THE EFFECT OF ZINC DEFICIENCY ON THE GROWTH PROMOTING ACTIONS OF GROWTH HORMONE AND INSULIN-LIKE GROWTH FACTOR-I

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ABSTRACT

The effect of zinc deficiency on the growth promoting effect of circulating IGF-I and the direct growth effect of GH on long bone growth were investigated. Food intake was decreased by lack of zinc in the diet. Tissue zinc content and plasma alkaline phosphatase activity were reduced by zinc deficiency. Systemic administration of human IGF-I increased the body weight, tail length and tibia epiphyseal cartilage width of control animals. This somatogenic action was impaired by zinc deficiency, as evidenced by continued weight loss, no increase in tail length and decreased tibial epiphyseal cartilage width of zinc deficient animals. Unilateral arterial infusion of GH increased the tibial epiphyseal width of the treated limb but not of the non-treated limb in control rats. However, no difference was found between the infused and the non-infused limb of zinc deficient animals, suggesting the occurrence of GH resistance on long bone growth in zinc deficiency. We conclude that zinc deficiency inhibits the growth promoting action of circulating IGF-I and the direct growth effect of GH on long bone growth.

RÉSUMÉ

L'étude des impacts d'une carence en zinc sur le fonctionnement normal de IGF-I a été effectuée chez des rats. La corrélation entre le développement des as longs et l'hormone de croissance a été étudiée. On a constaté que les animaux consommaient moins de nourriture lorsque celle-ci était déficiente en zinc. La concentration en zinc des tissus ainsi que l'activité de la phosphatase alcaline du plasma ont été considérablement réduites dues au manque de zinc dans le régime alimentaire. L'administration systémique de IGF-I humain à un groupe de rats témoins a accéléré la croissance de ces derniers, ils ont gagné du poids, leur queue s'est allongée et la largeur de l'épiphyse du tibia a augmenté. Lorsque ces animaux étaient soumis à un régime alimentaire déficient en zinc, cette action somatogénique du IGF-I a été grandement affectée. Les animaux perdaient constamment du poids, leur queue ne grandissait plus, l'épiphyse tibiale a perdu de la largeur. L'expérience a démontré que suite à l'administration d'hormones de croissance (façon unilatérale) par voie artérielle, l'épiphyse tibiale du menbre traité a connu une croissance méliorée par rapport aux membres non-traités chez des animaux témoins. Pour les animaux sous un régime alimentaine déficient en zinc, aucune différence nà été observée que le membre soit traité ou non. En conclusion, une carence en zinc empêche le fonctionnement normal du IGF-I et rend inexistante la relation positive qui existe habituellenment entre la croissance des as longs et l'hormone de croissance.

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STATEMENT OF ORIGINALITY

The following aspects described in this thesis are considered contributions to original knowledge.

1. This is the first report that the growth promoting action of systemically administered IGF-I was impaired by zinc deficiency, suggesting that the growth retardation associated with zinc deficiency may be, in part, due to the impairment of the growth promoting effect of circulating IGF-I rather than reduced concentrations of plasma transported IGF-I.

2. It is the first demonstration that the direct growth effect of GH on skeletal growth, which is believed to be mostly mediated by locally produced IGF-I, was impaired by zinc deficiency. This finding provides further evidence to support the contention that GH resistance, rather than decreased GH concentration is responsible for the retarded growth in zinc deficient animals.

3. It is the first report of dietary intolerance by hypophysectomized rats to semipurified diets prepared according to the AIN-76 rodent diet formulation, questioning the suitability of the AIN formulations for all types of laboratory rats.

CHAPTER I. LITERATURE REVIEW

I. Introduction

Zinc was shown to be essential for microorganisms more than a century ago (Raulin, 1869). Sixty-five years later, two groups of workers showed convincingly that zinc was required for the growth and well-being of rats (Bertrand and Bhattacherjee, 1934; Todd et al, 1934). Since 1955, when zinc deficiency was noticed to be responsible for parakeratosis in pigs (Tucker and Salmon, 1955), the effects of zinc deficiency have been reported in many deficient animal species such as chicken (O'Dell et al, 1958), cattle (Dynna and Havre, 1963) and lambs (Mills et al, 1967). The first report of conditioned zinc deficiency in humans was published in 1956 by Vallee et al, who studied zinc metabolism in Laennec's cirrhosis. Later, Prasad et al (1961) observed a clinical syndrome consisting of growth retardation, male hypogonadism, mental lethargy, hepatosplenomegaly and skin changes in a study related to iron-deficiency anaemia in Iranian children. This syndrome was attributed to zinc deficiency by the investigators. Although the essentiality of zinc for animal and human growth and development has long been beyond dispute, the exact mechanism still remains unclear.

II. An Overview of Zinc

1. Dietary source and absorption

Dietary sources of zinc include meat, poultry, seafood, dairy products, eggs, legumes, grains, vegetables and fruits. Foods vary widely in their zinc content, with meat, fish and poultry being the best sources. According to the report of Solomons (1982), approximately 10 to 40 percent of dietary zinc is available for absorption. The absorption of zinc appears to occur primarily in the proximal portion of the small intestine. Whether the greatest absorption occurs in the duodenum or the proximal jejunum is still unclear. Transport across the brush border membrane is the first phase of zinc absorption which is thought to be a carrier facilitated passive process (DiSilvestro and Cousins, 1983). Solomons (1988) proposed three possible fates for the zinc in enterocytes. The zinc ion may (1) be used for the formation of cellular metalloenzymes and stabilization of cell and organelle membrane; (2) pass on through the cell and the basolateral membrane into the plasma, an active transport process ; (3) be captured and held as metallothionein. Several factors can influence zinc absorption. Among them, the amino acid, histidine appears to enhance its absorption. It is postulated that histidine forms a ligand with zinc that is readily absorbed (Scholmerich et al, 1987). Phytate, in plant foods, is believed to be the main inhibitor of zinc bioavailability (Forbes and Erdman, 1983). However, a later investigation demonstrated

that phytate alone had little adverse effect on zinc absorption even at high concentration (Ellis et al, 1987). It is in the presence of a high intraluminal calcium that phytate exerts its inhibitory effect on zinc absorption. Other possible inhibitors of zinc absorption are iron, copper, phosphate and fiber. The total amount of zinc absorbed appears to be homostatically regulated. Absorption efficiency tends to be increased in a diminished supply of dietary zinc and decreased during zinc excess (Dinsdale and Williams, 1977).

2. Transport and distribution

Zinc passing into portal blood is transported by its plasma carrier, albumin to the liver (Smith and Cousins, 1980), which is the major organ of zinc metabolism (Cousins, 1985). About 40 percent of the zinc entering the liver is released back into the blood (Aamodt et al,1979). Over 95% of zinc in plasma is carried by two transport proteins: approximately two thirds is complexed with albumin, while most of the remaining, to an α-2 macroglobulin (DiSilvestro and Cousins, 1983). The remainder is loosely bound to transferrin and certain amino acids, particularly histidine and cysteine (Giroux and Henkin, 1972). The estimated content of zinc ion in the adult human body ranges from 1.5 to 3 g (Sandstead and Evans, 1984). Zinc is present in all organs, tissues, fluids and secretions of the body with average concentrations ranging from 1 to 150 µg/g wet weight (Jackson, 1980). Although retina and prostate have the highest zinc content (approximately 600 to 800µg/g), most of the zinc in

human body is found in the skeletal muscle, bone, liver and skin (Solomons 1988). Muscle and bone account for more than 80 percent of the total body zinc. Zinc is primarily an intracellular ion, the intracellular compartment containing well over 95% of the total body zinc. Blood plasma contains about 1μg zinc/ml, representing only approximately 0.1 percent of total body content (Jackson et al, 1982). There is considerable lack of information regarding the distribution of zinc within tissues and the nature of its intracellular binding. At least a small amount of zinc is present in all organelles of the cell because zinc-containing enzymes are so widely distributed (Jackson, 1980). It appears that the major proportion of the zinc within cells is protein-bound (Jeffreys et al, 1982) and different proteins have different affinities for zinc (Pedrosa et al, 1977). Bettger and O'Dell (1981) have suggested that there is a fraction of the intracellular zinc specifically bound to membranes which may be very important in terms of the onset of deficient symptoms.

3. Function and excretion

The most widely known role of zinc in metabolism is its role as a component of numerous metalloenzymes. More than 70 enzymes coming from nearly every enzyme class have been identified to require zinc (DiSilvestro and Cousins, 1983). Among them carbonic anhydrase was the first zinc containing enzyme to be isolated (Keilin and Mann, 1939). Other important metalloenzymes requiring zinc include deoxythymidine kinase, DNA and RNA polymerase and alkaline phosphatase. By way

of its primary effect on zinc-dependent enzymes, zinc plays an important role in the metabolism of proteins, carbohydrates, lipids and nucleic acids. The physiological role of zinc includes tissue and bone growth, skin integrity, cell-mediated immunity and sexual maturity (DiSilvestro and Cousins 1983). The effects of zinc on protein synthesis, cell replication and tissue growth are apparently due to its vital role in nucleic acid metabolism. Zinc has been used to isolate intact cell membrane and neurotubules from rat brain, suggesting that it may have a role in stabilizing plasma membranes (Chvapil, 1976). Prasad (1983) pointed out that the activity of several enzymes which attached to and control the structures and functions of cell membranes are influenced by zinc. Zinc is physiologically involved in keratogenesis and integrity of the skin and play a role in wound healing. Hsu et al (1974) demonstrated that hyperkeratosis of the skin and effusion of serum are characteristically seen in young zinc deficient rats. Zinc deficiency reduces the amount of zinc accumulated at the wound site and delays wound healing (Senapati and Thompson, 1985). The importance of zinc for adequate cell-mediated immunity is universally accepted (Fraker et al. 1986). Thymic function is especially sensitive to zinc. Reduced natural killer cell activity in experimental human zinc deficiency is restored to normal after zinc supplementation in humans (Tapazoglou et al, 1985). Other immune activities such as lymphocyte responsiveness, neutrophil chemotaxis, and antibody-dependent cell-mediated cytotoxicity are also zinc dependent. Zinc deficiency in males is associated with both reduced sperm count and motility. Zinc has been demonstrated to be essential for spermatogenesis and testosterone steroidogenesis (Piesse, 1983). Zinc also affects

all phases of reproduction in females, from estrus to parturition and lactation (Hambidge et al, 1986). Clinical zinc depletion is often associated with abnormal appetite, taste and smell. Henkin et al (1975) demonstrated that zinc is present in taste bud, and that gustin, a zinc metalloprotein exists in saliva. Zinc is required to activate an alcohol dehydrogenase that catalyzes the conversion of retinol to retinal in the visual cycle (Smith, 1982).

The major routes of zinc loss from the body are the gastrointestinal tract, the kidneys and the skin. The primary zinc excretion is via the faeces (Bunker et al, 1984). Most of the endogenous zinc entering the gastrointestinal tract is secreted by the salivary glands, intestinal mucosa, pancreas and liver. Among them, pancreatic secretions contributes the most (Hambidge et al, 1986). A small amount of the plasma zinc is excreted via the kidneys, which is approximately 0.5 mg per day, representing <10% of that normally excreted via the faeces. Urinary zinc appears to be quite sensitive to the alterations of dietary intake (Prasad, 1982). About 5 μg zinc is lost from body surface, which is primarily contributed by sweating, and can increase considerably with increased temperatures (Prasad, 1981).

III. Zinc Deficiency in Humans

After Vallee et al (1956) first suggested the possibility that alcoholic cirrhosis may be associated with a secondary zinc deficiency state, the interest in studying the role of zinc in human nutrition has been growing steadily. Prasad and co-workers (1961) described a clinical syndrome consisting of growth retardation, male hypogonadism, mental lethargy, hepatosplenomegaly and skin changes in a study related to iron-deficiency anaemia in Iran. This syndrome was attributed to zinc deficiency. Two years later, Prasad and his coworkers (1963) reported comparable clinical findings in Egypt. Mild zinc deficiency was confirmed by decreased zinc concentrations in plasma, red cells and hair, as well as radio-labeled tracer studies.

The role of zinc in prepubertal growth was subsequently demonstrated by Sandstead and coworkers (1967), who reported cessation of prepubertal development in Egyptian children due to insufficient zinc intake. Zinc supplementation with adequate animal protein and calorie intake was sufficient to produce a growth response. Similar findings were obtained in Iran. Halsted et al (1972) showed that the development in 19- or 20-year-old subjects receiving a well-balanced diet alone was slow, and the effect on height increment and onset of sexual function was strikingly enhanced in those receiving zinc.

The reports of human zinc deficiency are not restricted to the Middle East. Hambidge and co-workers (1972) demonstrated that impaired taste acuity, poor appetite and low growth percentiles in Denver children were associated with low hair zinc

levels. Subsequent investigations indicated that zinc deficiency may be quite common in otherwise normal infants and children in the United States (Hambidge, 1976; Sandstead, 1973; Walravens and Hambidge, 1975). The existence of a growth-limiting, mild zinc-deficiency syndrome in some apparently healthy children in Southern Ontario was reported by later investigators (Gibson et al, 1985; Vanderkooy and Gibson, 1987). Zinc deficiency was also documented in other areas throughout the world including Australia (Cheek et al, 1982), China (Chen et al, 1985) and former Yugoslavia (Buzina et al, 1980).

Zinc deficiency can be subdivided into primary and secondary deficiencies according to etiology. Primary zinc deficiency is of dietary origin. Inadequate zinc in the diet or childhood protein-calorie malnutrition are among the common contributing factors. Symptoms include growth retardation, skin lesions, delayed secondary sexual maturity and diarrhea. Plasma zinc is generally low, and the symptoms are reversed after zinc supplementation. Thus, diagnosis of primary zinc depletion is not difficult to make. Secondary zinc depletion is a result of abnormal metabolism of zinc in the presence of potentially adequate dietary zinc intake. Abnormal zinc metabolism includes decreased zinc absorption caused by excess dietary phytate and fiber, increased zinc requirement such as in surgery and chronic infection, and increased zinc excretion resulting from burns and renal disease. The manifestations of secondary zinc depletion may not be as obvious as primary depletion. Indicative symptoms such as growth retardation and delayed sexual maturation can not be applied to adults. The less reliable characteristics such as low plasma zinc, defective taste/smell, male infertility

or reduced dark adaptation may then become important in diagnosing zinc deficiency.

It appears that mild nutritional deficiency of zinc in humans is fairly prevalent throughout the world (Prasad, 1982). However, a mild or marginal deficiency is more difficult to diagnose because there are no specific clinical features. Impaired growth velocity in the infant, child and adolescent, as well as poor appetite and depressed immune status are among the possible ones. Depressed plasma GH concentrations were also reported in children suffering from mild zinc deficiency (Collipp et al, 1982; Yoshikazu et al, 1989). Randomized and controlled trials of zinc supplementation are considered to be the most reliable way of confirming the existence of mild zinc deficiency and for assessing its effects on clinical status (Walravens et al, 1983).

Severe zinc deficiency occurs in patients with acrodermatitis enteropathica (Moynahan, 1974), following total parenteral nutrition (Kay et al, 1976) and gastrointestinal as well as hepatic disease (McClain et al, 1980). Dermatological manifestations, diarrhea, alopecia, mental disturbances and intercurrent infections are predominant in severe zinc deficiency. If there is no prompt and adequate zinc supplementation, the condition becomes fatal.

IV. Zinc Deficiency in Rat

Rat is the most commonly used laboratory animal model to demonstrate zinc deficiency and more is known about zinc depletion in the rat than in any other species. The NRC (1978) zinc requirement for the rat maintained in a zinc-free environment and fed a diet that contains casein or egg white is 12 mg/kg for maximum weight gain. The commonest means of inducing zinc deficiency in the rat is to feed it a semisynthetic diet in which spray-dried egg white is the protein source, since egg white is very low in zinc.

Reduced growth rate and loss of appetite are the first observable signs of zinc deficiency in the growing rats. Williams and Mills (1970) observed that weanling rats ceased to gain weight 4 to 5 days after receiving a basal diet containing 0.9 ppm of zinc. Declined food consumption happened at the same time and a characteristic cyclical pattern of food intake developed thereafter. As the deficiency progressed, alopecia, immature hair coats, dermal lesions, fissures at the corners of the mouth and scaly feet appeared (Swenerton and Hurley, 1968). At the height of the deficiency state, the rats were extremely emaciated with edema around the eyes and mouth, and an abnormal "kangaroo-like" posture. Such animals died soon unless zinc supplementation was provided.

Virtually every organ system can be affected by zinc deficiency. Barney et al (1968) demonstrated that the esophagus of zinc deficient rats developed parakeratosis. This is thought to impair swallowing and pose a physical limitation to food intake. Koo

and Turk (1977) have described in detail the effects of zinc deficiency on the pancreas, including the reduction of zymogen granules, lipid and lysosome accumulation, and defects in ribosomes and endoplasmic reticulum. Morphological and functional changes in the intestine of zinc deficient rats have also been reported (Elmes and Jones, 1980). Lema and Sandstead (1970) investigated the effect of zinc deficiency on epiphysis growth. They found bones from zinc deficient rats had lower synthesis of DNA, collagen and glycoprotein and decreased resistance of the epiphysis to shearing. The large decreases in total zinc and zinc concentration of femur of zinc deficient rats suggest that bones appear to be a major source of zinc during zinc depletion (Anon, 1978).

One of the systems most affected by zinc deficiency is the immune system. Thymic atrophy, reduced number of thymocytes, and decreased thymic hormone levels are characteristic in zinc-deficient rats and may be due in part to increased levels of circulating steroids (Gross et al, 1979). Circulating triiodothyronine and thyroxine are decreased in zinc deficiency, as is hypothalamic thyroid-releasing hormone (Gordon et al, 1979). Insulin secretion in response to glucose is decreased in zinc deficient rats (Huber and Gershoff, 1973). The investigation done by Prasad and coworkers (1971) strongly suggested an effect of zinc deficiency on zinc metalloenzymes and zinc-dependent enzymes such as alkaline phosphatase and deoxythymidine kinase. A dietary concentration of less than 0.5 mg/kg zinc fed to growing males arrested spermatogenesis, resulted in atrophy of the germinal epithelium, and reduced growth of the pituitary and accessory sex organs. When less than 2 mg/kg of zinc was fed to

females, a severe disruption of the estrous cycle occured, and in most cases no mating with normal males occured (Hurley and Swenerton, 1966).

