THE EFFECT OF GROWTH HORMONE TREATMENT ON GROWTH IN ZINC DEFICIENT RATS

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ABSTRACT

The ability of human growth hormone (hGH) to alleviate the effects of zinc deficiency on growth was investigated in the rat. Human GH treatment had no significant effect on food consumption, growth parameters or plasma IGF-I. Food consumption, tail length, liver weight, and tibia weight were significntly lower in the zinc deficient group. Body weight was significantly reduced in the zinc deficient and pair fed groups compared to the control. A significant interaction between zinc and hGH was found for tibial epiphyseal cartilage width but there were no significant differences between the groups receiving hGH and the respective shams. Plasma IGF-I was numerically lower in the zinc deficient rats compared to the pair fed rats but this difference did not reach statistical significance. Tissue zinc content and plasma alkaline phosphatase were significantly decreased by the dietary zinc deficiency. Plasma zinc was higher in the groups receiving hGH. Significant interactions between zinc and hGH was found for liver iron, tibial zinc, and tibial copper. Copper and iron showed a competitive interaction with zinc and were lower in the rats receiving the control diet. Both lowered zinc and food intake contributed to the effects of the zinc deficiency; however, these effects were not equally distributed. Food intake had the greater effect on growth and plasma IGF-I while tissue mineral content showed a greater effect for zinc intake.

RÉSUMÉ

On a étudié la capacité de la somatotrophine humaine à atténuer les effets d'une carence en zinc sur la croissance du rat. Un traitement à la somatotrophine humaine n'a eu aucun effet significatif sur la consommation d'aliments, les paramètres de croissance ou l'IGF-I. La consommation d'aliments, la longueur de la queue, le poids du foie et le poids du tibia étaient nettement inférieurs dans le groupe qui affichait une carence en zinc. Le poids corporel était nettement plus bas chez les sujets souffrant d'une carence en zinc et chez les animaux témoins recevant une alimentation identique par rapport au groupe témoin de l'étude dont les animaux étaient nourris à volonté. On a noté une interaction significative entre le zinc et la somatotrophine pour ce qui est de la largeur du cartilage épiphysaire du tibia, mais on n'a observé aucune différence significative entre les groupes recevant de la somatotrophine humaine et les sujets témoins qui leur étaient appariés. Le taux d'IGF-I plasmatique était inférieur chez les rats présentant une carence en zinc et les rats nourris de façon identique, mais cette différence ne revêtait pas d'importance statistique. La teneur en zinc des tissus et la phosphatase alcaline plasmatique ont affiché une diminution marquée liée à une alimentation pauvre en zinc. Les taux de zinc plasmatiques étaient plus élevés dans les groupes qui recevaient de la somatotrophine humaine. On a noté des interactions significatives entre le zinc et la

somatotrophine humaine au niveau du fer hépatique, du zinc du tibia et du cuivre du tibia. On a observé une interaction compétitive du cuivre et du fer avec le zinc et ces taux ont été moins élevés chez les rats qui ont reçu le régime de référence. La réduction de l'apport de zinc ainsi qu'une diminution de la ration alimentaire ont contribué à la carence en zinc; toutefois, ces effets n'ont pas été répartis également. L'alimentation a été le facteur qui a le plus influé sur la croissance et l'IGF-I plasmatique tandis que la teneur en minéraux des tissus a fait apparaître un effet plus marqué de la prise de zinc.

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INTRODUCTION

Raulin (1869) first demonstrated the importance of zinc for a biological system when he reported that zinc was essential for the growth of Aspergillus niger. Sixty-five years later, Todd et al. (1934) demonstrated the essentiality of zinc for the rat. During the 1950's, Tucker and Salmon (1955) showed that zinc cured and prevented parakeratosis in swine while O'Dell et al. (1958) found zinc to be essential for the chicken. In the early 1960's, studies in the Middle East provided the first evidence for the occurrence of a nutritional deficiency of zinc in humans (Prasad et al., 1963). While the importance of zinc for growth and development has been demonstrated, the exact mechanisms have yet to be established. One possible mechanism is an adverse effect of zinc deficiency on the generation of insulin-like growth factor I (IGF-I), a growth factor believed to mediate some of the effects of growth hormone (GH) on longitudinal growth (Cossack, 1986; 1988). Growth hormone is also the major regulator of circulating IGF-I. The objective of the present study was to examine the efficacy of GH treatment to alleviate the adverse effects of zinc deficiency on growth in the rat.

CHAPTER I. LITERATURE REVIEW

OVERVIEW OF ZINC NUTRITURE

Health and Welfare Canada (1990) recently published the revised nutrition recommendations for Canadians. Recommended intakes for zinc were as follows: 12 mg/day for males, 9 mg/day for females, and 15 mg/day for pregnant and lactating females. Based on the zinc content of breast milk, 2 mg/day was suggested for infants aged 0-4 months. No firm recommendations were established for older children since available data was believed to be inadequate. Food sources of zinc include red meat, liver, egg yolk, shellfish, poultry, milk, cheese, and whole grains with zinc from animal sources being more readily available. The mixed Canadian diet provides approximately 5 mg of zinc per 1000 kcal with about one-half derived from meat, fish, and poultry (Srivistava et al., 1977). More recently, Smit Vanderkooy and Gibson (1987) showed an average zinc intake of 4.6±1.0 mg/1000 kcal for Canadian preschool children. The suboptimal zinc status of boys in this study was associated with decreased zinc intake from animal sources. While animal foods are important sources of zinc for affluent countries, less developed countries depend upon grains which have a high phytate and phosphate content (Prasad, 1988). Prasad stated that the "predominant use of cereal proteins by the majority of the world's population is an important predisposing factor for zinc deficien-

cy".

DISTRIBUTION

The estimated zinc content of a standard 70 kg man is approximately 1.5-2 g (Prasad, 1982). It is present in all organs, tissues, fluids, and secretions of the body with average zinc concentrations ranging from 1-150 μg/g wet weight (Jackson, 1989). Skeletal muscle contains the greatest portion of body zinc because of its large mass. Muscle and bone together account for more than 80% of the total body zinc. The liver, kidney, retina, and prostate also have high concentrations of zinc. Plasma zinc represents approximately 0.1% of total body zinc. Most plasma zinc is bound to albumin although other plasma proteins can bind significant amounts (Prasad, 1982; Jackson, 1989).

There is little information regarding the distribution of zinc within tissues and the nature of its intracellular binding (Jackson, 1989). Small amounts of zinc can be found within all organelles of the cell which corresponds to the wide distribution of zinc-containing enzymes. Within the cell, zinc appears to be bound to proteins with the affinity for zinc varying among the different proteins. It is unclear whether substantial amounts of free zinc or zinc bound to amino acids exist within the cell. The susceptibility of various subcellular fractions to zinc depletion is unknown.

ABSORPTION

Approximately 10-40% of dietary zinc is available for

absorption (Solomons, 1982). The exact sites of absorption are unknown although the majority probably occurs in the duodenum and ileum (Hambidge et al., 1986). Information on the mechanism of absorption, whether it be passive, active, or facultative transport, is also scarce (Prasad, 1982). Factors affecting zinc absorption include body size, zinc status, level of zinc in the diet, and presence in the diet of potential inhibiting substances such as phosphate, phytate, fiber, or chelating agents. Cousins (1979) proposed a four-phase model to explain the intestinal absorption of zinc: uptake by the intestinal cell, movement through the mucosal cell, transfer to the portal circulation, and secretion of endogenous zinc back into the intestinal cell.

Zinc absorbed in the intestine is carried to the liver in the portal plasma bound to albumin (Hambidge et al., 1986). The liver is the major organ involved in zinc metabolism and contains various zinc binding components of different molecular weights and lability, including metallothionein. About 30-40% of the zinc entering the liver is released back into the blood. The circulating zinc is then incorporated into various extrahepatic tissues. Entry of zinc into the cells occurs in two phases: an early, rapid uptake that is saturable and probably carrier mediated followed by a slower phase that is apparently passive.

EXCRETION

The feces are the major route of excretion for zinc

(Hambidge et al., 1986). A healthy adult who consumes 12-15 mg of zinc per day and is in zinc balance will excrete approximately 90% of this ingested zinc in the feces. Endogenous zinc can amount to 1-2 mg per day (Prasad, 1982) with a major contributor being pancreatic secretions (Hambidge et al., 1986). Urinary zinc loss is approximately 0.5 mg per day but can vary with the level of dietary intake (Prasad, 1982). It arises from the ultrafilterable portion of plasma zinc. Sweat is another route of zinc excretion and approximately 0.5 mg may be lost daily. This zinc loss can increase considerably under conditions of profuse sweating.

METABOLISM

The most widely known function of zinc is its involvement in enzyme function and structure (Hambidge et al., 1986; Prasad, 1982). Carbonic anhydrase was the first zinc metalloenzyme to be isolated and purified. Over 200 zinc enzymes or other proteins have been identified from various sources (Hambidge et al., 1986). Consequently, zinc plays an important role in the metabolisms of proteins, carbohydrates, lipids, and nucleic acids. Other zinc-containing enzymes include alkaline phosphatase, alcohol dehydrogenase, superoxide dismutase, carboxypeptidase, and RNA polymerase.

Zinc, both alone and as a metalloenzyme, plays a role in many functions in the body (Prasad, 1982; Hambidge et al., 1986). As an ionic species, zinc stabilizes biomembrane structures and polynucleotide conformation (Bettger and

O'Dell, 1981). The involvement of zinc in nucleic acid and protein metabolism makes it important for cell differentiation and replication (Prasad, 1982; Hambidge et al., 1986). Zinc participates in the production, storage, and secretion of hormones as well as in the effectiveness of receptor sites and end organs. In particular, zinc deficiency adversely affects testicular function and appears to be essential for spermatogenesis and testosterone synthesis. Zinc is important for the growth of both the skeleton and body tissues of young animals, including man, with growth retardation being one of the first signs of zinc deficiency. Recovery from malnutrition appears to be optimized with the addition of zinc to the diet (Golden and Golden, 1981). Zinc appears to be required for appetite and normal taste perception. Zinc is essential to the integrity of the immune system (Prasad, 1982; Hambidge et al., 1986). The predominant influence is on T-cell functions but other reported functions include thymic hormone production and activity, lymphocyte function, natural killer cell function, antibody-dependent cell-mediated cytotoxicity, immunological ontogeny, neutrophil function, and lymphokine production. Zinc is required for keratogenesis and normal integrity of the skin and it may also play a role in wound healing (Hambidge et al., 1986). Zinc is essential for normal sexual development of males and normal reproduction, from estrus to lactation, in females. The retina-choroid complex has the highest zinc concentration with zinc being

involved in several processes related to vision.

ZINC DEFICIENCY IN THE RAT

The NRC (1978) zinc requirement for the rat maintained in a zinc-free environment and fed a casein or egg white diet is 12 mg/kg for maximum weight gain. A severe deficiency will result with a diet containing less than 1 ppm (1 mg/kg) of zinc with deficiency signs occurring rapidly.

Reduced growth rate and feed intake are the first effects of zinc deficiency in the growing rat (Swenerton and Hurley, 1968; Williams and Mills, 1970). As the deficiency progresses, alopecia, loss of the hair follicles, and dermal lesions occur (Swenerton and Hurley, 1968). The lesions involve thickening or hyperkeratinization of the epit. elial cells and can also occur in the esophagus. Some deficient rats maintain the immature hair of the weanling. At the height of the deficiency state, the rat is extremely emaciated with an abnormal "kangaroo-like" posture. When less than 2 mg/kg of zinc is fed to females, a severe disruption of the estrus cycle occurs, and in most cases no mating with normal males occurs (Hurley and Swenerton, 1966). A dietary concentration of less than 0.5 mg/kg zinc fed to growing male rats arrested spermatogenesis, resulted in atrophy of the germinal epithelial, and reduced growth of the pituitary and accessory sex organs. Teratogenic effects can include hydrocephaly and other central nervous system malformations, cleft palate,

fused or missing digits, and urogenital abnormalities. Dietary intakes of zinc are reflected in the zinc concentrations of some tissues (e.g., blood, hair, bone, testes, liver) but others (e.g., brain, lung, muscle, heart) are insensitive to marked reductions or increases in zinc intake (Hambidge et al., 1986).

Rats given a zinc deficient diet will voluntarily restrict their food intake (Chesters and Quarterman, 1970; Chesters and Will, 1973). Day-to-day food intake varies and a cyclical pattern of eating develops (Chesters and Quarterman, 1970; Williams and Mills, 1970). This appears to be a necessary adaptation since force-feeding of zinc-depleted rats with 140% of their voluntary intake rapidly causes signs of ill health (Chesters and Quarterman, 1970). The reasoning behind the anorexia remains uncertain. It may be the result of a zinc-protein interaction since zinc deficient rats will selectively choose a diet with the lower protein content (Chester and Will, 1973; Reeves and O'Dell, 1981). Decreased food intake may also be an adaptation to limit growth so the available zinc is used for more essential functions. Altered taste perception may also be involved. As a result, it becomes difficult to separate the effects due to lowered food intake with those of decreased zinc intake. To overcome this problem, pair feeding is usually included for control of food intake. However, this is not a "perfect" control since pair-fed animals develop a meal-eating pattern while the zinc-

deficient animals remain nibblers (Chesters and Quarterman, 1970; Wallwork et al., 1981). This altering of the frequency of food ingestion may result in differences in nutrient metabolism and body composition (Fabry and Braun, 1967; Leveille, 1972).

