AN ABSTRACT OF THE THESIS OF

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Title: Effect of Dietary Ethanol and Zinc on Vitamin B-6 Metabolism in the Rat

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It is generally believed that the zinc metalloenzyme alkaline phosphatase is required for the hydrolysis of phosphorylated vitamin B-6 prior to its use by tissue. To test this hypothesis, pregnant rats were fed a liquid diet containing either adequate (10 μg/mL) or low (2 μg/mL) zinc with and without 30% kcal from ethanol to determine if a moderate zinc deficiency, possibly exaggerated by alcohol ingestion during the reproductive period, would be of sufficient magnitude to impair vitamin B-6 metabolism.

Zinc deficiency was evident by significant reductions in maternal plasma alkaline phosphatase activity (48%), and reductions in zinc concentrations of maternal plasma zinc (49%), liver (25%), and femur (24%). Maternal plasma pyridoxal-5'-phosphate (PLP) concentration significantly increased (61%) in these same rats and was negatively correlated ($r=-0.74$, $P<0.02$) with plasma alkaline phosphatase activity.

The offspring appeared to be largely spared the effects of zinc deficiency, despite significant reductions in zinc concentration of liver and femur, because low maternal dietary zinc did not significantly depress offspring plasma alkaline phosphatase activity. There was a trend for the offspring plasma PLP concentration to be higher due to low maternal dietary zinc, but it was not statistically significant.

Stimulation of erythrocyte alanine transaminase activity by exogenously-added PLP in vitro was measured to determine if vitamin B-6 functional impairment occurred. There was a trend for increased erythrocyte alanine percent stimulation in zinc-deficient dams. Offspring erythrocyte alanine transaminase percent stimulation in the low versus high zinc group by exogenous PLP in vitro was similar to that seen in dams.

Ethanol did depress maternal zinc status (plasma alkaline phosphatase and plasma zinc concentration) when zinc was adequate, but not when it was deficient. Maternal plasma PLP concentration was significantly higher in the low zinc plus ethanol group compared to high zinc without ethanol. Ethanol failed to depress offspring plasma, liver and femur zinc. Ethanol did, however, disrupt vitamin B-6 utilization in the offspring, evidenced by decreased plasma PLP, concentration in offspring originating from zinc-deficient mothers.

The results of the present study suggest that zinc

status of an individual should be determined when plasma PLP is to be used as an indicator of vitamin B-6 status because a pre-existing zinc deficiency could conceivably mask the presence of an actual vitamin B-6 deficiency.

Effect of Dietary Ethanol and Zinc on Vitamin B-6 Metabolism in the Rat

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INTRODUCTION

Many nutrient interactions are known to exist that have a significant impact on human nutrient requirements (Bodwell and Erdman, 1988; Anonymous, 1984). One possible interaction is between the essential nutrients, zinc and vitamin B-6. Although zinc is a trace element and vitamin B-6 is a vitamin, the nutrients have some similarities. There are over 24 mammalian zinc enzymes that participate in diverse functions including protein synthesis. Similarly, the active coenzyme form of vitamin B-6, pyridoxal 5'-phosphate (PLP), is required for many reactions especially those involving amino acid metabolism. Zinc and vitamin B-6 also have similar functions with respect to immune response, nucleic acid synthesis, and glucose metabolism. The common function of vitamin B-6 and zinc is further illustrated by the fact that a deficiency of either nutrient produces

growth retardation, alocepia, dermal lesions and decreased immunocompetence.

Evidence already exists to document that an interaction between zinc and vitamin B-6 can occur. Vitamin B-6, for example, is required in the degradation pathway of the essential amino acid tryptophan that yields the minor metabolite picolinic acid. Picolinic acid, by virtue of its ability to form a water soluble zinc chelate, has been shown to increase zinc absorption (Evans, 1980). Zinc and vitamin B-6 interaction also occurs in the phosphorylation of vitamin B-6, an important reaction in both absorption and post-absorption vitamin B-6 metabolism, because the enzyme required, pyridoxal phosphokinase, requires zinc (Ink and Henderson, 1984).

A specific example of a zinc and vitamin B-6 interaction that has the most revelance to the hypothesis pursued in the present study involves the zinc metalloenzyme alkaline phosphatase. Alkaline phosphatase is necessary for the dephosphorylation of vitamin B-6 (Ink and Henderson, 1984). This dephosphorylation is required for both the intestinal uptake of vitamin B-6 and for maintaining the cellular concentration of vitamin B-6. Few studies, however, have actually attempted to define the effect of a dietary zinc deficiency on vitamin B-6 metabolism (Anonymous, 1990). The present study was therefore designed to define the extent to which a dietary zinc deficiency will

disrupt vitamin B-6 metabolism in rats. Ethanol was also included as a dietary variable because of its known antagonism to both zinc (McClain and Su, 1983) and vitamin B-6 (Bonjour, 1980) metabolism. Pregnancy plus lactation was selected as the period of study because requirements for both zinc and vitamin B-6 are high at this time (Kirksey and Udipi, 1985; Solomons et al, 1986). The specific aims of this thesis were designed to determine if:

1) a moderate zinc deficiency possibly exaggerated by alcohol ingestion during the reproductive period would be of sufficient magnitude to impair vitamin B-6 metabolism

2) the zinc deficiency produced will inhibit utilization of pyridoxal 5'-phosphate(PLP)

3) the zinc deficiency produced is sufficient of magnitude to impair vitamin B-6 function

The following sections review the metabolism of zinc, vitamin B-6 and alcohol and develops the rationale for the research hypothesis.

REVIEW OF LITERATURE

ZINC

Food Sources

The major biologically available dietary source of zinc is animal products. Muscle, organ meats, and some seafood are rich sources of the trace mineral (Osis et al., 1972). Whole grain cereals are high in total zinc, but most of the zinc is lost in the milling process. The zinc content of some American wheats and products are 24.0 ± 4.5 μg/g dry weight basis(mean ± SD) (common hard wheat), 21.0 ± 7.0 μg/g (soft wheat), and 8.9 ± 0.5 μg/g (white bread) (Zook et al., 1970). Nuts and legumes contain large amounts of zinc, but are also high in phytate and the zinc may be less available than that from non-phytate containing foods (Cheryan, 1980).

Variation in zinc content in certain foods may be due to the effects of soil types and fertilizers. One researcher has shown increased zinc content of plants grown in soils treated with a zinc-containing fertilizer (Welch et al., 1974).

Zinc losses due to cooking and processing appear to have minimal influence on total zinc content of food because the zinc content in cooked meats is similar to the zinc content of raw muscle and fish (Osis et al., 1972).

Absorption

Transport across the brush border of the upper small intestine is the first phase of zinc absorption and is affected by diet composition (Jackson et al., 1981; Becker and Hoekstra, 1971). Interaction of zinc with intra-cellular components is the second phase and is dependent on zinc status. The third phase of zinc absorption involves the efflux from the intestinal cells to the plasma carrier. The major carrier in the portal circulation is albumin and the extent of absorption is dependent on saturation of albumin with zinc. The last phase involves the secretion of endogenous zinc back through the intestinal cells to the lumen (Jackson et al., 1981).

The absorption of zinc is homeostatically regulated and may involve all processes described above. Zinc deficiency and pregnancy & lactation (Davies and Williams, 1977) increase the absorption of zinc. Zinc absorption in pregnant and lactating rats is increased in the latter part of pregnancy and is designed to meet the increased need for zinc of the fetus.

Dietary components affect uptake of zinc by intestinal cells. Methionine, histidine and reduced gluthatione increase zinc uptake while cysteine and arginine have no appreciable effect (Smith et al., 1978). Other dietary factors that have been reported to have an influence on intestinal absorption of zinc are phytate, formation of an

insoluble complex between phytate and calcium and zinc, folic acid and picolinic acid.

Phytic acid has an inhibitory effect on zinc absorption. Single meal studies using radionuclide and stable isotope showed a negative effect of phytic acid on zinc absorption (Turnland et al., 1984) and a decrease in zinc absorption occurred when phytate was added to white bread (Navert et al., 1985). Foods high in phytic acid also tend to be high in dietary fiber and fiber is also implicated in decreased zinc absorption (Reinhold et al., 1976). A molar ratio between zinc and phytate of greater than 10 has an adverse effect in humans. For instance, consumption of some Middle Eastern breads that had a phytate/zinc molar ratio of 23 caused clinical zinc deficiency despite zinc intakes of 30 mg/d in humans (Oberleas, 1983). A high phytate to zinc molar ratio also interferes with the reabsorption of secreted zinc. Secreted zinc is endogenous zinc that is secreted into the intestines primarily from the pancreas.

Another dietary component that affects zinc absorption is calcium (Cunnane, 1988). The potentiating effect of calcium on the antagonistic effect of phytate on zinc availability has been confirmed in animals (Morris and Ellis, 1980). There is some debate about the occurrence of insoluble complex formation of calcium, phytate and zinc in humans because the levels of calcium and phytic acid in most

human diets may not cause the formation of an insoluble complex, but it may result if calcium supplements are taken with phytic acid rich foods. Secreted zinc, primarily from pancreatic juice, is also potentially affected by the formation of an insoluble complex involving calcium, zinc and phytate.

One vitamin which affects zinc absorption is folic acid. Male subjects fed 400 μg of pteroylheptaglutamate had a greater fecal zinc loss and reduced zinc excretion than control subjects (Milne et al., 1984).

The effects of picolinic acid on zinc absorption has been studied extensively (Evans, 1980). Picolinic acid is a minor metabolite of tryptophan metabolism. Picolinic acid coordinates with zinc to form a soluble complex that facilitates passage of zinc through the lumenal membrane, across the cell and through the basolateral membrane. A human metabolic study has shown that picolinic acid supplementation increases zinc absorption. Male subjects were fed a diet limited in tryptophan (approximately 235 mg/day). The same diet was then supplemented with picolinic acid(10 mg/day) for 4-7 weeks. During picolinic acid supplementation, the mean percent zinc absorption was 68.1 $\pm$ 13.9 (mean $\pm$ SD) versus a significantly lower mean zinc percent absorption of 55.7 $\pm$ 15.9 during the supplemented period (Johnson et al., 1988).

Transport

Within the plasma, zinc appears to be bound to albumin with a small amount bound to alpha-macro globulin. The proportion of plasma zinc bound to the alpha-macro globulin ranges from 20 to 40% (Giroux, 1975). Amino acids also bind between 0.2-1.0% zinc (Hallman et al., 1971). Albumin also functions as the zinc carrier in the portal plasma. Albumin-bound zinc represents the metabolically active pool of the plasma.

Metabolism

Zinc absorbed from the intestines is carried to the liver in portal plasma bound to albumin. About 30 to 40% of zinc entering the hepatic venous circulation is extracted by the liver. Intracellular zinc (60-80%) is found in the cytosol and most of zinc in the cytosol is bound to protein.

The main organ involved in zinc metabolism is the liver. Human liver cytosol contains zinc binding components of differing molecular weight and lability. The amount of binding component varies with zinc status and age. When the liver zinc content exceeds normal levels, the additional zinc is associated with metallothionein, a cysteine-rich protein of approximately 6,000 molecular weight. Its synthesis is induced by a range of essential metals including zinc. Radioactive zinc included in the diet, for example, was recovered in association with newly formed metallothionein (Richards and Cousins, 1976).

Post Hepatic Tissue Distribution and Uptake

Zinc is found in every human tissue and tissue fluid. Extensive analyses of human tissue by emission spectrography found that the retina of the eye contained the highest zinc concentration. However, skeletal muscle and bone account for about 80% of total body zinc (Tipton and Cook, 1963). Factors which affect the body distribution of zinc are development and growth.

The uptake of zinc occurs in two phases. The first phase is rapid. It is probably saturable and carrier-mediated. The second phase is slower and more passive. Hepatic uptake of zinc occurs rapidly after absorption and heptocytes take up zinc from albumin-containing medium in vitro (Failia and Cousins, 1978). Early experiments with fish liver slices showed zinc uptake was passive (Saltman and Boroughs, 1960). Later experiments with rat hepatocyte showed that zinc uptake involved two phases (Pattison and Cousins, 1986).

Excretion

The major route of zinc excretion is the feces. In addition to unabsorbed zinc, fecal excretion also includes endogenous zinc that is secreted into the gut from the body and subsequently not reabsorbed. Pancreatic secretions are a major contributor to endogenous zinc secretions in many species including humans. The other major pathway of endogenous zinc excretion is provided by the intestinal

mucosa cells which includes both unabsorbed zinc taken up from the intestinal lumen and zinc reentering the cells from the plasma. The amount of zinc excreted in the feces is dependent on the zinc intake (Jackson et al., 1984). In human subjects, fecal zinc excretion increased from 7 to 32 mg/d when diet zinc intake increased from 7 to 30 mg/d. A healthy adult man whose zinc balance is in equilibrium and has an intake of 10 to 15 mg zinc/day, will excrete about 90% of the ingested zinc.

Functions

Zinc can play either a structural role or a catalytic role within an enzyme. The binding of zinc to an enzyme-protein at the site of catalysis in a metalloenzyme results in a distorted and partial coordination sphere around the metal ion. The energy from this distortion is released to the substrate. In its structural role, zinc stabilizes the quaternary structure of the enzyme (Vallee and Galdes, 1984).