The decrease in food intake in zinc-deficient animals can be very important for survival of the animal because the limited available zinc will be used for vital functions of the body instead of being diverted to the growing tissues (Chesters and Quarterman 1970). In addition to the low food intake, cyclic eating pattern may slow the development of zinc deficiency by providing plasma zinc for vital functions of the body through the catabolism of tissues when food intake is low (Park et al, 1986). However, this makes it difficult to separate the effects due to lowered food intake from those of decreased zinc supply. To overcome this problem, pair feeding is usually included to control for food intake in studies dealing with zinc deficiency. Although this approach is not a "perfect" control, as pair-fed animals soon become meal eaters while the zinc deficient animals remain nibblers.

V. Growth Hormone and Insulin-like Growth Factor-I

1. Growth hormone

Growth hormone (GH, Somatotropin) is a single chain polypeptide with a molecular weight of about 22,000D in all mammalian species. Human GH is a polypeptide with 191 amino acid residues. GH is synthesized in pituitary gland by somatotropes which is a subclass of the pituitary acidophilic cells.

GH secretion is markedly pulsatile, a pattern which is important for optimal body growth as well as for the regulation of certain hepatic functions (Muller, 1987). GH secretion is autoregulated by GH itself, somatomedins (IGFs) and the two specific hypothalamic hypophysiotropic peptides: growth hormone releasing hormone (GHRH) and somatostatin. In freely moving rats, intracerebroventricular administration of rat GH caused a severe suppression in amplitude of spontaneous GH secretory bursts. In these rats, restoration of normal pulsatile GH release was permitted only by a combination of a supramaximal bolus of GHRH by systemic route and antiserum against somatostatin (Gurd et al, 1985), implying that GH inhibits its own secretion by decreasing output of GHRH and increasing release of somatostatin, a regulatory loop defined as short feed back. In a series of elegant studies in freely moving rats, Sato et al (1989) have provided data suggesting that GH pulsatile secretion trigger somatostatin

release from the hypothalamus, thereby contributing to suppression of plasma GH levels during the subsequent period. In such a way, GH regulation via somatostatin release plays an important role in preventing pituitary desensitization and allowing production of the next large GH surge. GH may also control its own secretion through IGFs, so called long-loop feedback. When injected intracerebroventricularly into conscious rats, IGF-I inhibits GH secretion. This effect appears to be due in part to decreased responsiveness to GHRH and increased somatostatin release from the hypothalamus, although IGFs may also inhibit hypothalamic GHRH release (Shibasaki et al, 1986).

There is also a third regulatery mechanism called ultrashort feed-back loop mechanism which is represented by neuronal influences either between or within GHRH and somatostatin neurons (Daikoku et al, 1988). Somatostatin may inhibit GHRH release whereas GHRH may increase the release from and expression of somatostatin in hypothalamic and nonhypothalamic tissues (Arimura et al, 1986).

In circulation, GH binds to a specific protein - GH binding protein (GHBP). GH binding activity has been identified in the serum of rats, mice, rabbits, sheep, pigs and man (Hochberg et al, 1990). At least two kinds of binding proteins were identified. The main GHBP in plasma was shown to have a molecular weight of about 60 kD and a high affinity. Approximately 40% of human GH circulates complexed to this binding protein. Another binding protein is larger (100 kD), has a lower affinity and is present in the plasma of patients with Laron dwarfism (Baumann et al, 1987). It is generally believed that these binding proteins are cleaved from GH receptor. Leung et al (1987)

demonstrated that the sequence of the rabbit GHBP is identical to the N-terminal sequence (extracellular binding domain) of the GH receptor. In man, the evidence suggests that the high-affinity GHBP is produced by enzymatic cleavage of the hydrophillic extracellular portion of the GH membrane receptor (Trivedi and Daughaday, 1988). But the larger, low affinity binding protein appears not to be a GH receptor fragment. The functions of the GHBPs are unclear. They do increase the half-life and decrease the degradation of GH, but also they can compete with GH on the GH membrane receptor (Herington et al, 1986). As the pulsatile nature of GH secretion is an important element in its action on target tissues, the likely role of the serum binding proteins must be interpreted in terms of their effect on pulsatile GH secretory patterns. At normal binding protein levels, only 25-45% of hGH is bound, so the effect may be one of dampening GH pulse height and maintaining GH availability during the inter-peak period (Jansson et al, 1985).

Circulating GH needs a mediator to affect cellular function. It is generally accepted that binding of GH to the membrane-bound receptor initiates the biological action of GH , at least in relation to growth enhancement. GH receptors have been found in many vertebrate cell-types including hepatocytes, adipocytes, fibroblasts, chondrocytes, osteoblasts, ß-islet cells, macrophages, lymphocytes and ventral prostatic epithelial cells (Waters, 1990).

In the rat liver there are at least two types of GH binding sites, defined on the basis of cross-reactivity with prolactin (purely lactogenic) or with bovine GH (purely somatogenic). The receptors have an unusually short half-life of around 45 minutes

(Baxter, 1985). Several investigators have studied the regulatory effect of GH on its own receptors. Hypophysectomy was found to result in a two- to three-fold increase in the number of rat liver membrane GH binding sites (Picard and Postel-Vinay, 1984). The effect of GH administration on the GH receptor depends on the time-scale of GH treatments. Chronic exposure to GH induces receptor up-regulation whereas acute administration induces down-regulation (Maiter et al, 1988). The plasma membrane receptor down-regulation is closely paralleled by an increase in receptor number in the Golgi apparatus (Bick et al, 1989). The cellular mechanisms involved in the processing of GH and its receptor start with the formation of a hormone-receptor complex. The membrane then becomes refractory and GH binding decreases markedly until a new supply of receptors arrives from de novo synthesis or from recycling of processed receptors. This cycle seems to be in harmony with the secretory pattern of GH from the pituitary.

The functions of GH include its effects on body growth and regulation of energy metabolism. The GH dependent growth develops during postnatal life. The absence of GH secretion leads to dwarfism in the young child, whereas overproduction of GH during development leads to gigantism. In the adult excess GH secretion leads to acromegaly. GH influences directly or indirectly the rate of cell multiplication and differentiation in different organs and tissues. It is generally believed that GH does not have a direct effect on different growth processes but rather stimulates growth by way of somatomedins (IGFs). A failure of the liver to respond to GH and synthesize somatomedins leads to Laron dwarfism. The width of the epiphyseal cartilage has been

used traditionally in a bioassay for GH. In the hypophysectomized animal, the cartilage at the ends of long bones is very narrow. It widens on treatment with GH in a dose-dependent manner.

GH can influence protein, carbohydrate and lipid metabolism. GH increases the transport of amino acid into muscle cells and increases protein synthesis. Thus, it produces a positive nitrogen and phosphorus balance and a concomitant fall in blood urea nitrogen and amino acid levels. Urinary excretion of sodium and potassium is also decreased, probably due to the increased uptake of these ions by growing tissue. These effects of GH on protein metabolism and electrolyte balance are also shown to be mediated indirectly by IGFs. GH generally antagonizes the effects of insulin. Hyperglycaemia after GH administration is the combined result of decreased peripheral utilization of glucose and increased hepatic production via gluconeogenesis. In the case of lipid metabolism, GH promotes the release of free fatty acids and glycerol from adipose tissue, increases circulating free fatty acids and causes increased oxidation of fatty acids in the liver. The GH effects on carbohydrates and lipids probably are not mediated by somatomedins.

2. Insulin-like growth factor-I

Insulin-like growth factors (IGFs) comprise a family of peptides with cell

proliferation and differentiation promoting and insulin-like metabolic effects. They are single-chain polypeptides chemically related to insulin (about 40% amino acid sequence homology with insulin). Two distinct but chemically related (about 60% amino acid sequence homology) IGFs have been purified from human plasma, that is insulin-like growth factor-I and -II (IGF-I and IGF-II). They are polypeptides of approximately 7.5 kD which occur in plasma at concentration of 20 -80nM and at lower concentration in most other tissues of the body. The single-chain peptides consist of the peptide domains B, C, A and D, in which domains A and B are structural homologies of the insulin A and B chains. Domain C is analogous to the connecting C peptide in proinsulin whereas the D domain is not found in insulin (Humbel, 1984). The amino acid sequences of six species (human, bovine, porcine, ovine, rat and mouse) have been determined. IGF-I in all six species consists of 70 amino acid residues while IGF-II has 67 residues(Daughaday and Rotwein, 1989).

More than 95% of serum IGFs are bound to specific IGF binding proteins (IGFBP). (Humbel, 1990) At least three different classes of IGFBPs have been distinguished on the basis of biochemical characteristics and amino acid sequence. IGFBP-1 is a 25 kD protein, whose concentrations are inversely related to those of GH, whereas the IGFBP-2 is a 31-kD protein. IGFBP-3 is a 150 kD protein consisting of an acid-labile 85 kD subunit and a 53 kD acid-stable binding subunit bound to IGF-I or -II (Baxter and Martin, 1989). It is this 150 kD complex which binds most of the IGFs in the circulation and which is GH-dependent. IGFBP-3 is lacking in hypophysectomized rats, but it can be induced by infusion of IGF-I. However, the

induced binding protein-3 needs the presence of GH to form the 150-kD complex. Although IGF induces the binding protein 3, GH is needed for the formation of the acid-labile subunit (Clemmons et al, 1989)).

IGFBP-1 has been shown to have a higher affinity for IGF-I than for IGF-II, whereas IGFBP-2 preferentially binds IGF-II. IGFBP-3 appears to bind both IGFs with equal affinity (Baxter and Martin, 1989). Binding protein-1 seems to occur mostly in amniotic fluid, binding protein-2 in fetal serum and binding protein-3 in adult serum.

IGFBPs can act as carriers for IGFs. They prevent IGFs crossing the capillary endothelium, protect them from degradation and stabilize their plasma levels. The circulating IGF-IGFBP complex is supposed to be biologically inactive. The complex acts as a reservoir, releasing continuously low amounts of IGF-I and thereby creating a steady receptor occupancy. It is a better mitogenic stimulus than temporary large concentration of IGF-I. So, IGFBPs may be synergistic with IGFs action at low molar ratios but inhibitory when present in excess (Clemmons, 1991).

Three distinct types of receptors can bind the peptides of IGF family. The insulin and type I IGF receptors have a high structural homology (Ullrich et al, 1986). The primary amino acid sequences show approximately 50% homology. Both the above two kinds of receptors are glycosylated heterotetramers, composed of two α subunits (135 kDa) and two β subunits (90 kDa) linked by disulphate bonds. The α subunits contain the extracelluar ligand binding site, the β subunits a transmembrane domain and the intracellular tyrosine autophosphorylation site (Kasuga et al, 1982). There is a high degree of specificity with the type I receptor binding IGF-I>IGF-II>insulin whereas,

the insulin receptor binds insulin>IGF-I>IGF-II (Nissley and Rechler, 1984).

Following binding there are multiple transmembrane signalling events that are believed to be mediated through the β subunit. The β subunits of both receptors contain tyrosine protein kinase activity and autophosphorylate tyrosine residues in response to IGF or insulin binding. Following autophosphorylation the kinase is activated and phosphorylates other cellular proteins. Insulin or IGF binding causes a down regulation of each receptor and this may function as a mechanism for protecting cells from over stimulation.

Although IGF-II can bind to the type I IGF and insulin receptor, another type of receptor, the type II IGF receptor has also been identified. In contrast to the other two receptors, it is a single chain of 260 kD monomeric unit. It binds IGF-II with approximately three times greater affinity than IGF-I and does not bind insulin. The protein is a multifunctional protein which binds not only the IGFs but also mannose-6-phosphate and following mannose-6-phosphate binding there is an increase in affinity for IGF-II (Tong et al, 1987). The type II receptor can be cleaved from the cell surface resulting in 200 kD form that is water soluble and circulates in blood (Kiess et al, 1987a). This cleavage occurs at the transmembrane domain junction and the cleaved fragment has been shown to function as an IGF-II binding protein in rat plasma. Antibody blocking studies have shown that both IGF-I and II mediate their growth promoting effects through the type I receptor and not through binding to the type II receptor (Kiess et al, 1987b).

IGF-I appears to be one of the primary regulators of post natal growth. During

puberty, there is a 2-3 fold rise of serum IGF-I whereas no significant changes of IGF-II. IGF-I stimulates both amino acid transport and protein, RNA and DNA synthesis in skeletal tissues and has the potential to fulfil an important role as mediator of tissue anabolism (Van Wyk et al, 1974). Originally, it was hypothesized that pituitary GH stimulates the liver to synthesize IGF which then mediates the somatogenic actions of GH in the target tissues in an endocrine manner (Salmon and Daughaday, 1957).It is now generally accepted that IGF-I acts both as an endocrine hormone via the blood and as paracrine and autocrine growth factor locally. GH stimulates the biosynthesis of IGF-I in the liver and in the other organs and tissues (D'Ercole et al, 1984). Humbel (1990) proposed the following modifications to the original hypothesis: the growth promoting activity of GH is due, on the one hand, to direct effects on the periphery enabling cells to produce and to respond to IGF-I and, on the other hand, to indirect effect, mostly on the liver, to increase serum concentrations of IGF-I; IGF-I can mimic most but not all the effects of GH.

IGF-I can mimic the actions of insulin by stimulating glycogen synthesis in the liver or glucose oxidation and lipid synthesis in the fat cells when its concentration is in excess of 100 ng/ml. It is at this concentration that it binds to the insulin receptors (King and Kahn, 1981). When a bolus of 40 µg of recombinant human IGF-I was injected into rats (120-140 g) intravenously, a transient hypoglycaemia within 15 min was observed (Zapf et al, 1986). Obviously, acute injections lead temporarily to such high concentrations of IGF-I that they exceed the binding capacity of the IGF binding proteins. Under physiological conditions, free IGF never rises to such concentrations

as seen after bolus injections (Zapf et al, 1989).

3. Endocrine and paracrine/autocrine action of Insulin-like growth factor-I

The initial classical studies of IGFs focused on the endocrine mechanisms. The original somatomedin hypothesis suggested that GH exerts its effects by stimulating IGF-I release from the liver which then mediates the somatogenic actions of GH in the target tissues (Salmon and Daughaday, 1957). Early reports demonstrated that partially purified somatomedins extracted from human plasma caused an increase in body weight and sulphate-incorporating activity of cartilage in snell dwarf mice when administered three times per day for 2-4 weeks (Van Baul-Offers and van den Brande, 1979). Later it was found that the IGFs were low in patients with GH deficiency and high in patients with growth hormone excess. Hypophysectomy reduced the level of IGF-I in the liver while GH treatment of hypophysectomized rats restored circulating levels (Schwander et al, 1983). Schoenle et al (1982) demonstrated a significant increase in body weight, tibial epiphyseal width and ^{3}H-thymidine incorporation into costal cartilage DNA of hypophysectomized rats by systemic administration of human IGF-I for 6 days. These results were assumed to be direct support of the somatomedin hypothesis.

However, later studies demonstrated that IGF-I can be produced within many extrahepatic tissues. For example, IGF-I is produced in culture by a number of fetal

mouse tissues (D'Ercole et al, 1980) by porcine smooth muscle cells (Clemmons and Van Wyk, 1985), and by fibroblasts derived from human skin and lung (Atkison et al, 1980). GH-dependent IGF-I mRNA has been identified in many rat tissues (Murphy et al, 1987). Treatment of hypophysectomized rats with GH increased the concentration of IGF-I in a number of nonhepatic tissues such as kidney, lung, heart and testis. The maximal increase in tissue concentration preceded the maximal elevation of IGF-I in serum, suggesting that IGF-I was produced at multiple local sites in response to GH (D'Ercole et al, 1984). Immunohistochemical studies have shown the existence of IGF-I-like peptides in a number of tissues including proliferative epiphyseal chondrocytes (Andersson et al, 1986; Nilsson et al, 1986). Isaksson and his colleagues (1982) administered GH locally into one tibial epiphysial plate of hypophysectomized rats and subsequently measured bone growth with a tetracycline labelling technique. There was significant increase in bone growth only on the injected side. In a subsequent study, Isgaard et al (1986) demonstrated that human GH stimulated local bone growth of hypophysectomized rats when administered by a catheter with tip inserted into the epiphysis, or into the knee joint intraarticularly. In contrast, hGH administration into the metaphysis did not cause such a stimulation. The effect of hGH was dose dependent, and the lowest daily dose of hGH that caused a stimulation was 50 ng. A direct action of GH injected into epiphyseal cartilage growth plate in stimulating growth of the cartilage was confirmed by Russell and Spencer (1985).