ZINC DEFICIENCY IN HUMANS

Zinc deficiency can be thought of as a continuum with mild or marginal deficiency on the left and severe deficiency on the right (Prasad, 1988). A severe zinc deficiency is the result of unusual circumstances such as a zinc-free parenteral feeding or the result of a genetic disorder of zinc metabolism such as acrodermatitis enteropathica. The clinical features are easily recognizable: neuropsychiatric changes, dermal lesions, diarrhea, and alopecia (Aggett, 1989). A mild or marginal deficiency is more difficult to diagnose because there is no clinical feature which is unique to it. Possible ones are slowing of physical growth, poor appetite and diminished taste acuity, and depressed immune status. Growth is the outcome variable frequently measured. There is no reliable, sensitive laboratory index of zinc status (Solomons, 1979). 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).

It was originally believed that zinc deficiency could not

occur in man. This view has now changed. In the early 1960's, Prasad and coworkers (1963) described a syndrome of "nutritional dwarfism" in Egypt. Clinical features included growth retardation, hypogonadism in males, rough and dry skin, anemia responsive to iron, enlarged liver and spleen, mental lethargy, and susceptibility to infections. Mild zinc deficiency was confirmed from blood zinc values and radio-labelled tracer studies.

Other work followed this report from Egypt. Carter et al. (1969) completed a zinc supplementation trial in Cairo but did not confirm an effect of zinc on growth. Studies in Iran gave both positive (Halsted et al., 1972; Ronaghy et al., 1974) and negative results (Mahloudji et al., 1975; Ronaghy et al., 1969). However, not all of the studies were "double-blind" or random and there was lack of good dietary information. It is unlikely that zinc deficiency was the only contributing factor. Coble et al. (1971) studied endocrine function in boys from the Nile Valley with retarded growth and delayed sexual maturation and concluded that it was not possible to attribute the delayed development of the boys solely to zinc deficiency. Other conditions associated with growth retardation and delayed puberty were also present in the region - a population of low socioeconomic status, poor hygiene, high incidence of infectious disease, poor protein and calorie nutrition, and other mineral deficiencies. Golden and Golden (1979; 1981) have documented the occurrence of zinc deficiency

with protein energy malnutrition in children.

Zinc deficiency has been investigated in other areas throughout the world including Colorado (Hambidge et al., 1972; Walravens and Hambidge, 1976; Walravens et al., 1983), southern Ontario (Smit Vanderkooy and Gibson, 1987; Gibson et al., 1989), China (Chen et al., 1985), Yugoslavia (Buzina et al., 1980), and Australia (Cheek et al., 1982). From the results of these studies, many investigators concluded that zinc deficiency, especially a mild or marginal one, is prevalent.

Hambidge et al. (1972) found low levels of hair zinc associated with anorexia, poor growth, and hypogeusia in young children from Denver which the authors attributed to zinc deficiency. Similar findings were more recently reported in Yugoslavia (Buzina et al., 1980) and China (Chen et al., 1985). Children in both of these groups were also in a poor nutritional status. Smit Vanderkooy and Gibson (1987) found preschool males with low hair zinc (<70 μg/g) had a lower mean height-for-age percentile. They suggested a mild zinc deficiency since these children had lower dietary intakes of readily available zinc.

Walravens and Hambidge (1976) demonstrated that zinc supplemented male infants were 2.1 cm greater in length ($p<0.025$) and 535 g greater in weight ($p<0.05$) than controls. Male preschool children who were supplemented had a 10% greater height velocity ($p<0.001$) than controls (Walravens et al.,

1983). No effect was demonstrated in middle-income children consuming zinc-fortified breakfast cereals (Hambidge et al., 1979). Subjects in this study were not selected on the basis of height or zinc status; therefore, they were probably not zinc deficient. Zinc does not have any pharmacological effects on growth or other functional measures of zinc status. In a double-blind, pair-matched study, boys with a hair zinc less than 1.68 μmol/g responded to the zinc supplement with a higher mean change in height-for-age Z scores ($p<0.05$); taste acuity, energy intakes, and attention span were unaffected (Gibson et al., 1989). In contrast, Krebs et al. (1984) reported significant improvements in children's appetite and food intake when zinc supplemented. Immune function was also reported to respond to zinc supplementation (Castillo-Duran et al., 1987).

THE SOMATOMEDIN HYPOTHESIS AND GROWTH

GROWTH HORMONE

Growth hormone (GH) is an anabolic hormone synthesized by the somatotropes of the anterior pituitary and is under hypothalamic control (Granner, 1990). It is a single polypeptide with an approximate molecular weight of 22,000 in mammalian species. Human GH (hGH) contains 191 amino acid residues. Although there is a high degree of sequence homology among various mammalian growth hormones, only hGH or that of other higher primates is active in humans. In the

rat, hGH is effective but antibodies will be produced after 14 days (A. Skottner, pers. comm.). Growth hormone is essential for postnatal growth and for normal carbohydrate, lipid, nitrogen, and mineral metabolism (Granner, 1990).

The action of GH on bone includes promoting growth of long bones at the epiphyseal plates in growing children and appositional or acral growth in adults and increasing cartilage formation in children (Granner, 1990). The "somatomedin hypothesis" states that the growth-promoting effects of GH are primarily mediated through the action of insulin-like growth factor I (IGF-I) (Salmon and Daughaday, 1957; Elders et al., 1975).

INSULIN-LIKE GROWTH FACTORS

Insulin-like growth factors are single-chain polypeptides of approximately 7.5 kD which occur in blood and most tissues of the body (Humbel, 1990). The two circulating forms are IGF-I, also known as somatomedin-C (Sm-C), and IGF-II. The peptides consist of four peptide domains, A, B, C, and D (Clemmons, 1989). IGF-I and IGF-II share 43% and 41% sequence homology with proinsulin respectively (Zapf and Froesch, 1986). The amino acid sequence of IGF-I for six species (human, bovine, porcine, ovine, rat, and mouse) has been determined. IGF-I in all six species consists of 70 amino acid residues while IGF-II has 67 residues (Humbel, 1990). There is a 62% sequence homology between the two growth factors.

Normal serum and plasma concentrations of IGF-I and IGF-II in adult man are 200 and 700 ng/ml respectively (Daughaday and Rotwein, 1989). Prior to puberty, IGF-I reaches adult levels and then rises 2-3 fold during puberty. IGF-II does not change significantly. Hypopituitary dwarfs, or persons with untreated GH deficiency, have reduced concentrations of IGF-I and IGF-II, 10 ng/ml and 200 ng/ml respectively (Zapf and Froesch, 1986). Comparisons of growth rates between normal and GH-deficient children suggest that IGF-I increases growth in later childhood and that it is particularly responsible for the pubertal growth spurt (Cara et al., 1987). Conversely, patients with GH-secreting hormones, ie gigantism or acromegaly, show increased concentrations of IGF-I (600-1000 ng/ml) but normal or subnormal levels of IGF-II (Zapf and Froesch, 1986; Granner, 1990).

Insulin-like growth factor I and IGF-II show both in vitro and in vivo insulin-like metabolic effects, such as on glucose transport and on blood glucose, but only at relatively high concentrations (Phillips and Unterman, 1984; Humbel, 1990). The binding of IGF-I to binding proteins in plasma probably prevents hypoglycemia under physiological conditions (Humbel, 1990). The biologically relevant effects of IGF-I at nanomolar concentrations are the stimulation of cell proliferation and, in certain tissues, cell differentiation. In vitro, IGF-II mimicks the effects of IGF-I. While some investigators consider IGF-II to be a fetal growth factor in

the rat, this view has not been supported for man. Therefore, the biological role of IGF-II has yet to be defined and its biological relevance is under dispute.

THE SOMATOMEDIN HYPOTHESIS

The liver is the most important source of circulating IGF-I with the major regulator being GH (Daughaday 1989; Humbel, 1990). Hypophysectomy reduces the level of IGF-I in the liver while GH treatment of hypophysectomized rats restores circulating levels (Schwander et al., 1983). Accordingly, GH releasing hormone is secreted into the portal venous system which connects directly to the anterior pituitary gland and stimulates the secretion of GH (Elders et al., 1975). According to the somatomedin hypothesis, GH then acts on the liver to stimulate the secretion of IGF-I which is transported via the plasma to the cartilage where a number of anabolic events are initiated. These include the stimulation of mitotic activity, deoxyribonucleic acid (DNA) synthesis, DNA-dependent ribonucleic acid (RNA) synthesis, protein synthesis, biosynthesis of glycosaminoglycans (GAGS), collagen synthesis, and increased thickness of the epiphyseal growth plate with resultant long bone growth. If the somatomedin hypothesis is true, IGF-I would act as an endocrine growth factor with circulating IGF-I being the major source for skeletal growth (Elders et al., 1975; Daughaday, 1989; Humbel, 1990).

Before the availability of a radioimmunoassay for measuring IGF-I specifically, the bioassay for IGF-I measured somato-

medin activity or sulfate incorporation into cartilage glycosaminoglycans (Elders et al., 1975). The biosynthesis of chondroitin sulfate, a major GAG found in cartilage, begins with the transfer of xylose from UDP-xylose to the serine residue of the receptor protein. Galactose and glucuronic acid are then added sequentially to form a polysaccharide chain. Sulfation occurs simultaneously with the polymerization of the polysaccharide chain. An alteration in any step of the biosynthesis will decrease sulfate uptake.

Circulating IGF-I is believed to act in a negative feedback system to inhibit the release of GH (Phillips and Unterman, 1984; Humbel, 1990). In a pituitary cell culture system, IGF-I inhibited the GH release stimulated by a synthetic GH releasing factor (Humbel, 1990). Therefore, IGF-I can self-regulate itself. Insulin and adequate protein and energy are important to maintain normal circulating levels while excess steroids, protein energy malnutrition, and certain illnesses adversely affect the activity of IGF-I (Figure 1.1) (Phillips and Unterman, 1984).

Lack of purified IGF-I made it difficult to study the in vivo action of IGF-I. Growth hormone improved growth and increased IGF-I when administered to hypophysectomized rats (Kemp et al., 1981). Early reports demonstrated that plasma peptide fractions containing somatomedin activity caused an increase in body weight and sulfate-incorporating activity of cartilage in Snell dwarf mice when administered subcutaneously

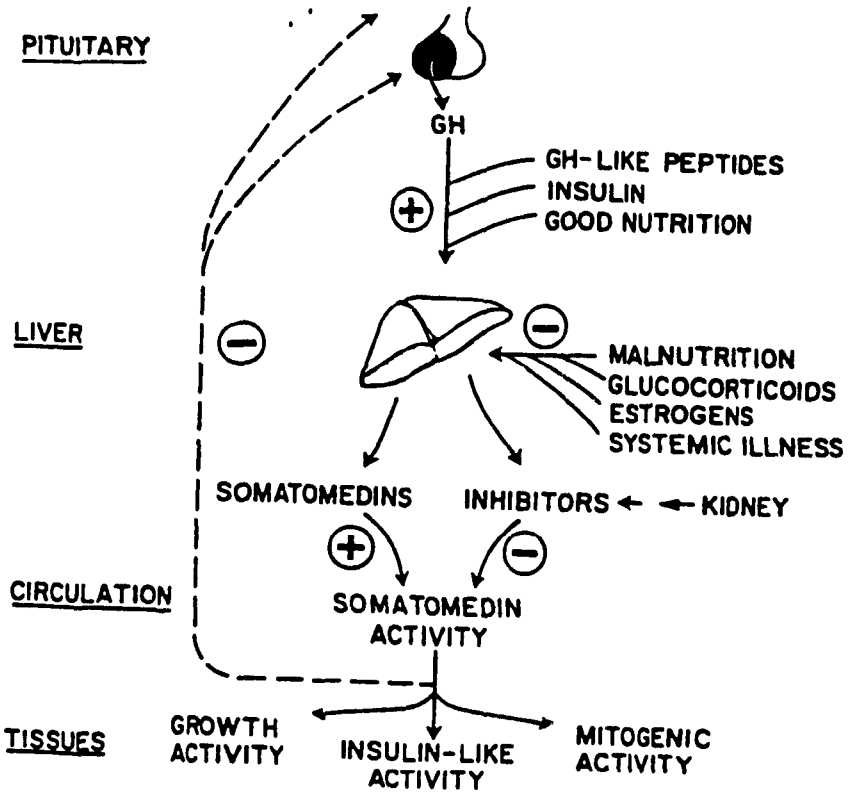


Figure 1.1 Regulation of somatomedin (IGF-I) (from Phillips and Unterman, 1984)

three times per day for 2-4 weeks (van Buul-Offers and van den Brande, 1979; Holder et al., 1981). However, growth responses were always smaller than those induced by GH. Schoenle et al. (1982) demonstrated small increases in tibial epiphyseal width, 3H-thymidine incorporation into costal cartilage DNA, and body weight compared to controls. The increases were similar to those obtained with hGH suggesting that IGF-I was capable of mimicking the effects of GH. These results were assumed to be direct support of the somatomedin hypothesis with IGF-I acting in a endocrine manner.