Over 200 zinc enzymes have been identified. Included in the 200 enzymes are over 24 mammalian enzymes that have been established as zinc metalloenzymes. Zinc metalloenzymes are enzymes in which zinc is part of the either structural site or catalytic site or both whereas in zinc dependent enzymes zinc is required as a co-factor (a co-factor is the nonprotein component that is required by the enzyme for activity and is either loosely or tightly attached to the

enzyme. The first metalloenzyme to be discovered was carbonic anhydrase (Keilin and Mann, 1940).

The zinc metalloenzymes have diverse functions. For instance, carbonic anhydrase catalyzes the reaction between carbon dioxide and bicarbonate. This reaction is important in acid-base balance which involves both lung and kidney metabolism. Superoxide dimutase catalyzes the dismutation of the superoxide anion radical thereby protecting cells against oxidative damage caused by molecular oxygen. Alcohol dehydrogenase is necessary for the detoxification of ethanol. It converts ethanol to acetaldehyde which is then converted into water, CO_2, and ATP. Fructose bisphosphatase is an enzyme involved in anaerobic glycolysis. Alkaline phosphatase is responsible for the non-specific hydrolysis of phosphate-monoesters. Alkaline phosphatase also functions in phosphate transfer reactions. Bone phosphatase is essential for bone calcification. Mathies (1958) was the first to demonstrate the presence of zinc in alkaline phosphatase. He showed that there was a direct relationship between concentration of zinc and enzymatic activity in vitro. Alkaline phosphatase has an absolute requirement for Zn(II). However, the Zn(II) ion does not appear necessary for the synthesis of the apoprotein nor does the zinc have to be present to assure the presence of the high affinity Zn(II) binding site (Vallee and Galdes, 1984). The Zn(II) content of native alkaline phosphatase has been shown to be

4 g-atom/mol of the enzyme dimer when isolated by methods that do not alter the intrinsic metal content.

Because zinc is present in so many enzymes, it has a variety of functions that are less understood compared to the metalloenzymes previously mentioned. Zinc plays a physiological role in the function and structure of biomembranes and plasma membranes (Bettger and O'Dell, 1981). A zinc deficiency can cause oxidative damage to membranes. Zinc is necessary for cell division and tissue growth, but the role is poorly defined. Zinc is involved in nucleic acid and protein metabolism. First zinc is detectable histochemically in the nucleus, nucleolus, and the chromosomes. Second, zinc stabilizes the structure of RNA, DNA, and ribosomes. Third, the enzymes required for nucleic acid synthesis are zinc dependent. Zinc is also apparently involved in glucose metabolism because impaired oral glucose tolerance has been found in human zinc deficiency (Sandstead et al., 1967). Zinc also has been shown to affect lipid metabolism in that the elongation-desaturation pathway of essential fatty acid metabolism is impaired in zinc deficiency (Bettger et al., 1979). Zinc is also involved in the production, storage, and secretion of individual hormones such as growth hormone (Root et al.,1979), thyroid hormone (Wada et al., 1983) and corticosteroids (Flynn et al., 1972).

Zinc plays a very important role in growth and

reproduction evidenced by the fact that dietary zinc deficiency is very teratogenic in mammals. Rats fed diets severely deficient in zinc (< 1 μg zinc/ g diet) throughout pregnancy, for example, had significantly fewer live fetuses. The surviving fetuses also had a variety of congenital defects (Hurley and Swenerton, 1966). The teratogenic effects of a zinc deficiency could be the result of depressed food intake. Hurley et al. (1971), however, found normally formed fetuses in pair-fed female rats. Other suggested mechanisms of teratogencity are decreased DNA synthesis and DNA polymerase activity due to zinc deficiency and decreased cellular membrane and cytoskeletal integrity due to zinc deficiency. Decreased cellular membrane integrity of zinc deficient rats was reflected in significantly elevated levels of malondialdehyde, an indicator of lipid peroxidation in maternal and fetal liver (Record, 1987). There is also evidence that zinc deficiency could be teratogenic in humans (Sever and Emanuel, 1973). Neural tubal defects are seen in parts of the world where zinc deficiency is prevalent. Women with acrodermtatis enteropathica, a genetic disorder of zinc, had pregnancies that resulted in either spontaneous abortion or birth of babies with malformations.

Homeostasis

The relatively constant level of zinc in tissues and body fluids, despite variation in composition of the diet,

suggests that there is whole body homeostasis for zinc. Increased dietary zinc results in a reduced fractional absorption of zinc and an increase in the rate of gastrointestinal excretion (Jackson et al., 1984). A key component in homeostasis is metallothionein. Richards and Cousins (1975) proposed that metallothionein regulates the flux of zinc within the mucosal cells and hence influences absorption. Reduction in zinc absorption has been correlated with an increase in metallothionein-bound zinc. Excretion of zinc into the urine contributes very little to whole body homeostasis, but urinary zinc does fall when there is overt depletion of tissue zinc. An excess intake of zinc also result in excessive loss of zinc via non-physiological routes such as hair (Natl. Res. Council, 1979).

Assessment of Zinc Status

There are several methods to assess zinc status. By far the most widely used approach of zinc assessment is the determination of circulating levels of zinc in either serum or plasma. Many investigators, however, feel that circulating plasma zinc concentration can only provide a portion of the information necessary to assess zinc status of an individual because it is affected by levels of carrier proteins and hemolysis (Prasad, 1982). Zinc in the erythrocytes has been proposed as an approach of zinc assessment because there is evidence that zinc concentration

in red blood cells declines in primary zinc deficiency (Solomons, 1979). Red blood cell zinc reflects long term status because the average life-time of the human red blood cell is 120 days.

Another valid method to assess zinc status is the measurement of alkaline phosphatase, a zinc metalloenzyme. Low serum alkaline phosphatase has been shown in human zinc deficiency caused by acrodermatitis enteropathica (Kay et al., 1976). The magnitude of the change in serum alkaline phosphatase activity during zinc therapy correlated with the severity of zinc deficiency. A rise in serum alkaline phosphatase activity after zinc supplementation has been considered to be supportive evidence for the diagnosis of zinc deficiency (Kasarikas and Schuna, 1980). Serial determinations of serum alkaline phosphatase and plasma zinc together therefore is one valid tool for the diagnosis of a zinc deficiency (Weismann and Hyer, 1985). A complete assessment of zinc status, however, can only be made when more than one biochemical indicator is combined with dietary intake information and a medical history. If several of the indicators are depressed including plasma zinc and serum alkaline phosphatase activity then most researchers would consider this a zinc deficiency.

VITAMIN B-6

Food Sources

Vitamin B-6 is the name used for three derivatives of 3-hydroxy-5-hydroxymethyl-2-methylpyridine. The most common forms of vitamin B-6 found in a mixed diet are pyridoxal 5'-phosphate(PLP) and pyridoxine(PN). There are significant amounts of phosphorylated pyridoxamine(PMP) and pyridoxal(PL). Animal products are rich source of vitamin B-6 (Kant and Black, 1990) and comprise about 35% of dietary vitamin B-6 intake. Poultry and some fish tend to contain higher levels of vitamin B-6 than do red meats (Agricultural Research Service, 1976). Liver is a concentrated source of the vitamin. Kidney and muscle tissue contain significant amounts of vitamin B-6. Whole grain cereals, legumes, nuts, and seeds are rich sources of vitamin B-6. Fruits and vegetables contain some vitamin B-6. Fruits and vegetables represent more than 30% of the vitamin B-6 in the U.S. food supply (Agricultural Research Service, 1976). Potatoes, bananas, and avocados are significant vitamin B-6 sources.

In plant foods, the major forms of vitamin B-6 are pyridoxine and some phosphorylated pyridoxamine. Glycosylated conjugates of pyridoxine are also found in plants and in some plant foods are the most significant form (Leklem, 1984). In animal products, the primary form is pyridoxal 5'-phosphate.

Absorption

PLP and PMP are hydrolyzed to PL and PM by intestinal phosphatase in the intestinal lumen. Specifically, alkaline phosphatase is responsible for the dephosphorylation of PLP and PMP at the intestinal site (Middleton, 1979). Hydrolysis of the vitamin B-6 forms is necessary because phosphorylated compounds do not readily cross cell membrane. It has been shown that a zinc deficiency decreases alkaline phosphatase activity in rat intestine (Leecke et al., 1967). At high concentrations, however, PLP can be absorbed slowly without hydrolysis (Henderson, 1985). PN was shown to be absorbed by passive diffusion in experiments using whole animals (Middleton, 1979), everted sacs (Tsuji et al., 1973), isolated loops and intestinal rings (Lumeng and Li, 1975).

Portal Vein Transport

As shown in Figure 1, the major forms entering the portal vein from the gastrointestinal tract are PL, PN, and PM. PL and PN can be taken up from the portal vein by erythrocytes and be phosphorylated by a kinase (Ink and Henderson, 1984).

Liver Metabolism

As shown in Figure 1, PL, PM and PN are phosphorylated by vitamin B-6 phosphokinase, a zinc-dependent enzyme (EC 2.7.1.35), when they are taken up by tissue to metabolically trap them and to maintain a low intracellular concentration

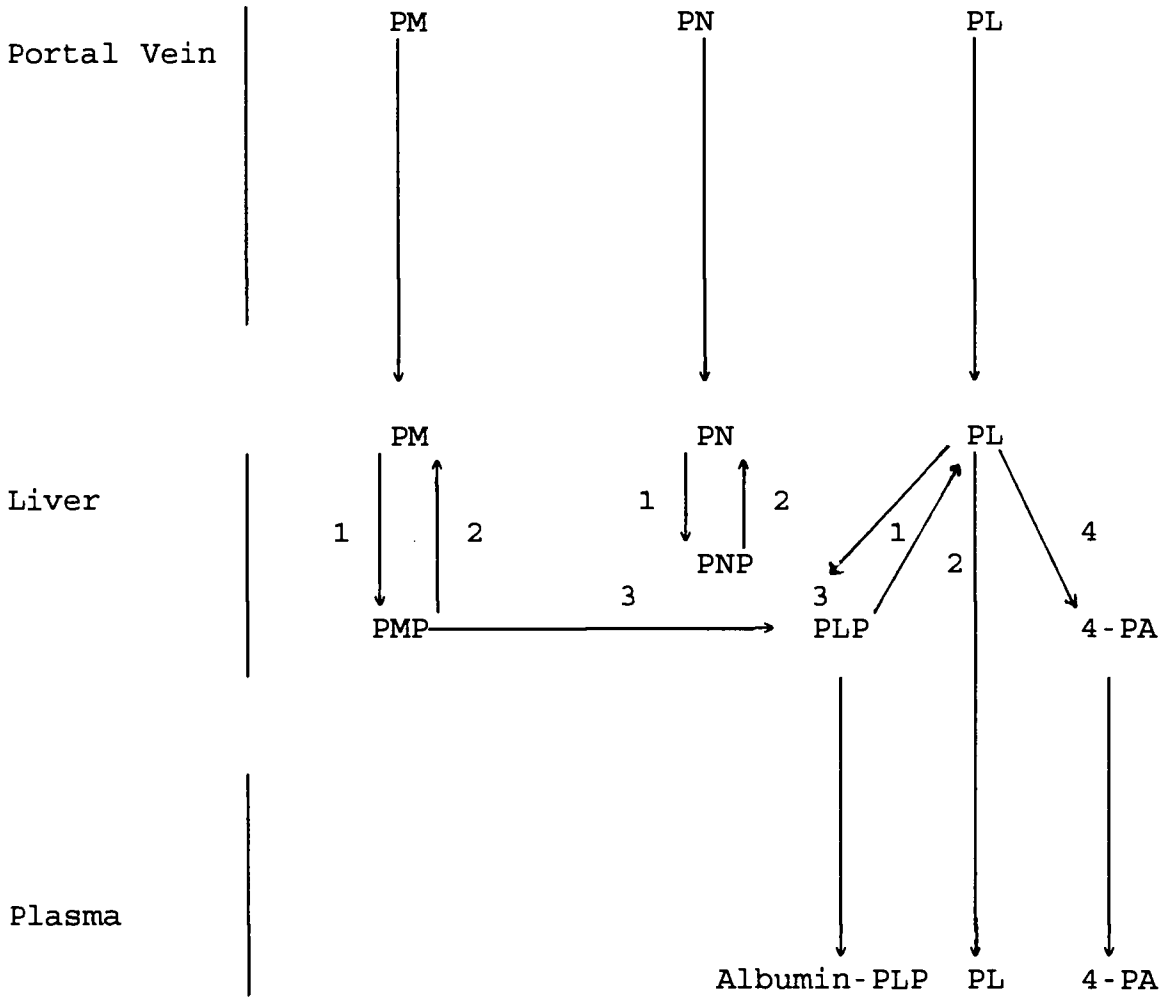

Figure 1. Vitamin B-6 Metabolism in Liver. [1] Vitamin B-6 phosphokinase (Zn dependent enzyme). [2] Alkaline phosphatase (Zn metalloenzyme). [3] PMP(PNP) oxidase. [4] pyridoxal oxidase.