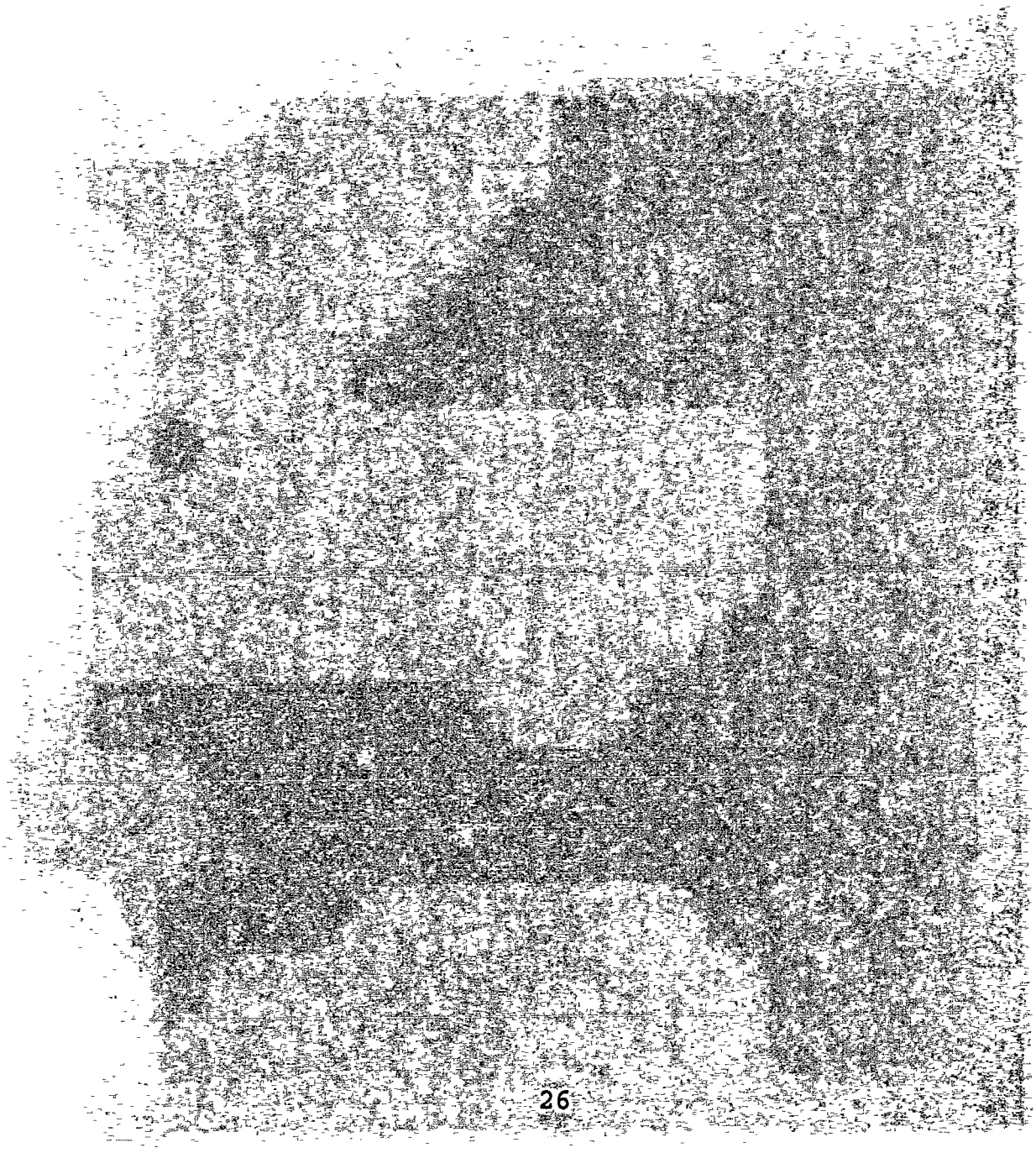
Schlechter et al (1986) resolved some of the apparent conflicts between the direct action of GH and the intermediary role of IGFs. When either hGH or hIGF-I was

infused into one femoral artery, there was a widening of the growth plate width only on the injected side. IGF-I antiserum infused concurrently with GH completely abolished this effect. The clear implication of this experiment was that GH was acting directly on tibial cartilage plate, but that this action required the local production of IGF-I. Additional evidence for this intermediary role of local IGF-I was provided by Isgaard et al (1988), who found that GH treatment of hypophysectomized rats resulted in an increase in the concentration of IGF-I mRNA in the costal cartilage growth zone, and by Nisson et al (1986), who found that both local and systemic GH increased IGF-I detected in the epiphyseal growth plate. The accumulating evidence suggests that IGF-I may act locally on the cells in which it is produced (autocrine action), or on neighbouring cells (paracrine action). Based on the concept that growth of tissues is a product of number of precursor cells that have started to differentiate and the number of cells that are formed through clonal expansion from one progenitor cell, a dual effector theory of GH action was proposed (Green et al, 1985). According to this theory, GH directly stimulates the differentiation of various mesenchymal precursor cells (e.g. prechondrocytes in cartilage and growth plates, preadipose tissue). Then IGF-I promotes the clonal expansion of cells that have started their program of differentiation.

Although conclusions from research on autocrine/paracrine role of IGF-I emphasize that local IGF-I production is the primary mediator of GH action on skeletal growth, none of these arguments exclude a classic endocrine role for IGF-I. Daughaday (1989) put forth several arguments concerning the importance of circulating IGF-I in

skeletal growth. They are as follows: (1) the plasma concentration of IGF-I is much higher than that of any other tissue; (2) plasma IGF-I is biologically active. Normal rat serum stimulates anabolic processes in isolated cartilage segments; (3) the ability of mesenchymal and skeletal tissues to synthesize IGF-I is much less than that of the liver. The concentration of IGF-I mRNA in rat costal growth plate after GH treatment is only 4-6 percent of that in liver; (4) the in vitro addition of GH stimulates anabolic processes and DNA synthesis to a much smaller degree than does IGF-I in all experiments with cartilage and bone tissue; and (5) the in vivo actions of GH on cartilage growth establish the local production and action of IGF-I but they do not prove that autocrine/paracrine actions of IGF-I are sufficient to explain normal growth. In addition, systemic injections of GH achieve a greater growth response than do local injections into the growth plate. Various studies have consistently shown that systemically administered IGF-I can promote growth. Skottner et al (1987) demonstrated that recombinant human IGF-I (hIGF-I), whether given by subcutaneous infusion, twice daily injection, or intravenous infusion, had a small but significant effect on growth parameters in hypophysectomized rats. Similar results were found by Schoenle et al (1985). Guler et al (1988) administrated rhIGF-I to hypophysectomized rats by subcutaneous infusion for 18 days, a significant increase in body weight, tibial epiphyseal width, longitudinal bone growth and trabecula bone formation was found. They concluded that hIGF-I was a potent anabolic and growth-promoting hormone in hypophysectomized rats in the absence of GH. Exogenous IGF-I has also been shown to stimulate growth in adult rats (Hizuka et al, 1986), growing rats (Philipps et al,

1988), diabetic rats (Carlsson et al, 1989) and undernourished rats (Schalch et al, 1989). It appears certain that systemic IGF-I mediates at least some of the actions of GH on skeletal growth and on other tissues. Thus it is reasonable to postulate that coordinated musculoskeletal development depends on both paracrine and endocrine actions of IGF-I.



VI. Effects of Hypophysectomy

The pituitary is necessary for postnatal growth, for adaption to starvation and many stresses and for reproductive behaviour and function (Hadley, 1992). Hypophysectomy induces disorders of endocrine system and results in a number of malfunctions. Young hypophysectomized animals exhibit a decline in normal growth rate as evidenced by a suppression of skeletal growth, reductions in lean body mass and body weight gain (Schoenle et al, 1985), decreases in longitudinal bone growth and tibial epiphyseal width (Thorngren et al,1973), and suppression of normal DNA synthesis in heart, kidney, skeletal muscle and costal cartilage (Goldspink and Goldberg, 1975). Additional biological changes associated with hypophysectomy are the cessation of proliferation and maturation of growth plate cartilage and a delay of osteogenesis reflected by an inhibition of mesenchymal cell proliferation with a reduction in bone formation and mineralization (Reddi and Sullivan, 1980). The growth failure associated with hypophysectomy is due to deficiencies in important hormones including GH, adrenocortical hormone, thyroid hormone and gonadal hormones.

Adaptation to starvation, trauma and other stresses as well as redistribution of body nutrients are regulated by the adrenal cortex. Corticotrophin (ACTH) secreted by the adenohypophysis is essential for maintenance of the adrenal cortex function. Hypophysectomy results in atrophy of the adrenal cortex and causes deficiency of the glucocorticoid hormone. Secondary hypothyroidism is due to the deprivation of thyrotropin (TSH) which is a peptidehormone produced by the pituitary. A continuing

supply of TSH is necessary for maintenance of the thyroid hormone-producing follicular cell, and the capacity to adapt to cold exposure requires an intact hypothalamo-hypophyseal response system (Hadley, 1992). Secondary hypogonadism is caused by hypophysectomy in both sexes. If the operation is performed in sexually immature animals of either sex, the gonads fail to develop. If pituitary failure occurs after sexual maturation, gonadal function regresses (Imura, 1985). Anemia and immunodeficiency are also seen in hypophysectomized animals (Berczi, 1986).

VII. Nutritional Regulation of Growth Hormone and Insulin-Like Growth Factor-I

1. GH and its receptors

Nutrition, specifically protein and energy, appears to play a role in the regulation of GH synthesis and secretion. During fasting in man and ruminants, GH secretion increases dramatically. Ho et al (1988) demonstrated that in adult males, the mean 24-hour GH secretion was increased 3-fold after 1 day of fasting and remained elevated after 5 days of fasting. In adult humans, striking increases in GH are also seen in hypoglycemia. However, procedures that normally evoke GH secretion (exercise, arginine infusion) fail to do so if a continuous intravenous infusion of glucose is maintained (Tepperman, 1987). Merimee et al (1982) reported that elevated GH secretion during fasting was associated with a decrease in circulating IGF-I and no IGF-I response happened subsequent to intravenous GH administration. This suggests that fasting induces a state of hepatic resistance to GH. Studies in rats have indicated that fasting induces a rapid decrease in hepatic GH binding sites which correlates, both quantitatively and temporarily, with the observed changes in circulating IGF-I levels (Maes et al, 1983).

In studies with ruminants, both high affinity and low affinity GH receptors were present in well-fed animals, whereas only the low affinity GH receptor was present in animals fed at a body maintenance level of nutrition, and the concentration of this

receptor was not markedly affected by the nutritional state. Thus, it is the high affinity GH receptor that is under active nutritional regulation (Gluckman et al, 1990).

It may be that there is different sensitivity to the nutritional influences in different tissues. For example, it appears that the GH receptor in adipose tissue is less sensitive or resistant to the effects of undernutrition (Gluckman et al, 1990). Such a differential sensitivity would allow mobilization of fat as a metabolic reserve when GH secretion rises during undernutrition, while at the same time restricting anabolism.

2. IGF-I and its binding proteins

While circulating IGF-I levels are clearly responsive to changes in GH secretion, they are also influenced by other factors, particularly nutritional factors. Grant et al (1973) observed that serum IGFs bioactivity was low in children with protein calorie malnutrition, despite high GH values. This uncoupling between serum GH concentrations and GH action was further confirmed by Merimee et al (1982), who showed that after three days of fasting, normal subjects showed no change in serum IGF-I in response to GH injections, suggesting that the low IGF observed in malnourished children was due to GH resistance. In adults, fasting causes a rapid and progressive fall in serum IGF-I levels, with values declining 40 - 70% within 5 days; Refeeding a control diet increases the IGF-I values to more than 70% of control values in 5 days (Clemmons et al, 1981).

Isley et al (1983) assessed the roles of specific dietary components on regulation

of serum IGF-I levels. They found that in adult human adult both the energy intake and the protein content of the diet affected serum IGF-I levels. However, the minimal requirement for calories was absolute and a threshold quantity of energy was necessary to increase the plasma concentrations of IGF-I during refeeding. In contrast, at each level of protein intake (adequate energy) an incremental increase in serum IGF-I occurred.

IGF-I has a direct role in mediating the fasting-induced changes in body mass. IGF-I infusion into fasted rats causes a reduction in protein degradation but produces no change in protein synthesis (Jacob et al, 1989). Likewise, infusion of IGF-I into rats fed a 5% protein diet does not result in the normal rate of increase in tail length, body weight, or tibial epiphyseal width (Thissen et al, 1991). This observation suggests that protein deficiency not only lowers serum IGF-I concentrations in response to GH but also produces refractoriness to IGF-I action. Interestingly, protein-deficient rats infused with IGF-I increase their serum IGFBP-3 concentrations (Thissen et al, 1991), suggesting that some actions of IGF-I are not impaired.

In fasted rats, decreased blood level of IGF-I are associated with decreased transcription of hepatic IGF-I mRNA (Emler and Schalch, 1987). Refeeding produces a prompt increase in steady-state mRNA levels.

Nutrient intake is a major regulator of the plasma concentrations of IGFBPs. Prolonged fasting and/or protein deficiency result in significant reduction in serum IGFBP-3 concentrations (Clemmons et al, 1989). Refeeding a diet adequate in energy or protein will increase the concentrations of this peptide. However, IGFBP-3 is not

affected by acute changes in nutrient intake, and its plasma concentrations remain stable throughout the day (Baxter and Martin, 1989). Protein restriction was also shown to increase hepatic IGFBP-2 mRNA abundance, although its effects on the serum concentrations of IGFBP-2 are unknown (Straus and Takemoto, 1990). Acute alterations in caloric intake have minimal effects on plasma IGFBP-2 concentrations. Unlike IGFBP-2 and -3, plasma concentrations of IGFBP-1 are markedly reduced by ingestion of food. This change is believed to be due to insulin secretion, since infusion of insulin into normal subjects suppresses IGFBP-1 (Suikkari et al, 1988). Ooi et al (1990) demonstrated that fasting and/or insulin deficiency resulted in increasing amounts of IGFBP-1 mRNA in rat liver, and this mRNA was reduced by manipulations that raised insulin secretion.

VIII. Zinc, Growth Hormone and Insulin-Like Growth Factor-I

One of the possible mechanisms to explain the growth retardation associated with nutritional zinc deficiency is an adverse effect of zinc deficiency on the endocrine regulating of growth. Coble et al (1971) studied 18 Egyptian boys with zinc deficiency who had retarded growth and delayed sexual maturation. The most striking endocrine abnormality was a poor growth hormone response to insulin-induced hypoglycaemia. Collipp and coworkers (1982) reported two children with growth hormone deficiency, as determined by their appearance, height, bone age and growth hormone assay, were also zinc deficient by hair analysis. Following oral zinc therapy, their growth rate and growth hormone assays improved. Ghavami-Maibodi et al (1983) supplemented short children who had a retarded bone age and low hair zinc concentration with oral zinc supplements for one year. There was a significant increase in the growth rate of the children whose hair zinc concentration increased. Growth hormone and somatomedin C (IGF-I) also increased after zinc supplementation. Some children with constitutional growth delay have been reported to have low serum growth hormone levels (Gourmelen et al, 1979). Reduced hair and serum zinc concentrations were also reported in children with constitutional growth delay (Castro-Magana et al, 1981). Administration of GH to children with GH deficiency has been found to significantly increase hair zinc concentration and reduced urinary zinc excretion (Cheruvanky et al, 1982). It appears that there are some children with GH deficiency who are also zinc-deficient, and some children with zinc deficiency who are also growth hormone-

deficient. From these studies it was concluded that the effect of zinc on growth may, in part, be mediated through its effect on GH levels. Similar results are found in animal study. Root et al(1979) demonstrated that zinc deficiency decreased serum concentrations of GH in both sexually mature and immature rats. When compared to pair-fed control rats receiving a zinc supplemented diet, both adult and juvenile zinc deficient rats had significantly lower body weights, tail lengths and ventral prostate weights. It was hypothesized that GH supplementation might relieve the retarded growth in zinc deficiency.

Prasad et al (1969) investigated the effect of GH administration on the growth of zinc deficient rats and of zinc on the growth of hypophysectomized rats. For the first part of the study, rats were given a zinc deficient diet for three weeks and then continued on the zinc deficient diet or given supplemental zinc with or without GH for an additional two weeks. In the second part, hypophysectomized rats were treated for two weeks as in part one. Dietary zinc supplementation increased the growth rate of nonhypophysectomized zinc deficient rats considerably while GH administration was without effect in the presence or absence of supplemental zinc. GH was effective in promoting growth in the hypophysectomized rats regardless of the dietary zinc status, although the weight gain was small in the zinc deficient group. Growth from zinc supplementation without GH in hypophysectomized rats was greater than that produced by GH alone. It was concluded that zinc and GH regulate growth in rats by independent mechanisms. Oner et al (1984) also looked at the effect of GH treatment in zinc deficient rats. After three weeks of a zinc deficient diet, rats received

supplemental zinc, GH or saline. Treatment with bovine GH did not increase skeletal growth in zinc deficient rats. Serum level of IGF-I was lower in zinc deficient rats and restored to normal by zinc repletion but not by GH treatment.

Cossack (1984) was the first to established the importance of zinc for maintaining normal circulating somatomedin C (IGF-I) levels. A significant decrease in the plasma somatomedin C level was observed in the rats after 14 days of zinc deprivation. Plasma somatomedin C levels correlated significantly with zinc status and body weight gain. Subsquently he found that rats fasted for three days showed an immediate decrease in plasma somatomedin C (Cossack, 1988). Again, adequate zinc was required in the refeeding diet to restore the plasma sometomedin C levels to normal. It was suggested that the declined plasma somatomedin C levels were responsible for the growth retardation associated with zinc deficiency.

All the previous studies have focused on the effect of zinc on plasma concentrations of GH and IGF-I and their relationship to growth. These studies have not distinguished between the growth promoting effect of circulating IGF-I and the direct growth effect of GH which seems to be expressed primarily by locally produced IGF-I. It is not clear whether the direct growth effect of GH, and/or the growth promoting action of circulating IGF-I are inhibited in zinc deficiency.

STATEMENT OF PURPOSE

Insulin-like growth factor-I (IGF-I) is a growth hormone (GH) dependent single chain polypeptide which appears to be one of the primary regulators of post natal growth. Pituitary GH stimulates the liver to synthesize IGF-I. The latter is then transported via the blood circulation to stimulate tissue growth as an endocrine factor (endocrine IGF-I). GH also can act on many other tissues directly to promote growth (direct growth effect of GH), which is likely to be mediated primarily by locally produced IGF-I. This locally produced IGF-I acts in an autocrine or paracrine manner. Both endocrine IGF-I and the direct growth effect of GH contribute to the coordinated tissue development.

Experimental evidence shows that dietary zinc deficiency leads to retarded growth in rats. Zinc deficiency reduces both serum GH and endocrine IGF-I levels. Supplements of GH to zinc deficient rats fail to improve circulating levels of IGF-I and growth. Based on these facts, it was hypothesized that zinc deficiency would lead to decreased efficacy of endocrine IGF-I and impairment of the direct growth effect of GH. The objectives of this study were to investigate the effect of zinc deficiency on the growth promoting action of endocrine IGF-I and the direct growth effect of GH. Two separate experiments were conducted. The hypophysectomized rat was used as the experimental animal model since there was little naturally occurring systemic or locally produced IGF-I in this animal.