However, Skottner et al. (1987) found that recombinant human IGF-I (hIGF-I), whether given by subcutaneous infusion, twice daily injections, or continuous intravenous infusion, had a very small effect on growth parameters in hypophysectomized rats compared to GH. Even when given in combination with GH, hIGF-I had little effect, particularly on skeletal growth , which raised the question of the importance of circulating IGF-I as an endocrine mediator of the growth-promoting actions of GH. However, hypophysectomized rats may not be the most appropriate model since they lack other pituitary hormones besides GH, and some of these can influence growth in hypophysectomized animals. In a mutant dwarf rat with isolated GH deficiency, both hGH and hIGF-I significantly increased body weight but hIGF-I resulted in a significantly smaller stimulation of bone growth (Skottner et al., 1989). The pattern of growth produced also differed with hGH

resulting in proportional growth while hIGF-I increased the size of the kidneys, adrenal, and spleen. It was concluded that GH and IGF-I differed quantitatively and qualitatively in their pattern of action and IGF-I was not acting as a simple mediator of GH. In neonatal rats, however, IGF-I but not GH showed an effect on somatic and organ growth (Philipps et al., 1988). Embryonic and neonatal growth is not believed to be GH-dependent.

Investigators have also administered GH and IGF-I locally into the tibiae of rats using daily injections or arterial infusion. The contralateral tibia serves as an internal control. When injected into the tibial epiphyseal plate of hypophysectomized rats, both rat GH (rGH), hGH, and hIGF-I caused unilateral tibial growth (Russell and Spencer, 1985). Isgaard et al. (1986) also stimulated local bone growth using GH from various species. The effect for IGF-I was smaller than that of GH. When infused into the arterial supply of the hindlimb, rGH and hIGF-I stimulated growth of the epiphyseal plate of the infused limb but not of the noninfused leg (Schlechter et al., 1986a). IGF-I showed a dose related response. An IGF-I antibody completely abolished the effect of rGH when it was co-infused with the hormone. High doses of either rGH or hGH resulted in a systemic effect with epiphyseal growth occurring in the noninfused leg (Schlechter et al., 1986b; Nilsson et al., 1987). Therefore, it was concluded that GH in vivo directly stimulates epiphyseal car-

tilage growth through stimulating the local production of IGF-I which then acts in a paracrine or autocrine manner. Some researchers questioned the need for circulating IGF-I.

Growth hormone significantly enhances the increase of IGF-I in a culture of embryonal rat tibiae (Stracke et al., 1984). Growth hormone will restore the number of IGF-I mRNA copies in the rib growth plate of hypophysectomized rats which further supports the paracrine/autocrine hypothesis (Isgaard et al., 1988). However, these results do not exclude the possibility that circulating IGF-I may be involved in the growth process. It also appears that IGF-I mediates the growth-promoting effect of GH and not GH's action on bone resorption (Spencer et al., 1991).

Longitudinal bone growth results from the production of new cells in the growth plate and from the expansion of these cells as they are displaced into the maturation zone by continuing cell division (Isaksson et al., 1987; Robertson, 1990). The growth plate consists of several cell layers: the germinal or reserve cell layer, the proliferative cell layer, the hypertrophic cell layer, and the calcification layer.

In support of the need for circulating IGF-I, early investigators failed to show any stimulatory effect of GH on cell proliferation in vitro (Ash and Francis, 1975; Ashton and Francis, 1977; 1978). However, Madsen et al. (1983) found that hGH did stimulate DNA synthesis in chondrocytes isolated from rabbit ears and rat rib growth plates. When looking at

the distribution of IGF-I in the growth plate, cells in the proliferative zone contained more IGF-I (Nilsson et al., 1986). It was also found that the gene for IGF-I was being expressed in chondrocytes from the proliferative and hypertrophic zones (Nilsson et al., 1990). Hypophysectomy decreased the expression of IGF-I which could be restored with GH. It appeared that GH regulated the synthesis of IGF-I in the growth plate which further supported the paracrine/autocrine hypothesis.

Human GH and hIGF-I was added to cultures of rat epiphyseal chondrocytes and the resultant colony size and number examined (Lindahl et al., 1987a). Both hGH and hIGF-I induced colony formation; however, hGH potentiated the formation of large size colonies while hIGF-I resulted in a higher proportion of small size chondrocyte colonies. Lindahl et al. (1987b) then looked at colony formation of chondrocytes isolated at different layers of the growth plate, corresponding to different stages of maturation. Human IGF-I increased the number of colonies in chondrocytes isolated from the proximal and intermediate zones. Low concentrations of hGH (10-40 ng/ml) stimulated colony formation in chondrocytes from the proximal zone whereas higher concentrations were ineffective. Again, hIGF-I potentiated small size colonies while hGH increased the number of large size colonies in the proximal zone. The distal zone showed no significant response to either hGH or hIGF-I. It was concluded that GH is capable of

interacting with a limited number of cells which exhibit a high proliferative capacity (Lindahl et al., 1987a; 1987b). Pretreating hypophysectomized rats with hGH increased the responsiveness of cultured epiphyseal chondrocytes to the growth-promoting effect of subsequently added hIGF-I (Lindahl et al., 1987c).

Based on the above work, Isaksson and coworkers (1987; 1990) suggested that GH directly stimulates the differentiation of prechondrocytes or young differentiating cells. During the process of cell differentiation, cells, directly stimulated by GH, become responsive to IGF-I. Concomitantly, the gene encoding for IGF-I is expressed, which results in an increased synthesis of IGF-I in the differentiating cells. The locally produced IGF-I is externalized and subsequently interacts with receptors on the proliferating chondrocytes by autocrine or paracrine mechanism(s). Circulating IGF-I is of limited value in the growth process.

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% 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 establish the local production and action of IGF-I but they do not establish that autocrine/paracrine actions of IGF-I are sufficient to explain normal growth. Systemic injections of GH achieve a greater growth response than do local injections into the growth plate.

Daughaday (1989) statistically analyzed the data from several studies to predict the growth response to local and systemic GH injections. Using a regression line calculated from Thorngren and Hansson (1974) as comparison, Daughaday (1989) showed that the results obtained by Isgaard *et al.* (1986) with local injections of 0.5 μg hGH per day only accounted for 12% of the longitudinal growth response that could have been induced by subcutaneous GH. A near maximal response is achieved with 400 μg of hGH subcutaneously (Daughaday, 1989). The response was equivalent to that predicted for a daily dose of 14 μg given subcutaneously. Analysis of data from Russell and Spencer (1985) demonstrated that the injection of rGH at 5 μg per day into the tibial growth plate for 4 days achieved only 22% of the increase in width as predicted for 400 μg of GH subcutaneously (Daughaday, 1989). The regression line was based on work from Geschwind and Li. Scheven and Hamilton (1991) studied longitudinal bone

growth using an *in vitro* model system of intact rat long bones. Growth arrest occurred in neonatal metatarsals after 1-2 days in a serum-free medium. Since they were able to respond to exogenously added IGF-I and GH, the authors suggested that endogenous factors were not sufficient to promote growth. Systemic, blood-borne, factors are probably required for *in vivo* regulation of long bone growth.

Daughaday (1989) concluded that "hormonal somatomedins reaching skeletal tissues from the circulating plasma have a major, if not predominant, role in the regulation of skeletal growth". 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 effects, mostly on liver, to increase serum concentrations of IGF-I.* This is illustrated in Figure 1.2. IGF-I can mimic most, but not all, effects of GH. The relative contributions of circulating IGF-I, locally produced IGF-I, and direct action of GH are at present disputed (Daughaday, 1989; Humbel, 1990).

NUTRITIONAL REGULATION OF IGF-I

Nutrition, specifically protein and energy, appear to play a role in the regulation of IGF-I. Grant *et al.* (1973) observed that serum somatomedins bioactivity was low in children with protein-calorie malnutrition, despite high GH

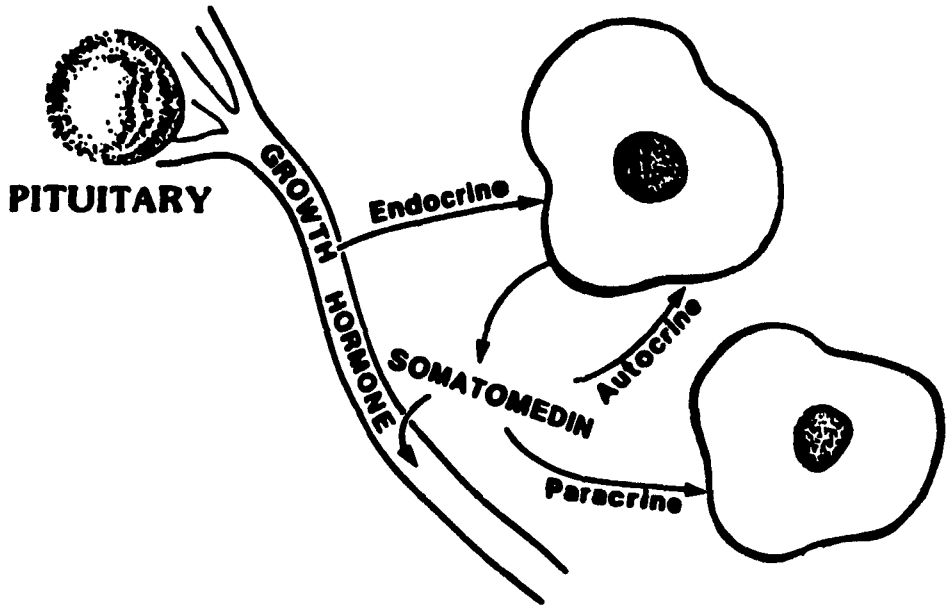


Figure 1.2 Activities of somatomedins (IGF-I)
(from Clemmons and Underwood, 1991)

levels. Merimee et al. (1982) later showed that after three days of fasting, normal subjects showed no change in serum IGF-I in response to GH injections. In fasted rats as well, bGH is not able to prevent the fall in serum somatomedin activity (Phillips and Young, 1976). Refeeding returns somatomedin levels and cartilage growth activity to control values. Prewitt et al. (1982) found that serum Sm-C was influenced by both protein and energy intake, although protein appeared to be the most important variable. Reeves et al. (1979) observed an inverse relationship between dietary fat and plasma somatomedin activity.

The effect of fasting on IGF-I may be regulated through a reduction of GH receptors in the liver. When rats are fasted for three days, they experience marked reductions in serum IGF-I and in binding of GH to its hepatic receptor (Maes et al., 1983). During refeeding, hepatic GH binding increased at approximately the same rate as the serum IGF-I concentrations. Furthermore, the decrease in GH receptors that occurs during fasting is accompanied by a parallel decrease in hepatic GH receptor mRNA abundance (Straus and Takemoto, 1990). Steadystate hepatic IGF-I mRNA levels also decrease in fasted rats compared to controls indicating an effect on transcription of IGF-I mRNA (Emler and Schalch, 1987). Refeeding produces a prompt increase in steadystate mRNA levels (Straus and Takemoto, 1990).

Dietary protein restriction appears to lower serum IGF-I

through a different mechanism. While serum IGF-I is consistently reduced in rats subject to protein restriction, only modest reductions in GH binding occur (Maes et al., 1984a). Also, the serum IGF-I response to exogenous GH is attenuated in protein deficient rats (Maes et al., 1988). Protein restriction appears to cause post-receptor resistance to the action of GH, and the low IGF-I is not mediated via reduced GH binding (Maiter et al., 1988). Protein restriction also decreases hepatic IGF-I mRNA commensurate with the decline in serum IGF-I (Moats-Staats et al., 1984). It also appears that protein restriction may cause resistance to IGF-I since infusion of IGF-I into rats fed 5% protein diets does not restore the normal rate of increase in tail length, weight, or tibial epiphyseal width despite normalization of serum IGF-I (Thissen et al., 1991). The effect of protein deprivation appears to be age-dependent, since it is less profound in older animals (Fliesen et al., 1989). These effects of energy and protein on IGF-I are illustrated in Figure 1.3.