Adapted from Merrill, A.H. and Burnham, F.S 1990. Vitamin B-6. In: Present Knowledge in Nutrition, 6th edition (M.L. Brown, ed.), pp.155-162, Nutrition Foundation: Washington, D.C.

of the free forms of the vitamin. Based on in vitro studies using ion-purified pyridoxal kinase from rat and turkey it was shown the Zn^{2+} was the superior cation for a maximal activity of pyridoxal phosphokinase at a concentration of 200 micromol (McCormik et al., 1961). Therefore, zinc is the primary cation in the pyridoxal kinase reaction under physiological conditions. Neary and Diven (1970) speculated that formation of a ZnATP complex may participate in the phosphorylation of vitamin B-6 compounds in the pyridoxal kinase reaction. Because zinc is the primary cation in pyridoxal kinase reaction, a zinc deficiency would be expected to cause a reduction in pyridoxal phosphokinase activity. However, Swenerton and Hurley (1968) showed that even a severe zinc deficiency did not significantly reduce pyridoxal phosphokinase activity. Zinc ions do stimulate enzyme activity in vitro, but the concentration required exceeds normal physiological intracellular zinc concentration (Nutr. Rev., 1990).

As seen in Figure 1, conversion of PMP and PNP to PLP, catalyzed by the activity of the flavin mononucleotide requiring enzyme PMP(PNP) oxidase, also occurs in the liver (et al., 1984). PMP(PNP) oxidase is sensitive to product inhibition by PLP (Pogell, 1958). The PLP formed in the liver is released into the plasma.

Figure 1 also shows that PLP can be hydrolyzed to PL by liver alkaline phosphatase, a zinc metalloenzyme (and

Henderson, 1990; Li et al., 1974; Lumeng and Li, 1975). However, it has been shown that a severe zinc deficiency (1 ppm zinc) does not produce a change in the liver alkaline phosphatase activity (Huber and Gershoff, 1973).

PL may either be released into circulation or be oxidized to 4-pyridoxic acid(4-PA), the major vitamin B-6 excretion metabolite, by a FAD dependent aldehyde oxidase (Stanulovic et al., 1976).

Regulation of liver vitamin B-6 metabolism has been primarily studied by enzyme activity experiments (et al., 1984; 1986; 1990) Under optimal assay conditions, PL kinase exhibited considerably greater activity than the phosphatase. At physiological pH, however, the activities of the two enzymes in human liver were found to be similar. Nevertheless, kinase activity still was slightly greater than the phosphatase which apparently ensures adequate delivery of PLP to tissues such as muscles.

Post Hepatic Metabolism

Vitamin B-6 is transported in the plasma, primarily as a PLP-albumin complex. PLP, a major source of vitamin B-6 for tissue, typically represents 55-75% of the total vitamin B-6 in plasma (Lumeng and Li, 1975). PL represents the majority of the other 20% of vitamin B-6 in the plasma and is the only other form of vitamin B-6 in significant amounts in the plasma(Lumeng and Li, 1975). PL also will bind to albumin but with less affinity than PLP.

PL can pass readily into the cell and is known to clear rapidly from the circulation following either a dose of vitamin B-6 or long term supplementation with vitamin B-6 (Kant et al., 1988). PL and PM are also taken up by diffusion in most tissues. Clearance of PLP from the plasma is not as rapid as PL following a vitamin B-6 dose (Kant et al., 1988). As shown in Figure 2, hydrolysis of albumin-bound PLP by plasma alkaline phosphatase is considered to be important for both tissue uptake and catabolism of vitamin B-6.

Muscle is one tissue that plays a significant role in vitamin B-6 metabolism. As seen in Figure 2, PL taken up by muscle is phosphorylated to PLP by pyridoxal phosphokinase which can then serve as a coenzyme for a variety of reactions involving amino acid metabolism as well as for glycogen phosphorylase.

Excretion

The oxidation of PL to 4-PA in the liver (Fig. 1) and kidney (Fig. 2) and its subsequent urinary excretion is considered to be an important route for the disposal of excess vitamin B-6. Most dietary vitamin B-6 is excreted as 4-PA in the urine. In healthy individuals, 40-60% of dietary vitamin B-6 will be excreted (Schultz and Leklem, 1991). With a vitamin B-6 deficiency, the percent of B-6 intake that is excreted decreases. The form of vitamin B-6 ingested also affects 4-PA excretion. More 4-PA is excreted

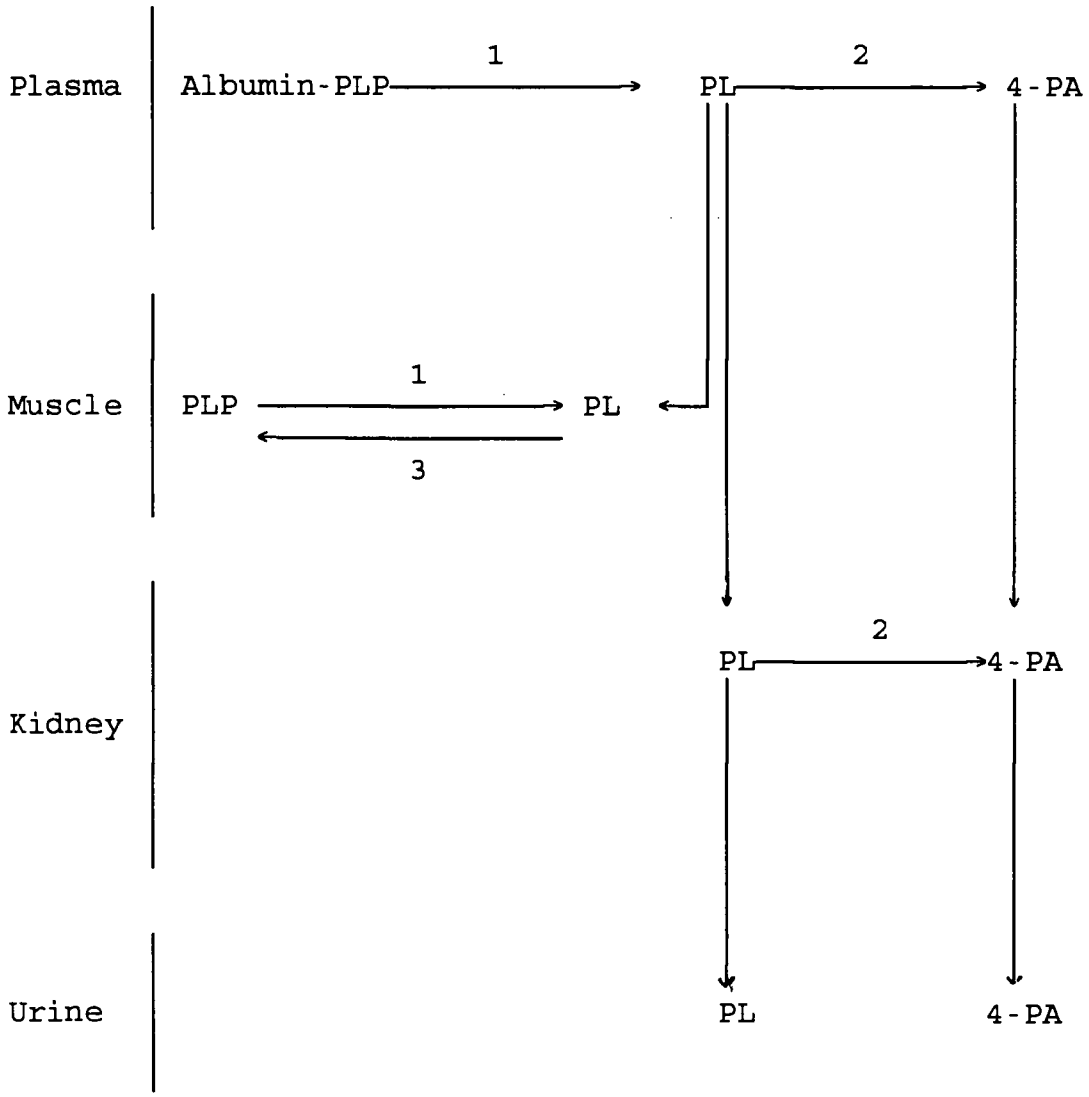

Figure 2. Post-Hepatic Utilization of Vitamin B-6. [1] Alkaline phosphatase (Zn metalloenzyme). [2] Pyridoxal oxidase. [3] Pyridoxal phosphokinase.

Adapted from Merrill, A.H. and Burnham, F.S. 1990. Vitamin B-6. In: Present Knowledge in Nutrition, 6th edition (M.L. Brown, ed.), pp. 155-62. Nutrition Foundation, Washington, D.C.

following the consumption of PL than any other vitamin B-6 form (Wozenk et al., 1980).

Functions

The most important role of vitamin B-6 is its function as a coenzyme. More than 100 enzymes require PLP as a coenzyme. These enzymes function in a variety of reactions especially those involving amino acid metabolism including amino transfer, decarboxylation, aldol cleavage, anhydride condensation and amino acid racemization.

PLP is also involved with carbohydrate metabolism, lipid metabolism and in immune and nervous system function. PLP may have also a role in the modulation of steroid hormone action (Litwick et al., 1985). One role of PLP is in tryptophan metabolism. Kynurenine aminotransferase, a PLP requiring enzyme, converts kynurenine to kynurenic acid. Most of the tryptophan is metabolized into carbon dioxide and pyridine derivatives such as niacin, an essential water-soluble B vitamin. Tryptophan degradation also yields the minor metabolite picolinic acid. Picolinic acid, by its ability to form a water soluble zinc chelate, has been shown to increase zinc absorption (Evans, 1980).

Serine transhydroxymethylase and glycine synthase are two PLP-dependent enzymes that convert tetrahydrofolate(THF) to 5,10 methylene-THF (Shane and Stokstead, 1983). Single carbon transfer is essential for nucleic acid synthesis.

PLP plays a role in gluconeogenesis because the

aminotransferases are PLP-dependent enzymes. Aspartate aminotransferase catalyzes the reactions that results in the shuttling of oxaloacetate from the mitochondria to cytosol, where it enters the gluconeogenic pathway.

PLP plays a role in neurotransmitter synthesis. Taurine, dopamine, norepinephrine, histamine, gamma-aminobutyric acid and 5-hydroxytryptamine(serotonin) are synthesized by PLP-dependent enzymes (Dakshinamurti, 1982).

Assessment of Vitamin B-6 Status

Indicators of vitamin B-6 status can be divided into three categories: dietary intake, direct methods and indirect biochemical indices (Leklem, 1990; Leklem and Reynolds, 1987). Direct methods of vitamin B-6 status assessment involve either the measurement of one or more vitamin B-6 forms or the measurement of urinary 4-PA or a combination of these measures. The indirect methods of status assessment measure the ability of vitamin B-6 to perform in its functional role as a coenzyme. Leklem and Reynolds (1987) have suggested that assessment of vitamin B-6 status in an individual should include dietary intake determination and at least two biochemical indices.

Plasma PLP represents a direct and versatile indicator of vitamin B-6 status. Plasma PLP also correlates well with PLP content of the skeletal muscle. Plasma PLP concentration reflects the degree of undernutrition and the degree of storage of vitamin B-6 (Leklem, 1990). Two factors that

influence plasma PLP are dietary protein and vitamin B-6 intake (Leklem, 1990). There is a negative correlation between protein intake and plasma PLP concentration. Urinary 4-PA excretion, another direct measure of vitamin B-6 status, reflects short term vitamin B-6 status.

One indirect measure that assesses the function of vitamin B-6 is erythrocyte alanine transaminase. Measurement of the erythrocyte enzyme is preferable to plasma aminotransferase because the erythrocyte aminotransferase activities are higher and less variable between individuals than plasma aminotransferase. Erythrocyte aminotransferase is used as a long-term measure of vitamin B-6 status and is thought to be responsive to vitamin B-6 intake. Determination of in vitro stimulation of the enzyme by incubation with PLP is a better indicator of vitamin B-6 status than measuring the unstimulated activity.

Tryptophan and methionine load tests, which reflect vitamin B-6 status in the liver, also are two indirect measures of vitamin B-6 status. In the methionine load test, usually 3 g of methionine are given and the urinary excretion of cystathionine is measured. Because of the limited number of studies utilizing this test, no definitive values are available for urinary cystathionine excretion as an indicator of adequate vitamin B-6 status. However, basal cystathionine excretion of > 350 μmol/d has been shown to be indicative of vitamin B-6 deficiency (Leklem, 1990).

ALCOHOL

Sources

The primary source of ethyl alcohol, commonly referred to as alcohol, is fermented beverages such as beer, wine, and distilled spirits. The bulk of beer sold in the United States is about 3.6% alcohol by weight(this is equal to about 12.6 gm alcohol) or 4.5% by volume. Low calorie beer is about 3% alcohol by weight. Table wines contain between 11.5-12.5% alcohol. However, fortified wines usually contain about 18-20% alcohol. Most distilled spirits contain 43% alcohol, while grain alcohol contains 95% alcohol (Darby, 1979). The non-alcoholic general population derives about 4.5% of its calories from alcohol (Scheig, 1970).