The specific objectives of each experiment were:

Experiment 1 (presented in manuscript 1):

(1) to determine the effect of dietary zinc deficiency on the growth promoting effect of endocrine IGF-I by measuring:

a. body weight

b. tail length, a measure of skeletal growth in the rat

c. tibial epiphyseal cartilage width, a measure of longitudinal bone growth

(2) to determine the effect of dietary zinc deficiency and systemic administration of hIGF-I on zinc status by measuring:

a. plasma zinc concentration

b. liver zinc concentration

c. tibial zinc concentration

d. plasma alkaline phosphatase activity

Experiment 2 (presented in manuscript 2):

(1) to determine the effect of dietary zinc deficiency on the direct growth effect of GH on tibia growth by measuring tibial epiphyseal cartilage width.

(2) to determine the effect of dietary zinc deficiency and local administration of GH on zinc status by measuring:

a. plasma zinc concentration

b. femur zinc concentration

c. plasma alkaline phosphatase activity

CHAPTER II.

FAILURE OF INSULIN-LIKE GROWTH FACTOR-I INFUSION TO PROMOTE GROWTH IN ZINC DEFICIENT HYPOPHYSECTOMIZED RATS

ABSTRACT

The retarded growth of zinc deficient rat is associated with low plasma insulin-like growth factor-I (IGF-I). To determine whether the low circulating IGF-I concentrations are responsible for the stunted growth, the growth response of zinc deficient hypophysectomized rats to the supplementation of recombinant human IGF-I (hIGF-I) was investigated. There were three dietary groups: zinc deficient (0.9 mg zinc/kg diet), control (66 mg zinc/kg diet) and zinc adequate pair-fed group (66 mg zinc/kg diet). All rats in each group received hIGF-I (150 μg/day) by subcutaneous infusion for 12 days, except for half of the animals in the control group which were sham treated receiving vehicle infusion for the same period of time. Food intake was reduced by lack of zinc in the diet but not increased by hIGF-I administration. hIGF-I infusion significantly increased the body weight , tail length, liver weight and tibia epiphyseal width of control animals although the magnitude of change was small. Tissue zinc content and plasma alkaline phosphatase were lowered by zinc deficiency.

In contrast to control animals, administration of hIGF-I did not stimulate body growth in zinc deficient rats, as shown by continuous weight loss, no increase in tail length and lowered tibial epiphyseal width. We conclude that systemic supplementation of hIGF-I can promote growth in hypophysectomized rats. The absence of weight gain and bone growth in zinc deficient animals during the infusion period suggestes that the growth promoting effect of circulating IGF-I is impaired by zinc deficiency.

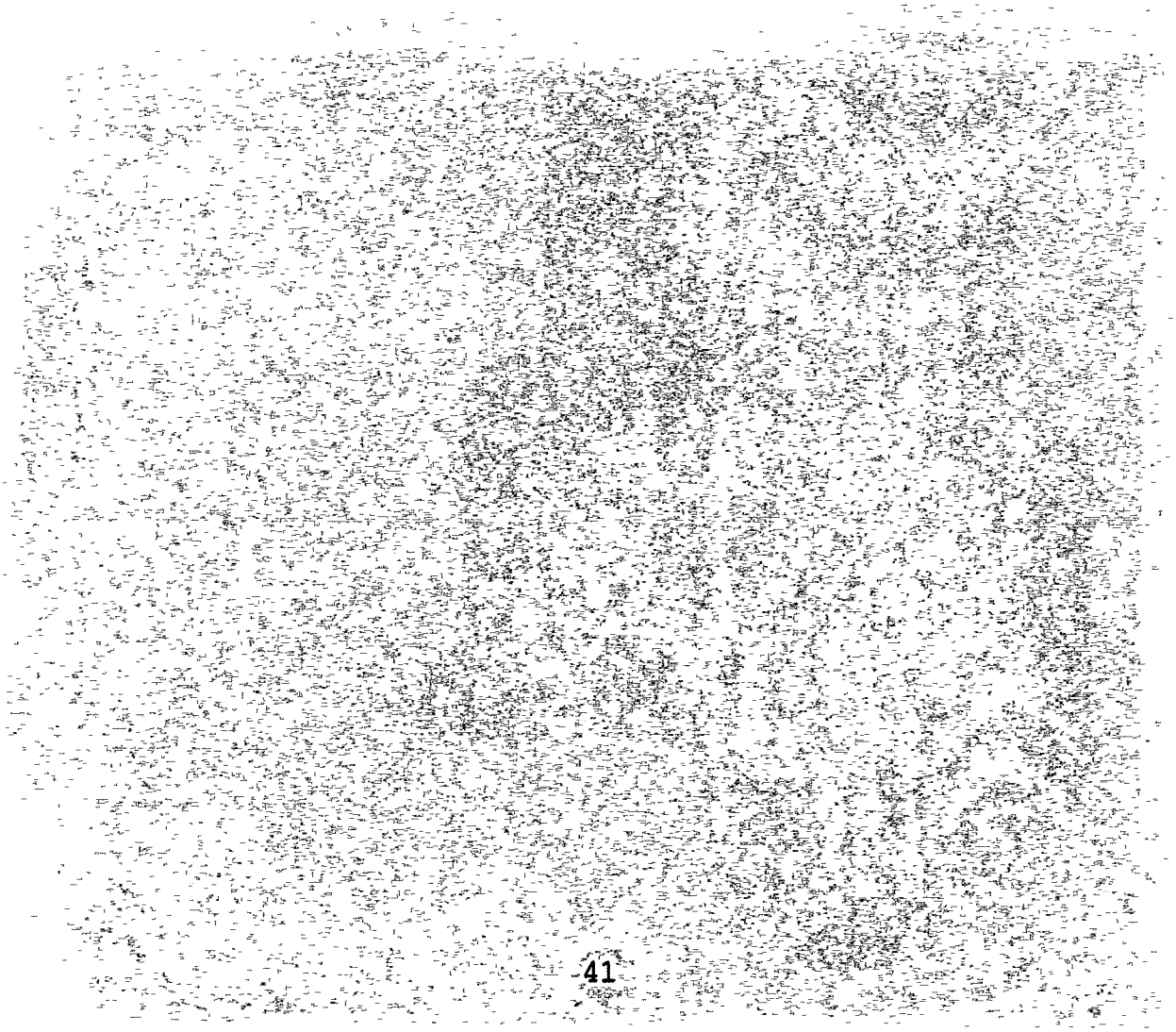
I. INTRODUCTION

Insulin-like growth factor-I (IGF-I) basically is a growth hormone (GH) dependent single chain polypeptide which appears to be one of the primary regulators of post natal growth. Initially it was postulated that GH acted on the liver to release IGF-I, which then promoted growth as the endocrine mediator of GH (Salmon and Daughaday, 1957). Subsequent investigation showed that IGF-I is produced within many tissues in addition to the liver (D'Ercole et al, 1984). GH has direct growth action on tissues such as the growth plate via local production of IGF-I (Schlechter et al, 1986). Although the major effect of GH on skeletal growth may be mediated by IGF-I of local origin, several studies consistently demonstrated that systemically administered IGF-I can promote growth, as reflected by an increase in body weight, tail length and epiphyseal width (Guler et al, 1988; Schoenle et al, 1982). Circulating IGF-I has also been implicated in regulating protein metabolism in a manner coordinated with skeletal and organ growth (Douglas et al, 1991). It thus seems clear that systemic IGF-I mediates at least some of the actions of GH on skeletal and other tissues growth (Gluckman et al, 1991).

Zinc deficiency in animals and humans is associated with growth retardation. Zinc deficiency reduces both serum GH (Root et al, 1979) and IGF-I levels (Cossack, 1984) in rats,and supplementation of GH to zinc deficient rats fails to increase plasma levels of IGF-I and body growth (Dick et al, 1992; Oner et al, 1984). A strong correlation between serum IGF-I levels and longitudinal growth was reported in

diabetic rats (Binz et al, 1989), indicating that low blood IGF-I concentrations may be the causal factor for the growth retardation. It has been suggested that reduced concentrations of circulating IGF-I is responsible for the growth retardation associated with zinc deficiency (Cossack, 1984; Dorup et al, 1991; Oner et al,1984).

The current study was undertaken to examine whether subcutaneous supplementation of IGF-I could promote growth in zinc deficiency and thereby to determine the effect of zinc deficiency on the growth promoting effect of circulating IGF-I.



II. MATERIALS AND METHODS

Animals and diets. Male Sprague-Dawley hypophysectomized rats were purchased from Charles River Inc. (Montreal, PQ, Canada). Hypophysectomy was performed when the body weight of the rats was in the range of 130 - 140g. The rats were received in our animal care facility five days after hypophysectomy. Upon arrival the rats were weighed and housed individually in stainless steel hanging cages in temperature (24 ± 1°C) and light-controlled (12h light, 0800-2000h) environment. All rats were given free access to Purina rat chow and a 5% glucose solution for the first seven days. Rats gaining more than 3g during this period were excluded from the study. On day eight, three days before implanting the pump, thirty-six rats were selected and randomly divided into three dietary groups and one sham group. The rats in the zinc deficient group (ZD) were fed a low zinc diet ad libitum, the pair fed (PF), control(C) and sham (S) groups received the control diet. The rats in the PF group were given the same amount of food as their zinc deficient counterparts ate the previous day, whereas the rats in the C and S groups were allowed free access to food. Deionized distilled water was available from plastic bottles ad libitum. Food intake was recorded daily.

The composition of the control and low zinc diets are shown in **Table 2.1.** Both were semi-purified powdered diets prepared in our laboratory. The low zinc diet contained 0.9 mg of zinc per kg of diet, while the control diet was the same ration but supplemented with zinc carbonate to contain 66 mg zinc per kg of diet. Zinc content

of the experimental diets was ascertained by atomic absorption spectrophotometry.

The purified diets used in this study differed from the AIN-76 recommendations (AIN,1977) due to unexpected intolerance shown by the hypophysectomized rats to these common semi-purified diets (prepared by ICN Biomedicals according to the AIN formulation). The diets contained the following (g/kg diet): egg white solids, 180; corn oil, 100; cornstarch, 440.3; sucrose, 200; cellulose, 30; choline bitartrate, 3; biotin, 20 mg/kg; AIN-76 mineral mix without zinc, 35; AIN-76 vitamin mix, 10. Zinc carbonate was added to the control diet at the level of 139 mg/kg diet. In the preliminary trial to determine the length of time needed to induce zinc deficiency in hypopohysectomized rats, 90% of the animals placed on both the low zinc and control semi-purified diets developed a mucoid diarrhea within the first 3-5 days, Mortality would commence, if kept on these diets an additional 2-3 days. Necropsy report revealed that the animals were suffering from an enteritis of unknown etiology. The payers patches and the mesenteric lymph nodes were also markedly enlarged. Other organ systems were grossly normal. No evidence of bacterial pathogens or fungus were found in samples of the diet and rectal swabs. When the animals were placed back on the Purina rat chow, the diarrhea ceased and the overall physical condition of the animals improved. To address this problem, a literature search brought to our attention where hypophysectomized rats were used and which were fed a low energy powered diet (1360 joules/100g) containing 15% protein, 45% carbohydrate and 5% lipid (Maes et al, 1988) . It was obvious to us that the carbohydrate content of the diet was reduced and the diet was diluted. Since high carbohydrate regimens are known to cause osmotic

diarrhea, we reasoned that the mucoid diarrhea of our rats may possibly be due to the inability to handle the carbohydrate load of the diet. The latter is possibly a side effect of hypophysectomy. We tested our idea by preparing diets that were diluted (1285 joules/100g, based on the standard physiological fuel values for protein, fat and carbohydrate) by increasing the fiber content and decreasing the carbohydrate content. Previous research has shown that the rat in general eats in relation to its energy requirement. A maximum 40% dilution of the diet could be made in weanling female rats before caloric intake was reduced, and a 50% dilution could be made in mature females (Person and Baumgardt, 1971). These results indicate that rats can increase their food intake to compensate for the energy dilution of the diet. Our observations with hypophysectomized rats confirmed these early findings. The high cellulose diets (5.4 times more than the amount in AIN-76 formulation) were tolerated well by the hypophysectomized rats. The diarrhea rate dropped from 90% to about 27%. In those animals that were afflicted with diarrhea, in general the onset was later and the symptoms were less severe. To our knowledge, there is no published report in the literature addressing the problem of intolerance to semipurified diets made according to the AIN-76 formulation by hypophysectomized rats. Our limited observations do not allow us to speculate on the exact cause of the problem or the physiological and pathological mechanisms involved. Obviously this is a problem that deserves further investigation.

Egg white was used as the protein source in the diet since it is a complete protein with the lowest zinc content. Excess biotin was added to control for the avidin,

a biotin-chelating agent present in egg white. High fiber diets are known to decrease bioavalability of minerals. However, the quantitative aspect of this inhibition is unknown. Reports about the interaction between dietary fiber and the bioavailability of vitamins are controversial. As a results, in preparation of the diets, we used the same quantity of vitamins and minerals as that recommended by the American Institute of Nutrition (AIN, 1977).

Hormone solution and subcutaneous infusion. Bacterially produced recombinant human insulin-like growth factor-I (hIGF-I) was generously provided by Ciba-Geigy Corporation (Summit, NJ). The Alzet osmotic minipump (model 2002, Alza, Palo Alto, CA) was used as the subcutaneous delivery system. According to the pump specifications, the hIGF-I solution was prepared to be delivered at 150μg per day. The minipumps filled with either hIGF-I dissolved in 0.1M acetic acid or acetic acid alone were incubated at 37^{0}C in saline for 4 hrs before implantation. The pumps were implanted on day eleven, two days after starting the feeding trial.This was designated as day one of the experiment. The animals were lightly anaesthetized with ether. The back of each animal was shaved and a 1 cm incision was made. The skin and underlying tissues were separated and the pump filled with the infusion solution was implanted. The skin was then sutured with wound clips. By way of this infusion system, rats in ZD, PF and C groups received hIGF-I infusion for twelve days while rats in S group were sham treated receiving vehicle solution infusion for the same period. Body weight and tail length were measured on day 1 of the experiment before implanting the pump and every other day thereafter. Tail length was measured in

millimetres from the anus to the tip of the tail. At the end of the twelve day infusion period, the rats were sacrificed and the pumps were removed. The pumps were weighed before implanting and after removing from the rats to confirm the delivery of their contents. The implantation sites of the pumps showed no signs of inflammation.

Tissue collection and analytical methods. On day thirteen of the infusion trial, all rats were lightly anaesthetized with ether and blood was drawn by cardiac puncture. Subsequently all animals were killed by decapitation. Blood was collected in vacutainer Trace Element Tube (Bectin Dickinson, Fisher Scientific, Montreal, PQ) coated with sodium heparin. Trace Element Tubes have a minimal zinc content (0.2ppm) and are recommended for trace element determinations. Blood was centrifuged at 2500 rpm for 15 minutes and the plasma transferred to microeppendorf centrifuge tubes. Plasma zinc concentrations were determined by flame atomic absorption spectrophotometry (Perkin-Elmer Model 3100, Perkin Elmer (Canada) Ltd., Montreal, PQ) after dilution with distilled deionized water. Plasma alkaline phosphatase activity (U/L) was determined by the hydrolysis of p-nitrophenyl phosphate (Sigma chemical Co, St. Louis. Mo) and read on the Abbott VP Super system discrete analyzer (Abbott Diagnostic, Mississauga, ON). The liver was removed and freeze - dried to a constant weight. The hind part of each animal was removed. Tibias from both legs were carefully dissected free from surrounding flesh. Left tibia was freeze-dried and used for zinc determination. The epiphyseal growth plate width of the right tibia was determined by the method described by Greenspan et al (1949). Tibias were sectioned and stained for calcified bone with silver nitrate. The width of the cartilage band was measured with a

microscope equipped with a micrometer. The dried liver and left tibia samples were wet digested using nitric acid according to the method of Clegg et al (1981). Liver and tibia zinc concentrations were determined using flame atomic absorption spectrophotometry.

Statistics. The experimental design was an unbalanced 3x2 factorial design with 3 levels of dietary zinc (zinc deficient, pair-fed and control) and 2 hIGF-I treatments (hIGF-I and sham). However, only the control dietary group included both levels of hIGF-I treatment whereas the animals in zinc deficient and pair-fed groups were all hIGF-I treated. As a result, no interactions between dietary zinc and hIGF-I treatment could be analysed. Unbalanced data was analyzed by a two way analysis of variance (dietary zinc and hIGF-I) using a SAS general linear model program (SAS Version 6. SAS Institute Inc. Cary, NC). Unpaired t-test was used to separate means between treatment groups and controls. Differences between means were considered to be significant at $p<0.05$. The data in the text are means ± SEM.

III. RESULTS

Cumulative food intake and growth parameters were analyzed and the results shown in **Table 2.2.** Dietary zinc had a significant effect on the food intake of hypophysectomized rats ($P<0.001$) as evidenced by a 35% reduction in food intake of ZD rats as compared to C animals ($P<0.0002$). There was no difference in food intake between ZD and PF animals. The difference in cumulative food intake between ZD and C animals occurred on day 3 of experiment and became significant on day 7($p<0.003$), 10 days after the initiation of the feeding trial (**Fig 2.1**). Systemic administration of hIGF-I by subcutaneous infusion had no effect on food intake ($p=0.54$).

Continuous infusion of hIGF-I affected weight gain of hypophysectomized rats ($p<0.001$). Supplementation of 150 µg/d of hIGF-I resulted in gain of about 9 grams in 12 days by C rats while S animals lost weight during this period. This difference in weight gain between C and S rats was significant ($p<0.0002$). Both dietary zinc deficiency and food restriction inhibited the growth promoting effect of hIGF-I ($p<0.001$) and the effect of zinc deficiency was independent of restricted food intake. Weight gain was less in PF animals as compared to C rats ($p<0.01$) but significantly higher than the weight lost by the ZD rats ($p<0.001$). There was no difference in weight change of ZD as compared with S animals suggesting that the growth effect of hIGF-I on weight gain was completely abolished by zinc deficiency. During the first 5 days, the effect of zinc on body weight change was not apparent. On day 5, the body weights of hIGF-I treated animals, including the ZD rats were significantly higher than

that of S animals (**Fig 2.2**). As zinc deficiency and food restriction aggravated, ZD rats began to lose weight while PF animals maintained their weights. In contrast, the body weights of C animals increase continuously. This difference among the three groups of animals became apparent on day 11 of infusion and continued for the remainder of the feeding trial. During this period, the body weight of ZD rats fell to the level of S animals (Fig 2.2).

