to 80 g, were divided into three groups of eleven rats each such that the mean weight of each group was similar.

Group A, the control group, was fed the adequate diet containing 2.42 mg/kg of vitamin B-6 in the form of PN.HCl.
Group B, the pair-fed animals, received the same diet as group A, but their rations were restricted to the average amount consumed the previous week by the deficient group.
Group C, the deficient group, was fed the PN-deficient diet (0 mg PN/kg).

The need for a pair-fed group in the experiment was evident since a decrease in food consumption by the PN-deficient animals was anticipated. The pair-fed animals were introduced into the experiment a week after the commencement of feeding of the control and deficient groups, thus enabling the determination of the average dietary consumption of the deficient group for the week.

The rats were housed individually in perspex metabolic cages (Techniplast) in an air-conditioned room at the BRC. The average humidity and temperature were 55% and 22°C respectively and automatically controlled illumination with twelve hour light and dark cycles was maintained. The perspex food containers, attached to the cages, were designed such that only the head of the animal could be inserted into the container while feeding, thus minimizing food spillage and contamination. The metabolic cages had stainless steel grid floors which allowed the faeces and urine to be separately collected in pastic containers below the cage, to prevent coprophagy. The cages and food containers were regularly washed with disinfectant and rinsed with deionised water by the BRC.

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 diet were supplied ad libitum to the animals.

Food consumption was recorded daily and all animals were weighed once a week for the duration of the study. The animals were also examined daily for any physical signs of deficiency or disease. Each group of rats was maintained on its particular diet for a period of eight weeks.

3.4. Tissue isolation

At the end of eight weeks, all food containers were removed from the metabolic cages and the rats were held overnight without food but with access to deionised water.

All surgical procedures and sample collection were performed on the following morning at the post-mortem laboratory in the BRC, UDW. All surgical instruments used were washed and rinsed in deionised water to avoid contamination.

Rats were anaesthetised with halothane and blood samples removed by cardiac puncture using disposable plastic syringes and stainless steel needles. The samples were immediately transferred to labelled 5 ml vacutest tubes with EDTA, which were then placed in crushed ice.

After the blood was removed, the hind limbs were excised and the tibias extracted. Tibias were cleaned of adhering soft tissue, blotted (ashless filter paper) and then placed individually in labelled plastic vials. All vials were immediately placed in crushed ice before storage at -7°C.

3.5. Reagents and laboratoryware

Zinc is ubiquitous in the environment, and it is therefore important that the utmost care is taken to prevent contamination of samples from water, glassware and reagents. To minimize contamination, all glassware and plasticware used in the experiment were first acid-washed by soaking in 20% nitric acid for 48 hours. Glassware and plasticware were then rinsed several times using deionised water, thoroughly dried and stored in plastic bags until required.

The reagents used were as follows:

Double deionised water, made using an Elgastat B114 water deioniser (Elga Products, England).

Zinc stock standard (Spectrosol) from BDH Chemicals, Poole, England.

Nitric acid (suprapure) from Merck, Darmstadt, Germany.

3.6. High Performance Liquid Chromatography (HPLC) - Analysis of plasma PL and PLP

3.6.1. General principles of HPLC

The techniques of chromatography are relatively new. "Their value lies in their versatility - providing methods not only for the separation and purification of compounds but also for product identification and quantitative analysis" (Doyle and Mungall, 1980). Chromatographic methods involve the reversible transfer of a compound that is adsorbed on a stationary phase into a mobile phase that is flowing past the stationary phase (Doyle and Mungall, 1980). The separation of mixtures into their various components is due to the difference in equilibrium distribution of these components between two different phases.

There are various types of chromatography:

- Paper
- Gas (GC)
- Thin layer (TLC)
- Open column
- High performance liquid (HPLC)

All the above forms, except GC, are forms of liquid

chromatography. The basic methods that apply to all forms of liquid chromatography are as follows: liquid chromatography comprises a stationary phase and a mobile phase and as a result of the various affinities of the sample for each, a separation occurs. Due to interactions between sample molecules, stationary phase and mobile phase, individual molecules are retained by the stationary phase. The unique chemical interactions of each component determines the length of time for which the individual molecules are retained. Since these interactions differ, the retention times differ and separation is achieved.

HPLC is ordinary liquid chromatography done with sophisticated equipment. Pumps employed for HPLC are usually designed to provide a constant flow and to be pulse-free. The pump drives injected solvent under high pressure through columns packed with an adsorbent and thus brings about a specific separation. Detectors "see" the various components as they elute and integrators collect, quantitate and interpret results. Results are presented as a series of peaks on a chart and retention time is used to identify unknown components in the sample (Williams and Wilson, 1984).

Various separation mechanisms are employed in HPLC, including:

1) Size separation, which is a form of non-interactive chromatography i.e. no chemical reaction takes place within the system. Molecules are instead separated by a porous gel on the basis of molecular size.

2) Ion-exchange chromatography, which is used for the separation of substances that are in an ionized form. The stationary phase consists of a porous organic or inorganic gel or resin carrying functional groups on its surface. The eluent contains ions of opposite charge to ions of the stationary phase. Sample ions exchange places with eluent ions and remain on the column for a certain period of time depending on the relative affinity of the packing for the eluent or sample ions.

3) Liquid-liquid or partition chromatography, where the column consists of a bed support (usually inert) on which the stationary partitioning phase is adsorbed. The mobile phase flowing through the column is in contact with the stationary phase over a large interphase. In this process, equilibrium distributions of the solute between the two phases take place rapidly. Separation of the different components of a mixture results from the differing distributions of the various solutes in these two unlike phases.

4) Liquid-solid or absorption chromatography, involves a relatively non-polar liquid mobile phase and a solid polar stationary phase. Separation of polar molecules occurs between the two phases.