The response of circulating IGF-I to dietary protein and/or energy restriction has also been explored in humans. Fasting causes reductions in plasma IGF-I which return to normal with refeeding (Isley et al., 1983; 1984). Restricting protein and/or energy in the refeeding diet causes smaller increases in circulating IGF-I compared to the control diet. In malnourished hospital patients, IGF-I appears to respond more quickly to nutritional interventions compared to conventional

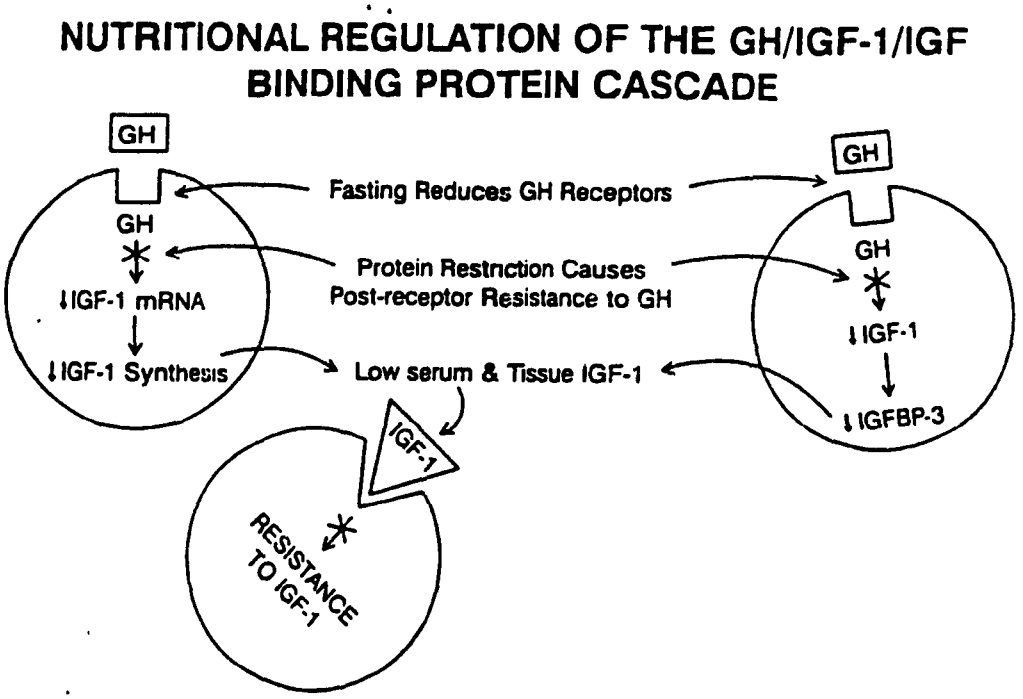


Figure 1.3 (from Clemmons and Underhill, 1991)

indices of nutritional status, such as albumin, prealbumin, transferrin, and retinol binding protein (Clemmons et al., 1985; Donahue and Phillips, 1989). Thus, it may be a useful tool for monitoring short-term changes in nutritional status.

ZINC, GROWTH HORMONE, AND IGF-I

Several mechanisms have been proposed to explain the growth retardation seen with zinc deficiency. These include: (1) altered activities of zinc enzymes adversely affecting nucleic acid metabolism, protein synthesis, and cell division; (2) decreased immune function leading to increased infection and stress on the individual; and (3) decreased taste acuity and appetite resulting in decreased food intake. A fourth possible mechanism is an adverse effect on the generation of IGF-I which would alter cartilage metabolism (Cossack, 1984; 1986; Bolze et al., 1987).

Oner and Bor (1978) observed a marked reduction in serum somatomedin A (later found to be IGF-I) activity in zinc deficient rats compared to controls. However, the authors failed to use a pair-fed group so it is difficult to attribute the fall in somatomedin A activity entirely to a lack of zinc. Cossack (1984) demonstrated that both decreased zinc and decreased food intake contribute to the fall in Sm-C. Zinc deficient rats had significantly depressed growth (as measured by body weight gain) and plasma Sm-C compared to both pair-fed groups and the ad libitum control group. As well, values for

the pair-fed groups were also significantly lower than the ad libitum controls indicating an effect of decreased food intake alone. It was also observed that plasma Sm-C correlated with tibial zinc concentrations ($r=0.79$, $p<0.005$) and with body weight gain ($r=0.96$, $p<0.001$). Cossack (1986) further established the importance of zinc for maintaining normal Sm-C levels. Growing rats were given one of six test diets based on combinations of two levels of protein and three levels of zinc. All rats were given the same amount of diet consumed by the low-zinc-low-protein group. An additional control group was given the medium-zinc-high-protein diet ad libitum. In rats given the low-zinc-low-protein, the level of plasma Sm-C increased as the amount of zinc or zinc and protein increased in the diet. However, no change was observed when the level of protein alone was increased. Among all groups tested, the ad libitum fed rats showed the highest level of plasma Sm-C. Rats fasted for three days showed an immediate decrease in Sm-C (Cossack, 1988). Again, adequate zinc was required in the refeeding diet to ensure that plasma Sm-C returned to normal. The zinc deficient and 30 ppm zinc groups experienced transient increases in Sm-C followed by further declines. The transient increase was attributed to food intake and not food composition.

Bolze et al. (1987) investigated the influence of zinc on growth, somatomedin activity, and glycosaminoglycan metabolism in the rat. Weanling male rats were fed control ad libitum,

zinc deficient (1 ppm zinc), or pair-fed control diets for 13 days. Rats were then refed the control diets for up to eight days and serially killed. Bioassayable somatomedin activity was 0.81, 0.42, and 0.33 ± 0.09 relative activity for ad libitum, pair-fed and zinc deficient rats at the end of depletion. Radiolabelled sulfate uptake by glycosaminoglycans (GAG) was significantly less in the zinc deficient rats compared to either ad libitum or pair-fed groups. Xylosyltransferase activity, an enzyme involved in GAG synthesis, was also significantly depressed in the zinc deficient group compared to the other two groups. Somatomedin activity and GAG metabolism returned to normal after refeeding for 2-5 days in the pair-fed and for 5-8 days in the zinc deficient rats again suggesting separate effects of food intake and zinc on somatomedin and cartilage activity. This study by Bolze et al. (1987) was important since it demonstrated an effect of zinc on the specific activity of IGF-I whereas others were investigating circulating levels and growth only.

Since GH is considered the major regulator of IGF-I, the effect of zinc on IGF-I may be mediated through GH. Root et al. (1979) demonstrated that zinc deficiency decreased serum and pituitary levels of GH in both sexually mature and immature rats. Body weight and tail lengths were concomitantly reduced. In GH deficient children, GH treatment significantly increased hair zinc concentrations and decreased

urinary zinc excretion suggesting a role for GH in zinc metabolism in children (Cheruvanky et al., 1982). The addition of 50 mg/day of zinc to some of the children taking GH increased their rate of growth to 7.3 ± 2.5 from 5.2 ± 2.5 of the previous year. Ghavami-Maibodi et al. (1983) supplemented healthy short children who had low hair zinc concentrations with oral zinc supplements for one year. Children with other reasons for growth retardation were excluded. There was a significant increase in growth rates for the children whose hair zinc concentrations increased. Growth hormone and Sm-C also significantly increased after the zinc supplement. Collipp et al. (1982) and Nishi et al. (1989) both reported cases of poor growth and GH deficiency apparently due to zinc deficiency.

From these studies, some investigators concluded that the effect of zinc on growth may, in part, be mediated through its effect on GH levels. It is also possible that GH treatment increases the requirement for zinc because of the deposition on new tissue. Richards and Marshall (1983) supplemented GH treatment with zinc and found no effect on growth or serum and hair zinc compared to nonsupplemented controls. The authors concluded that GH does not alter zinc requirements and zinc does not improve growth in children with GH deficiency. Zinc does not have an effect on growth unless a deficiency is present. In the other studies, some improvement was seen in the indices of zinc status indicating a mild zinc deficiency

may have been present. Solomons et al. (1976) also concluded that zinc deficiency was not a contributory factor in all cases of short stature. However, five patients with Crohn's disease who experienced growth arrest had some evidence of zinc deficiency. The authors concluded that a normal pattern of growth is consistent with normal zinc nutrition but that a plateau in growth is observed when zinc deficiency is present.

From these results, it can be hypothesized that zinc deficiency adversely affects GH levels or synthesis leading to decreased levels of circulating IGF-I. If so, GH treatment would be expected to alleviate the effects of zinc deficiency on growth. Prasad et al. (1969) investigated the effect of bovine GH (bGH) treatment in zinc deficient rats and of zinc in 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 for two weeks. One half of each group received bGH. The control groups were rats given the supplemented diet with or without growth hormone. 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 bGH treatment was without effect in the presence or absence of zinc. The growth rate of the hypophysectomized rats was affected by both zinc and GH and these effects were independent. It was concluded that zinc and GH regulate growth in rats by indepen-

dent mechanisms. Oner et al. (1984) also investigated the effect of growth hormone treatment in zinc deficient rats. After three weeks of a zinc deficient diet, rats received supplemental zinc, bGH, or saline. As libitum and pair-fed control groups were also included. The zinc deficiency diminished skeletal growth (as measured by tibial epiphyseal width) and decreased serum somatomedin which treatment with bGH did not improve. Zinc repletion restored somatomedin levels to normal and improved body weight gain and skeletal growth. It was observed that despite similar serum somatomedin levels, the GH-treated zinc deficient rats had smaller tibial epiphyseal widths than pair-fed control rats. The authors concluded that zinc deficiency impairs skeletal growth through decreased somatomedin production as well as decreased action of somatomedin on skeletal growth. Zinc was not acting through GH since GH treatment failed to increase epiphyseal widths in zinc deficient rats.

The study design of both studies had limitations. Prasad et al. (1969) did not use pair-fed control groups in his study and Oner et al. (1984) did not have a pair-fed group that received bGH. The lack of response may be partially explained by protein energy malnutrition with the rats being unable to respond by increasing growth. The rats were on the zinc deficient diets for five and six weeks respectively with a concomitant decrease in food intake. Swenerton and Hurley (1968) reported that 9% of male rats died after three weeks on

a zinc deficient diet if not zinc repleted. Prasad et al. (1969) used a pituitary extract which may have been contaminated with other growth factors which could influence growth. Oner et al. (1984) gave the GH for three weeks. Antibody formation occurs when rats are given hGH for two weeks or longer. Neither study specifically measured IGF-I. Oner et al. (1984) measured somatomedin activity while Prasad et al. (1969) did not do any somatomedin measurements.

STATEMENT OF PURPOSE

It has been shown that dietary zinc deficiency leads to retarded linear growth in the rat. One mechanism for this impaired growth may be a reduction in circulating growth hormone which, in turn, reduces insulin-like growth factor I (IGF-I). Circulating IGF-I is believed to be a mediator of some of the effects of GH on linear growth. Previous studies used a bovine GH preparation to study the effect of GH treatment on growth in zinc deficiency. Considering the species differences in GH, the suitability of bGH for the rat may be questionable. Human GH is effective in the rat and can be used for 14 days before the production of antibodies by the animal. Based on the evidence, it was hypothesized that hGH treatment would increase circulating IGF-I and alleviate the effects of zinc deficiency on growth in rats.

Specific objectives in the experiment were:

(1) to determine the effect of dietary zinc deficiency on nutritional status by measuring:
   (a) liver zinc, copper, and iron concentrations
   (b) tibial zinc and copper concentrations
   (c) plasma zinc concentration
   (d) plasma alkaline phosphatase activity, a zinc-dependent metalloenzyme, as a functional test of zinc deficiency:

(2) to determine the effect of dietary zinc deficiency on

growth 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 growth;

(3) to determine the effect of dietary zinc deficiency on plasma IGF-I levels;

(4) to determine the effect of hGH treatment on nutritional status, growth, and plasma IGF-I by comparing parameters between GH-treated and untreated animals.

CHAPTER II. MATERIALS AND METHODS

EXPERIMENTAL DESIGN

The experiment was carried out as a completely randomized block design with a 2x3 factorial combination. The experimental design consisted of three dietary groups: a zinc-deficient (ZD) group fed a diet containing < 1.5 ppm zinc ad libitum; a control (C) group fed a diet containing 85 ppm zinc ad libitum; and a pair-fed (PF) group fed the 85 ppm diet to the level of intake of the ZD group. The PF group was included to control for the effects of decreased food intake accompanying zinc deficiency. After 7 days, one-half of each dietary group began to receive hGH dissolved in saline or the saline alone (sham-treated). The experimental design and sample sizes within each block is shown in Figure 2.1.

	hGH-treated	sham-treated
Zn Deficient (<1.5 ppm)	12	12
Control (85 ppm)	12	12
Pair-fed (85 ppm)	12	11

Figure 2.1 Experimental Design

EXPERIMENTAL PROTOCOL

All animals were acclimatized for four days on rat chow. After the acclimatization period, the rats were weighed and randomized by weight into the ZD, C, or PF dietary groups. The ZD and C groups immediately began their respective diets. The PF rats began their diets one day later since they were fed the 85 ppm diet in the amount that their ZD counterparts consumed the previous day. The first day of the experimental diets is Day 1 of the study.