Similar to body weight , hIGF-I infusion had a small but significant effect on tail growth ($p<0.001$). Tail length of C group increased about 2.7 mm, significantly higher than the S group ($p<0.0002$). Dietary zinc had a significant effect on tail growth ($p<0.001$). The largest gain in tail length occurred in the C group and smallest in the ZD group. The change in the PF group was intermediate. There was no difference in tail length between ZD and S rats (Tab 2.2). **Fig 2.3** shows the changes in tail length. By day 7, differences in tail length between PF and ZD groups were apparent ($p<0.02$) and continued for the remainder of the feeding trial. The difference in tail length between PF and C groups became significant on day 9 ($p<0.01$).

Supplementation of hIGF-I had no effect on liver weights when examined per unit body weight ($p=0.22$) as shown by no differences in liver weights of hIGF-I infused C animals as compare to S animals ($p=0.22$). Liver weights of ZD and PF rats were significantly lower than those of C ($p<0.002$) and S($P<0.01$) animals, suggesting that decreased food intake reduced the liver weight and hIGF-I infusion could not reverse this decline. Dietary zinc deficiency did not have an independent effect on liver weight as evidenced by no difference ($p=0.82$) between ZD and PF animals.

Infusion of hIGF-I had a significant effect on tibial epiphyseal cartilage width ($p<0.0002$) (**Fig 2.4**). Tibial cartilage width of all the hIGF-I treated rats in all three dietary groups were significantly higher than that of S animals ($p<0.05$). Tibial epiphysial width of hIGF-I treated C animals (160.0 ± 8.3μm) increased by 91% as compared with S animals (84.0 ± 11.9μm) ($p<0.0002$). This growth promoting effect of hIGF-I administration was significantly affected by lack of zinc in the diet ($p<0.0005$). The C group had larger epiphyseal cartilage width compared to either the PF group (136.3 ± 4.1μm) ($p<0.05$) or ZD group (108.0 ± 6.7μm) ($p<0.002$) while the PF group had larger epiphyseal width than the ZD group ($p<0.02$), the latter suggested that zinc deficiency inhibited the growth promoting effect of hIGF-I infusion on bone growth independent of decreased food intake. However, this growth effect was not completely abolished by lack of zinc in the diet, as evidenced by the larger cartilage width of ZD rats as compared to S animals ($p<0.05$).

No difference in tibia weight was found across the dietary and treatment groups. When examined as per 100 g body weight, a negative correlation between the tibia weight and body weight was found, suggesting that dietary zinc and hIGF-I administration had no effect on tibia weight.

To examine the effect of dietary zinc and hIGF-I infusion on the zinc status of the experimental animals, plasma zinc, liver zinc, tibia zinc and plasma alkaline phosphatase activities were determined (**Table 2.3**). Dietary zinc had an effect on plasma zinc ($p<0.01$). Plasma zinc was 49% lower in ZD rats than in the PF and C animals ($p<0.01$). No difference existed between PF and C animals ($p=0.40$),

suggesting that decreased food intake did not affect plasma zinc concentrations. Continuous infusion of hIGF-I did not affect plasma zinc concentrations ($p=0.84$). There was no difference in plasma zinc values between C and S groups ($p=0.83$). Similar to plasma zinc, liver zinc was affected by zinc in the diet ($p<0.02$) but not by hIGF-I infusion ($p=0.08$). Liver zinc concentrations were significantly lower in ZD than in PF groups ($p<0.02$), while the difference between ZD and S groups did not reach statistical significance ($p=0.08$). Liver zinc concentrations were unexpectedly low in C rats, being significantly lower ($p<0.02$) than in the PF animals. There was an effect of zinc in the diet on tibia zinc concentrations ($p<0.001$), but no effect of hIGF-I treatment ($p=0.75$). Tibia zinc concentrations were lower in ZD animals than in PF, C and S groups ($p<0.002$), while the differences among PF, C and S groups were not significant ($p>0.30$).

Both dietary zinc ($p=0.07$) and hIGF-I infusion ($p=0.54$) had no effect on plasma alkaline phosphatase activities. However, a lowered value of alkaline phosphatase activity in ZD as compared with PF animals ($p<0.05$) was found.

TABLE 2.1

Composition of the Experimental Diets

Ingredient	(-) Zn diet	(+) Zn diet
	g/kg diet	
Egg white[1]	150	150
Corn oil[1]	70	70
Cornstarch[1]	320	320
Sucrose[1]	140	140
Cellulose[1]	270	270
Choline bitartrate[1]	2	2
Biotin[1]	0.02	0.02
AIN-76 Mineral mix[1,2,3]	35	35
AIN-76 Vitamin mix[1,4]	10	10
BHT[1]	0.007	0.007
Zinc carbonate[5]	--	0.09

[1] ICN Biomedicals Inc., Cleveland, OH.

[2] Special mineral mix without zinc.

[3] Provided (g/kg mineral mix): calcium phosphate dibasic, 500; sodium chloride, 74.0; potassium citrate monohydrate, 220; potassium sulfate, 52.0; magnesium oxide, 24.0; manganous carbonate, 3.50; ferric citrate, 6.0; cupric carbonate, 0.30; potassium iodate, 0.01; sodium selenite, 0.01; chromium potassium sulfate, 0.55; sucrose, finely powdered, 118.

[4] Provided (mg/kg vitamin mix): thiamin hydrochloride, 600; riboflavin, 600; pyridoxine hydrochloride, 700; nicotinic acid, 3000; D-calcium-pantothenate, 1600; folic acid, 200; D-biotin, 20; cyanocobalamin, 1.0; retinyl palmitate, 1600; DL-alpha tocopherol acetate, 20,000; cholecalciferol, 250; menaquinone, 5.0; sucrose, 972.9 (g/kg vitamin mix).

[5] Fisher Scientific, Montreal, PQ.

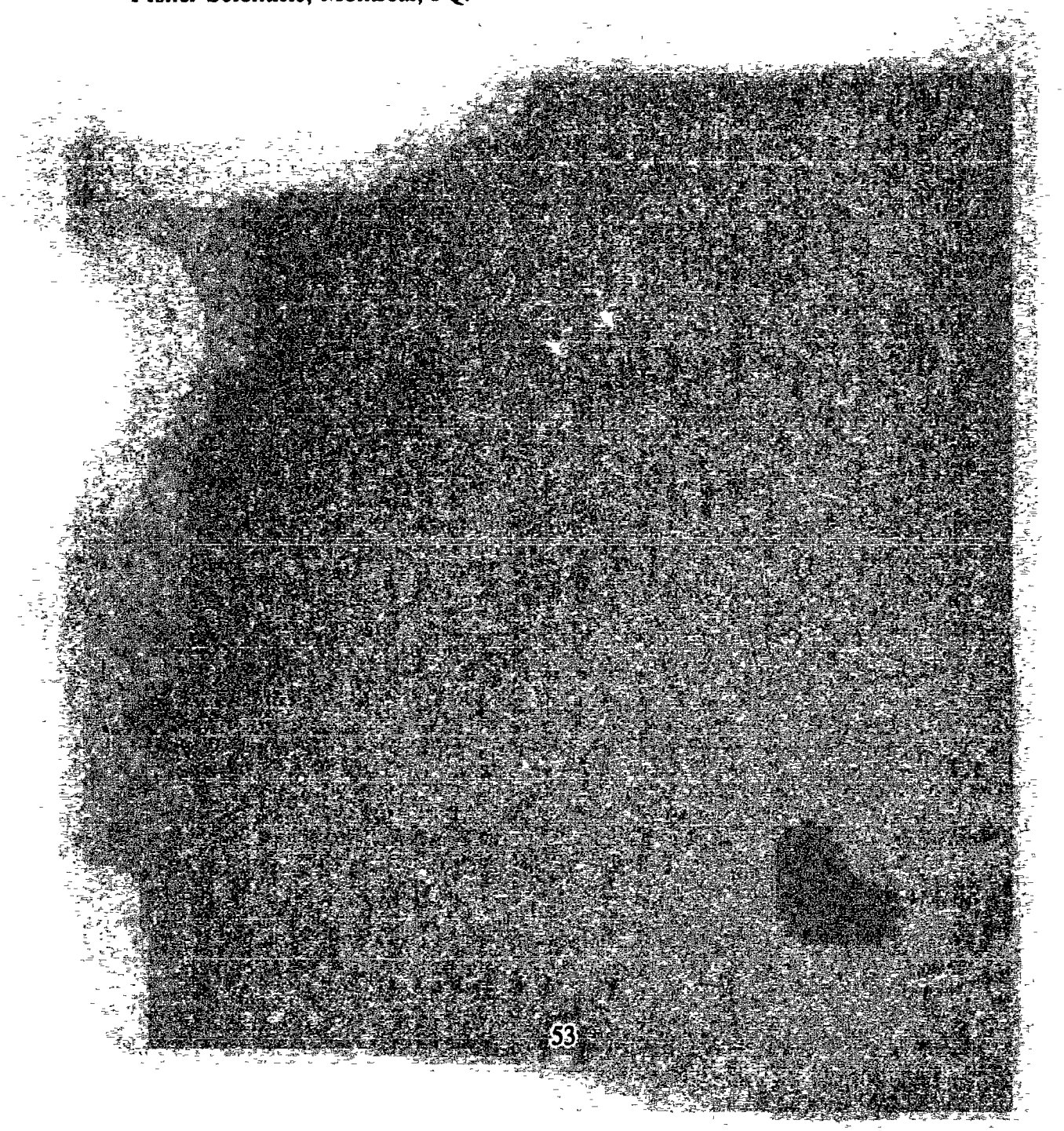


TABLE 2.2

Effects of dietary zinc and hIGF-I subcutaneous infusion on food intake and growth parameters in hypophysectomized rats[1,2]

	Zn deficient	Pair fed	Control	Sham
Cumulative food intake (12 days g)[3]	67.5 ± 4.6[a]	70.9 ± 1.8[a]	103.4 ± 3.6[b]	107.6 ± 7.6[b]
Cumulative weight gain (g)[4]	-10.6 ± 1.6[a]	0.3 ± 0.8[b]	8.9 ± 2.7[c]	-7.1 ± 2.0[a]
Cumulative tail length (mm)[5]	-0.4 ± 0.3[a]	1.1 ± 0.4[b]	2.7 ± 0.6[c]	-0.4 ± 0.2[a]
Liver weight (g dry wt)[6]	0.95 ± 0.04[a]	0.97 ± 0.05[a]	1.54 ± 0.10[b]	1.24 ± 0.06[c]
Liver weight (% body wt)[7]	0.80 ± 0.02[a]	0.77 ± 0.04[a]	1.15 ± 0.08[b]	1.07 ± 0.05[b]
Tibia weight (g dry wt)[8]	0.25 ± 0.01[a]	0.24 ± 0.01[a]	0.24 ± 0.01[a]	0.24 ± 0.01[a]
Tibia weight (% body wt)[9]	0.21 ± 0.01[a]	0.19 ± 0.01[b]	0.18 ± 0.01[c]	0.20 ± 0.01[a]

[1] Values are means ± SEM; there were 5 -7 rats in each treatment group.

[2] Means within each parameter with different letter superscripts differ significantly ($p<0.05$).

[3] ANOVA: zinc ($p<0.001$); hIGF-I ($p=0.54$).

[4] ANOVA: zinc ($p<0.001$); hIGF-I ($p<0.001$).

[5] ANOVA: zinc ($p<0.001$); hIGF-I ($p<0.001$).

[6] ANOVA: zinc ($p<0.001$); hIGF-I ($p<0.01$).

[7] ANOVA: zinc ($p<0.001$); hIGF-I ($p=0.22$).

[8] ANOVA: zinc ($p=0.27$); hIGF-I ($p=0.88$).

[9] ANOVA: zinc ($p<0.001$); hIGF-I ($p<0.001$).

TABLE 2.3

Effects of dietary zinc and hIGF-I subcutaneous infusion on zinc status in hypophysectomized rats[1,2]

	Zn deficient	Pair fed	Control	Sham
Plasma zinc (µg/ml)[3]	0.64 ± 0.12[a]	1.25 ± 0.09[b]	1.42 ± 0.21[b]	1.37 ± 0.05[b]
Liver zinc (µg/g dry wt)[4]	116.5 ± 4.1[a]	140.7 ± 4.3[b]	116.3 ± 7.1[a]	134.0 ±10.2[a]
Tibia zinc (µg/g dry wt)[5]	148.3 ± 4.5[a]	183.7 ± 3.3[b]	188.0 ± 5.4[b]	190.1 ± 3.0[b]
Alkaline phosphatase (U/L)[6]	105.1 ± 10.1[a]	180.0 ± 27.6[b]	178.9 ± 21.0[b]	200.6 ±26.6[b]

[1] Values are means ± SEM; there were 5 - 7 rats in each treatment group.

[2] Means within each parameter with different letter superscripts differ significantly ($p<0.05$).

[3] ANOVA: zinc ($p<0.01$); hIGF-I ($p=0.84$).

[4] ANOVA: zinc ($p<0.02$); hIGF-I ($p=0.08$).

[5] ANOVA: zinc ($p<0.001$); hIGF-I ($p=0.75$).

[6] ANOVA: zinc ($p=0.07$); hIGF-I ($p=0.54$).

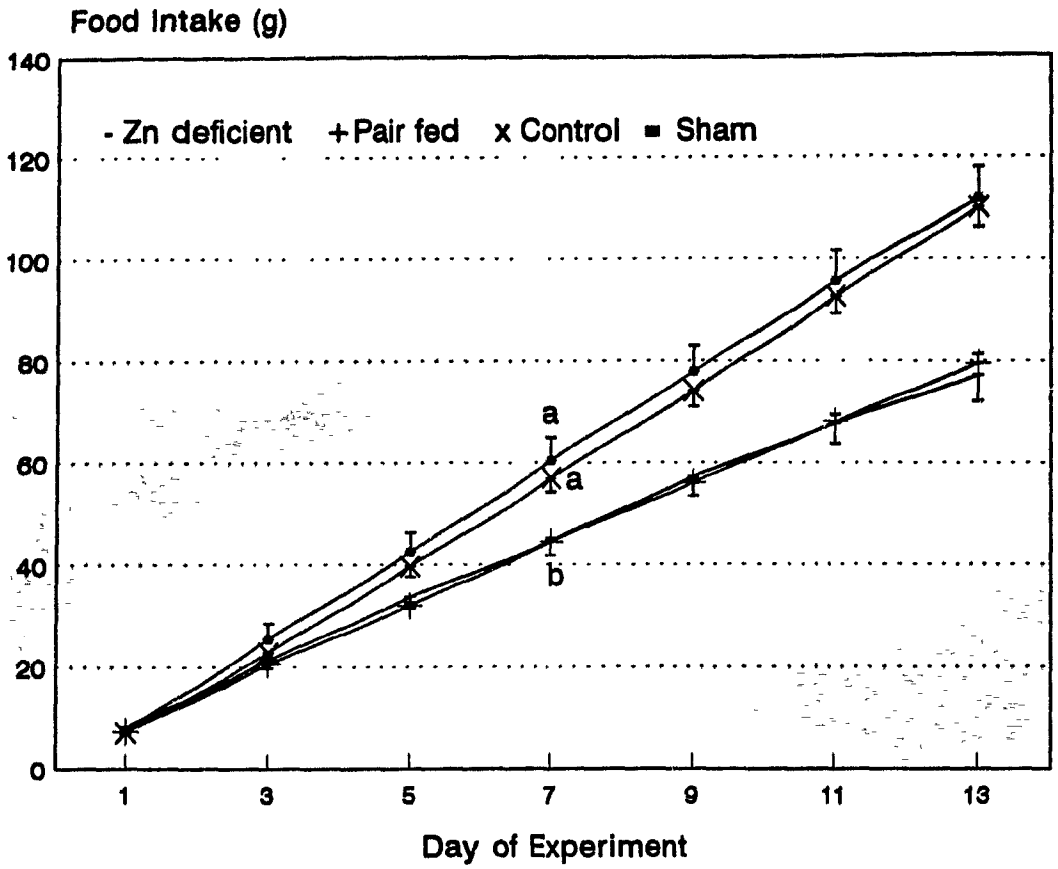


Figure 2.1. Effects of dietary zinc and hIGF-I subcutaneous infusion on cumulative food intake in hypophysectomized rats. Each data points (means ± SEM) represents the mean of individual observations for a group of 5-7 rats. Means on the same day with different letter superscripts differ significantly ($p<0.05$).