Absorption

Alcohol, a fat-soluble, nonelectrolyte is readily absorbed from the gastrointestinal tract and the mucosal membranes in the mouth. Alcohol absorption after oral ingestion is affected by many factors including the type of beverage, the presence of food in the gut, body temperature and physical exercise. Alcohol from distilled spirits is absorbed more readily than alcohol from either beer or wine whereas the presence of food delays gastric emptying and reduces the rate and efficiency of alcohol absorption. Diets containing high protein, fat and carbohydrate foods slow alcohol absorption. Lower body temperature also decreases

alcohol absorption. There is also decreased absorption of alcohol with decreased physical exercise. Alcohol is absorbed into the circulation by diffusion across the duodenum, jejunum, and to a lesser extent from the stomach and large intestine (Agarwal and Gedde, 1980).

Tissue Distribution

Alcohol accumulates in tissues with the highest water content and tends to be distributed in organs where there is good blood flow. Organs such as the brain, lung and kidneys show the highest alcohol level while muscles generally have a lower alcohol content.

Metabolism

Alcohol is metabolized primarily in the liver, but there is evidence that some alcohol is metabolized in the stomach (Frezza et al., 1990). Metabolism of alcohol in the stomach occurs via gastric alcohol dehydrogenase and is known as first pass metabolism of alcohol. First pass metabolism of alcohol may contribute to the sex-related differences in blood concentration between men and women because women have been shown to have less alcohol dehydrogenase activity than men (Frezza et al., 1990).

In the liver, the first step of alcohol metabolism is the conversion of ethanol to acetaldehyde by the zinc metalloenzyme, alcohol dehydrogenase. By products generated during the conversion enter the mitochondria to help create energy. Acetaldehyde is oxidized to acetate which is broken

down into carbon dioxide, water, and energy. The microsomal ethanol-oxidizing system (MEOS), which normally plays only a minor role in the metabolism of ethanol, can be activated when there is chronic consumption of high alcohol levels. Once the MEOS pathway is activated it increases the conversion of ethanol to acetaldehyde. Acetaldehyde is a cytotoxic agent that is largely responsible for the symptoms comprising what is commonly referred to as a hangover. Acetaldehyde is oxidized to acetate by acetaldehyde dehydrogenase. Reduced nicotinamide-adenine dinucleotide (NADH) is produced as a result of the oxidation of acetaldehyde to acetate.

Excretion

At low intakes of alcohol, approximately 90% of ingested alcohol is eliminated by oxidation to carbon dioxide and water. About 100-300 mg alcohol can be eliminated in this way. Alcohol can be eliminated unchanged in expired air(5%), urine(0.5-2.0%) and sweat(0.5%). At alcohol intakes that exceed the capacity of liver metabolism, blood alcohol levels rise resulting in depression of the central nervous system.

Nutritional Effects of Ethanol

Ethanol has a profound effect on nutritional status (Lieber, 1988). Because alcohol supplies 7.1 kcal/g, it provides extra kcal in the diet of light drinkers, but often replaces other energy sources in diets of moderate and heavy

drinkers. Heavy drinkers derive more than half their total daily energy from ethanol. Thus as alcohol consumption increases, the percentage of protein, fat and carbohydrate decreases from the diet and the nutritional quality of the diet decreases (Lieber, 1988). Alcohol also can interfere with the digestion, absorption, transport, metabolism, and storage of nutrients. This can led to serious nutritional deficiencies. Alcohol has a negative effect on both zinc and vitamin B-6 metabolism (Prasad, 1977; Lumeng, 1977). The deleterious effect of alcohol on zinc status is well documented (McClain and Su, 1983). Ethanol induced zinc deficiency has also been implicated in the Fetal Alcohol Syndrome (Flynn et al., 1981). Fetal Alcohol Syndrome is a collection of alcohol-related defects and malformations caused by maternal alcohol abuse. These defects include microencephaly, microopthlamia and depressed prenatal and postnatal growth.

Ethanol has also been shown to have an effect of vitamin B-6 metabolism (Lumeng and Li, 1988). The plasma PLP content of 66 alcoholic subjects was compared to 94 control subjects. The control subjects had an average plasma PLP content of 15 ng/ml (61 nmol/L) while the alcoholic population had a plasma PLP content of 5 ng/ml (20 nmol/L).

Hypothesis and Expected Results

During the last decade, evidence has accumulated to define that ethanol is antagonistic to the absorption, utilization, and function of the essential trace element zinc (McClain and Su, 1983; Silverman et al., 1979; Dinsmore, 1985; Henderson et al.,1980). In one study, for example, ethanol ingestion at 30% of kcal during gestation and lactation significantly depressed zinc status as evidenced by decreased serum alkaline phosphatase activity and serum zinc (Yeh and Cerklewski, 1984).

Because alkaline phosphatase is generally believed to be required for hydrolysis of albumin-bound pyridoxal-phosphate in circulation (Henderson, 1984), it seems likely that a consequence of a zinc deficiency would be impaired vitamin B-6 metabolism. However, no one has systematically attempted to define the relationship between a zinc deficiency and vitamin B-6 revolving around alkaline phosphatase and PLP utilization by tissues. Therefore, the present study was designed to define the extent to which a dietary zinc deficiency will impair vitamin B-6 metabolism. The specific aims of the study were to determine if a moderate zinc deficiency, possibly exaggerated by alcohol ingestion during the reproductive period, would be sufficient to disrupt vitamin B-6 metabolism.

Given that a zinc deficiency exaggerated by dietary

ethanol is expected to cause a decrease in plasma alkaline phosphatase (Yeh and Cerklewski, 1984) and alkaline phosphatase is required for hydrolysis for albumin-bound PLP in circulation (Henderson, 1984), the expected results of the study are an increase in plasma PLP and a decreased PL uptake into tissue. Decreased utilization of PL by tissue will cause a decrease in PLP-dependent enzyme activity.

MATERIALS AND METHODS

Animals/Conduct of Study

Forty-two outbred virgin female Sprague-Dawley rats (Cr1 CD BR COBS), initially weighing 187-202 gm, and eleven male rats of the same strain, initially weighing 292-314 gm, were obtained from Charles River Breeding Laboratories, Wilmington, MA. Rats were approximately 75 days old when shipped. Upon arrival, female rats were individually housed in suspended stainless steel cages in a humidity-and temperature-controlled room with a 12 hour light-dark cycle. During a 20-day adjustment period, a powder type diet which met the nutritional requirement of the laboratory rat (Subcommittee on Laboratory Animal Nutrition, 1978) was provided in glass food jars. The composition of this adjustment diet in g/kg was casein vita-free (US Biochemical Corporation, Cleveland, OH.), 150; DL-methionine (Sigma Chemical Company), 3; cellulose powder (Alphacel, ICN Nutritional Biochemical, Cleveland, OH.), 40; vitamin mix, 50; mineral mix, 30; corn oil (0.01% BHT), 50; cornstarch (CPC International, Summit Argo, IL.), 150; and dextrose (Cerulose, CPC International, Summit Argo, IL.), 527. The vitamin mix contained (in grams/kilogram dextrose) thiamin·HCl, 0.20; Pyridoxine·HCl, 0.15; riboflavin, 0.12; calcium pantothenate, 0.32; d-biotin, 0.04; niacin, 0.60;

folic acid, 0.04; vitamin B-12 (0.1% in mannitol), 1.00; menadione, 0.01; retinyl palmitate (250,000 units/g), 0.50; ergocalciferol (500,000 units/g), 0.08; d-α-tocopheryl succinate (1210 units/g), 0.8: choline chloride, 30.00. The mineral mixture contained (in grams/kilogram) $CaHPO_4$, 556.48; $CaCO_3$, 50.00; NaCl, 78.21; K_2CO_3, 45.96; K_2SO_4, 60.60; $K_3C_6H_5O_7 \bullet H_2O$, 145.51; $MgCO_3$-n-hydrate (26% Mg), 51.28: $ZnCO_3$, 0.8950; $MnCO_3$; 3.6955; $CuCO_3$; 0.3044; Ferric citrate (18.42%), 6.3355; KIO_3, 0.0112; $Na_2SeO_3 \bullet 5H_2O$, 0.0112; $CrK(SO_4)_2 \bullet 12H_2O$, 0.6403; NaF, 0.0626. The zinc content of the adjustment diet (20 mg/Kg) originated from the combination of that supplied by the mineral mix mixture plus that from casein. Drinking water was provided as distilled-deionized water in glass water bottles fitted with a stainless steel sipper and a silicone rubber stopper. Male rats were group-housed in the same animal room as female rats and were fed lab-chow plus tap water.

Three days prior to breeding, male rats were individually housed in solid bottom polyproplylene cages (51 x 41 x 20 cm) fitted with stainless steel wire tops. The design of the experiment was to bred four female rats with one male rat. Because forty-two female rats and eleven male rats were obtained there was an extra cage with two cage with two females and one male. The rats were allowed to mate during four overnight periods. All rats during breeding received the adjustment diet provided in stainless steel

pendulum feeders (Lab Products, Inc. Maywood, NJ). A vaginal smear taken each morning was fixed with Spray-cyte (Clay Adams, Parsippany,NJ), stained with Wright Stain (Humason, 1972) for four minutes, and examined with a light microscope (Zeiss Light Microscope). Presence of spermatozoa in the smear was considered to be day 0 of gestation.

The twenty-nine pregnant rats obtained were fed a liquid diet, described in Table 1-3, containing either low (2 μg/mL) or adequate zinc (10 μg/mL) with and without 30% of the kcal supplied from ethanol. The experimental design was a 2 x 2 factorial experiment involving two factors, zinc and ethanol, with two levels of each factor (Table 4). Seven rats each were assigned to the low zinc group without ethanol, the high zinc group without ethanol, and the high zinc plus ethanol group. Eight rats were assigned to the low zinc plus ethanol group because it was anticipated that this group would be at greatest risk for developing zinc deficiency.

Pregnant rats were individually housed in suspended stainless steel cages modified to received a liquid diet feeding tube fitted with a solid silicone rubber stopper. The liquid diet feeding tubes were similar to Richter tubes, but were made by a glassblower at Oregon State University. On day 18 of gestation, each pregnant rat was transferred to polycarbonate solid-bottom cage (20 x 48 x 27 cm) containing wood-chip bedding material. The cages had a hole drilled in

Table 1. Composition of Liquid Diet

Component	g/L Control	Ethanol
Casein, micropulverized	46.50	46.50
Egg-white, spray dried[1]	23.00	23.00
DL-Methionine[2]	0.30	0.30
Vitamin mixture[3]	12.50	12.50
Mineral mixture[4]	8.00	8.00
Cellulose powder[5]	10.00	10.00
Dextrose[6]	144.00	64.00
Corn oil[7](0.01% BHT)	13.00	13.00
Xanthan Gum[8]	2.00	2.00
Distilled-Deionized Water (to make 1L when blended)		

[1] U.S. Biochemical Corporation, Cleveland, Ohio

[2] Sigma Chemical Company, St. Louis, MO.

[3] See Table 2

[4] See Table 3

[5] ICN Nutritional Biochemical, Cleveland, Ohio

[6] Cerulose, CPC International, Summit-Argo, IL.

[7] Best Foods, CPC International, Englewood, NJ.

[8] Keltrol, Kelco Corp., Chicago, IL.

Table 2. Vitamin Mixture of Liquid Diet

Component	grams/Kg
Thiamin · HCl	0.20
Riboflavin	0.20
Pyridoxine · HCl	0.15
Calcium pantothenate	0.40
d-Biotin	0.04
Niacin	0.60
Folic acid	0.04
Vitamin B-12 (0.1% in mannitol)	1.00
Menadione	0.01
Retinyl palmitate (250,000 u/g)	0.80
Ergocalciferol (500,000 u/g)	0.08
d-α-tocopheryl Succinate (1210 u/g)	0.80
Choline Chloride	30.00
Dextrose (to make 1 Kg)	

Table 3. Mineral Mix of Liquid diet (No Zn added)[1]

Component	grams
$CaHPO_4$	549.16
$CaCO_3$	63.99
NaCl	88.02
K_2SO_4	43.09
K_2CO_3	56.81
$K_3C_6H_5O_7 \cdot H_2O$	136.41
$MnCl_2 \cdot 4H_2O$	5.625
$FeC_6H_5O_7 \cdot 5H_2O$ (18.42% Fe)	7.6345
$MgCO_3$-n-hydrate (26% Mg)	48.08
$CuCl_2 \cdot 2H_2O$	0.5031
$Na_2SeO_3 \cdot 5H_2O$	0.0105
$CrK(SO_4)_2 \cdot 12H_2O$	0.6003
KIO_3	0.0105
NaF	0.0587

[1] When the mineral mixture is used at 8 g/L, the liquid diet provides 2 μg/mL Zn. This concentration includes the zinc contributed by diet casein

Table 4. Experimental Design

Factor	A= Zinc (μg/mL)	
	Level	a_1 = 2 a_2 = 10
B= Ethanol (% kcal)	b_1 = 0	a_1 b_1 a_2 b_1
	b_2 = 30	a_1 b_2 a_2 b_2

the front and were fitted with a stainless steel clip to hold the glass feeding tube. Rats were allowed to normally deliver their pups, ingest the placenta and to clean the young. Litter size for all rats was reduced to eight within 2 days of lactation. Diets were fed until day 18 of lactation.