3.6.2. Determination of plasma PLP and PL levels by HPLC

Plasma pyridoxal-5-phosphate (PLP) content is employed with increasing frequency as an indicator of the nutritional status of vitamin B-6 (Lawrence et al, 1978). For the simultaneous quantification of plasma PLP and PL levels, the method devised by Ubbink et al, 1985/1986 was employed. This simple isocratic technique is a rapid and sensitive HPLC method that makes use of an internal standard and is based on post-column semicarbazone formation of PLP and PL, followed by fluoresence detection. An important feature of this method is the simultaneous addition of trichloroacetic acid (TCA) and semicarbazide to plasma to liberate PLP from albumin (Ubbink et al, 1986).

Reagents

The internal standard, 6-methyl-2-pyridine carboxaldehyde was obtained from Aldrich (Milwaukee, U.S.A.); PL

and PLP from Merck (Darmstadt, Germany); Chromatography grade acetonitrile and all other reagents used (analytical reagent grade) were also obtained from Merck.

Purification

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

Standards

Working standards containing 1-20 ng PLP and PL per ml were prepared by using purified PLP and PL. Equal volumes of standard solution and precipitation reagent (50 ml TCA, 30 ml semicarbazide) were added to labelled vials. The mixture was then incubated for 40 minutes at 45°C.

Sample preparation

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

Instrumentation

A Beckman (Beckman Instruments, Berkeley, CA, U.S.A.) Model 112 solvent delivery module was modified so that the pump was used for solvent delivery. An LS 4 Perkin-Elmer fluorescence pectometer (excitation wavelength: 367 nm); emission wavelength: 478 nm) was coupled to a Spectra Physics 4920 integrator (San Jose, CA, USA).

Columns

A Whatman (Clifton, NJ, USA) Partisphere C18 analytical column (110 x 4.7 mm I.D.; particle size 5 µm) was used. A Whatman reversed-phase 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.

3.7. Tissue analysis

3.7.1. Sample preparation

3.7.1.1. Drying of bones

Prior to wet ashing of tissue samples, the tibias were dried to a constant weight. Tibias were removed from refrigeration and allowed to thaw. The thawed samples were then blotted dry on ashless paper. The bones were then individually weighed (wet weight) using plastic weighing boats on an electronic balance (Mettler BB 300) and the masses recorded. Tibias were then transferred to separate labelled 20 ml beakers which were then placed in the oven at 110°C for 24 hours. It was determined that 24 hours was adequate time for the complete drying of tibia samples. After 24 hours, the samples were removed from the oven and transferred to a dessicator where they remained for approximately 30 minutes until cool. The dry weights of the tibias were then recorded.

3.7.1.2. Wet ashing

Atomic absorption spectrometry requires that samples be in a liquid form to enable analysis (Clegg et al, 1981). A variety of methods exist at present for the solubilization of biological tissues for atomic

absorption spectrometry. Wet ashing procedures are being used more extensively because of the increased interest in the biological role of trace metals. Several methods of wet ashing were examined by Clegg et al, 1981, to determine which acids, acid combinations, or bases should be used as digesting agents for accurate and precise measurement of iron, copper, zinc and manganese. It was found that nitric acid proved to be the most effective wet ashing agent. Nitric acid was therefore chosen for wet ashing in the present study.

Tibia samples were transferred to labelled 50 ml Kjeldahl flasks, each containing 5 ml of nitric acid and three glass beads. Samples were predigested for approximately 12 hours at room temperature. After predigestion, samples were heated and maintained at a constant slight boil for 4 hours and then allowed to cool. Digested samples were subsequently transferred to 50 ml volumetric flasks. The inside of the digestion flasks were rinsed several times with deionised water to ensure complete transfer of the acid digest. Samples were then brought to volume with deionised water (Clegg et al, 1981).

Digestion was carried out in sets of 6 samples, comprising 4 tibia samples (one each from groups A, B and C and one tibia from the control group for recovery

studies), a blank and a blank plus standard.

3.7.2. Recovery studies

Eleven tibias, one from each of the control group animals were used for recovery studies. The bones were dried and wet ashed as described above. A known concentration of Zn stock standard (Spectrosol, BDH Chemicals, Poole, England) was then added to the samples to enable the determination of percentage recoveries so as to standardize the ashing and atomic absorption methods.

Note - One of the tibias from each of the group A rats was used for recovery studies and the other tibia for determination of Zn levels in the control animals.

3.7.3. Atomic absorption spectrophotometry (AAS)

3.7.3.1. General principles of flame atomic absorption spectrophotometry (FAAS)

At present, the most widely employed methods to determine Zn concentrations in biological samples are based on atomic spectrometry - the interaction of analyte atoms with electromagnetic radiation. These procedures fall into three categories -

- atomic emission
- atomic absorption and
- atomic fluorescent spectrometry

FAAS (the method used in the present study) is the most widely used technique for routine analysis of most elements including zinc (Falchuk et al, 1988).

Briefly, the mechanism of FAAS may be summarized as follows: A hollow cathode tube emits a narrow line spectrum specific for the element to be measured. The particular wavelength to be employed is isolated by a monochromator (usually 213.9 nm for Zn). A detection system converts the radiant energy received into an electrical signal. In the absence of sample, the readout is shown as zero percent absorbance or hundred percent transmittance. A liquid sample (1-2 ml) is aspirated into a flame nebulizer system where the sample atoms are atomised. The radiation emitted by the hollow cathode tube is absorbed by the sample atoms in proportion to their concentration. The absorption decreases the incident radiation received by the detection system. For most instruments, the proportionality between Zn and absorbance follows Beers Law and is linear in the range of 0.1-1.0 µg Zn/ml.

Because of the specificity of the line spectra, it is possible to determine the amounts of a specific element present. Since the amount of radiation present is proportional to the number of excited atoms, standard solutions are used to calibrate the system. The flame most widely used is the air/acetylene flame (Welz, 1976).