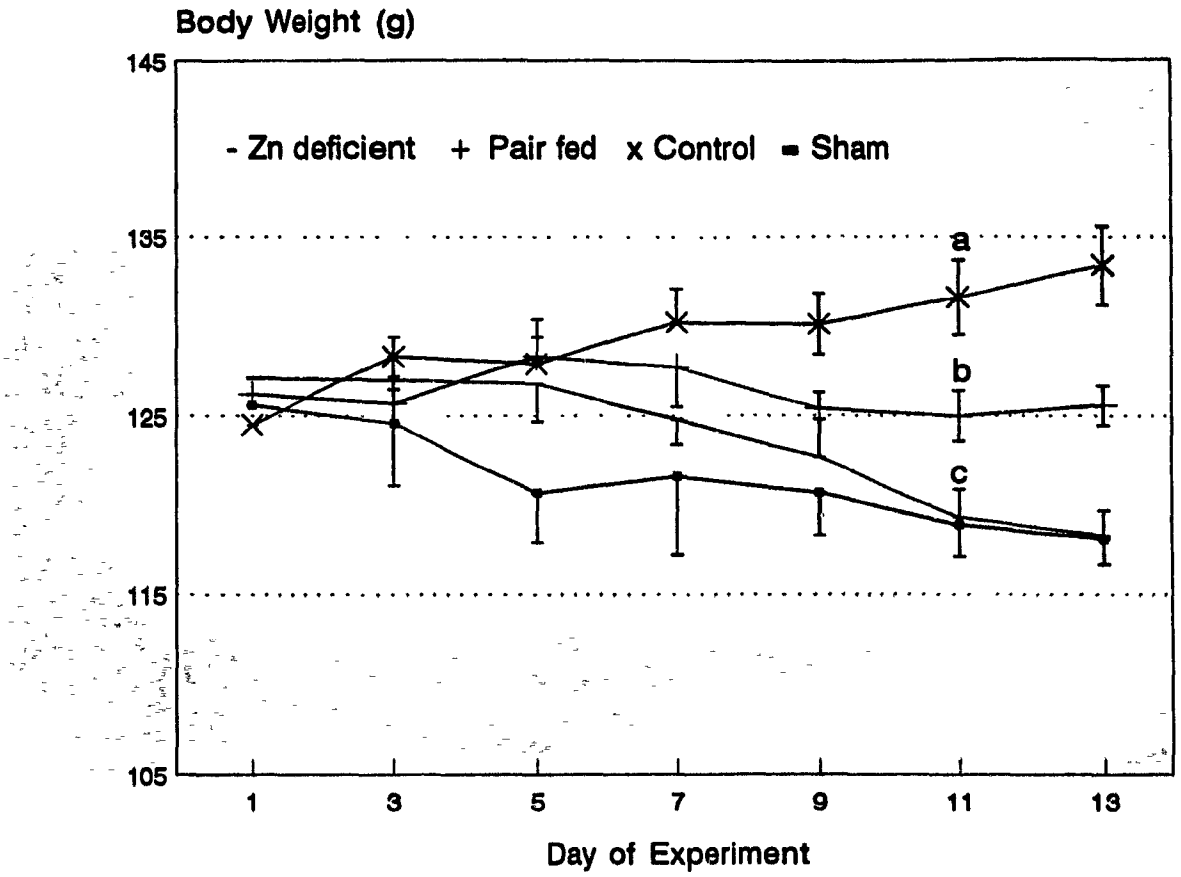


Figure 2.2. Effects of dietary zinc and hIGF-I subcutaneous infusion on cumulative body weight gain. Each data points (means ± SEM) represents the mean of individual observations for a group of 5-7 rats. Means on the same day with different letter superscripts differ significantly ($p<0.05$).

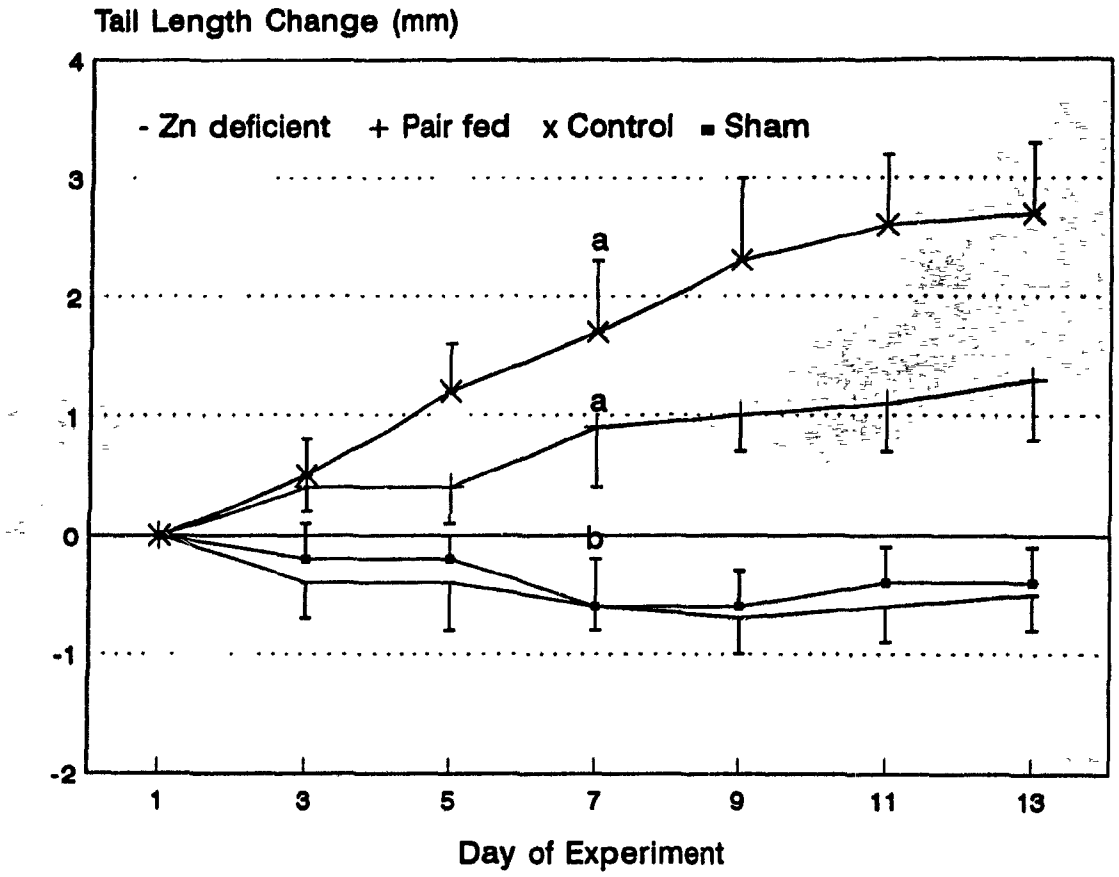


Figure 2.3. Effects of dietary zinc and hIGF-I subcutaneous infusion on cumulative tail length change in hypophysectomized rats. Each data points (mean ± SEM) represents the mean of individual observations for a group of 5-7 rats. Means on the same day with different letter superscripts differ significantly ($p<0.05$).

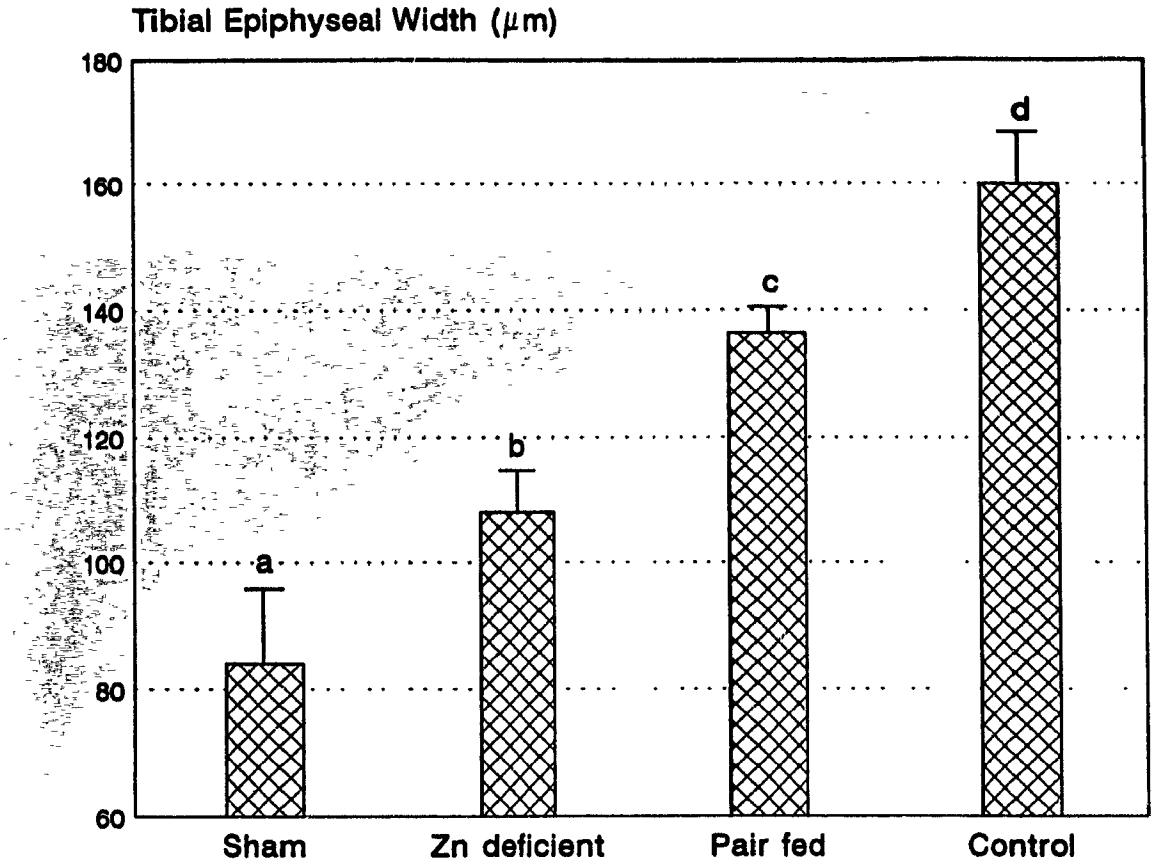


Figure 2.4. Effects of dietary zinc and hIGF-I subcutaneous infusion on tibial epiphyseal cartilage width in hypophysectomized rats. Values are means ± SEM for 5-7 rats. Means with different letter superscripts differ significantly ($p<0.05$).

IV. DISCUSSION

Although the primary actions of IGF-I might occur via paracrine or autocrine mechanism close to its site of production, many studies have shown that systemically administrated IGF-I could promote body weight gain and bone growth in hypophysectomized rats. Schoenle et al (1982) were the first to attempt to demonstrate that IGF-I could mimic the effects of GH in hypophysectomized rats. A purified human IGF-I infused over a period of 6 days stimulated body weight gain to a maximum of 7 grams and increased tibial epiphyseal width by 168% compared with controls. Guler et al (1988) administered recombinant hIGF-I to hypophysectomized rats for a period of 18 days, leading to a dose-dependent weight gain of 13.6 g with 300μg of hIGF-I per day. Tibial epiphyseal width widened up to 297 μm as compared with 135 μm of untreated hypophysectomized rats. The authors concluded that recombinant hIGF-I was a potent anabolic and growth promoting hormone in hypophysectomized rats in the absence of GH. The current study confirmed their findings. In the present study treatment with hIGF-I greatly increased the tibial epiphyseal cartilage width of C animals. During the 9-day pre-infusion period, rats in all treatment groups consistently lost weight. Continuous infusion of hIGF-I increased the body weight and tail length of C animals although the magnitude of this increase was small. In contrast, weight loss continued in S animals through the infusion period. An interesting finding was that food intake was not higher in C than in S groups. Despite similar food intake there was a 16 g difference in weight change between C and S groups. This is consistent with the

findings of Skottner and coworkers (1987), who reported a small but significant increase in weight gain after infusion of hIGF-I for 8 days at a daily dose of 180 μg, while food consumption remained unchanged. Salter et al (1957) showed that the weight gain obtained by treatment of hypophysectomized rats with insulin could largely be ascribed to an increase in their food intake. Therefore, it was supposed that since large doses of IGF-I have a pronounced insulin-like effect when given systemically, it is possible that IGF-I may affect some parameters of growth by an insulin-like action by way of stimulating food intake. The findings from present work do not support this contention. It appears that the anabolic effect of IGF-I increased the feed efficiency (weight gain/food intake) in hypophysectomized animals.

Previous studies have demonstrated that systemic administration of IGF-I had different effects on organ growth (Glasscock et al, 1992; Skottner et al, 1989). Marked weight gains were reported in kidney, spleen and adrenal following IGF-I administration. In the present study we couldn't show an increase in liver weight in response to IGF-I infusion. This is consistent with the findings of Guler et al (1988), who also did not find a difference in liver weight between IGF-I and saline treated rats. The exact mechanism for this different growth effect of IGF-I on organ growth is unknown.

Prasad and coworkers (1969) reported experimentally induced zinc deficiency in hypophysectomized rats by maintaining these animals on a low zinc diet for 2 weeks. Zinc content of bone decreased and activities of zinc dependent enzymes were diminished. However, no pair-fed control group was included in their study, and thus

the results might be confounded by the combined effect of lack of zinc in the diet and decreased food intake. The hypophysectomized animals in the present study were maintained on a zinc-deficient diet for a similar length of time. Anorexia, cyclical pattern of food intake and alopecia occurred within 5-6 days after the beginning of the feeding trial, comparable to what was reported in zinc deficient non-hypophysectomized growing rats (Swenerton and Hurley, 1968). The decreased zinc concentrations of the plasma, liver and tibia further confirmed the zinc deficiency.

Administration of hIGF-I had no effect on the measured indices of zinc status except for the liver zinc concentrations, which were significantly lower in C group as compared to PF group. Prasad et al (1969) demonstrated that hypophysectomized rats had a greater hepatic zinc content than non-hypophysectomized rats of similar age while bone zinc was greater in the non-hypophysectomized rats. GH administration decreased the zinc content of liver and bone in the hypophysectomized rats (Prasad et al, 1969). Since systemic IGF-I mediates at least some of the actions of GH on skeletal and other tissue growth, it is reasonable to postulate that liver zinc was mobilized and released to support the needs of other growing tissues.

Growth retardation is a prominent feature of zinc deficiency observed in man and animals. Root et al (1969) demonstrated the association of zinc deficiency and low serum levels of GH, suggesting that the depressed growth may be due, in part, to GH deficiency. However, later studies found that GH treatment of zinc deficient rats did not increase body weight gain or skeletal growth (Dicks et al, 1992; Oner et al, 1984; Prasad et al, 1969), providing evidence against the possibility that growth retardation

in zinc deficiency was due to decreased GH production.

Cossack (1984; 1988) was the first to examine the effect of dietary zinc deficiency on the circulating levels of IGF-I. Decreased plasma concentration of IGF-I was found in zinc deficient rats. Adequate zinc was required in the refeeding diet of previously starved rats to restore plasma IGF-I levels to normal. Our previous work (Dicks et al, 1992) and that of Oner et al (1984) have shown that GH administration fails to reverse the declined plasma IGF-I and the retarded growth. It was postulated that the growth retardation associated with zinc deficiency might be related to the adverse effect of zinc deficiency on the generation of circulating IGF-I. The results of current work do not support this hypothesis. Supplementation of IGF-I to zinc deficient rats could not promote growth, indicating that the decreased circulating IGF-I concentrations in zinc deficient rats may not be responsible for the retarded growth. Similar results were found in protein-restricted rats by Thissen et al (1991), who demonstrated that despite the normalization of serum IGF-I concentrations by 300 μg/day of hIGF-I infusion, body weight of the protein-restricted rats was not increased. The authors found that the decreased serum IGF-binding protein-3 (IGFBP-3) was restored to normal by IGF-I infusion. Similar finding was found by Zapf et al (1989), who reported increased plasma IGFBP-3 in hypophysectomized rats by IGF-I infusion. IGFBP-3 is the principal carrier protein of IGF-I in the serum. It modified the biological effects of IGF-I by control of clearance from plasma (Cohen and Nissley, 1976), transport to tissues and interaction with IGF receptors at the cell surface (Clemmons et al, 1986). Since protein synthesis is impaired in zinc deficiency

(Underwood, 1977), it is possible that the generation of IGFBP-3 is decreased by zinc deficiency and results in impaired biological action of IGF-I.

Despite the fact that the present work does not reveal the exact mechanism(s) for the impaired action of IGF-I in zinc deficiency, the results of this study provide evidence against the possible usage of IGF-I in the treatment of growth retardation in zinc deficient children without prior zinc supplementation.

In the preliminary trial to determine the appropriate dose of hIGF-I, all the zinc deficient rats receiving 250 μg or 300 μg daily of hIGF-I died within 2 -3 days while the control animals treated with the same dosage of IGF-I were healthy. Since hypoglycaemia has been reported following high doses of IGF-I administration and could be fatal in hypophysectomized rats (Zapf et al, 1989), we supplemented the drinking water of ZD rats with 10% glucose, but the condition of the animals remained unchanged. Our observations indicate that there is an interaction between zinc deprivation and hIGF-I supplementation. It is reasonable to hypothesize that the limited zinc supply in the zinc deficient animals is used for the vital life processes other than new tissue synthesis. The strong anabolic action caused by high dosage of IGF-I infusion interrupts this zinc homeostasis and leads to death.

In summary, the current work confirms the previous findings that systemically administered IGF-I can promote body growth. Systemic administration of IGF-I failed to promote growth of zinc deficient animals, suggesting that the growth promoting effect of circulating IGF-I was impaired by zinc deficiency.

CONNECTING STATEMENT

The study in Chapter II demonstrated that systemic supplementation of hIGF-I to zinc deficient hypophysectomized rats failed to promote growth, indicating that the growth promoting action of circulating IGF-I, which is an endocrine mediator of the growth promoting action of GH, was impaired by zinc deficiency. Evidence in literature indicates that in addition to the IGF-I transported in serum, another important route of regulation of growth by GH-IGF-I axis is the direct growth effect of GH, which is primarily mediated by locally produced IGF-I. The following study investigated whether the direct growth promoting effect of GH on longitudinal bone growth was also impaired by dietary zinc deprivation.