On lactation day 18, rats were fed a third of the diet to provide a partial fast. Distilled-deionized drinking water was also available to the rats from glass water bottles fitted with a silicone rubber stopper and a stainless steel sipper during late gestation and all of lactation. During the study, dietary intake was recorded daily and body weights were taken on day 0, 13, and 18 of gestation.

Preparation of the Liquid Diet

As formulated, the liquid diet contained 2 μg/mL Zn (30.5 μM). The additional zinc needed to yield 10 μg/mL (153 μM) was added as a stock solution at the time that the diets were blended. The stock solution (1000 μg/mL) was made by dissolving $ZnSO_4 \cdot 7H_2O$ in metal-free water. The level of vitamin B-6 in the liquid diet was 1.5 mg/L as pyridoxine. As seen in Table 5, ethanol was isocalorically substituted for dextrose in the ethanol diet. The liquid diet provided 1 kcal/mL during gestation and 1.5 kcal/mL for the last 4 days of lactation to meet increased energy needs.

The control liquid diets were prepared by blending

Table 5. Energy Content of Liquid Diet

Component	kcal/g	Liquid Diet, kcal/L	
		Control	Ethanol
Casein	4.27	199	199
Egg White	4.38	101	101
Dextrose	3.75	585	285
Corn oil	8.84	115	115
Ethanol	7.10	0	300
Total		1000	1000

259.3 g of the diet in 870 mL cold (0°C) distilled-deionized water (Waring, New Hartford, CT.) for 15 seconds at low speed. The ethanol-containing liquid diet was prepared by blending 179.3 g of diet, 870 mL cold distilled deionized water, minus the mLs of ethanol added and 56.5 mL of 95 % ethanol. Because ethanol is known to depress food intake (Vannuchi et al., 1986), the ethanol was supplied gradually. For the first two days of the treatment period, the diet supplied 10% of the kcal from ethanol, 20% on day 3 and 4, and 30% of the kcal on day 5 of gestation. Food grade antifoaming agent (Myvatem 92, Eastman Chemical Products, Kingsport, TN.) was added (0.04%) when diet was blended to prevent foaming due to spray-dried egg white especially in the presence of ethanol. Liquid diet was prepared 16-20 hours in advance to allow any remaining foam to dissipate.

Termination

On day 18 of lactation, dams were lightly anesthetized intraperitoneally with 3 mg sodium pentobarbital/100 g body weight (Anthony Products Co., Arcadia, CA.). Blood was withdrawn from the abdominal aorta into 12 mL syringes fitted with a 22 gauge x 1 inch needle (Monojet, Sherwood Medical Industries Inc., St. Louis, MO.) that contained 50 μL of ammonium heparin (2800 units/mL in saline). The offspring were lightly anesthetized intraperitoneally with sodium pentobarbital (diluted 1:5 with reagent water). For

pups, blood was withdrawn into 1 mL Tuberculin syringes fitted with a 25 gauge X 5/8 needle (Monojet, Sherwood Medical Industries Inc., St.Louis, MO.) that contained 10 μL of ammonium heparin (2800 units in saline). The syringes were inverted to mix the anti-coagulant with the blood. Blood was directly transferred to 10 mL trace-element-free vacuum blood collection tubes fitted with royal blue stopper (Termuno Medical Corp., Elkston, MD.)

Sample Collection

The blood collection tubes, cooled on ice for 5-10 minutes, were centrifuged at 2575 x g in a clinical centrifuge (Damon/IEC, Needham Hts., MA) for ten minutes. Plasma was transferred to multiple low-temperature 2 mL freezer vials (VWR Scientific Inc., San Francisco, CA.) using plasma separators (Medical Lab. Automation, Mount Vernon, New York). Vials were frozen (-5°C) immediately prior to returning to the laboratory but were eventually stored at -20°C. Femur, muscle, and liver were stored in Ziploc storage bags and frozen (-20°C) for later analyses. Red blood cells remaining in the blood collection tubes were placed on ice for transport. Red blood cells were washed three times with cold saline and were centrifuged each time at 1,500 x g (Beckman Model TJ-6-RS, Palo Alto, CA.) for 10 minutes. A one mL aliquot of red blood cells was placed in 1.5 mL microtube (VWR Scientific Inc., San Francisco, CA.) and frozen at -20°C.

Procedures to Minimize Trace Element Contamination

Rat diets were prepared with purified diet ingredients using plastic and stainless steel mixing equipment. Mixing bowls were rinsed with distilled water before use. Reagent grade chemicals and crystalline vitamins was used to prepare the mineral and vitamin mixture. For metal analyses, only reagent grade chemicals were used. All glassware used was Pyrex previously made metal-free. Metal-free plastic labware was used.

Analytical Methods

Most inorganic reagents were purchased from J.T. Baker, Phillipsburg, NJ through VWR Scientific, Seattle, WA. Most organic reagents were purchased from Sigma Chemical Company. St. Louis, MO. All dilutions were made with deionized water.

A. Alkaline Phosphatase

1. Principle of the Method

Alkaline phosphatase activity of maternal and offspring plasma was determined by the method of Roy (1970). Thymolpthalein monophosphate is hydrolyzed to chromogenic thymolpthalein and inorganic phosphate. The amount of thymolpthalein released is directly proportional to enzyme concentration and is quantified photometrically following the addition of an alkaline color developer. The alkaline color developer terminates enzyme function and raise the pH permitting full development of the chromphore.

2. Reagents

0.3 M $MgCl_2$ solution. $MgCl_2 \cdot 6H_2O$ (6.09 g) was dissolved in water and diluted to 100 mL.

Brij-35, 20% With gentle warming, Brij-35 (20 g) was dissolved in 60 mL of water, cooled, and diluted to 100 mL.

Concentrated buffer, 3.5M Enough 6 N HCl was added to 32.2 g of 2-amino-2-methyl-1-propanol to bring pH to approximately 10.30 to 10.35. To the solution, 1 mL of 0.3 M $MgCl_2$ and 10 mL of Brij-35 was added and diluted to 100 mL with water.

Buffered Substrate. Sodium thymolpthalein monophosphate (0.3 g) was dissolved in approximately 85 mL of water. Concentrated buffer (10 mL) was added and diluted to 100 mL with water.

Color developer. NaOH (4 g) and anhydrous Na_2CO_3 (10.6 g) was dissolved in water and diluted to 1 liter.

Thymolpthalein stock standard solution, 0.01M. Thymolpthalein (430.5 mg) was dissolved in sufficient ethanol to make 100 mL of solution.

0.35 M 2-amino-2-methyl-1-propanol buffer. Concentrated buffer was diluted 10-fold with water.

Working Thymolpthalein Standard. Thymolpthalein stock standard (0.50 mL) was diluted with 50 mL with 0.35M 2-amino-2-methyl-1-propanol (1 mL = 100 μmol of thymolpthalein).

3. Analytical Method

Into six labeled test tubes, 0.00, 0.02. 0.04. 0.06,

0.08 and 1.00 mL of the working standard respectively was added. Then 1.00, 0.08, 0.06, 0.04, 0.02, and 0.00 mL of 0.35M 2-amino-2-methyl-1-propanol buffer was added to each cuvet. To each test tube, 5.1 mL of color developer was added and mixed by inversion. The test tubes were read in a spectrophotometer (Beckman Instruments, Model DU 40, Irvine, CA.) at 590 nm. For maternal plasma samples, 1 mL of buffered substrate was added to the test tubes and 0.10 mL of plasma was added to test tubes. Less plasma (50 μL) was used for the offspring assay. Exactly 5 minutes after the addition of plasma, 5.0 mL of color developer was added. The test tubes were covered and inverted. The tubes were at 590 nm against a reagent blank prepared by substituting water for the plasma.

B. Plasma Pyridoxal 5'-Phosphate

1. Principle of the Method

Plasma pyridoxal 5'-phosphate (PLP) was determined by the method of Chabner and Livingston (1970) except that $^{14}CO_2$ was captured in 0.2 mL Nuclear Chicago solubilizer instead of 0.1 mL of 2.0 M potassium hydroxide and perchloric-acid borate, instead of trichloracetic acid, was used to precipitate the protein. This assay is based on an enzymatic reaction in which tyrosine is converted to tyramine and CO_2 by tyrosine decarboxylase. In the assay, the apo form of the enzyme is incubated with PLP to form the holoenzyme. The limiting factor is the reaction is PLP.

Thus the amount of tyrosine converted to $^{14}CO_2$ and tyramine is directly proportional to the amount of PLP present.

2. Reagents

Buffer No.1 Potassium acetate buffer, 10mM, containing 5 mM EDTA. Potassium acetate (982 mg) and 1.86 g of Na_2-EDTA was dissolved in water, the pH was adjusted to 5.50 with acetic acid, and diluted to 1000 mL.

Buffer No. 2 Potassium acetate buffer, 10mM, containing 5 mM L-tyrosine. Prepared as above for Buffer No. 1 plus 195 mg of mercaptoethanol and 25 mg L-tyrosine.

Saturated ammonium sulfate in dilute NH_4OH Concentrated NH_4OH (3 mL) was dissolved to 100 mL. Ammonium sulfate (75 g) was added to 100 mL of dilute NH_4OH.

L-Tyrosine -1-^{14}C substrate Fifty μCi of L-tyrosine (Amersham International, Arlington Heights, IL.) was transferred to a 125 mL flask. This was done using 0.5 mL of 0.1 N HCl to rinse the vial containing hot tyrosine. To this add 79.5 mL of "cold" tyrosine (The "cold" tyrosine was prepared by adding 1.017 g of L-tyrosine to 75 mL of 0.1 N HCl and 174 mL of deionized water). The "hot" tyrosine mixture is stored in 20 mL scintillation vials.

Pyridoxal 5'-phosphate stock standard Pyridoxal 5'-phosphate (15 mg) was weighed out on a micro-analytical balance, and made up to 500 mL with deionized water. This solution was protected from direct exposure to light.

Perchloric-acid borate, 1.0 M. Two grams of potassium

borate and 100.5 g of 70% perchloric acid were combined and diluted to 1000 mL with water.

Trichloroacetic acid (TCA), 50% Reagent grade TCA (250 g) was dissolved in water and dissolved to 500 mL. Store at 4°C.

Potassium Acetate, 5.0 M Potassium acetate (245.4 g) was added to 300 mL of water and made up to 500 mL.

Nuclear Chicago Solubilizer (Amersham Canada Limited, Oakville, Ontario). Tissue solubilizer used to trap $^{14}CO_2$.

Scintillant PPO (71.6 g) and 1.097 g of POPOP was mixed with 4 liters of spectral-grade toluene using a magnetic stirrer. The solution was stored in an amber bottle at room temperature.

3. Analytical Method

Apoenzyme was isolated from Streptoccous facealis cells. Once the enzyme was isolated, working PLP standard was prepared by diluting 1.00 mL of stock PLP standard with deionized, glass redistilled water in a 100 mL red glass volumetric flask and then diluting 8.00 mL of the first solution with 250 mL deionized, glass redistilled water. Plasma samples were thawed, mixed, and centrifuged at 2,480 x g for 3 minutes in a clinical centrifuge (Damon/IEC, Needham Heights, MA.). Plasma protein were then precipitated by combining 1.25 mL of water, 0.25 mL plasma, and 0.6 mL 1 M perchloric-acid borate in test tubes and stirred with a micro stir bar on a magnetic stirrer. All

tubes were mixed for 10 seconds by gentle shaking. Tubes were allowed to stand for one hour in the dark room at room temperature. Two reaction flasks for each precipitated sample and each standard curve point were set-up in a rack. Water (0.275 mL) was added to each reaction flask. Solubilizer (0.2 mL) was added to a 20 mL scintillation vial which was then attached to the reaction flask, 0.50 mL of tyrosine decarboxylase enzyme was added. At the end of one hour incubation, precipitated samples were centrifuged at 1,986 x g for 15 minutes (Beckman, J-21, Palo Alto, CA.). The supernatant was quantitatively decanted into ice-cold tubes containing 0.3 mL potassium acetate tubes (5.0 M). To each reaction flask, 0.20 mL of the working PLP solution and 0.1 mL of the sample solution form each potassium acetate tubes was added. The timer was set for 30 minutes and 30 seconds. Then 0.10 mL of ^{14}C-tyrosine was added to every two reaction flask every 30 seconds and stoppered with a rubber vacutainer stopper. The reaction was stopped at 15 minutes and 30 seconds by adding 0.5 mL 50% TCA to every 2 flask by a syringe every 30 seconds. The water bath was turned off and the flasks were allowed to sit for at least five hours. The scintillation vials were removed form the reaction flasks and ten mL of scintillant was added to the vials and shaken for two minutes. The samples were counted in duplicate in a scintillation counter (Beckman LS5000 TD Scintillation counter, Fullerton, CA.) for 50 minutes.