The food intake of each rat was recorded daily. On Day 1 and every 3 days thereafter, body weight and tail length was measured. Tail length was measured in millimeters from the anus to the tip of the tail. The final measurements were taken 1 day prior to the end of the feeding trial.

Growth hormone treatment began on Day 8. All animals were killed on Day 18 after an overnight fast. Blood was collected by cardiac puncture for determination of plasma zinc, alkaline phosphatase, hGH, and IGF-I. One tibia was removed for measurement of epiphyseal cartilage width. The other tibia and the liver were removed for trace element analysis.

EXPERIMENTAL TECHNIQUES

ANIMAL CARE

Male weanling rats (3 weeks old) of the Sprague-Dawley strain were obtained from Charles River Inc. (St. Constance, PQ). The rats were housed individually in stainless steel

cages with stainless steel grids on the bottom that prevented coprophagy. The room temperature was maintained at 22-25°C and lighting was provided automatically for a period of 12 hours daily from 0800 to 2000h. Control and ZD rats were allowed ad libitum access, and the PF group restricted access, to a pelleted semi-purified diet. Food was placed on the floor of each cage. It was weighed before being placed in the cages. Leftover food and spillage was weighed the next day to determine daily food intake. Deionized distilled water was available ad libitum from plastic water bottles. Single-use plastic gloves were used when handling experimental materials and animals.

HUMAN GROWTH HORMONE (hGH) TREATMENT

The hGH, an extract from human pituitaries, was obtained from Sigma Chemical Company (St. Louis, MO). The potency was 4 I.U. per mg. For the injections, the hGH was dissolved in saline and administered using a 1 cc Tuberculin syringe fitted with a 26½ gauge needle (Becton Dickinson, Fisher Scientific, Montreal, PQ). It was administered between 0900 and 1100h every day for 10 days beginning on Day 8. All hGH was used within 24 hours after being dissolved in saline since no preservatives were added. The dose was 20 μg per 100 g body weight. Individual dosages for each rat were calculated every 3 days based on changes in body weight. Three injections sites were used: the right hindflank, the left hindflank, and the back of the neck. The sites were rotated daily to prevent

bruising or other adverse reactions in one area. The injection sites were examined daily. The sham-treated rats were treated in the same manner as the hGH-treated rats.

DIETS

Experimental diets were purchased from ICN Biomedicals Canada, Ltd. (Mississauga, ON). The composition of the diets is shown in Tables 2.1 - 2.4. The zinc content of both diets was determined by flame atomic absorption spectrophotometry after wet digestion in a nitric:perchloric acid mixture. The method is described in Appendix 1. The zinc deficient diet contained < 1.5 ppm of zinc and the control contained 85 ppm of zinc. They were semi-purified, biotin-fortified, egg white diets in a pelleted form. Egg white was used as the protein source since it is a complete protein with the lowest zinc content. The diets were biotin-enriched to control for the avidin, a biotin-chelating agent found in egg white. A pelleted form was chosen for ease of handling and to control spillage. Both diets were stored in plastic bags in sealed buckets and kept in refrigerated storage.

TISSUE COLLECTION

All rats were killed on Day 18 of the feeding trial in a fasted state. The rats were anaesthetized with carbon dioxide and blood was withdrawn by cardiac puncture. If possible, two withdrawals were made on each animal. One used a Vacutainer (Becton Dickinson, Fisher Scientific, Montreal, PQ) Trace Element Tube coated with sodium heparin for the zinc and

alkaline phosphatase determinations. Trace Element Tubes have a minimal zinc content (0.20 ppm) and are recommended for trace element determinations. For the hGH and IGF-I determinations, a Vacutainer Tube coated with EDTA was used. EDTA is the recommended anti-coagulant for determination of IGF-I. All blood was centrifuged and the plasma transferred to microeppendorf centrifuge tubes. It was stored at -80°C until analyzed. The EDTA plasma was stored in two aliquots. Following cardiac puncture, the neck of each animal was broken to ensure it was dead. The abdomen was opened and the liver removed, weighed, and kept frozen at -80°C. The skin and fur was then removed from the hindflanks and legs. The hind part of each animal was removed intact and kept frozen at -80°C.

ANALYTICAL PROCEDURES

TIBIAL EPIPHYSEAL CARTILAGE WIDTH

The measurement of the tibial epiphyseal cartilage width was based on the method of Greenspan et al. (1949). One tibia, from either the right or the left leg of each animal, was dissected free of the surrounding tissue and bone. The calcified portion of the tibia was stained with silver nitrate and the width of the cartilage band was measured with a microscope equipped with a micrometer. The procedure is fully described in Appendix 2.

PLASMA HUMAN GROWTH HORMONE

Plasma samples for hGH were thawed and heavily hemolyzed samples were discarded. Plasma hGH was determined with a radioimmunoassay kit manufactured by Diagnostic Products Corporation (Los Angeles, CA). It is a competitive, double-antibody procedure using ^{125}I-labelled hGH. Since the kit was intended for human plasma, a tube to account for nonspecific binding by the rat plasma was included.

PLASMA INSULIN-LIKE GROWTH FACTOR I

Plasma samples for IGF-I were thawed and heavily hemolyzed samples were discarded. The determination of IGF-I was a two-step procedure using a radioimmunoassay kit manufactured by Nichols Institute (San Juan Capistrano, CA). The first step was the extraction of plasma samples and controls to separate binding proteins from the IGF-I. The acid:ethanol extraction procedure included with the kit was modified according to Takahashi et al. (1990). The modification was recommended since rat plasma contains more IGF binding protein 3 than does human plasma. The extraction procedure is outlined in Appendix 3. The IGF-I in the extracted samples and controls was then determined with a competitive, double-antibody assay using ^{125}I-labelled IGF-I.

PLASMA ZINC AND ALKALINE PHOSPHATASE

Plasma samples for zinc and alkaline phosphatase were thawed and heavily hemolyzed samples discarded. Plasma zinc concentrations were determined by flame atomic absorption

spectrophotometry (Perkin-Elmer Model 2380, 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 phophate (Sigma Chemical Co., St. Louis, MO) and the absorbance of p-nitrophenol read at 405 nm.

TIBIA AND LIVER TRACE ELEMENT ANALYSIS

The remaining tibia was dissected free from the surrounding tissue and bone and the fat was extracted with ether (see Appendix 4). The liver and the tibia were placed in acid-washed plastic vials and freeze-dried to a constant weight. They were then sealed until needed. All samples were wet digested using nitric acid according to the method of Clegg et al. (1981). Duplicate digestions were done for each liver. Zinc, copper, and iron concentrations in the liver and zinc and copper concentrations in the tibia were determined using flame atomic absorption spectrophotometry (Perkin Elmer Model 3100, Perkin-Elmer (Canada) Ltd., Montreal, PQ). The full procedure for the trace element analysis is outlined in Appendix 5.

STATISTICAL ANALYSES

The experimental design was a 2 by 3 factorial, arranged in a completely randomized design with 12 non-real replicates. A two-way analysis of variance was done using the general linear models (GLM) procedure of the SAS systems (SAS

Institute Inc., 1985), according to Steel and Torrie (1980). Means of variables were separated by Fisher's protected least significant difference (LSD). The level of significance was set at $p \leq 0.05$. The command statements are summarized in Appendix 6. ZINC represents the 3 dietary groups and GHORM represents the 2 hGH treatments.

Table 2.1 Zinc Deficient Diet (<1.5 ppm Zn)[1]	
Ingredients	**g/kg**
Egg White	180
Corn Oil	100
Corn Starch	443
Sucrose	200
Alphacel Hydrolyzed	30
Choline Bitartrate	2
Biotin	0.02
AIN-76 Mineral Mixture[2]	35
AIN-76 Vitamin Mixture[3]	10

[1]ICN Biomedicals Canada, Mississauga, ON.
[2]Composition of AIN-76 Mineral Mix without zinc is presented in Table 2.4.
[3]Composition of AIN-76 Vitamin Mix is presented in Table 2.3.

Table 2.2 Zinc Control Diet (85 ppm Zn)[1]

Ingredients	g/kg
Egg White	180
Corn Oil	100
Corn Starch	443
Sucrose	200
Alphacel Hydrolyzed	30
Choline Bitartrate	2
AIN 76 Vitamin Mix[2]	10
AIN-76 Mineral Mix[3]	35
Biotin	0.02
Menadione	0.00045
BHT	0.01
Zinc Sulfate $7H_2O$	0.44

[1]ICN Biomedicals Canada, Mississauga, ON.
[2]Composition of AIN-76 Vitamin Mix is presented in Table 2.3.
[3]Composition of AIN-76 Mineral Mix without zinc is presented in Table 2.4.

Table 2.3 AIN-76 Vitamin Mixture[1]

Ingredient	g/kg
Thiamin Hydrochloride	0.6
Riboflavin	0.6
Pyridoxine Hydrochloride	0.7
Nicotinic Acid	3
D-Calcium Pantothenate	1.6
Folic Acid	0.2
D-Biotin	0.02
Cyanocobalamin (Vit B-12)	0.001
Retinyl Palmitate (Vit A)(250,000 IU/g)	1.6
DL-alpha-Tocopherol Acetate (250 IU/g)	20
Cholecalciferol (Vit D_3)(400,000 IU/g)	0.25
Menaquinone (Vit K)	0.005
Sucrose, finely powdered	972.9

[1]ICN Biomedicals Canada, Mississauga, ON.

Table 2.4 AIN-76 Mineral Mixture without Zinc[1]

Ingredient	g/kg
Calcium Phosphate Dibasic	500
Sodium Chloride	74
Potassium Citrate Monohydrate	220
Potassium Sulfate	52
Magnesium Oxide	24
Manganous Carbonate (43-48% Mn)	3.5
Ferric Citrate	6
Cupric Carbonate (53-55%)	0.30
Potassium Iodate	0.01
Sodium Selenite	0.01
Chromium Potassium Sulfate	0.55

[1]ICN Biomedicals Canada, Mississauga, ON.

CHAPTER III. RESULTS

FOOD INTAKE AND GROWTH PARAMETERS

Cumulative food intake and growth parameters were analyzed and the data presented in Table 3.1. Growth parameters included body weight gain, change in tail length, liver wet weight, dry, fat-free tibia weight, and tibial epiphyseal cartilage width. Figures 3.1 - 3.4 illustrate patterns of food intake, weight gain, and tail length changes. Appendix 7 contains a summary of the analysis of variance for each dependent variable.

FOOD INTAKE

There was no effect of hGH treatment ($p>0.05$) on food intake while the effect for zinc in the diet was highly significant ($p=0.0001$; Table 3.1; Figure 3.1). The three dietary groups were pooled across hormone treatments and compared. There was no difference in food intake between the zinc deficient (ZD) and pair fed (PF) animals; however, food intake for the control (C) group was approximately twice that of both the ZD and PF groups. When compared on a daily basis, this difference in food intake became apparent on Day 4 and continued for the remainder of the feeding trial (Figure 3.2). Except for a slight difference on Day 1 (11.6 g vs 12.7 g), there were no significant differences in food intake on a daily basis between the PF and ZD animals.

BODY WEIGHT GAIN

The effect of zinc in the diet on body weight gain was significant ($p=0.0001$; Table 3.1) while no effect ($p>0.05$) was attributed to hGH treatment. When the dietary groups were pooled across hGH treatments, the C animals gained more weight ($p<0.05$) compared to the ZD and PF animals. There was no difference between the ZD and PF groups in body weight gain. By Day 4 of the feeding trial, weight gain for the ZD and PF animals began to plateau while the C animals continued to gain substantial weight (Figure 3.3). Initial body weight for the PF group was higher ($p<0.05$) on Day 1 because these animals began the experimental diet one day later than the ZD and C groups. As a result, body weight throughout the experimental period was approximately 7 grams higher in the PF group compared to the ZD group.

TAIL LENGTH

Human GH treatment had no effect ($p>0.05$) on tail length growth while zinc content of the diet was significant ($p=0.0001$; Table 3.1). After being pooled across hormone treatments and compared, changes in tail length were different among all three dietary groups ($p<0.05$). The largest gain in tail length occurred in the C group and the smallest in the ZD group. The change in the PF group was intermediate. By Day 10, differences in tail length among the three groups were apparent ($p<0.05$) and continued for the remainder of the feeding trial (Figure 3.4). Initial tail lengths were not

different.

LIVER AND TIBIA WEIGHT

Neither liver weight nor tibia weight showed an effect for hGH treatment ($p>0.05$). The effect of zinc was significant for the weight of both the liver and the tibia ($p=0.0001$; Table 3.1). When pooled across hGH treatments, liver and tibia weights were different among all three dietary groups ($p<0.05$). The C group had larger livers and tibiae compared to either the ZD or PF groups. Liver and tibia weights in the PF group were greater than those in the ZD group.