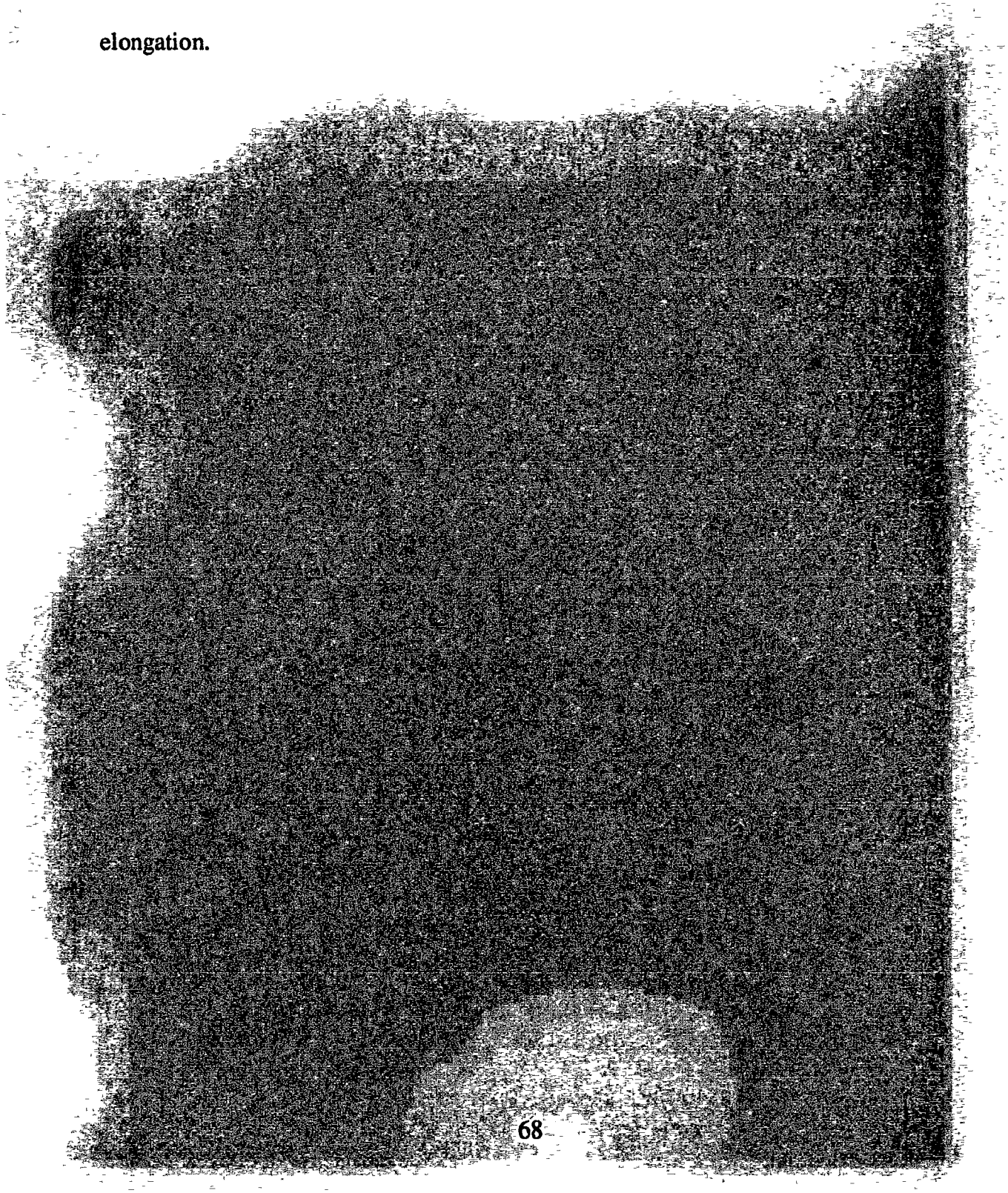
CHAPTER III.

ZINC DEFICIENCY INHIBITS THE DIRECT GROWTH EFFECT OF GROWTH HORMONE ON THE TIBIA OF HYPOPHYSECTOMIZED RATS

ABSTRACT

The effect of zinc deficiency on the direct growth effect of growth hormone on tibia growth in hypophysectomized rats was studied. There were three dietary groups: zinc deficient (0.9mg zinc/kg diet), control (66mg zinc/kg diet) and zinc adequate pair-fed groups (66mg zinc/kg diet). All rats in each group received local infusion recombinant human growth hormone (1μg/day), except for half of the animals in the control group which were sham treated receiving vehicle infusion only. The substances were infused continuously for 13 days by osmotic minipumps through a catheter implanted into the right femoral artery. Food intake was lower and body weight loss was greater in deficient and pair-fed animals as compared with control animals ($p<0.001$). Tissue zinc content and plasma alkaline phosphatase activity were decreased ($p<0.05$) by dietary zinc deficiency. Growth hormone infusion increased the tibial epiphyseal width of the treated right limb but not of the non-infused left limb in control

and pair-fed animals. However in zinc deficient rats, no difference was found between the infused and the non-infused limbs. These results demonstrate that zinc is an essential element for the expression of growth hormone effect in the process of bone elongation.



I. INTRODUCTION

The key role of pituitary growth hormone (GH) in the control of skeletal growth of most mammals has long been confirmed. Congenital failure to synthesize and secrete GH results in dwarfism whereas overproduction during early life leads to gigantism (Hadley 1992). Nevertheless, Salmon and Daughaday (1957) found that most of the somatogenic effects of GH may be mediated indirectly through the action of a single chain polypeptide, since then named insulin-like growth factor-1 (IGF-1). It is believed that pituitary GH acts on liver to synthesize and secrete IGF-1. The latter is then transported via the blood circulation to target tissues to mediate the somatogenic actions of GH. While the growth promoting effect of circulating IGF-1 is well established (Guler et al.1988, Schoenle et al.1982), D'Ercole and coworkers (1984) had shown that IGF-1 can be produced within many tissues in addition to liver. Specific IGF-1 mRNA has been detected widely among tissues both in human fetus (Han et al.1988) and rats (Murphy et al.1987). Isgaard et al. (1988) demonstrated that GH regulated IGF-1 mRNA levels in the bone growth plate of rats. By way of unilateral infusion of GH into the arterial supply of hind limb in hypophysectomized rats, Schlechter and co-workers (1986) clearly showed a direct growth effect of GH on longitudinal bone growth which is potentially mediated by locally produced IGF-1. It is suggested that this locally produced IGF-1 stimulates cartilage growth via

paracrine/autocrine mechanism and may be functionally more important than circulating IGF-1 for the somatogenic effect of GH on bone growth (Isaksson et al.1987).

Growth retardation is among the first signs of zinc deficiency in both animals and humans (Prasad 1977). One of the possible mechanisms is the adverse effect of zinc deficiency on the endocrine system of growth regulation. It has been shown that zinc deficiency decreases circulating levels of GH both in humans (Collipp et al. 1982) and in rats (Root et al. 1979). Zinc supplementation of zinc deficient rats increases the growth rate while bovine GH administration is without effect (Prasad et al. 1969). Zinc deficiency has also been shown to decrease plasma level of IGF-I (Cossack 1984). Although GH is considered the major regulator of IGF-1, administration of GH to zinc deficient rats cannot reverse the declined circulating levels of IGF-1 (Dicks et al. 1993, Oner et al.1984).

All the previous studies have focused on the effect of zinc on the circulating levels of GH and IGF-1 and their relationship with growth. These studies have not distinguished between the growth promoting effect of circulating IGF-1 and the direct growth effect of GH. The present study was undertaken to investigate the effect of zinc deficiency on the direct growth promoting effect of GH on longitudinal bone growth while excluding the growth effect of circulating IGF-1.

II. MATERIALS AND METHODS

Animals and diets. Male Sprague-Dawley hypophysectomized rats were purchased from Charles River Inc. (Montreal, PQ, Canada). Hypophysectomy was performed when the body weight of the rats was in the range of 150-160 g. The rats were received in our animal care facility five days after hypophysectomy. Upon arrival the rats were weighed and housed individually in stainless steel hanging cages in a temperature (24±1°C) and light-controlled (12h light, 0600-1800h) room. All aspects of animal handling were approved by the animal care and use committee of McGill University.

All rats were given free access to Purina rat chow and a 5% glucose solution for the first seven days. On day eight, two days before the catheterization, the rats were fed a powdered semi-purified diet (control diet). The composition of the control and the low zinc diets is shown in **Table 3.1.** Both diets were prepared in our laboratory. The low zinc diet contained 0.9 mg of zinc per kg of diet, while the control diet was the same composition but enriched with zinc carbonate to contain 66 mg zinc per kg of diet. Zinc content of the experimental diets was ascertained by atomic absorption spectrophotometry. Rats gaining more than 2 g during the pre-catheterization period were excluded from the study. On day 10, forty-four rats were selected and randomly divided into three dietary groups and one sham group. The femoral artery of all rats was catheterized and infusion of the solutions were started.

The rats in the zinc deficient group (ZD) were fed the low zinc diet containing 0.9 mg zinc/kg of diet. The pair fed (PF), control (C) and sham (S) groups received the control diet. The rats in the PF group were given the same amount of food as their zinc deficient counterparts ate the previous day, whereas the rats in the C and S groups were allowed free access to food. Deionized distilled water was available from plastic bottles ad libitum. Food intake was recorded daily. Body weight was measured on the day that the animals were divided into dietary groups before the operation and the day they were sacrificed.

Hormone and vehicle solutions. Bacterially produced recombinant human growth hormone (hGH) (Genotropin, 3IU/mg, generously supplied by Kabi Pharmacia AB, Stockholm, Sweden) was dissolved in a solvent described by Schlechter et al (1986). It consisted of 1.6% glycerol, 0.02% sodium azide and 100 USP U/ml porcine sodium heparin (Sigma Chemical Co., St. Louis, MO). The solution was adjusted to pH 8.0 with NaOH.

Growth hormone infusion system. The Alzet osmotic minipump (model 2002, Alza, Palo Alto, CA) was used. According to the specifications of the pump, the solution was prepared to deliver 1 µg per day of hGH. The catheter was prepared from a 4 cm piece of intramedic PE-10 tubing (Fisher-Scientific, Montreal, PQ) which was stretched at the distal end to fit into the vessel. The proximal end was inserted into a 5 mm piece of PE 50 tubing which was then inserted into a 5 mm piece of PE 60 tubing. The free end of the PE 60 tubing was connected to the flow moderator of the pump. The mini pumps and their connected catheters filled with hormone or vehicle

solutions were incubated at 37°C in saline for 8 hrs. before catheterization. The catheterization technique was modified from the method described by Nilsson et al (1987). The animals were anesthesized with sodium pentobarbital. The proximal medial part of the right hind limb was shaved and a 12 mm skin incision was made. The femoral artery was identified and dissected free from the femoral vein and nerve. A small hole was made in it about 8 mm below the abdominal wall. The tip of the stretched catheter was carefully inserted into the artery. The catheter was fixed in place with 4-0 silk to adjacent tissue. A drop of nexaband (cyanoacrylate glue, MTC Pharmaceuticals, Cambridge, Ont.) was used in the catheterization spot to further secure the catheter at the point of entry into the vessel and prevent blood leakage. The pump was then placed subcutaneously in the right side of the abdominal region. The skin was sutured with 3-0 silk. By way of this infusion system rats in ZD, PF and C groups received hGH infusion for thirteen days while rats in S group were sham treated receiving infusion of vehicle solution for the same period. At the end of the infusion period, the rats were sacrificed, the pumps and catheters were removed and inspected. About 43% of the animals were excluded from the study because of presence of coagulated blood in the catheters. The implantation and catheterization sites showed no signs of inflammation.

Tissue collection and analytical methods. On day 13 of the infusion trial, all rats were killed by decapitation. They were anaesthetized with ether. Blood was drawn by cardiac puncture and immediately centrifuged. Plasma zinc concentrations were determined by flame atomic absorption spectrophotometry (Perkin-Elmer Model 3100,

Perkin Elmer (Canada) Ltd., Montreal, PQ) after dilution with distilled deionized water. Plasma alkaline phosphatase activity (U/L) was determined by the hydrolysis of p-nitrophenyl phosphate (Sigma Chemical Co., St. Louis, MO) and read on the Abbott VP Super System discrete analyzer (Abbott Diagnostic, Mississauga, ON). The hind part of each animal was removed. Tibias and femurs from both legs were carefully dissected free from flesh. Femurs were freeze dried to a constant weight and wet ashed with nitric acid according to the method of Clegg et al (1981). Femur zinc concentrations were determined using flame atomic absorption spectrophotometry. The tibial epiphyseal growth plate width was determined as previously described (Dicks et al, 1993). Plasma GH was determined with a radioimmunoassay kit (Diagnostic Products Corporation, Los Angeles, CA).

Statistics. Unbalanced data was analyzed by a two way ANOVA (dietary zinc level and hGH treatment) using a SAS general linear model program (SAS Version 6, SAS institute Inc., Cary, N.C.). Unpaired t-test was used to differentiate means between treatment and control groups. Differences between means were considered to be significant at $p<0.05$. The data in the text are means ± SEM.

III. RESULTS

Dietary zinc had a significant effect on the food intake of hypophysectomized rats($p<0.001$). Food intake was 31% lower ($p<0.001$) in the ZD than in C animals (**Table 3.2**). There was no difference in food intake between the PF and ZD animals. Right hindlimb hGH arterial infusion had no effect on the food intake($p=0.12$). The differences in the body weight on the day of catheterization before the operation and on day 13 of the infusion trial are also given in Table 2. The body weights of animals in all dietary groups decreased continuously throughout the experimental period. There was no difference ($p=0.65$) between hGH infused C and sham treated animals, suggesting that no somatogenic effect of GH on body growth was produced by arterial infusion of 1 μg of hGH daily. On the other hand, body weight loss was significantly higher in ZD rats (11 times more, $p<0.001$) than in C animals. Even though the difference in body weight of ZD and PF animals was not statistically significant, the smaller magnitude of weight loss in PF rats as compared with ZD animals suggested that zinc may play a role independent of food intake in keeping body mass of hypophysectomized animals. Dietary zinc, food intake and hGH treatment had no effect on dry femur weight of the rats in this study (Table 3.2).

Tibial epiphyseal cartilage width was measured in this study to examine the effect of dietary zinc on the direct growth effect of hGH on tibia growth of the experimental animals (**Figure 3.1**). Vehicle infusion for 13 days had no effect on tibia

growth in S animals (96.5 ± 3.1 vs 99.0 ± 6.8 μm for the right and left leg, respectively; p=0.82). Infusion of 1 μg of hGH daily into the right femoral artery of C animals for the same period of time significantly increased the tibial epiphyseal width of the infused leg (138.5 ± 7.7 μm) as compared with the non-infused left leg (105 ± 8.0 μm, p<0.01) and the sham treated leg (p<0.001) of S animals. No difference was found (p=0.59) between the non-infused left leg and the left leg of S rats. This suggested that no systemic growth effect of hGH on tibia growth existed. Similar results were obtained with PF animals (116.2 ±2.2 vs 91.3 ± 3.9 μm for the right and left leg, respectively; p<0.02), except that the difference between the infused leg and the sham treated leg of S animals was not statistically significant (p=0.07). Zinc deficiency inhibited the direct growth effect of hGH on tibia growth. Tibial epiphyseal cartilage width of the hGH infused leg and the non-infused control leg in ZD animals were not significantly different (114.2 ± 9.5 vs 108 ± 11.7 μm for right and left leg, respectively; p=0.56). In addition, no difference was seen (p=0.10) between the hGH infused leg of ZD rats and the sham treated leg of S rats. Dietary zinc did not significantly affect plasma GH concentrations (p=0.54) (**Table 3.3**). When data were pooled across dietary groups, plasma GH level of hGH treated animals was significantly higher than sham treated animals (p<0.05).

To examine the effect of dietary zinc and local infusion of hGH on the zinc status of experimental animals, plasma zinc, femur zinc and plasma alkaline phosphatase activities were determined (**Table 3.4**). Plasma zinc was significantly decreased (p<0.05) in ZD rats as compared to the PF and C animals. No difference

existed between PF and C animals, suggesting that decreased food intake did not affect plasma zinc concentrations. Local hGH infusion had no effect on femur zinc concentrations ($p=0.90$). When femur zinc concentrations were pooled across infused and non-infused control legs, a significantly lower ($p<0.0001$) concentration of zinc was found in the femur of ZD animals as compared to PF and C animals. No difference was found between PF and C animals ($p=0.91$). Plasma alkaline phosphatase activities were significantly different ($p<0.05$) among the three dietary groups with the lowest values in the ZD, highest values in the C and intermediate values in the PF animals, suggesting that dietary zinc and food intake both affected this parameter. In addition to laboratory analysis, clinical signs of zinc deficiency, such as cyclic eating pattern and alopecia were apparent in ZD animals five days after feeding the deficient diet.

TABLE 3.1

Composition of the Experimental Diets

Ingredient	(-) Zn diet	(+) Zn diet
	g/kg diet	
Egg white[1]	150	150
Corn oil[1]	70	70
Cornstarch[1]	320	320
Sucrose[1]	140	140
Cellulose[1]	270	270
Choline bitartrate[1]	2	2
Biotin[1]	0.02	0.02
AIN-76 Mineral mix[1,2,3]	35	35
AIN-76 Vitamin mix[1,4]	10	10
BHT[1]	0.007	0.007
Zinc carbonate[5]	--	0.09

[1] ICN Biomedicals Inc., Cleveland, OH.

[2] Special mineral mix without zinc.

[3] Provided (g/kg mineral mix): calcium phosphate dibasic, 500; sodium chloride, 74.0; potassium citrate monohydrate, 220; potassium sulfate, 52.0; magnesium oxide, 24.0; manganous carbonate, 3.50; ferric citrate, 6.0; cupric carbonate, 0.30; potassium iodate, 0.01; sodium selenite, 0.01; chromium potassium sulfate, 0.55; sucrose, finely powdered, 118.

[4] Provided (mg/kg vitamin mix): thiamin hydrochloride, 600; riboflavin, 600; pyridoxine hydrochloride, 700; nicotinic acid, 3000; D-calcium-pantothenate, 1600; folic acid, 200; D-biotin, 20; cyanocobalamin, 1.0; retinyl palmitate, 1600; DL-alpha tocopherol acetate, 20,000; cholecalciferol, 250; menaquinone, 5.0; sucrose, 972.9 (g/kg vitamin mix).

[5] Fisher Scientific, Montreal, PQ.