C. Erythrocyte Alanine Transaminase

1. Principle of the Method

Erythrocyte alanine transaminase, a pyridoxal phosphate-dependent enzyme, catalyzes the reaction: alanine + α-ketoglutarate → pyruvate + glutamate. Pyruvate is measured after it is reacted with dinitrophenylhydrazine to form pyruvate dinitrophenylhydrazine which in turn is extracted into toluene. When the toluene extract is treated with a strong alkali, a red color results. Absorbance of this compound is proportional to the level of erythrocyte alanine transaminase. The assay is done with and without the addition of PLP to measure the stimulation of the erythrocyte transaminase.

2. Reagents

Pyridoxal phosphate (1 mg/mL). $PLP \bullet 2H_2O$ (107.3 mg) was diluted to 100 mL with redistilled water.

DL-Alanine substrate DL-Alanine (1.78 g), 2 g of K_2HPO_4 and 0.6 g α-ketoglutarate was dissolved in 100 mL of redistilled water.

Sodium Pyruvate Pyruvic acid (200 mg) was dissolved in 100 mL of redistilled water.

Dinitrophenylhydrazine (DNPH). Dinitrophenylhydrazine (100 mg) was dissolved in 20 mL of concentrated HCL and 80 mL of redistilled water.

Trichloroacetic acid (TCA), 100 percent. Trichloroacetic acid (100 g) was dissolved in and made to 100 mL with

reagent water.

Ethanolic potassium hydroxide, 2.5 percent. KOH (2.5 g) was dissolved in 95 percent ethanol.

0.033M Tris buffer, pH 7.4. 7.98 g of Trizma base was titrated with 6 M HCL to pH 7.4.

3. Analytical Method

Erythrocyte alanine transaminase was determined by the method of Woodring and Storvick (1970). For working sodium pyruvate standards were prepared by adding 1,2,3, and 4 mL sodium pyruvate and were made up to 10 mL with distilled water in volumetric Pyrex flasks. A standard curve was obtained by adding 0.1 mL of each working into test tubes containing 0.9 mL of buffer. Red blood cell samples, hemolyzed by the 10-fold addition of reagent water, were added to all other test tubes. Pyridoxal phosphate (0.1 mL) was added to in vitro stimulation test tubes. Alanine (0.5 mL) was added to all test tubes. Samples were incubated for 60 minutes at 37°C. At the end of the incubation, TCA,and dinitrophenylhydrazine were added to the test tubes in that order. After five minutes, 1.0 mL toluene was added and each test tube was mixed for 10 seconds. Samples were centrifuged for 5 minutes at 1,500 x g (Beckman TJ-6-RS, Palo Alto, CA.). A fraction of the toluene layer (0.3 mL) was transferred to a 13 x 100 mm test tube containing 3.0 mL ethanolic KOH. Samples were read at 490 nM. Hemoglobin content of the samples were determined to standardize the

assay. Hemoglobin was determined by the cyanomethemoglobin method (Maile, 1972). Enzyme units were expressed as μg pyruvate/mg Hemoglobin/hr. Results were presented as percent stimulation.

D. Femur Zinc and Magnesium

Femurs were steam autoclaved for 10 minutes at 15 psi to facilitate removal of muscle. Femurs from the dams were split into two pieces, wrapped with cheesecloth, and extracted 24 hours each first with 95% ethanol and then with petroleum ether. The femurs were dry-ashed for 24 hours at 590°C in an electric furnace and stored dry in a desiccator. All weighed samples were dissolved with 3 mL of 3 M HCl and were made up to 10 mL with distilled water in a volumetric Pyrex flask. For zinc analysis, 0.1 mL of the 10 mL solution was added to 1.9 mL of 0.1 N HCl. For magnesium analysis, 0.02 mL of the 10 mL was added to 9.98 mL of 0.1 N HCl. For offspring femur zinc and magnesium analysis, the same procedure described above was followed. Zinc and magnesium analyses were carried out by atomic absorption spectroscopy (Perkin-Elmer Model No. 2380, Norwalk, CT.) according to standard conditions described by Perkin-Elmer Corporation.

E. Plasma Zinc and Magnesium

Plasma zinc was measured by direct measurement of zinc in plasma by atomic absorption spectroscopy (Smith et al., 1979). For zinc analysis, 0.5 mL plasma was added to 2.0 mL reagent water. The zinc standard was diluted to volume with

a 5% glycerol solution to match the viscosity of the sample. For magnesium analysis, sample were diluted 1:50 with 0.1 N HCl (Dawson and Heaton, 1961). For zinc analysis, a three-slot burner head was used to prevent clogging of the burner.

F. Soft Tissue Zinc and Magnesium

Liver and muscle were wet-ashed with reagent grade nitric acid followed by 30% hydrogen peroxide. Liver and muscle samples from dams and litters were placed into 100 mL Pyrex beakers and 25 mL of reagent grade nitric acid was added. The beakers were covered with a watch glass and were allowed to sit overnight. Heat was applied to the samples to complete digestion. Completeness of digestion was tested by adding 30% hydrogen peroxide to the samples. Digestion was complete when samples turned white. Five mL of 3 M HCl was added to samples until the solution turned green-yellow. The solution was quantitatively transferred by rinsing the beaker three times distilled water into a 10 mL Pyrex volumetric flask. For liver zinc analysis, 0.1 mL of the 10 mL was added to 4.90 mL of 0.1 mL HCl in a test tube. For the determination of magnesium in liver, 0.02 mL of the 10 mL was added to 4.98 mL of 0.1 N HCl in a test tube. For muscle zinc analysis, 0.02 mL of the 10 mL added to 4.98 mL of 0.1 N HCl. For muscle magnesium analysis, 0.1 mL of the 10 mL was added to 4.90 mL of 0.1 N HCl. Zinc and magnesium analyses were carried out by atomic absorption spectroscopy.

Statistical Analysis

The experimental design of this experiment was a 2 X 2 factorial experiment involving two factors, zinc and ethanol, with two levels of each factor. If there was a significant F-value for treatment, effects were partitioned into effects due to zinc, ethanol, and interaction of these two factors. Differences between individual means were tested by Fisher's protected least significance difference only if a significant F-value was found for the treatment effect. By using this procedure, the possibility of making a type I error (null hypothesis rejected but it is actually true) was reduced while still being able to detect real differences between means. Effects were considered to significant at $P<0.05$.

During the conduct of this study, some rats were lost because of either poor lactational performance, especially in the low zinc group, or because pregnancy did not actually occur (false positive). Therefore the actual number of rats per group shown in the results section is $n = 6$ except for the low zinc group ($n = 4$). To continue to use factorial analysis of variance to evaluate the effects, it was necessary to estimate missing data (Zar, 1984). This procedure penalized the total degrees of freedom by $n = 2$ which enlarged the error mean square and decreased the probability of finding a significant difference.

RESULTS

Energy Intake and Growth Response

The effect of dietary zinc and ethanol on maternal body weight gain and energy intake, ethanol intake, litter size and growth response during gestation and lactation is summarized in Table 6. Maternal body weight gain for the first 18 days of gestation and maternal caloric intake during day 0 through day 18 gestation and day 3 through day 17 of lactation were unaffected by either the level of zinc or ethanol. Caloric intake on day 21 through day 22 of gestation (10-100 kcal) and day 1 through day 2 of lactation (10-100 kcal) was variable and therefore was not included in the total caloric intake. Ethanol intake during day 0 through day 20 of gestation and day 3 through day 17 of lactation was unaffected by the level of zinc administered in the diet.

Litter size was unaffected by the level of either zinc or ethanol. Litter weight on day 1 of lactation was significantly ($P<0.05$) depressed in the low zinc (2 μg/mL Zinc) group compared to the high zinc (10 μg/mL Zinc) group in the absence of ethanol. Litter weight on day 18 of lactation was depressed by ethanol, but the effect of ethanol ($P<0.05$) was significant only in the presence of low zinc.

Table 6

Effect of Dietary Zinc and Ethanol (ETOH) on Maternal Weight Gain and Total Energy Intake, Maternal Ethanol intake, Litter size, and Pup Weight[1,2,3,4]

Measures	Dietary Treatment: % kcal from ethanol				Significance Levels		
	0		30				
	10 µg/mL Zn (n=6)	2 µg/mL Zn (n=4)	10 µg/mL Zn (n=6)	2 µg/mL Zn (n=6)	Zn	ETOH	Zn X ETOH
Maternal weight gain, g.							
Gestation Day 0-18	112 ± 9	116 ± 7	111 ± 17	107 ± 9	NS	NS	NS
Energy intake, kcal							
Gestation Day 0-20	1849 ± 19	1866 ± 14	1829 ± 49	1812 ± 59	NS	NS	NS
Lactation Day 3-17	2181 ± 19	2205 ± 20	2018± 109	2015± 135	NS	NS	NS
ETOH intake, kcal							
Gestation Day 0-20	0	0	549 ± 14	544 ± 9	NS	NS	NS
Lactation Day 3-17	0	0	606 ± 33	605 ± 40	NS	NS	NS
Litter size	13 ± 3	14 ± 1	13 ± 3	13 ± 3	NS	NS	NS
Pup weight, g.							
Lactation Day 1	7.9 ± 1.0[a]	6.7 ± 0.5[b]	7.1 ± 0.8[a,b]	6.9 ± 0.9[a,b]	<0.05	NS	NS
Lactation Day 18	37.0 ± 3.0[a]	36.0 ± 2.0[a,b]	34.0 ± 5.0[a,b]	31.0 ± 3.0[b]	NS	<0.05	NS

[1] mean ± SD [2] Litter reduced to 8 on day 2 of lactation [3] Different superscript letters indicate significant ($P<0.05$) differences of means. If any letter combination matches, the difference between means is not significant [4] Liquid diet supplied 10% of kcal form ethanol on day 1 and 2 of gestation and 20% of kcal from ethanol on day 3 and 4 of gestation. Liquid diet supplied 30% of kcal from ethanol on day 5-20 of gestation and during all of lactation.

Maternal Zinc Status

Zinc deficiency was produced in dams demonstrated by a significant reduction in maternal alkaline phosphatase activity (Fig. 3), and in maternal plasma zinc concentration (Fig. 4). In the absence of ethanol, maternal plasma alkaline phosphatase activity was depressed by 48% ($P<0.02$) in the low zinc group compared to the high zinc group. The % reduction in maternal plasma zinc concentration (49%, $P<0.001$) was similar to that observed for maternal plasma alkaline phosphatase activity. Maternal plasma zinc concentration was reduced by low zinc regardless of ethanol level, but the magnitude was greater in the absence of ethanol. Ethanol only depressed maternal plasma alkaline phosphatase activity and plasma zinc concentration when diet zinc was high. Therefore, there was a significant interaction effect of zinc and ethanol for maternal plasma alkaline phosphatase ($P<0.05$) and plasma zinc ($P<0.01$).

With regard to other indicators of zinc status (Table 7), maternal femur zinc concentration was depressed in the low zinc group regardless of the ethanol level whereas maternal liver zinc was only depressed by low zinc in the absence of ethanol. Zinc content of maternal femur muscle was unaffected by either level of zinc or ethanol.

Maternal Vitamin B-6 Status

The effect of dietary zinc and ethanol on maternal plasma pyridoxal 5'-phosphate (PLP) during gestation and

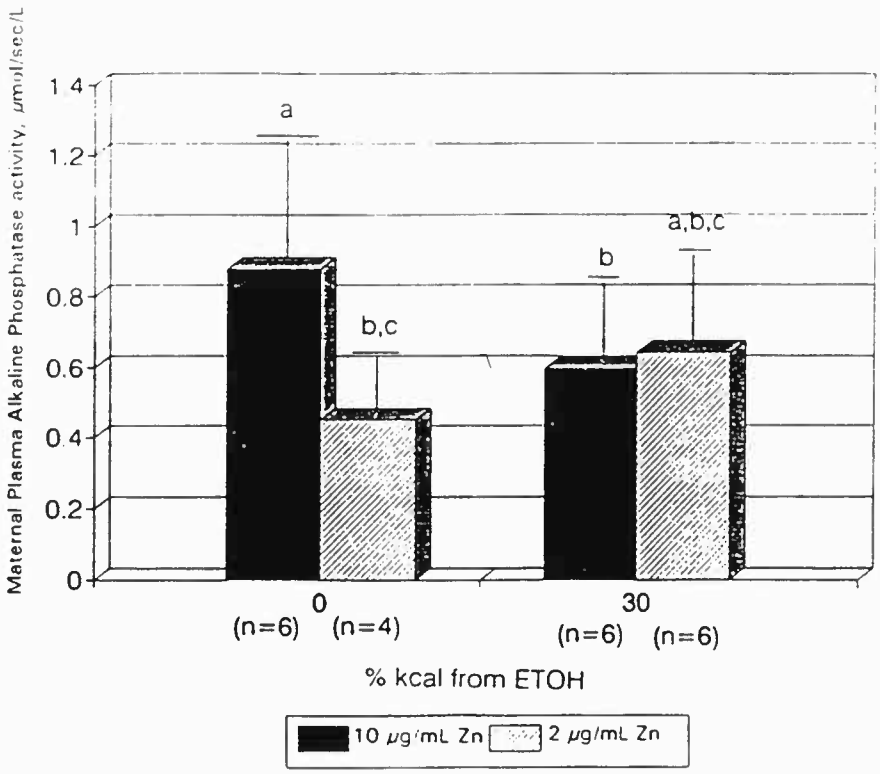

Figure 3. The Effect of Dietary Zinc and Ethanol (ETOH) on Maternal Plasma Alkaline Phosphatase activity during Gestation and Lactation. Values are mean ± SD. Different superscript letters indicate significant differnces of means. If any letter combination matches, the differences between means is not significant. Signficance levels for the effects of Zinc, ETOH, and Zinc x ETOH are NS, NS, $P<0.05$.