3.7.3.2. Analysis of tibia Zn levels by FAAS

Zinc levels of tibias were determined by FAAS. The Perkin-Elmer Model 2380 microprocessor controlled atomic absorption spectrophotometer (Perkin-Elmer Corporation, Norwalk, C.T., U.S.A.) was used. Results were expressed in µg/gram of dry tissue.

Set-up procedure

The hollow cathode lamp specific for Zn was installed into the lamp compartment as specified by the instruction manual (Perkin-Elmer, 1976 b) and the lamp control was adjusted until the lamp current as specified on the lamp was reached. A ten minute lamp warm-up period was allowed.

The appropriate instrumental settings, as specified for Zn by the instruction manual (Perkin-Elmer, 1976 a) was

selected.

wavelength	-	213.9 nm
slit setting	-	0.7 nm

The gain setting was used to optimize both the wavelength and lamp current.

Prior to ignition, the air pressure was adjusted to 280 kPa and the acetylene pressure to 140kPa. The air and acetylene tanks were equipped with pressure regulators which were regularly checked to ensure that the pressures remained constant. Ignition resulted in a lean blue oxidizing air-acetylene flame.

Before sample analysis, deionised water was aspirated for a few minutes to clear the burner slot of any contaminants.

Calibration

A Zn working standard was prepared daily from Spectrosol grade Zn nitrate stock standard having a concentration of 1000 µg/ml (BDH Chemicals, Poole, England). To establish a linear standard curve, a 1 µg/ml Zn standard was used. The calibration standard was analysed after three sample readings were recorded and recalibration was

performed if the known concentration varied by >5%.

Operating parameters that apply were as follows:

linear working range	: 1 µg/ml
sensitivity limit	: 0.018 µg/ml
sample introduction rate	: 4 ml/min
number of readings	: 3
integration time	: 1 sec

To obtain maximum sensitivity, the burner head and the nebulizer were adjusted as specified by the instruction manual (Perkin-Elmer, 1976a).

3.8. Statistical analysis

Growth

When several measurements are taken on the same experimental unit (rat), the measurements tend to be correlated with each other. When the measurement can be thought of as responses to levels of an experimental factor of interest such as time, the correlation can be taken into account by performing a repeated measures analysis of variance (used in the present study).

In this analysis, the dependent variable was growth at day one, week 1 to week 8 after receiving the diet. Logarithms were applied to the growth to minimize correlation between the mean and variance of the data.

Individual analysis of variance (ANOVAS) was performed on the growth at each week (for differences between the groups at each week). Duncan's multiple range tests were used for multiple comparisons.

Food consumption

Repeated measures analyses were performed on mean weekly food consumption and Duncan's multiple range tests were used for multiple comparisons.

Zn concentration in rat tibias

The three groups were compared using analysis of variance with Duncan's multiple range test.

Plasma PL and PLP levels.

ANOVAS were also performed on PLP and PL levels.

Note: All statistical analyses were done in collaboration with Ms E.Gouws (Institute of Biostatistics of the South African Medical Council) and Professor G.P.Y. Clarke (University of Natal, Pietermaritzburg, Department of Statistics and Biometry).

CHAPTER FOUR

RESULTS

4.1. The effect of vitamin B-6 deficiency on food consumption

The mean weekly food consumption for the rats in groups A and C, over the eight week period, is shown in table 2. The food consumption of the group C animals was found to decline significantly from the second week onwards. A significant difference in the food intake between groups A and C was observed from weeks two to eight ($p < 0.05$). The pair-fed rats (group B), fed the PN-sufficient diet in amounts equivalent to the mean weekly intakes of the deficient animals, were found to consume all of their food. Their weekly food intake was thus equivalent to that of the PN-deficient rats.

Two-tailed P values are given in table 3.

Figure 12 represents the food consumption trends for groups A and C.

Table 2: Effect Of Vitamin B-6 Deficiency on Weekly Food Consumption (g).

GRP	WEEK 1	WEEK 2	WEEK 3	WEEK 4	WEEK 5	WEEK 6	WEEK 7	WEEK 8
A	8.29 a ±0.90	10.64 a ±0.58	12.21 a ±0.86	14.66 a ±0.66	16.51 a ±1.85	15.77 a ±2.24	15.36 a ±2.19	15.69 a ±1.64
C	7.99 a ±0.57	8.06 b ±0.39	7.71 b ±0.26	7.12 b ±0.39	6.58 b ±0.42	6.34 b ±0.44	6.36 b ±0.44	5.97 b ±0.70

Data are presented as mean ±SD and were obtained from 11 rats per group. Means for each week not followed by the same superscript letter are significantly different ($p < 0.05$). Groups A and C were fed 2.42 and 0 mg/kg of vitamin B-6 in the diet respectively.