TIBIAL EPIPHYSEAL CARTILAGE WIDTH

For the tibial epiphyseal cartilage width, a significant interaction occurred between the zinc and hGH ($p=0.0331$; Table 3.1); therefore, the main effects of zinc and hGH could not be considered and the data could not be pooled. Each mean was compared to all other means and the least significant difference calculated ($p<0.05$). Significant differences occurred between pairs of means when different dietary groups were compared. The means of the ZD groups were significantly different from both PF groups and both C groups. The means of the PF groups were significantly different from both C groups. No significant difference was found between hGH treated and sham animals within the same dietary treatment. The C groups had larger epiphyseal cartilage widths compared to either the ZD and PF groups while the ZD groups had larger epiphyseal widths than the PF groups. The ZD group receiving hGH had a

larger epiphyseal cartilage width compared to its respective sham. For the PF and C groups, however, the hGH treated animals had smaller epiphyseal widths compared to the shams.

PLASMA VALUES

All blood measurements were performed on the rat plasma and included zinc, insulin-like growth factor I (IGF-I), alkaline phosphatase, and human growth hormone. Table 3.2 summarizes the data.

PLASMA ZINC

There was an effect of zinc in the diet ($p=0.0001$; Table 3.2) and of hGH treatment ($p<0.05$; Table 3.2) on plasma zinc concentrations. When pooled across dietary groups, rats that received hGH had higher concentrations of plasma zinc compared to the sham animals ($p<0.05$). Plasma zinc also differed among the three dietary groups ($p<0.05$). The C animals had higher plasma zinc concentrations than either the ZD or PF animals while plasma zinc in the PF group was almost twice that in the ZD group.

PLASMA ALKALINE PHOSPHATASE

Plasma alkaline phosphatase was affected by the zinc level in the diet ($p=0.0001$; Table 3.2) but not by hGH treatment ($p<0.05$). When dietary groups were pooled, alkaline phosphatase values were different among the three dietary groups ($p<0.05$). The PF group had higher alkaline phosphatase activity when compared to the C and ZD animals. It was also

higher in the C animals compared to the ZD animals.

PLASMA INSULIN-LIKE GROWTH FACTOR I

There was no effect of hGH treatment ($p>0.05$) on plasma IGF-I while the effect of zinc in the diet was significant ($p=0.0001$; Table 3.2). When pooled across hormone treatments, the C group had higher plasma IGF-I compared to either the ZD or PF groups ($p<0.05$). While plasma IGF-I was numerically higher in the PF rats compared to that in the ZD rats, this difference did not reach statistical significance.

HUMAN GROWTH HORMONE

Human GH in the rat plasma was not affected by zinc in the diet ($p>0.05$) but was affected by hGH treatment ($p<0.001$; Table 3.2). When dietary groups were pooled, the rats receiving hGH had higher hGH concentrations compared to the sham animals ($p<0.05$).

TISSUE MINERAL CONTENT

The concentration of zinc, copper, and iron was measured in the liver and of zinc and copper in the tibia. The data are summarized in Table 3.3.

LIVER ZINC

Liver zinc concentrations were affected by zinc in the diet ($p<0.001$; Table 3.3) but not by hGH treatment ($p>0.05$). When pooled across hGH treatment, the ZD rats had lower liver zinc compared to either the C or PF animals ($p<0.05$). There was no difference between liver concentrations in the C and PF

groups.

LIVER COPPER

There was an effect of zinc in the diet on liver copper concentrations ($p=0.0001$; Table 3.3) but no effect attributable to hGH treatment ($p>0.05$). Liver copper was higher in the PF group compared to either the C or ZD groups ($p<0.05$). Liver copper concentrations were lower in the C group compared to the ZD group but the difference was not significant.

LIVER IRON

A significant interaction between zinc and hGH treatment occurred for liver iron concentrations ($p<0.05$; Table 3.3). Therefore, main effects of zinc and of hGH were ignored and the data was not pooled. When differences between individual means were examined by LSD ($p<0.05$), differences occurred when the ZD group not receiving hGH was compared to the other groups. The exception was the comparison with the PF group receiving hGH. The other significant differences occurred between the PF group receiving hGH and the two C groups. All other differences were not significant. In general, the C animals had lower liver iron concentrations compared to the ZD and PF groups. Larger differences in the means occurred between the hGH treated and sham animals in the ZD and PF groups.

TIBIAL ZINC

For tibial zinc concentrations, a significant interaction

occurred between zinc and hGH treatment ($p<0.01$; Table 3.3). Main effects were not considered and individual means were compared by LSD ($p<0.05$). All comparisons were significantly different except for the comparisons between hGH treated and sham animals in the ZD and PF groups. Tibial zinc tended to be numerically higher in the C groups, intermediate in the PF groups, and lower in the ZD groups.

TIBIAL COPPER

There was a significant interaction between hGH treatment and zinc in the diet for tibial copper concentrations ($p<0.05$; Table 3.3). Main effects were ignored and the animals were not pooled. Individual means were compared using the LSD ($p<0.05$) and the ZD group receiving hGH was found to be significantly different from all other groups. Mean tibial copper was highest in this group. The ZD and C groups not receiving hGH were also significantly different. There were also significant differences between the PF group not receiving hGH and the C groups. Tibial copper concentrations tended to be lower in the C groups than in either the ZD or PF groups.

Table 3.1 The effect of zinc and hGH on food intake and growth parameters.[1,2]

	Dietary Groups		
hGH Trt	**Zn-Deficient**	**Control**	**Pair-fed**
	Cumulative Food Intake (g)[3]		
hGH	(12) 131.4±2.4	(12) 268.5±5.9	(12) 131.9±2.1
sham	(12) 131.2±2.3	(12) 264.0±7.1	(11) 128.8±1.8
pooled	(24) 131.2±1.6[a]	(24) 266.2±4.5[b]	(23) 130.4±1.4[a]
	Cumulative Weight Gain (g)[4]		
hGH	(12) 25.8±1.2	(12) 136.3±7.2	(12) 26.2±2.3
sham	(12) 22.9±1.3	(12) 129.1±3.8	(11) 23.2±2.2
pooled	(24) 24.3±0.9[a]	(24) 132.7±4.1[b]	(23) 24.8±1.6[a]
	Liver Wet Weight (g)[5]		
hGH	(12) 2.8±0.1	(12) 6.9±0.1	(12) 3.5±0.3
sham	(12) 2.8±0.1	(12) 6.7±0.2	(11) 3.3±0.1
pooled	(24) 2.8±0.1[a]	(24) 6.8±0.1[b]	(23) 3.4±0.2[c]
	Tibia Weight (dry, fat free) (g)[6]		
hGH	(8) 0.14±.003	(12) 0.23±.004	(12) 0.18±.003
sham	(12) 0.15±.002	(12) 0.23±.005	(11) 0.17±.002
pooled	(20) 0.15±.002[a]	(24) 0.23±.003[b]	(23) 0.17±.002[c]
	Cumulative Tail Length (mm)[7]		
hGH	(12) 33.5±0.9	(12) 63.3±1.6	(12) 38.0±1.1
sham	(12) 31.8±0.9	(12) 62.8±1.0	(11) 36.8±1.7
pooled	(24) 32.6±0.6[a]	(24) 63.1±0.9[b]	(23) 37.4±0.8[c]
	Cartilage Epiphyseal Width (μm)[8]		
hGH	(11) 331± 9.9	(12) 446±11.5	(12) 219± 8.1
sham	(12) 299±14.7	(12) 465±15.0	(10) 248±12.0

[1]Values are means ± SEM. Numbers in parentheses equals the number of rats.
[2]Means within each parameter having different letter superscripts following the number differ significantly

($p<0.05$) based on the least significant difference.
[3-8]Significance of main effects from analysis of variance (AOV):
[3]AOV: zinc ($p=0.0001$); hGH (NS); zinc*hGH (NS).
[4]AOV: zinc ($p=0.0001$); hGH (NS); zinc*hGH (NS).
[5]AOV: zinc ($p=0.0001$); hGH (NS); zinc*hGH (NS).
[6]AOV: zinc ($p=0.0001$); hGH (NS); zinc*hGh (NS).
[7]AOV: zinc ($p=0.0001$); hGH (NS); zinc*hGH (NS).
[8]AOV: zinc ($p=0.0001$); hGH (NS); zinc*hGH ($p<0.05$).

Table 3.2 The effect of zinc and hGH on plasma values.[1,2,3]

hGH Trt	Dietary Group: Zn-Deficient	Control	Pair-fed
	Zinc (μg/ml)[4]		
hGH[a]	(12) 0.77±0.05	(11) 2.11±0.13	(6) 1.59±0.09
sham[b]	(12) 0.72±0.03	(12) 1.89±0.08	(4) 1.28±0.07
pooled	(24) 0.75±0.03[a]	(23) 1.99±0.08[b]	(10) 1.46±0.08[c]
	Alkaline Phosphatase (U/L)[5]		
hGH	(12) 66.8±4.8	(11) 133.2±9.1	(7) 166.8±15.0
sham	(12) 75.1±4.4	(12) 144.1±6.3	(4) 164.4±13.6
pooled	(24) 70.9±3.3[a]	(23) 138.9±5.4[b]	(11) 164.4±10.3[c]
	Insulin-like Growth Factor I (ng/ml)[6]		
hGH	(12) 141±8	(12) 764±54	(9) 200±23
sham	(12) 159±11	(12) 744±78	(10) 244±19
pooled	(24) 150±7[a]	(24) 754±47[b]	(19) 223±15[a]
	Human Growth Hormone (ng/ml)[7]		
hGH[a]	(7) 1.9±0.5	(7) 3.9±1.0	(7) 2.3±0.5
sham[b]	(8) 1.0±0.0	(8) 1.0±0.1	(5) 1.1±0.1

[1]Values are means ± SEM. Number in parentheses equals the number of rats.
[2]Means within each parameter having different letter superscripts following the number differ significantly ($p<0.05$) based on the least significant difference.
[3]Different letter superscripts following "hGH" and "sham" indicates that the hGH treated animals, pooled across the three dietary groups, differ significantly ($p<0.05$) from the sham animals pooled.
[4-7]Significance of main effects from analysis of variance (AOV):
[4]AOV: zinc ($p=0.0001$); hGH ($P<0.05$); zinc*hGH (NS).
[5]AOV: zinc ($p=0.0001$); hGH (NS); zinc*hGH (NS).
[6]AOV: zinc ($p=0.0001$); hGH (NS); zinc*hGH (NS).
[7]AOV: zinc (NS); hGH ($p<0.001$); zinc*hGH (NS).

Table 3.3 The effect of zinc and hGH on tissue mineral content.[1,2]

hGH Trt	Zn-Deficient	Control	Pair-fed
		Dietary Group	
		Liver Zinc (μg/g dry weight)[3]	
hGH	(12) 99.4±4.2	(12) 111.4±2.5	(11) 109.6±2.8
sham	(12) 98.8±3.0	(12) 110.7±2.7	(11) 113.0±5.6
pooled	(24) 98.8±2.5[a]	(24) 111.0±1.8[b]	(22) 111.3±3.1[b]
		Liver Copper (μg/g dry weight)[4]	
hGH	(12) 19.7±0.7	(12) 18.6±0.4	(10) 23.5±1.1
sham	(11) 20.5±0.5	(12) 18.8±0.4	(11) 21.9±0.9
pooled	(23) 20.1±0.4[a]	(24) 18.7±0.3[a]	(21) 22.7±0.7[b]
		Liver Iron (μg/g dry weight)[5]	
hGH	(10) 469.8±45.0	(12) 412.9±23.4	(11) 552.9±42.3
sham	(12) 614.4±33.7	(12) 417.0±23.0	(10) 484.5±37.6
		Tibia Zinc (μg/g fat-free, dry weight)[6]	
hGH	(12) 132.5±3.9	(12) 333.2±9.9	(12) 258.3±6.6
sham	(12) 132.6±6.6	(12) 294.2±7.3	(11) 265.8±6.8
		Tibia Copper (μg/g fat-free, dry weight)[7]	
hGH	(12) 11.5±0.4	(12) 9.6±0.2	(12) 10.0±0.3
sham	(11) 10.4±0.3	(12) 9.5±0.3	(11) 10.5±0.3

[1]Values are means ± SEM. Number in parentheses equals the number of rats.
[2]Means within each parameter having different letter superscripts following the number differ significantly ($p<0.05$) based on the least significant difference.
[3-7]Significance of main effects from analysis of variance (AOV):
[3]AOV: zinc ($p<0.001$); hGH (NS); zinc*hGH (NS).
[4]AOV: zinc ($p=0.0001$); hGH (NS); zinc*hGH (NS).
[5]AOV: zinc ($p<0.001$); hGH (NS); zinc*hGH ($p<0.05$).
[6]AOV: zinc ($p=0.0001$); hGH (NS); zinc*hGH ($p<0.01$).
[7]AOV: zinc ($p=0.0001$); hGH (NS); zinc*hGH ($p<0.05$).