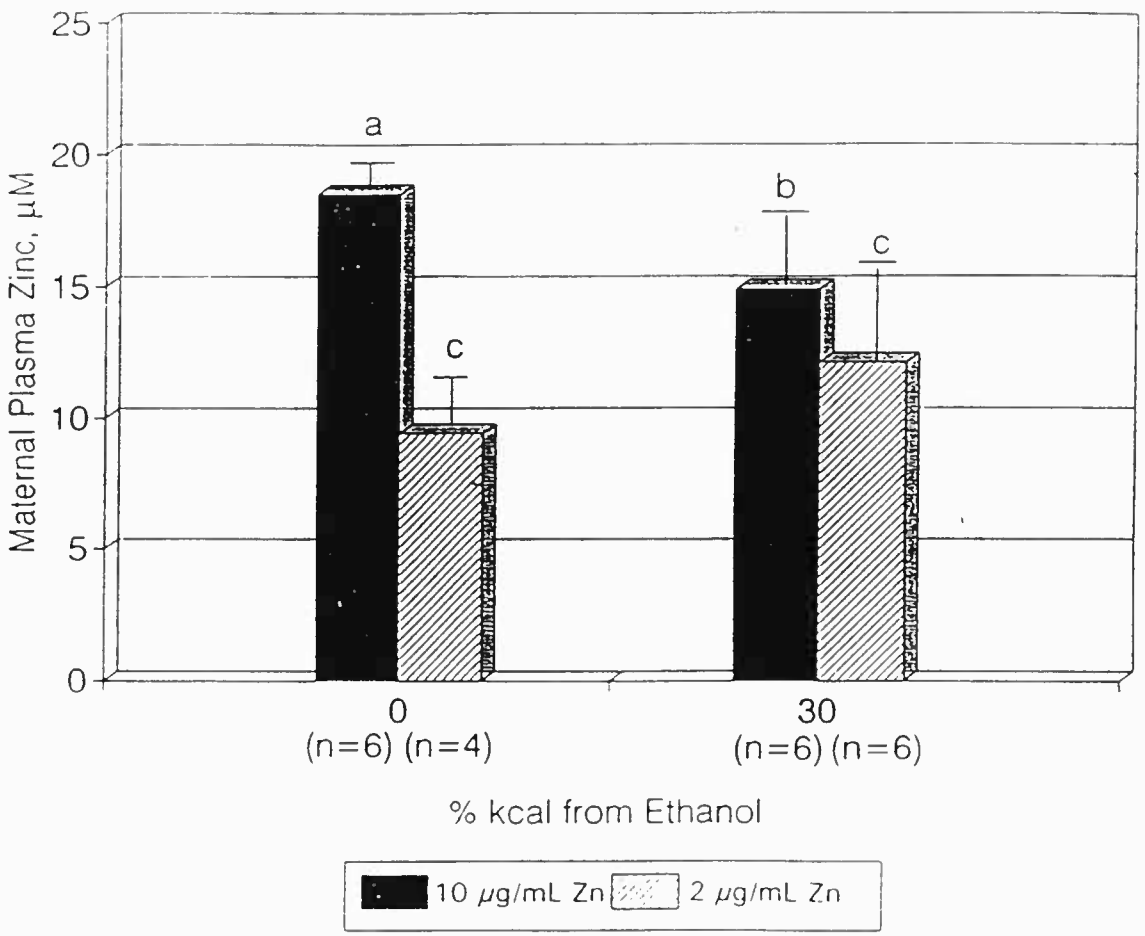

Figure 4. The Effect of Dietary Zinc and Ethanol (ETOH) on Maternal Plasma Zinc during Gestation and Lactation. Values are mean ± SD. Different superscript letters indicate significant ($P<0.05$) differences of means. Significance levels for the effects of Zinc, ETOH, and Zinc X ETOH are $P<0.001$, NS, and $P<0.01$.

Table 7

Effect of Dietary Zinc and Ethanol(ETOH) on Maternal Tissue Zinc and Erthryrocyte Alanine Transaminase (EALT) Percent Stimulation during Gestation and Lactation[1,2]

	Dietary Treatment % kcal from ethanol				Significance Levels		
	0		30				
	10 µg/mL Zn (n=6)	2 µg/mL Zn (n=4)	10 µg/mL Zn (n=6)	2 µg/mL Zn (n=6)	Zn	ETOH	Zn X ETOH
Measures							
Femur Zn, µmol/g ash	5.7 ± 0.5[a]	4.3 ± 0.3[b]	5.3 ± 0.3[a]	4.5 ± 0.7[b]	<0.001	NS	NS
Liver Zn µmol/g ash	0.53 ± 0.03[a]	0.39 ± 0.1[b]	0.46 ± 0.1[a,b]	0.43 ± 0.1[b]	<0.05	NS	NS
Muscle Zn, µmol/g wet wt.	0.2 ± 0.04	0.2 ± 0.04	0.2 ± 0.05	0.2 ± 0.01	NS	NS	NS
EALT, % stimulation	10.7 ± 22.1	14.6 ± 17.7	0.5 ± 10.7	12.0 ± 8.5	NS	NS	NS

[1] mean ± SD [2] Different superscript letters indicate significant (P<0.05) differences of means. If any letter combination matches, the difference between means is not significant.

lactation is shown in Figure 5. Maternal plasma PLP was significantly higher (61%, $P<0.01$) in low zinc group compared to the high zinc group in the absence of ethanol. Maternal plasma PLP concentration was significantly higher in the low zinc plus ethanol group compared to the high zinc without ethanol. An interactive effect between zinc and ethanol failed to occur with regard to maternal plasma PLP concentration. There was a negative correlation ($r=-0.74$, $P<0.02$) between the plasma alkaline phosphatase activity and levels of plasma pyridoxal phosphate in the low zinc group compared to the high zinc group in the absence of ethanol.

The effect of dietary zinc and ethanol on maternal erythrocyte alanine transaminase percent stimulation during gestation and lactation is summarized in Table 7. There was a trend for increased erythrocyte alanine percent stimulation in the low zinc group compared to the high zinc group regardless of the level of ethanol; however it was not statistically significant. In the low zinc without ethanol group, there was both a non-significant increase in EALT percent stimulation (Table 7) and an increase in maternal plasma PLP (Figure 5) compared to the high zinc without ethanol group. There was also both a non significant increase in EALT percent stimulation and a slight increase in maternal plasma PLP concentration in the low zinc plus

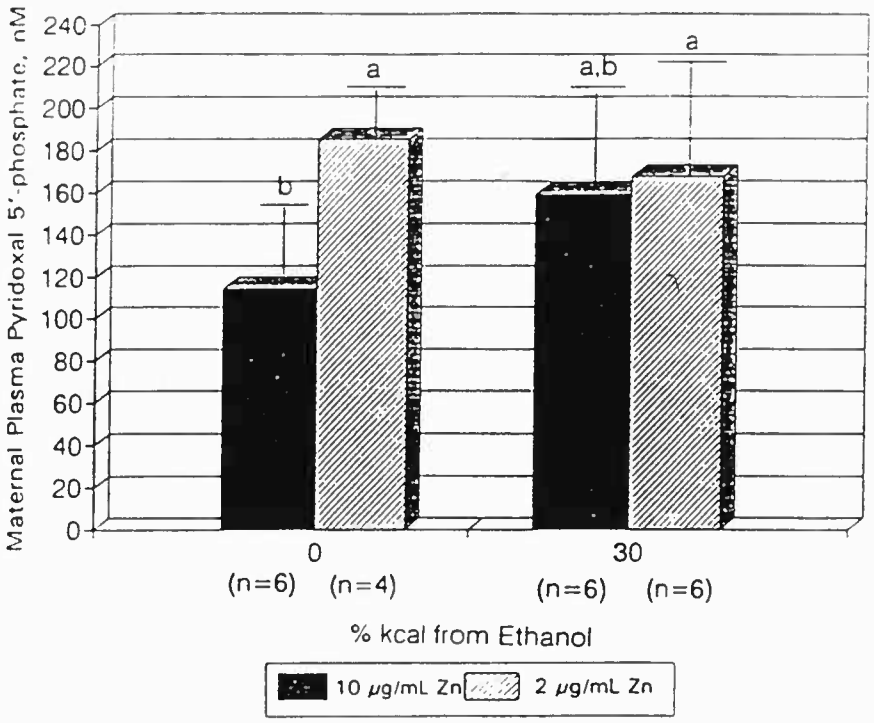

Figure 5. The Effect of Dietary Zinc and Ethanol (ETOH) on Maternal Plasma Pyridoxal 5'-Phosphate during Gestation and Lactation. Values are mean± SD. Different superscript letters indicate significant ($P<0.05$) differences of means. If any letter combination matches, the differences between means is not significant. Significance levels for the effects of Zinc, ETOH, and Zinc X ETOH are $P<0.05$, NS, and NS.

ethanol compared to the high zinc plus ethanol. There was much variability in the EALT percent stimulation.

Offspring Zinc Status

Offspring plasma zinc (Table 8) was significantly ($P<0.05$) reduced by the low zinc compared to the high zinc group although this effect just missed achieving significance in the absence of ethanol. A similar but non-significant zinc effect was seen in the absence of ethanol. Offspring plasma alkaline phosphatase activity tended to be reduced by low zinc in the absence of ethanol, but the effect was non-significant. Offspring liver zinc content was significantly ($P<0.005$) depressed in the low zinc group compared to the high zinc group, but the zinc effect only achieved significance in the absence of ethanol. Offspring femur zinc content was significantly ($P<0.001$) depressed in the low zinc group compared to high zinc regardless of the level of ethanol. Significant differences in zinc content of offspring muscle due to either zinc level or ethanol level were not demonstrated. Ethanol failed to depress offspring plasma zinc concentration, liver zinc, and femur zinc. There was no significant interaction effect of zinc and ethanol for any of the offspring zinc status indicators.

Offspring Vitamin B-6 Status

The effect of dietary zinc and ethanol on offspring plasma pyridoxal 5'-phosphate is shown in Figure 6. There was an inconsistent effect of ethanol because ethanol

Table 8

Effect of Dietary Zinc and Ethanol on Offspring Alkaline Phosphatase, Plasma Zinc, Tissue Zinc Content, and Erthrocyte Alanine Transaminse (EALT) Percent Stimulation[1,2]

	Dietary Treatment						
	% kcal from ethanol						
	0		30		Significance Levels		
Measures	10 µg/mL Zn (n=6)	2 µg/mL Zn (n=4)	10 µg/mL Zn (n=6)	2 µg/mL Zn (n=6)	Zn	ETOH	Zn X ETOH
Alkaline Phosphatase Activity, µmol/sec/Liter	2.3 ± 0.1	2.0 ± 0.2	2.3 ± 0.3	2.3 ± 0.4	NS	NS	NS
Plasma Zn, µM	26.4 ± 2.9[a]	23.2 ± 1.0[a,b]	25.9 ± 3.3[a]	22.1 ± 3.7[b]	<0.05	NS	NS
Liver Zn, µmol/g wet wt.	0.60 ± 0.1[a]	0.44 ± 0.04[b]	0.56 ± 0.0[a,c]	0.47 ± 0.07[b,c]	<0.005	NS	NS
Femur Zn, µmol/g ash	7.6 ± 0.6[a]	5.1 ± 0.3[b]	7.7 ± 0.6[a]	5.6 ± 0.7[b]	<0.001	NS	NS
Muscle Zn, µmol/g wet wt.	0.2 ± 0.03	0.2 ± 0.1	0.2 ± 0.03	0.2 ± 0.1	NS	NS	NS
EALT, % stimulation	0.9 ± 8.3	2.7 ± 5.7	-6.8 ± 6.8	0.7 ± 10.8	NS	NS	NS

[1] mean ± SD [2] Different superscript letters indicate significant ($P<0.05$) differences of means. If any letter combination matches, the difference between means is not significant