VITAMIN B-6 & FOOD CONSUMPTION

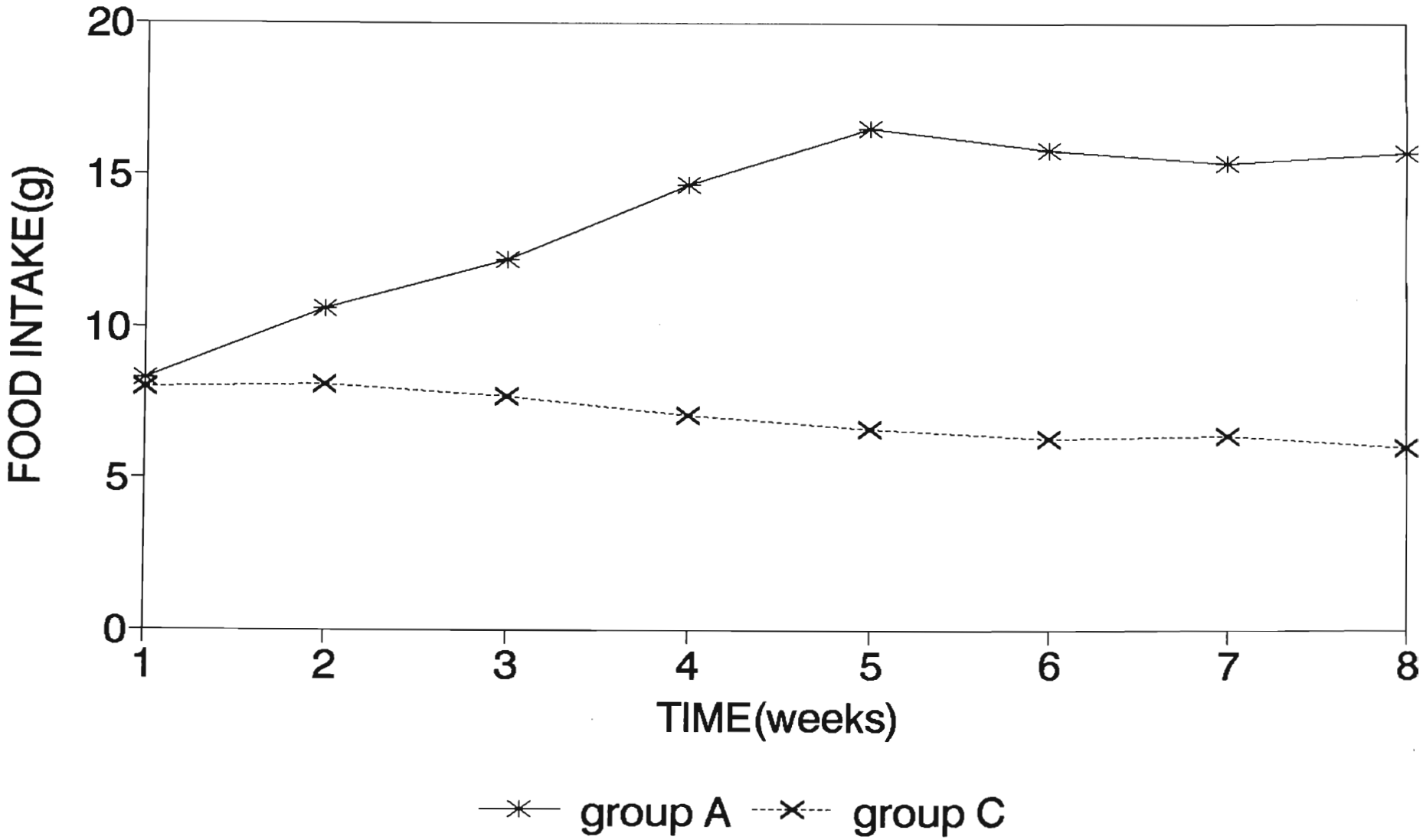


Figure : 12

Table 3: Two-tailed P-values - Effect of vitamin B-6 deficiency on weekly food consumption

GRP	WEEK 1	WEEK 2	WEEK 3	WEEK 4	WEEK 5	WEEK 6	WEEK 7	WEEK 8
A vs C	0.3655	0.0001	0.0001	0.0001	0.0001	0.0001	0.0001	0.0001

A significant difference exists between groups if the P value is < 0.05.

4.2. The effect of vitamin B-6 deficiency on growth

Data on the mean body mass of the 3 groups of rats are given in table 4. In table 5, the mean body masses (given in table 4) are expressed in logarithmic form to minimize correlation between the mean and variance of the data.

The mean weights of the rats in all three groups were similar at the beginning of the experiment. However, by the second week a significant difference in mass was noted between groups A and C and groups A and B ($p < 0.05$). No significant difference in body weight was noted between groups B and C ($p > 0.05$). This pattern continued for the next two weeks as well. By the fifth week, however, a significant difference in mass was observed between all three groups. This trend continued for the duration of the experiment. The difference in mean body mass between groups A and C was greater than the difference between groups A and B from the third week onwards.

Figure 13 shows the growth patterns of the three different groups.

Table 4: Effect of vitamin B-6 deficiency on growth (g)

GROUP	DAY 1	WEEK 1	WEEK 2	WEEK 3	WEEK 4	WEEK 5	WEEK 6	WEEK 7	WEEK 8
A	71.54 ±6.17	88.00 ± 8.31	117.09 ±11.13	161.82 ±10.23	199.36 ±11.99	233.36 ±19.45	263.18 ±26.65	289.18 ±34.72	302.09 ±35.67
B	70.18 ±7.56	85.55 ±12.38	101.91 ±15.99	131.82 ±10.79	151.00 ±14.16	165.64 ±14.90	186.18 ±18.27	192.09 ±17.39	196.36 ±16.70
C	72.09 ±6.63	88.91 ±9.75	104.36 ±11.51	127.00 ±11.69	143.45 ± 9.97	153.64 ±11.29	168.73 ±11.59	172.18 ±12.94	173.82 ±13.88

Data are presented as means ±SD and were obtained from 11 rats per group. Group A (control group) received 2.42 mg/kg of vitamin B-6 in the diet. Group B is the pair-fed group. Group C (deficient group) received 0 mg/kg of vitamin B-6 in the diet.

Table 5: Effect of vitamin B-6 deficiency on body mass.

GROUP	WEEK 1	WEEK 2	WEEK 3	WEEK 4	WEEK 5	WEEK 6	WEEK 7	WEEK 8
A	a 4.47	a 4.76	a 5.08	a 5.29	a 5.45	a 5.57	a 5.66	a 5.70
B	a 4.44	b 4.61	b 4.88	b 5.01	b 5.11	b 5.22	b 5.25	b 5.28
C	a 4.48	b 4.64	b 4.84	b 4.96	c 5.03	c 5.13	c 5.15	c 5.16

Data are presented as logarithms of mean and were obtained from eleven rats per group. Values for each week not followed by the same superscript letter are significantly different ($P < 0.05$). Group A (control group) received 2.42 mg/kg of vitamin B-6 in the diet. Group B is the pair-fed group. Group C received 0 mg/kg of vitamin B-6 in the diet.