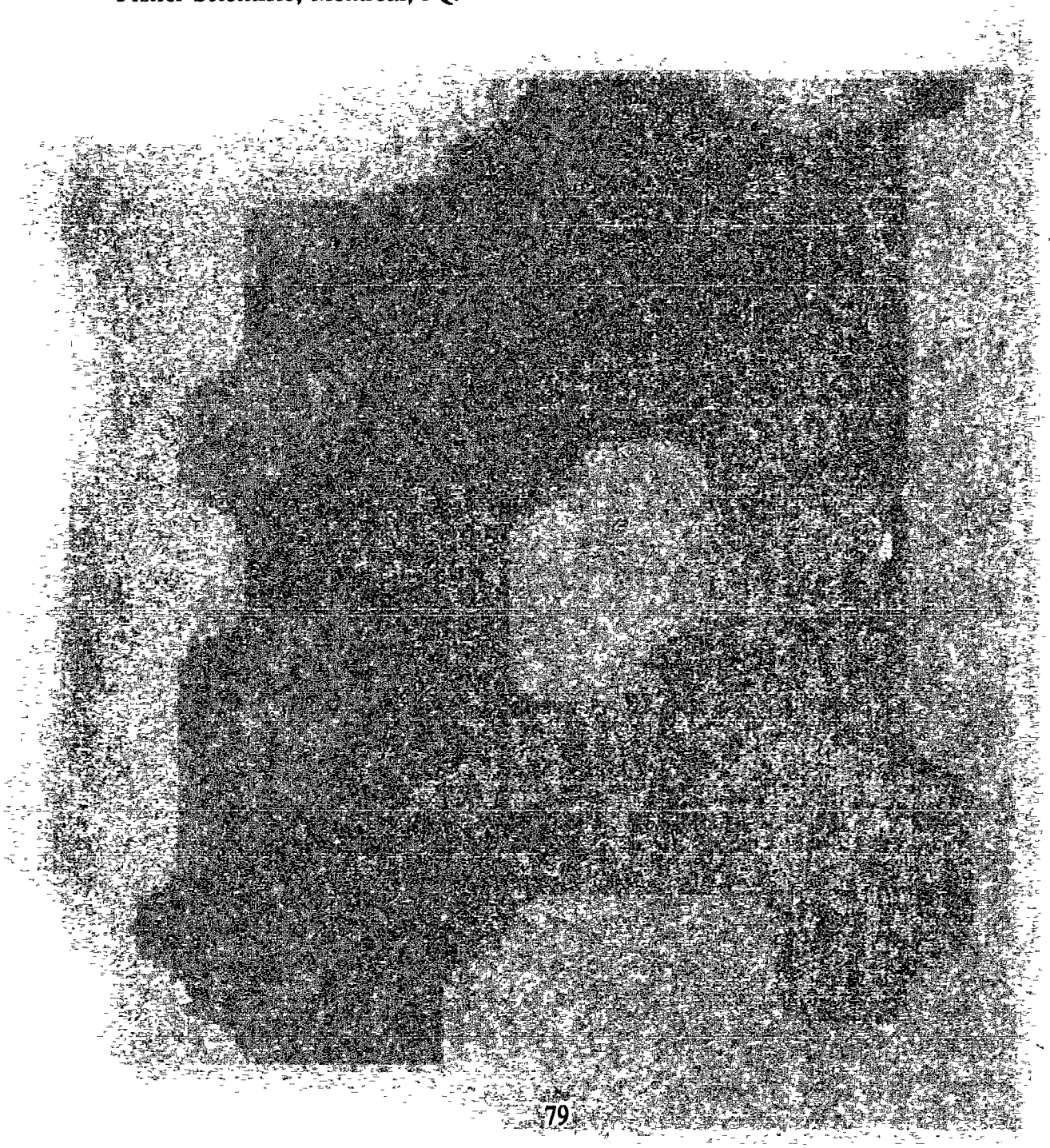


TABLE 3.2

Effects of dietary zinc and hGH right femoral artery infusion on food intake body weight change and femur weight in hypophysectomized rats [1,2]

	Zn deficient	Pair fed	Control	Sham
Cumulative food intake (12 days g)[3]	79.7 ± 5.7[a]	78.0 ± 4.2[a]	115.2 ± 8.3[b]	99.2 ± 1.8[b]
Body weight change (g)[4]	-19.8 ± 4.1[a]	-11.0 ± 2.9[ab]	-1.7 ± 3.2[b]	-4.3 ± 1.3[b]
Femur weight (g dry wt)[5]				
Right	0.29 ± 0.01	0.27 ± 0.01	0.29 ± 0.01	0.30 ± 0.02
Left	0.29 ± 0.01	0.28 ± 0.01	0.30 ± 0.01	0.30 ± 0.02
Pool	0.29 ± 0.01[a]	0.28 ± 0.004[a]	0.30 ± 0.02[a]	0.30 ± 0.05[a]

[1] Values are means ± SEM; there were 5-7 rats in each treatment group.

[2] Means within each parameter with different letter superscripts differ significantly ($p<0.05$).

[3] ANOVA: zinc ($p<0.001$); hGH ($p=0.12$).

[4] ANOVA: zinc ($p<0.01$); hGH ($p=0.65$).

[5] ANOVA: zinc ($p=0.25$); hGH ($p=0.85$)

TABLE 3.3

Effects of dietary zinc and hGH right femoral artery infusion on plasma GH in hypophysectomized rats [1,2,3]

	Plasma GH
	ng/ml
Zn deficient	3.9 ± 0.4[a]
Pair fed	3.9 ± 0.2[a]
Control	3.2 ± 0.7[ab]
Pool	3.6 ± 0.3[a]
Sham	2.3 ± 0.5[b]

[1] Values are means ± SEM; there were 5-7 rats in each treatment group.

[2] Means with different letter superscripts differ significantly ($p<0.05$).

[3] ANOVA: zinc ($p=0.54$); hGH ($p=0.22$).

TABLE 3.4

Effects of dietary zinc and hGH right femoral artery infusion on zinc status in hypophysectomized rats[1,2]

	Zn deficient	Pair fed	Control
Plasma zinc (μg/ml)[3]	0.83 ± 0.11[a]	1.19 ± 0.09[b]	1.18 ± 0.1[b]
Femur zinc (μg/g dry wt)[4]			
Right	196.1 ± 6.8	226.3 ± 2.5	225.6 ± 6.2
Left	196.8 ± 7.1	225.0 ± 5.2	224.4 ± 5.2
Pool	196.4 ± 4.7[a]	225.7 ± 2.7[b]	225.0 ± 3.8[b]
Alkaline phosphatase (U/L)[5]	73.1 ± 5.1[a]	103.0 ± 7.2[b]	140.1 ± 19.3[c]

[1] Values are means ± SEM; there were 5 -7 rats in each treatment group.

[2] Means within each parameter with different letter superscripts differ significantly ($p<0.05$).

[3] ANOVA: zinc ($p<0.05$).

[4] ANOVA: zinc ($p<0.001$); hGH ($p=0.90$); zinc*hGH ($p=0.98$).

[5] ANOVA: zinc ($p<0.001$).

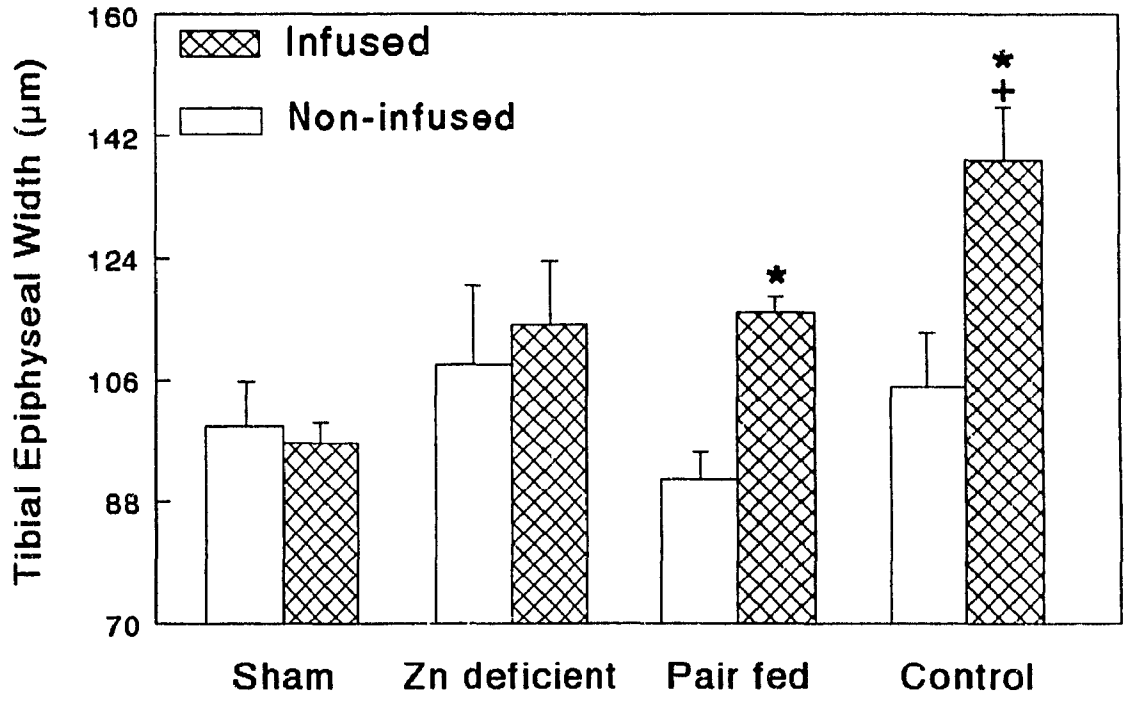


Figure 3.1. Effects of dietary zinc and hGH right femoral artery infusion on tibial epiphyseal cartilage width in hypophysectomized rats. Values are means ± SEM for 5-7 rats. * $p<0.05$ compared with non-infused leg; + $p<0.05$ compared with sham treated leg.

IV. DISCUSSION

Experimentally induced zinc deficiency in hypophysectomized rats is rarely seen in literature. Prasad et al. (1969) reported successful induction of zinc deficiency in hypophysectomized rats within two weeks, as evidenced by decreased zinc content in bone and lowered activities of zinc-dependent enzymes at the end of the experiment. The present study confirms the results of Prasad and his coworkers. Although the rats in ZD group were maintained on zinc deficient diet for only 13 days, the data on food intake, body weight change and zinc status indicated that we were able to induce zinc deficiency in these animals.

Symptoms of zinc deficiency were rapid in onset in ZD rats. Anorexia, cyclic feed consumption pattern and hair loss were observed within the first 5-6 days and continued for the remainder of the study. These findings are similar to what has been reported in zinc deficient non-hypophysectomized growing rats (Bolze et al. 1987, Dicks et al. 1993).

The decrease in the body weight of the ZD rats appeared to be due to the anorexia accompanying zinc deficiency, since there was no significant difference between the body weights changes of PF and ZD animals. Our findings in this respect confirms our earlier observations in non-hypophysectomized zinc deficient rats (Dicks et al.1993) and is in agreement with those of Oner et al (1984), but is at variance with the results of Cossack (1986) and Bolze et al (1987) who reported a significant

decrease in weight gain per day for zinc deficient non-hypophysectomized rats compared to their pair fed counterparts. Both of these studies were longer in duration than the present investigation. Since the mean weight loss in PF animals was numerically lower than the ZD animals, an independent effect of zinc on maintenance of body weight of hypophysectomized rats could not be completely ruled out. Although plasma GH concentrations were significantly higher in GH infused rats compared to sham treated animals, no difference in body weight loss between C and S animals was found. This indicates that administration of 1µg hGH daily is insufficient to promote growth in hypophysectomized rats.

Local administration of hGH and dietary treatment had no effect on femur weight in present study. This is in agreement with the observation of Nilsson et al (1987), who also couldn't show differences between femur weight of the infused and non-infused limbs after continuous local infusion of hGH for two weeks.

Food restriction in PF animals did not decrease the plasma and femur zinc concentrations compared to C animals. This is in contrast with our earlier observations in non-hypophysectomized rats (Dicks et al.1993) and results reported by other investigators (Bolze et al. 1987, Oner et al. 1984). It is possible that the zinc requirement for growth is dramatically decreased in hypophysectomized animals. As a result, the reduced dietary zinc supply accompanying restricted food intake in PF rats may be still within the limits necessary to maintain zinc homeostasis in these animals. The smaller magnitude of difference for both parameters between ZD and PF animals is apparently due to the short duration of this study.

Plasma alkaline phosphatase activity was decreased in ZD animals compared to PF and C animals. It appeared that the lack of zinc in the diet and the lowered food intake had equal impact on decreasing plasma alkaline phosphatase activity. Oner et al (1984) reported similar findings in non-hypophysectomized rats.

The hind limb arterial infusion approach used in this study is a novel system that permits delivery of a substance to one leg of an animal via its arterial blood supply, while the contralateral leg acts as a control. This delivery approach has been used in recent years by several laboratories to study the direct effect of GH on long bone growth. Nilsson et al (1987) demonstrated that local arterial infusion of GH stimulates unilateral longitudinal bone growth of hypophysectomized rats. This observation confirmed the earlier work of Schlechter et al (1986) who found that continuous arterial infusion of small doses of GH into the arterial blood supply of the right hind limb of hypophysectomized rats maintained tibial cartilage width following hypophysectomy. By direct injection of GH into the proximal tibial epiphyseal plate of hypophysectomized rats, Isaksson et al (1982) and Russel and Spencer (1985) also showed a unilateral tibial cartilage growth. Taken together these observations indicate that GH has direct local stimulatory effect on cartilage growth when delivered to the appropriate target cells. However, these findings do not clarify the precise mechanism(s) for the involvement of GH in the regulation of bone growth.

It is generally accepted that the liver produces and secretes IGFs in response to GH, and perhaps is the main organ contributing to the circulating pool of IGF-1 (Daughaday et al. 1975). However, in recent years it has been shown that a number

of nonhepatic tissues, both pre- and postnatally, produce IGF-1 like peptides (Clemmons and Vanwyk 1985, D'Ercole et al. 1980). The existence of IGF-I in epiphyseal chondrocytes of rats is regulated by growth hormone (Nilsson et al. 1986). By coinfusing antibodies to IGF-1, Schlechter et al (1986) demonstrated that the stimulatory effect of locally administered GH on the growth plate was completely abolished. These findings provide support for the idea that the direct effect of GH on bone growth is mediated by locally produced IGF-1.

Previous studies that have examined the efficacy of GH in alleviating the growth retarding effects of zinc deficiency have administered GH to non-hypophysectomized rats via the systemic circulation and attempted to measure the general growth indices and the circulating levels of IGF-1 (Oner et al. 1984, Prasad et al. 1969). All three studies including the most recent one from our laboratory (Dicks et al.1993) have consistently shown that GH treatment of zinc deficient rats does not increase body weight gain, skeletal growth or decreased circulating IGF-1 levels. However, in terms of endocrine regulating system of growth, whether the growth arrest associated with zinc deficiency is due to decreased circulating level of IGF-1, or impaired direct growth effect of GH, or both, still remains unknown.

The animal model and the infusion system used in this study enabled us to investigate the effect of zinc deficiency on the direct effect of GH on bone growth while excluding the systemic growth effect of circulating IGF-1. We conducted a preliminary trial to determine the appropriate dose of hGH. Infusion of 1 μg hGH daily via the femoral artery was found to be adequate to promote bone growth as evidenced

by the increase in the tibial epiphyseal width of the infused right leg as compared to the non-infused left leg. There was no difference betweer the tibial epiphyseal width of the non-infused left leg and the legs of the non-treated animals. Increasing the dose of hGH up to 3 or 5 µg daily significantly increased the tibial epiphyseal width of the non-infused control leg, similar to the previous reports by Isgaard et al (1986) and Nilsson et al (1987). Nilsson et al (1987) also found that systemic administration of 1 µg hGH daily could not stimulate longitudinal bone growth.

The width of the epiphyseal cartilage plate is believed to be an extremely sensitive indicator of GH activity (Geschwind et al. 1955). By way of measuring tibia epiphysial width, the direct growth effect of GH was clearly shown in C and PF animals in this study. Our results demonstrate that dietary zinc deficiency impaired the direct growth effect of GH on tibial growth. Epiphyseal cartilage width of hGH infused and non-infused legs in ZD rats were identical and lower compared to hGH treated leg in C animals. Since previous research has shown that the direct growth effect of GH on bone growth is mediated by locally produced IGF-1 (Schlechter et al. 1986), this strongly suggests that zinc deficiency impairs the production and / or action of locally produced IGF-1. Compared to zinc deficiency, food intake appears to have a greater impact on the tibial epiphysial width as shown by numerically lower values in both legs of PF rats compared to C animals. This is in agreement with Oner et al.'s (1984) and our earlier findings (Dicks et al. 1993).

We can conclude from present results that zinc deficiency inhibits the direct growth effect of GH. However, due to the limitation of this study design, we could not

investigate the exact mechanism(s) of zinc's action on the epiphyseal cartilage growth involving locally produced IGF-1. The advent of new methods (D'Ercole et al. 1984) for extracting and measuring tissue concentrations of IGF-1 makes it possible to study the regulation of this peptide after alterations in nutritional intake. Future research must focus on examining the effect of zinc deficiency on the content and activity of IGF-1 in the epiphyseal growth plate.

In conclusion, the results of this study supports the contention that GH stimulates longitudinal bone growth directly. Zinc deficiency inhibits the direct growth effect of GH on bone growth, which is most probably due to the impairment of production and/or action of locally produced IGF-1.

CONCLUSION

Pituitary GH is responsible for post natal growth. It is generally believed that GH does not have a direct effect on different growth processes but rather stimulates growth by its mediator IGF-I. Evidence in literature suggests that GH acts on liver to synthesize and release IGF-I. The latter is then transported via blood circulation to target tissues to mediate the somatogenic action of GH. Another way by which GH regulates growth is its effect on tissue growth without the action of plasma transported IGF-I. This direct growth effect of GH is thought to be mediated primarily by locally produced IGF-I. The study presented in Chapter II supports the view that circulating IGF-I can promote growth. Systemic supplementation of hIGF-I to hypophysectomized rats increased body weight, tail length and tibial epiphyseal width. We have also successfully demonstrated a direct growth effect of GH on the tibia of hypophysectomized rats while excluding the possible action of circulating IGF-I (Chapter III). The cartilage width of GH infused leg was significantly higher than the non-infused leg.

Zinc deficiency leads to growth retardation both in children and young animals. Previous studies have shown that zinc deficiency reduces both serum GH and endocrine IGF-I levels. Supplement of GH to zinc deficient rats fails to improve circulating levels of IGF-I and growth, suggesting the presence of GH resistance rather than GH deficiency. Our results demonstrated a resistance to endocrine IGF-I in zinc deficiency. Supplementation of exogenous IGF-I failed to promote growth in zinc deficient animals

(Chapter II). This finding is in contrast to the contention that the decreased circulating IGF-I levels are responsible for the retarded growth of zinc deficiency. Our investigations have further revealed that the direct growth promoting effect of GH is abolished by zinc deficiency. We have proved that in zinc deficiency GH resistance exists in the growth plate cartilage of bone (Chapter III). Although the exact mechanisms surrounding the blunted growth promoting actions of IGF-I and GH in zinc deficiency remain unknown, the results of present investigations provide evidence against the possible usage of IGF-I or GH for the correction of the retarded growth in zinc deficient children prior to sufficient zinc supplementation.

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