Figure 3.1 Cumulative Food Intake
Effect of Dietary Zinc

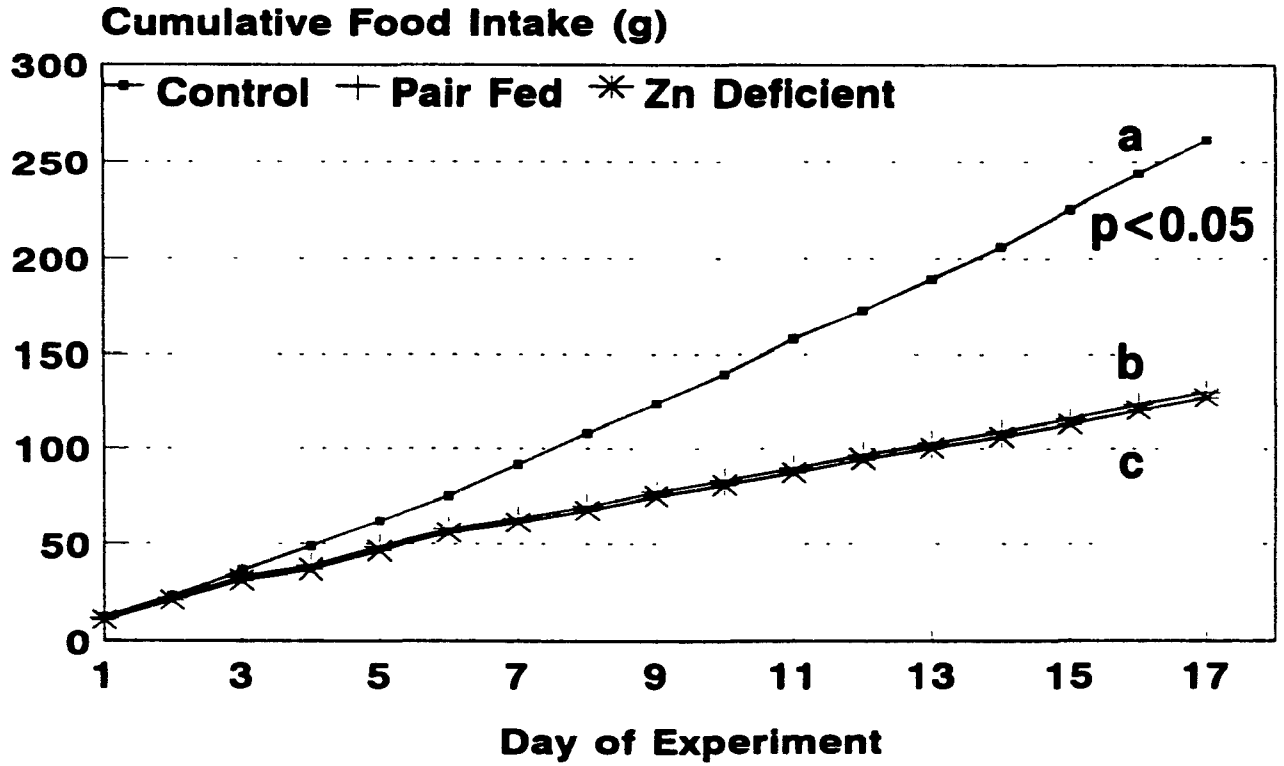


Figure 3.2 Daily Food Intake
Effect of Dietary Zinc

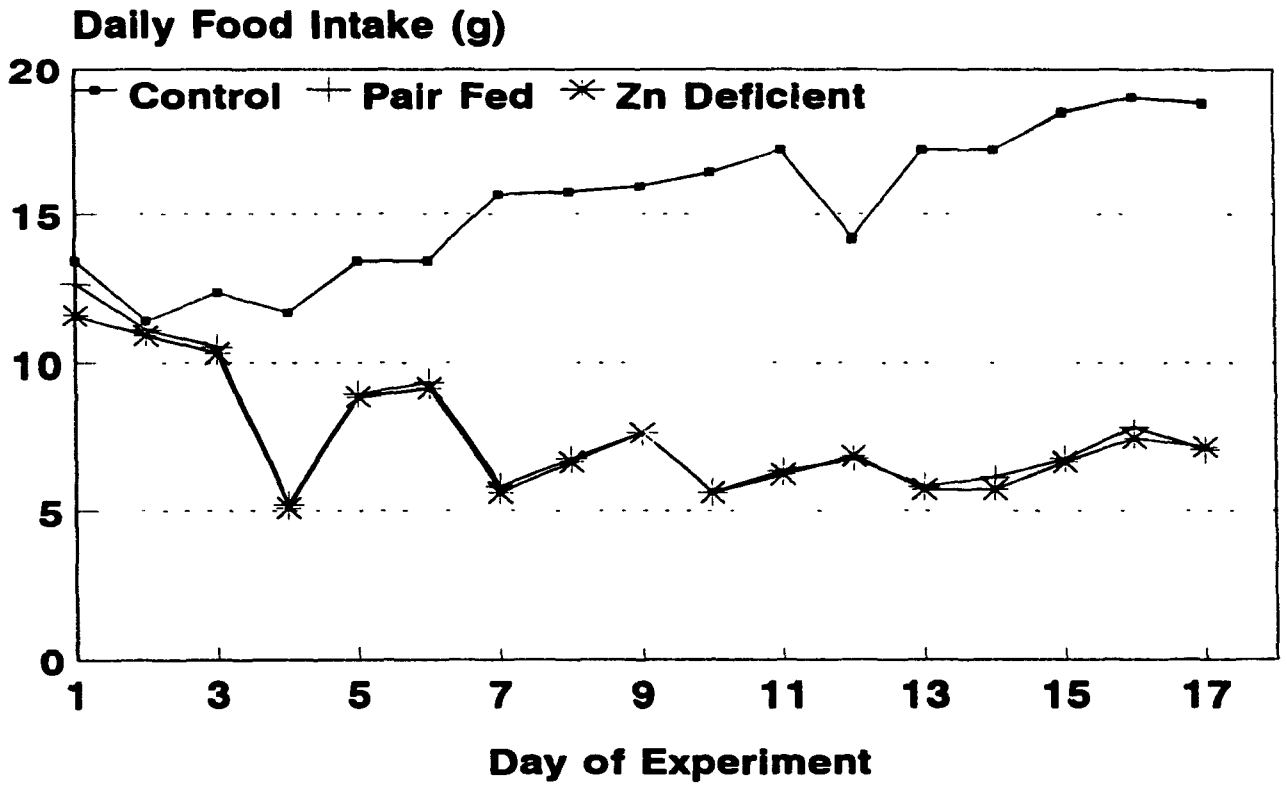


Figure 3.3 Cumulative Weight Gain
Effect of Dietary Zinc

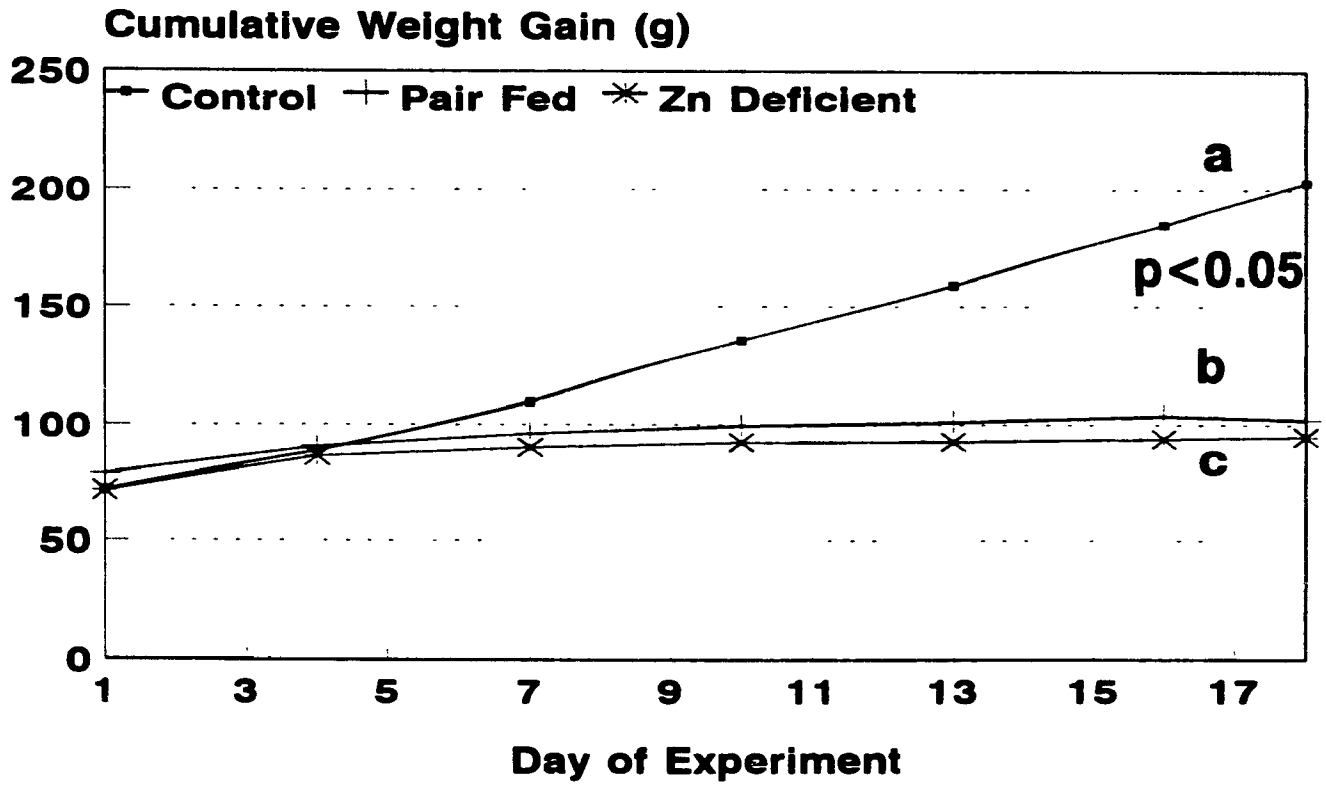
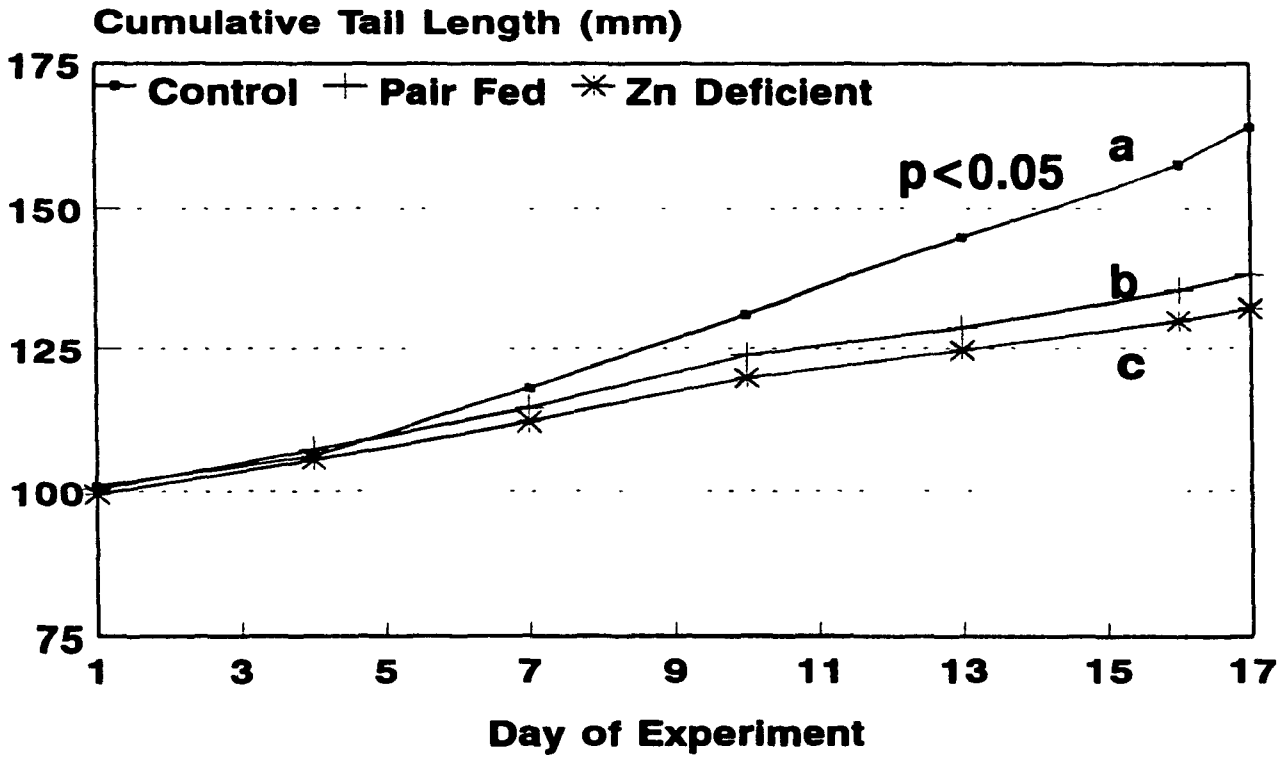


Figure 3.4 Cumulative Tail Length
Effect of Dietary Zinc



CHAPTER IV. DISCUSSION

The ability of human growth hormone (hGH) to alleviate the effects of zinc deficiency on growth was investigated. The effects of injecting hGH on growth and nutritional status were compared in three groups of rats: a zinc deficient group (ZD, < 1.5 ppm zinc, ad libitum); a control group (C, 85 ppm zinc, ad libitum); and a pair-fed group (PF, 85 ppm zinc, restricted fed). This was the first study to examine the efficacy of GH in zinc deficiency using a human GH preparation rather than a bovine GH preparation. It was also the first study to include a pair-fed group to control for the effects of inanition and to investigate the combined effects of hGH and zinc on insulin-like growth factor I (IGF-I) specifically.