VITAMIN B-6 & GROWTH

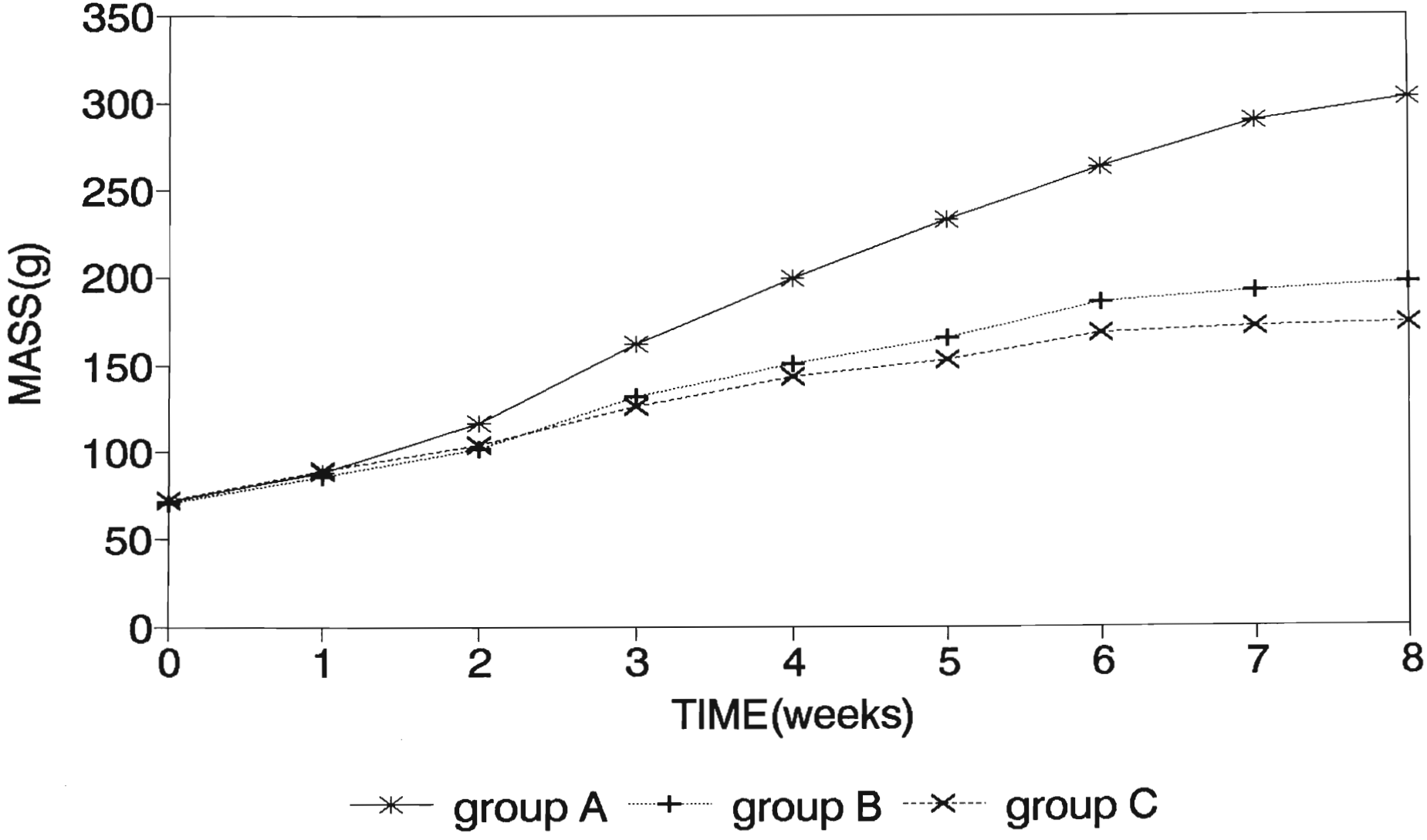


Figure : 13

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

Plasma PL and PLP levels were determined by HPLC at the end of the eight week experimental period. Duncan's Multiple Range Test was employed to determine the significance of difference between groups. There was a significant difference in both PL and PLP levels between groups A and C and between groups B and C ($P < 0.05$). There was no significant difference in PL or PLP levels between groups A and B.

Table 6 provides the mean PL and PLP values for groups A, B and C. Figure 14 shows the plasma PL and PLP levels of the three groups at the end of experimental period.

Table 6: The effect of vitamin B-6 deficiency on plasma PL and PLP levels.

Group	Plasma PLP ng/ml	Plasma PL ng/ml
A	331.70 ± 64.02[a]	202.90 ± 53.13[a]
B	337.00 ± 65.82[a]	227.30 ± 57.23[a]
C	41.50 ± 16.63[b]	21.50 ± 9.69[b]

Plasma PL and PLP values are given as means ± SD and were obtained from 10 rats in each group. Means for each group not followed by the same superscript letter are significantly different ($p < 0.05$). Group A received 2.42 mg/kg of vitamin B-6 in the diet. Group B is the pair-fed group and group C received 0 mg/kg of vitamin B-6 in the diet. The two-tailed P-values for groups A vs C and B vs C were equal to 0.0001. A significant difference exists between groups when $P < 0.05$.