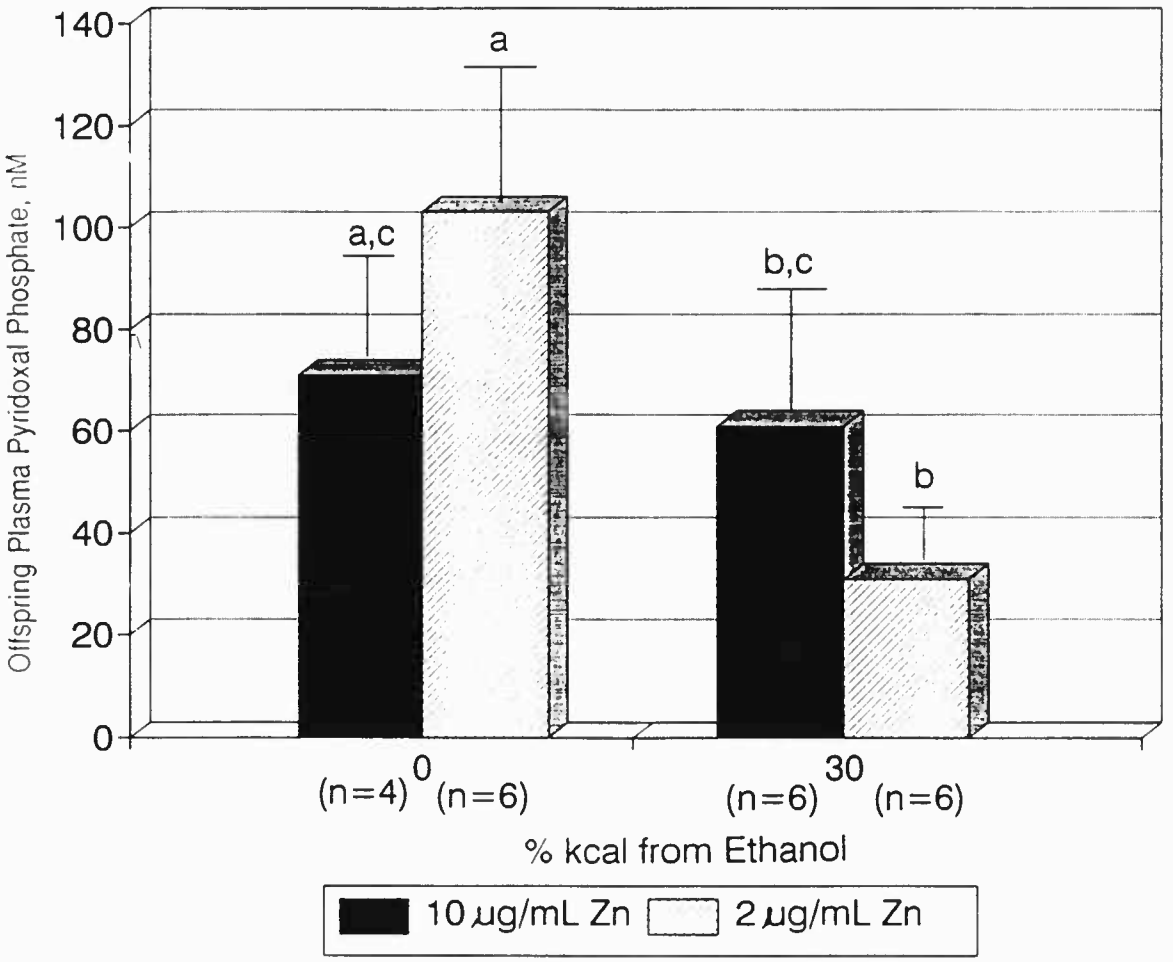

Figure 6. The Effect of Dietary Zinc and Ethanol (ETOH) on Offspring Plasma Pyridoxal 5'-Phosphate during Gestation and Lactation. Values are means ± SD. Different superscript letters indicate significant ($P<0.05$) differences of means. If any letter combination matches, the difference between means is not significant. Significance levels of the effects of Zinc, ETOH, and Zinc X ETOH are NS, $P<0.005$, and $P<0.05$.

significantly decreased offspring PLP concentration when diet was low in zinc, but not when diet zinc was high. In the absence of ethanol, offspring PLP concentration tended to be higher in the low zinc group compared to the high zinc group, however it was not significant. A significant ($P<0.05$) interactive effect of zinc and ethanol occurred for offspring plasma PLP.

The effect of dietary zinc and ethanol on offspring erythrocyte alanine transaminase (EALT) is summarized in Table 8. There was a trend for a slight increase in EALT percent stimulation in the low zinc group compared to the high zinc group regardless of ethanol level; however, it was not significant. In the low zinc without ethanol group, there was both a non-significant increase in EALT percent stimulation and an increase in offspring plasma PLP concentration compared to the low zinc plus ethanol group. There was also similar non-significant increase in EALT percent stimulation and a smaller increase in offspring plasma PLP concentration in the high zinc without ethanol group compared to the high zinc plus ethanol group. In the low zinc group without ethanol group, there was both a non-significant increase in EALT percent stimulation and an increase in offspring plasma PLP compared to the high zinc without ethanol group.

Magnesium Status of Dams and Offspring

Magnesium status of dams and offspring (Table 9) was

Table 9

Effect of Dietary Zinc and Ethanol(ETOH) on Maternal and Offspring Tissue and Plasma Magnesium during Gestation and Lactation[1,2]

Measures	Dietary Treatment: % kcal from ethanol: 0: 10 μg/mL Zn (n=6)	0: 2 μg/mL Zn (n=4)	30: 10 μg/mL Zn (n=6)	30: 2 μg/mL Zn (n=6)	Significance Levels: Zn	ETOH	Zn x ETOH
Maternal plasma Mg^{2+}, mM	0.93 ± 0.04[a,b]	0.96 ± 0.05[b]	0.82 ± 0.07[c]	0.87 ± 0.06[b,c]	NS	<0.001	NS
Offspring plasma Mg^{2+}, mM	0.9 ± 0.1	0.9 ± 0.1	0.9 ± 0.1	1.0 ± 0.1	NS	NS	NS
Maternal liver Mg^{2+}, μmol/g wet wt.	10.8 ± 0.8	9.4 ± 0.9	9.6 ± 1.2	9.5 ± 1.3	NS	NS	NS
Offspring liver Mg^{2+}, μmol/g wet wt.	9.9 ± 1.2	9.9 ± 0.3	9.0 ± 0.8	9.1 ± 3.9	NS	NS	NS
Maternal femur Mg^{2+}, mmol/g ash	0.3 ± 0.02	0.3 ± 0.01	0.3± 0.01	0.3 ± 0.01	NS	NS	NS
Offspring femur Mg^{2+}, mmol/g ash	0.3 ± 0.04	0.3 ± 0.1	0.4 ±0.03	0.3 ± 0.1	NS	NS	NS
Maternal muscle Mg^{2+}, μmol/g wet wt.	12.2 ± 1.6	11.9 ± 1.3	12.3 ±1.6	11.9 ± 0.9	NS	NS	NS
Offspring muscle Mg^{2+}, μmol/g wet wt.	13.6 ± 0.7	12.3 ± 0.5	13.4 ± 0.8	12.4 ± 1.0	NS	NS	NS

[1] mean ± SD. [2] Different superscripts letters indicate significant(P<0.05) difference of means. If any letter combination matches, the differences are not significant.

measured because of the role of magnesium in alkaline phosphatase activity and because of the known depressive effect of ethanol on magnesium status (Flink, 1986). Maternal plasma magnesium was slightly,but significantly lower ($P<0.001$) due to ethanol regardless of the dietary zinc level. Offspring plasma magnesium on the other hand was unaffected by the level of either zinc or ethanol administered in the diet. Significant difference in maternal and offspring liver, femur, and muscle magnesium due to either the zinc or ethanol level were not demonstrated.

SUMMARY AND DISCUSSION

The hypothesis of the present study was that a reduction in alkaline phosphatase activity due to a moderate zinc deficiency during the reproductive period would be of sufficient magnitude to disrupt vitamin B-6 metabolism by way of decreased hydrolysis of pyridoxal 5'-phosphate (PLP) in circulation. The results of the present study provided support for this hypothesis because a moderate zinc deficiency, as evidenced by a reduced zinc concentration in maternal plasma, femur and liver, and plasma alkaline phosphatase activity, significantly increased maternal plasma pyridoxal 5'-phosphate (PLP), the active coenzyme form of vitamin B-6 despite a constant vitamin B-6 intake. This increase in plasma PLP is in contrast to the observed fall in PLP concentration during pregnancy in both rats (Van Den Berg and Boggard, 1987) and in humans (Delport et al., 1991).

Depressed hydrolysis of PLP in a zinc deficiency would be expected to impair enzymatic function dependent upon the vitamin B-6 coenzyme. In the present study, there was a trend for increased erythrocyte alanine transaminase (EALT) stimulation by exogenous PLP in vitro in the low zinc group compared to the high zinc group. However, the effect was

non-significant. It is possible that a larger functional impairment of vitamin B-6 function was not seen because red blood cells may be able to take pyridoxal from the portal blood and convert it to PLP (and Burnham, 1990).

The offspring appeared to be largely spared the effects of the zinc deficiency, despite significant reductions in zinc concentration of liver and femur, because low maternal dietary zinc did not signifcantly depress offspring plasma alkaline phosphatase activity. Furthermore, the percent reduction seen in plasma zinc (non significant 12%) was much less than that seen in dams (49%) and both litter size and body weights of zinc adequate and zinc deficient pups were similar. There was a trend for the offspring plasma PLP concentration to be higher due to low maternal dietary zinc, but it was not statistically significant. Although not achieving significance, it was interesting that offspring erythrocyte alanine transaminase percent stimulation in the low versus high zinc group by exogenous PLP in vitro was similar to that seen in dams.

Unexpectedly, maternal ethanol ingestion did not uniformly depress zinc status as predicted by others (Flynn et al., 1981; Ghishan et al., 1982; Suh and Frisk, 1982; Ruth and Goldsmith, 1981; Ghishan and Greene, 1983; Antonson and Vanderhoof, 1983; Yeh and Cerklewsi, 1984). Ethanol did depress maternal zinc status (plasma alkaline phosphatase activity, and plasma zinc concentration) when zinc was

adequate, but not when it was deficient. Therefore, the lowest zinc status was not found in the present study when ethanol and low zinc were combined. A similar study did show that ethanol depressed maternal and offspring plasma alkaline phosphatase activity (Yeh and Cerklewski, 1984). One possible explanation for this difference was in study protocol. In the present study, virgin outbred Sprague-Dawley rats were used whereas in a similar study (Yeh and Cerklewski, 1984) proven breeder rats with one previous pregnancy were used. Furthermore the present study used a combination of micropulverized casein and spray dried egg-white to supply diet protein whereas the only dietary protein used in the comparison study was micropulverized casein. The combination of casein and dried egg white may have acted as a protective agent against the effects of ethanol. It is also possible that rats in the low zinc plus ethanol group were able to adjust their efficiency of zinc utilization to avoid developing a severe zinc deficiency (King, 1990).

Ethanol did, however, disrupt vitamin B-6 utilization in the offspring, evidenced by decreased PLP, especially when zinc was inadequate in the maternal diet. PLP concentration in the present study was similar to that observed in pups originating from dams fed a vitamin B-6 deficient diet (Sloger and Reynolds, 1980) even though dietary vitamin B-6 was adequate in the present study. One

explanation is that acetaldehyde can displace PLP in binding to albumin leading to PLP degradation (Lumeng, 1977). Because the depressive effect of ethanol on vitamin B-6 status was independent of plasma alkaline phosphatase activity, further research will be necessary to define the mechanism for this effect.

Although magnesium is required as a co-factor for alkaline phosphatase activity (Vallee and Galdes, 1984) and ethanol is known to depress magnesium status, (Flink, 1986) magnesium deficiency was not a factor in the present study. Alcohol did however slightly depress maternal plasma magnesium concentration.

With regard to overall significance, the rats used in the present study were fed a diet which supplied 2/3 of the minimum recommendation for zinc for laboratory rats (Subcommittee on Laboratory Animal Nutrition, 1978). Assuming that the rat can be used as a conceptual model for human nutrition, the effects of a marginal zinc deficiency on vitamin B-6 metabolism could have a significant impact on certain segments of the human population. Marginal zinc deficiency in the United States may not be uncommon because of poorly absorbable zinc (Sandstead, 1991). Furthermore, the fact that zinc and vitamin B-6 deficiency have some similarities (growth retardation, alocepia, dermal lesions, and decreased immunocompetence) suggests that at least part of the clinical symptoms of vitamin B-6 deficiency may be

attributed to a zinc deficiency. The results of the present study also suggest that zinc status of an individual should be determined when plasma PLP is to be used as an indicator of vitamin B-6 status because a pre-existing zinc deficiency could conceivably mask the presence of an actual vitamin B-6 deficiency.

Of course, conclusions made in the present study are limited by the use of rats as test subjects and by the fact that single point determinations of zinc and vitamin B-6 status indicators did not allow for measuring change of key indicators during the course of the experiment. Rats, for example, metabolize ethanol more efficiently than humans. In addition, alcohol was a component of a liquid diet whereas in alcoholic binge drinkers, alcohol is often consumed in the absence of food. Thus the results of the present study are not applicable to either chronic alcoholics nor to another case of excessive alcohol consumption during pregnancy, the fetal alcohol syndrome (FAS). On the other hand, these examples of non-applicability of present results are tempered by the fact that excessive alcohol only applies to a small segment of the population. Effects seen in the present study in the absence of confounding malnutrition thus would apply to the larger population of light to moderate alcohol user.

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