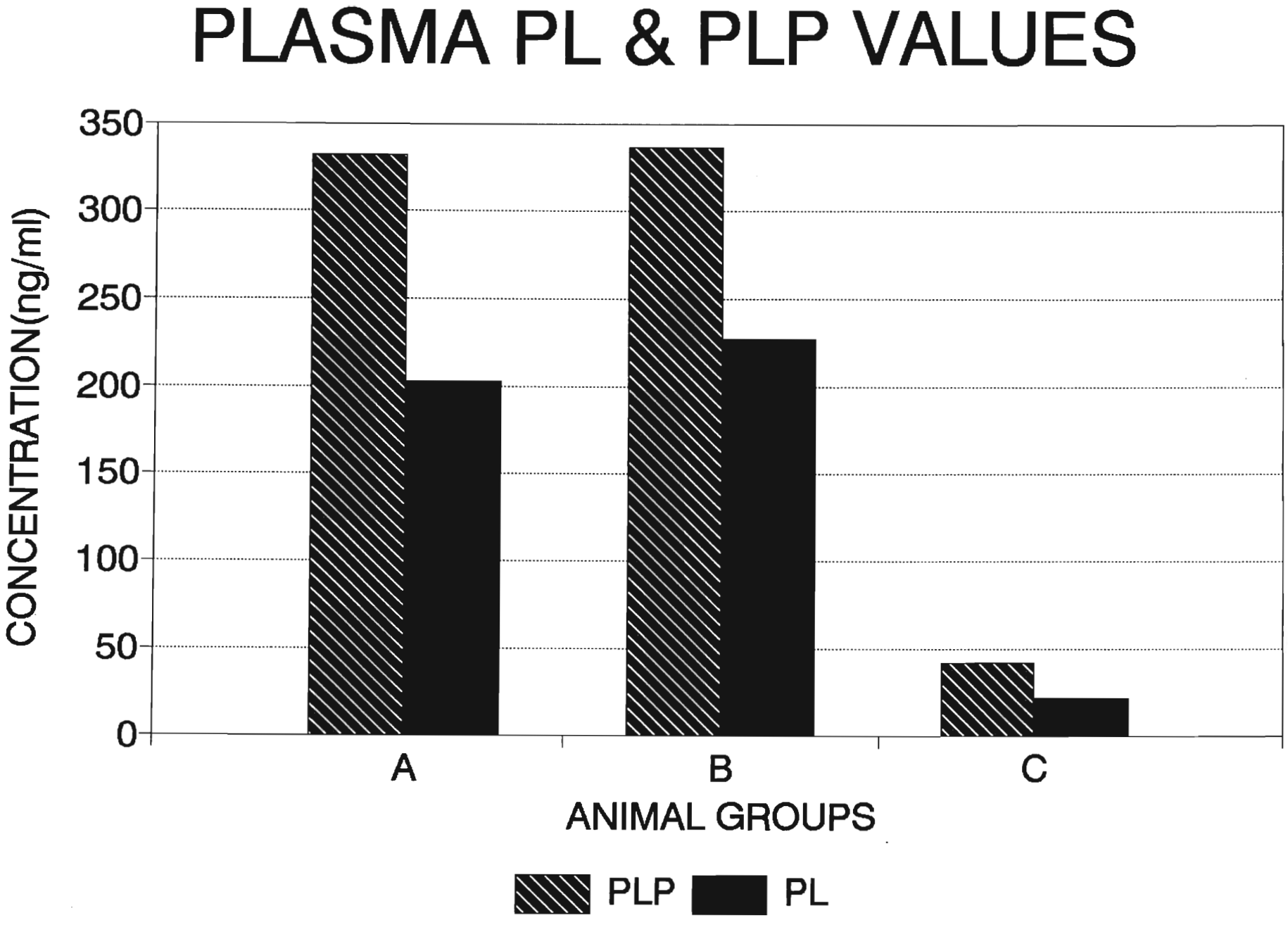

PLASMA PL & PLP VALUES
CONCENTRATION(ng/ml)
350
300
250
200
150
100
50
0
A
B
C
ANIMAL GROUPS
PLP
PL

Figure : 14

4.4. Vitamin B-6 deficiency and tibia Zn levels

Zn levels are expressed in µg/g dry tissue. Tibia Zn concentrations were calculated as follows:

Zn concentration in µg/g = Zn concentration in µg/ml x 50 (dilution factor) divided by the weight of the tissue.

Recovery studies

The percentage recovery of Zn from each of the eleven tibias used was determined after digestion. Individual values are given in Table 7 and were found to range between 95 and 101 percent.

Table 7: Percentage of Zn recovered after digestion.

Animal	Percentage recovery from tibia
1	101
2	99
3	98
4	97
5	97
6	97
7	99
8	98
9	95
10	99
11	98
	Mean: 98

Tibia Zn concentrations

The mean tibia Zn concentrations for groups A, B and C are given in Table 8.

Group C (deficient animals) were found to have a significantly lower tibia Zn concentration than groups A and B ($p < 0.05$). There was no significant difference in the tibia Zn levels between groups A and B ($p > 0.05$).

Table 8: Mean of Zn levels in tibias (µg/g).

Group	Tibia Zn concentration
A	161.34 ± 7.78^{a}
B	159.11 ± 9.07^{a}
C	141.12 ± 6.02^{b}

Values are expressed as means ± SD and were obtained from 11 rats per group. Means for each group not followed by the same superscript letter are significantly different. Group A (control rats) received 2.42 mg/kg of vitamin B-6 in the diet. Group B is the pair-fed group. Group C (deficient rats) received 0 mg/kg of vitamin B-6. The two-tailed P-values for Groups A vs. C and Groups B vs. C were equal to 0.0001. A significant difference exists between groups when $p < 0.05$.

CHAPTER FIVE

DISCUSSION

5.1. Appearance of rats

The most obvious physical symptom observed in the vitamin B-6 deficient animals was poor growth which became apparent from the second week onwards. By the fourth week, some of the rats in the deficient group showed signs of acrodynia (symmetrical scaling dermatitis) on the chin and upper thorax. Also noticeable on all deficient rats was the unkempt appearance of the fur when compared to control rats. These symptoms are in keeping with the vitamin B-6 deficiency symptoms observed by Rutishauser, 1972 and Labadarios and Shephard, 1985.

5.2. Food consumption and growth

The vitamin B-6 deficient rats (group C) consumed approximately 7g of food per day while the control animals consumed almost twice this amount per day (13.7g/d). This would in itself account for the significant difference in body mass observed between group A

(control) rats and the PN-deficient animals. However, the pair-fed rats (group B) also consumed only 7g/d and yet showed a significantly higher growth rate than the deficient group from the fifth week onwards. This would suggest that the decreased growth rate of the deficient rats was due largely to a factor other than the decreased caloric intake.

The deficient group received no pyridoxine in their diet while the control and pair-fed rats received 33 µg/d and 17 µg/d of pyridoxine respectively. The significant difference in growth rates observed between groups C and B from the fifth week onwards can therefore be attributed to a deficiency of pyridoxine in the group C animals, since both groups consumed the same amount of food. The significant difference in growth rates between group A and group C rats from the second week onwards is therefore due primarily to a pyridoxine deficiency in group C animals, although a decreased caloric intake is also a contributory factor.

The significant difference in growth between groups A and B can also be accounted for by pyridoxine levels. Group B rats consumed only 17 µg/d of pyridoxine while group A animals consumed almost double this amount per day.

A survey of the literature reveals that the vitamin B-6 requirement for rats as reported by individual investigators ranges from between 24 µg/d to 80 µg/d. Lumeng et al, 1987 found that maximal growth occurred in weanling rats receiving 29 µg/d of PN.HCl, while Van den Berg et al, 1982 reported that 24 µg/d of pyridoxine is required to produce maximal growth in rats. Other researchers have reported higher levels of pyridoxine (75 µg/d) as being necessary for optimal growth in rats (Mercer et al, 1984).

The above findings suggest that the pair-fed group in the present study received less pyridoxine than is required to allow maximal growth. This would explain the significant difference in body weights between the control and pair-fed rats in this study.

The various observations in the present study with regard to food consumption and growth suggests that both a decreased caloric intake and PN deficiency account for the decreased growth rats of group C and B rats when compared to group A animals; however since the significance of difference between groups A and C is greater than that between groups A and B, one can conclude that pyridoxine deficiency is the major contributory factor resulting in the decreased growth rates observed.

5.3. Plasma PL and PLP levels

Plasma PL and PLP levels were measured by a HPLC technique developed by Ubbink et al, 1985. The purpose of this analysis was to establish whether there was a significant difference in the levels of plasma PL and PLP between the deficient rates (group C) and the control rats (group A).

The levels of plasma PL and PLP in all three groups were higher than values reported in the literature for control and vitamin B-6 deficient rats (Channa, 1988). No explanation can be given at present for this observation.

The deficient rats were, however, found to have significantly lower plasma PL and PLP levels than the control and pair-fed animals. The plasma PLP and PL levels in the deficient animals were approximately eight and nine times lower than that in the control animals, respectively.

5.4. Tibia Zn concentrations

The vitamin B-6 deficient rats were observed to have significantly lower tibia zinc concentrations than the control and pair-fed animals. No significant difference

in tibia Zn concentrations was found between pair-fed and control rats.

These observations are in keeping with reports by Hsu, 1965 and Ikeda et al, 1979 who maintained that a vitamin B-6 deficiency results in decreased Zn levels in various tissues. Since there was no significant difference in tibia Zn levels between control and pair-fed animals, it can be concluded that the decreased zinc levels observed in the deficient group is due to a vitamin B-6 deficiency and not to the decreased caloric intake of the group.

The findings in the present study may be explained by the observations of Evans and Johnson, 1981, who reported an impairment of Zn absorption resulting from a vitamin B-6 deficiency. The decreased Zn absorption was attributed to a decreased production of a low molecular weight Zn-binding ligand viz. picolinic acid. Picolinic acid is a product of tryptophan metabolism and pyridoxal is an essential cofactor in the pathway from tryptophan to picolinic acid. A decrease in vitamin B-6 would therefore result in a decreased production of picolinic acid which in turn would result in decreased Zn absorption leading to decreased tissue zinc levels.

The observations in the present study are thus in

keeping with findings by Brown, 1985, who stated that the nutritional status of zinc is related to vitamin B-6 status.

5.5. Zinc bioavailability as affected by a vitamin B-6 deficiency

The bioavailability of zinc may be defined as that proportion of dietary Zn which is absorbed and utilized in Zn metabolism (Schaafma et al, 1988). Tibia Zn levels and growth were chosen as parameters to assess Zn bioavailability as affected by vitamin B-6 deficiency in this study.

A vitamin B-6 deficiency was found to result in decreased tibia Zn levels indicating a decreased bioavailability of zinc. One might however argue that vitamin B-6 was not the only limiting nutrient in the deficient rats (group C) since their daily food intake was approximately half that of the control animals. This would mean that the decreased tibia Zn levels observed in the present study may be due not only to the vitamin B-6 deficiency but also to the decreased caloric intake. The tibia Zn levels observed in the pair-fed group do, however, show that the decreased caloric intake did not affect tibia Zn levels. The mean tibia Zn levels between the control and pair-fed animals showed no significant

difference indicating that the caloric intake in the present study was not a limiting factor with regard to tibia Zn concentrations. From this, it can safely be concluded that the reduced tibia Zn levels of the deficient group are in fact due to vitamin B-6 deficiency.

Rosenberg and Solomons, 1982 have roughly divided factors that influence the bioavailability of trace elements into those which influence behaviour in the intestinal lumen and those which occur at the level of the intestinal cell.

In the present study, the influence of vitamin B-6 deficiency on tibia Zn concentrations may be traced to the absorptive level, that is, at the level of the intestinal lumen. The decreased bioavailability of Zn as a result of vitamin B-6 deficiency can thus be accounted for by the influence of vitamin B-6 on Zn absorption.

Evans et al, 1979 provided evidence suggesting that the dominant Zn-binding ligand in the rat intestine is picolinic acid. Picolinic acid has been shown to facilitate Zn absorption (Evans, 1980) by forming dipicolinate complexes with Zn^{2+}. Evans and Johnson, 1981 showed that a deficiency of picolinic acid impairs Zn absorption.

Since PA is a product of pyridoxine-dependent tryptophan metabolism, a deficiency of either pyridoxine or tryptophan would impair Zn absorption. Therefore, in the present study it can be hypothesised that the vitamin B-6 deficiency resulted in a decrease in the synthesis of picolinic acid. Pyridoxal is required as a cofactor for the enzyme kynureninase in the pathway from tryptophan to PA. The decreased synthesis of PA would result in a malabsorption of Zn which would account for the decreased levels of Zn observed in the tibias of the vitamin B-6 deficient rats.

The decreased tibia Zn concentrations in the vitamin B-6 deficient rats may be due not only to the decreased uptake of Zn at the absorptive level, but also as a result of Zn mobilization from bone during a zinc deficiency state. This would be in keeping with the findings of Calhoun et al, 1978 who reported significantly lower Zn levels in zinc deficient rats when compared to control animals. It was suggested that zinc is not "locked" in the bone in young animals but rather that the bone serves as a Zn store from which Zn may be mobilized during a deficiency state. These observations, however, still strongly point to an impaired Zn absorption which would result in zinc mobilization from bone.

It must be mentioned, however, that the involvement of picolinic acid in Zn absorption as suggested by Evans et al, 1979 has not as yet been conclusively demonstrated. Hurley and Lönnerdal have cast doubt on the involvement of picolinic acid in Zn absorption. They maintained that the effects of picolinic acid with regard to Zn absorption are not unique since other Zn-binding ligands are also present in the intestinal mucosa.

Although the exact mechanism whereby Zn absorption is affected by a vitamin B-6 deficiency may not be known at present, the findings in the present study do however suggest that a vitamin B-6 deficiency does have an effect on the Zn absorption in rats.

In addition to decreased tibia Zn levels, the vitamin B-6 deficient rats also showed significantly lower growth rates when compared to control animals. The growth pattern by itself may not, however, enable one to conclude that vitamin B-6 has an effect on Zn bioavailability since the caloric intake of the deficient rats was significantly lower than that of the control animals. However, as already discussed, the significant difference in body weights between pair-fed and deficient animals in the second half of the experimental period would suggest that vitamin B-6 deficiency does

reduce the growth rates of the deficient rats.

Both the decreased tibia Zn concentrations and the decreased growth rates in the vitamin B-6 deficient rats when compared to control and pair-fed animals would suggest that a vitamin B-6 deficiency does indeed lower the zinc bioavailablity in rats.

CHAPTER SIX

CONCLUSION

The results of this study point to a significant difference in the bioavailability of zinc between animals that receive adequate amounts of vitamin B-6 and animals that are deficient in vitamin B-6.

Both tibia Zn concentration and animal growth were found to be significantly lower in the vitamin B-6 deficient rats than the control animals, indicating a decreased bioavailability of Zn in response to a vitamin B-6 deficiency. The influence of vitamin B-6 on zinc bioavailability has been traced to the absorptive level of zinc. As already discussed, various studies have provided evidence which suggest that the dominant Zn-binding ligand in the rat intestine is picolinic acid, a product of pyridoxine-dependent tryptophan metabolism (Evans et al, 1979). The decreased zinc bioavailability observed in the present study has been attributed to a decrease in picolinic acid which results from a vitamin B-6 deficiency.

The findings in this study also support the suggestion

that bone serves as a zinc store from which Zn is mobilized during a Zn deficiency state (Calhourn et al, 1978). This would mean that bone Zn concentration may be employed as a parameter to determine Zn bioavailability in rats.

The exact mechanism whereby vitamin B-6 deficiency affects zinc bioavailability is incompletely understood and should provide an interesting avenue for future research.

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SUMMARY

Various studies have shown that both zinc and vitamin B-6 participate in a great variety of enzymatic reactions. A deficiency of vitamin B-6 has been observed to affect tissue zinc levels in rats. The present study was undertaken to determine the effect of a vitamin B-6 deficiency on the bioavailability of zinc in the rat. Tibia Zn levels and animal growth were used as parameters to assess Zn bioavailability.

Thirty-three young male Wistar rats were divided into three groups of eleven rats each. Group A, the control animals, received 2.42 mg PN.HCl/kg of diet. Group B, the control pair-fed rats, received the same diet as group A but their rations were restricted to the amounts consumed by the deficient rats. Group C, the vitamin B-6 deficient rats, received a vitamin B-6 deficient diet.

Plasma PL and PLP levels were determined to establish the vitamin B-6 status of each rat. A significant difference in PL and PLP levels was observed between the deficient and control rats.

Tibia Zn levels were determined by atomic absorption spectrophotometry. The tibia zinc levels in the

deficient animals were found to be significantly lower than tibia zinc concentrations of the control and pair-fed rats. The low tibia zinc levels of the deficient animals were attributed to a decrease in the concentration of picolinic acid. Picolinic acid, a product of pyridoxine-dependent tryptophan metabolism, is reported to be a major Zn-binding ligand in the rat intestine.

The food consumption and growth rates of the deficient group were found to be significantly lower than the control group.

These findings would suggest that the bioavailability of zinc is lowered during a vitamin B-6 deficiency in rats.

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