NUTRITIONAL STATUS OF THE RATS

The use of < 1.5 ppm zinc in the diet was successful in producing a zinc deficiency in the experimental animals. The ZD groups displayed many of the classical signs of a zinc deficiency, including anorexia, alopecia, poor growth, and tissue zinc depletion.

A decline in food intake occurs rapidly when rats are fed a zinc deficient diet (Swenerton and Hurley, 1968; Williams and Mills, 1970; Cossack, 1984; Hambidge et al., 1986). Within four days of beginning the experimental diet, a significant reduction in food intake occurred among the deficient animals and this decrease in food intake continued

for the remainder of the study. When the day to day variation in food intake was examined (Figure 3.2), a cyclical pattern was apparent in the intake of the ZD animals which has been reported to occur with a severe deficiency (Williams and Mills, 1970; Chesters and Will, 1973). No such cycling was apparent in the C animals which increased their daily food intake throughout the study except for a slight decrease on Day 12. This increase corresponded to their increasing body weight. The pair- feeding in the PF groups was successfully completed. The daily pattern of food intake was very similar between the ZD and PF groups and the cumulative food intake was only 1 gram higher in the ZD group compared to the PF group. This successful pair-feeding can be partly attributed to the use of a pelleted diet which helped to control spillage. As well, the PF rats consumed their feed immediately when it was placed in the cage. There was no opportunity for any wasting of the diet. Human GH did not alter food intake between the hGH and sham groups.

Upon visual inspection, it was apparent that the ZD rats in the study were beginning to experience mild alopecia, or hair loss, which is a well-recognized feature of zinc deficiency (Swenerton and Hurley, 1968; NRC, 1978). Their hair coats were more sparse and had a greyish tinge compared to that of the C animals. There was very little fur on the abdomen area of the ZD animals. The PF animals showed no sign of hair loss with hair coats identical to those of the C group. The

deficiency had not progressed to the point of causing dermal lesions in the ZD animals. Figure 4.1 is a photograph of a C rat (bottom, near ruler) and a ZD rat (top) one day prior to sacrifice. Both animals were injected with hGH; however, hGH had no apparent effect on the rats' hair coats. From this photograph, it is possible to distinguish the difference in the hair coats of the two animals.

Growth measurements included cumulative body weight gain, tail length gain, tibial epiphyseal cartilage width, liver weight, and tibial weight. While all measurements were affected by zinc in the diet, only cartilage epiphyseal width showed any effect of hGH injections.

The decrease in cumulative body weight gain, a measure of overall growth, appeared to be entirely the result of the decrease in food intake. While relatively small compared to the effect of lowered food intake, many investigators report an additional effect of zinc deficiency on weight gain. In the present study, the cumulative weight gain of the C group was five times that of the other two groups while the weight gains for the ZD and PF groups were identical indicating a lack of effect for zinc. Cossack (1984) and Bolze et al. (1987) in studies of similar length reported a significant decrease in weight gain per day for ZD rats compared to PF rats. Cossack (1986) also found that zinc depressed weight gain per day when food intake was held constant and the zinc content was varied among experimental groups. In contrast,

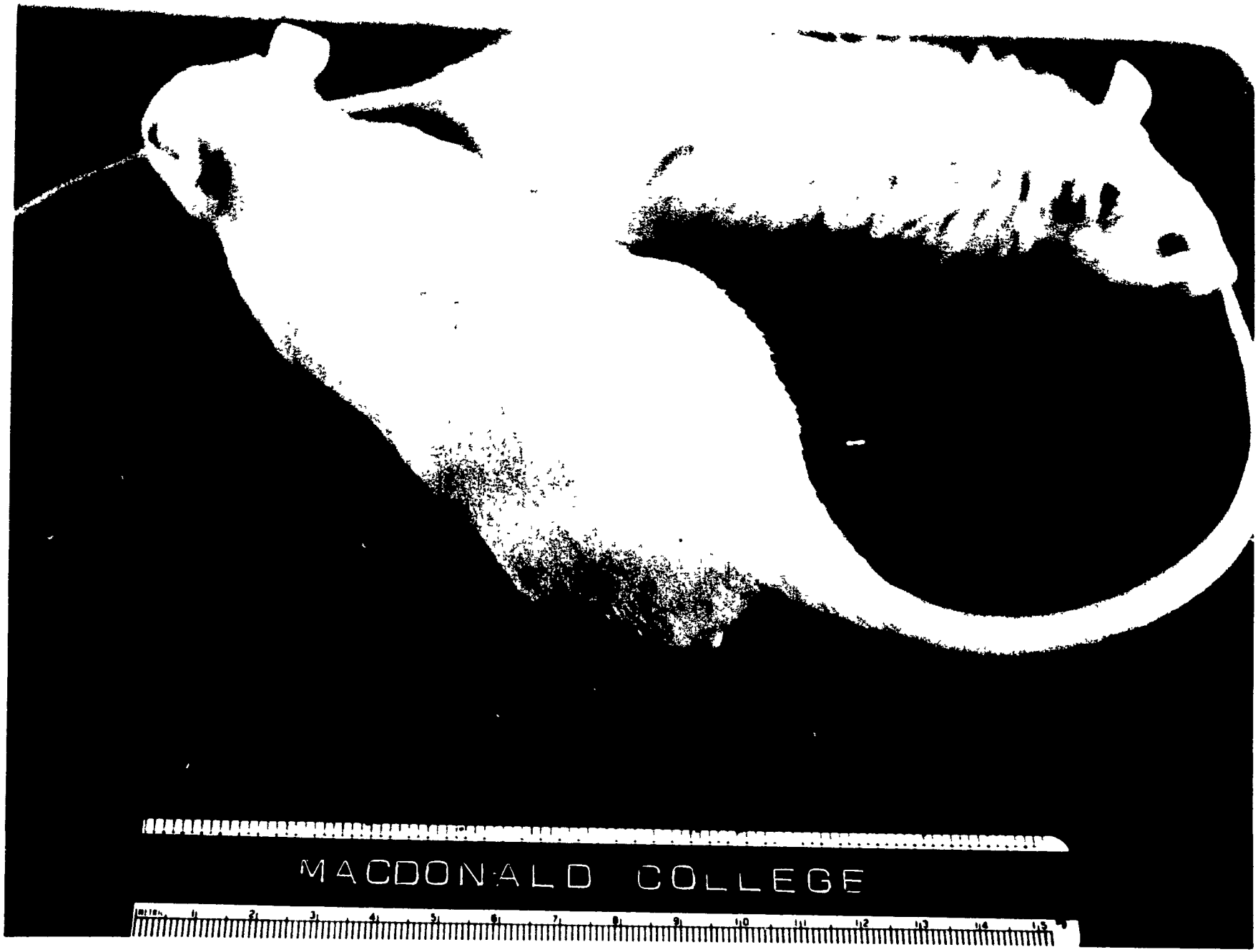


Figure 4 1 Comparison of a ZD (top) and C (bottom) rat

Oner et al. (1984) did not find a significantly greater weight gain in pair-fed rats compared to zinc deficient rats. It is interesting to note that the weight gain in the groups receiving hGH were slightly higher than their respective shams which may reflect the general anabolic nature of growth hormone.

Tail length is considered a measure of longitudinal growth in the rat since the tail contains vertebral bodies. Cumulative gain in tail length followed the expected pattern showing a small effect of zinc deficiency in addition to the larger effect of decreased food intake. Tail length was not measured in previous studies where zinc deficiency was induced; therefore, it is difficult to make comparisons. Final tail length in the C group (164 mm) was comparable to that reported in other literature (Prewitt et al., 1982; Fliesen et al., 1989). It can be safely assumed that growth was normal in the C animals and the effects in the ZD and PF groups were the expected results of the experimental diets. Decreased linear growth is the usual feature of zinc deficiency. In comparison to body weight, therefore, tail length may be a more sensitive index of growth in relation to zinc deficiency.

The decreased tibial weights and liver weights in the ZD rats compared to the C and PF animals also reflected the general growth retardation accompanying the zinc deficiency in these animals. These results agreed with Swenerton and Hurley

(1968) who reported lower femur weights and lower liver weights in zinc deficient rats. When comparing the mean tibial and liver weights for the three diet groups (Table 3.1), it is apparent that the lowered food intake had the greater effect on these organ weights. The effect of decreased zinc in the diet, as reflected by the difference between the ZD and PF groups, is relatively small although significant.

The final growth parameter was the measurement of the tibial epiphyseal cartilage width. The analysis of variance indicated a significant interaction between hGH and zinc pointing to a possible effect of hGH administration on growth. When the six group means (2 hGH treatments by 3 diets) were compared individually, the differences between the hGH treated animals and their respective shams were found not to be significant. The significant differences occurred between the various diet treatments. Human GH had no effect on epiphyseal cartilage width. These results are in agreement with those of Oner et al. (1984) who administered bovine GH treatment to zinc deficient rats and failed to show any GH effect on growth, alkaline phosphatase, or tissue zinc. In the present study, for the ZD animals, the epiphyseal width in the hGH group was approximately 32 μm larger compared to the shams. The opposite trend was found in the C and PF groups where the epiphyseal width was smaller in the hGH treated animals. Another unexpected result was the lower mean epiphyseal width

in the PF groups compared to the ZD groups. It was expected that the ZD rats would have the smallest epiphyseal widths of the three dietary groups (Oner et al., 1984). This was not the result of tibial weight since the PF group had larger tibia than the ZD group. Thus, the results in the PF rats are not readily explainable and may be a unique phenomenon in this study.

Plasma alkaline phosphatase, a zinc metalloenzyme used as a functional test of zinc deficiency, decreases in zinc deficiency (Hambidge et al., 1986). As was expected, alkaline phosphatase was decreased in the ZD animals compared to the C and PF groups; however, the enzyme was unexpectedly higher in the PF animals compared to the C animals. In the only other study similar to the one reported here, Oner et al. (1984) found that in the pair-fed rats, alkaline phosphatase was lower than the controls. A partial explanation of the present results may be the small number of samples available for analysis in the PF groups which may have altered the true mean. The variation was larger in the PF and C groups compared to the ZD rats. Overall, the values for alkaline phosphatase were much lower in this study compared to those of Oner et al. (1984).

The zinc content of the plasma, liver, and tibia confirmed the development of a zinc deficiency in the ZD animals and corresponded to the results reported in the literature (Swenerton and Hurley, 1968; Cossack, 1984; 1986; Hambidge et

al., 1986). In the ZD group, the values for these parameters were always lower compared to the C and PF groups. Values for the C and PF rats were similar or lower in the PF group. In comparison to the lowered food intake, the lack of zinc in the diet had the greater effect on decreasing tissue zinc content. Actual values for these parameters are comparable to those of Cossack (1984; 1986) who used similar analytical procedures for zinc determinations.

Plasma zinc responds relatively quickly to zinc depletion. Both the decreased zinc intake and food intake influenced plasma zinc values since the PF rats had intermediate values compared to the other animals. Human GH had an apparent effect on plasma zinc which was higher in the animals receiving hGH. Oner et al. (1984) showed no increase in serum zinc when bGH was given to ZD rats. Studies in humans also have reported no increase of serum zinc in response to GH therapy (Cheruvanky et al., 1982; Richards and Marshall, 1983). The increase in plasma zinc levels for the ZD rats in this study was also much smaller than the increases for the C and PF groups; therefore, the effect may not be as great for the deficient animals.

While liver zinc in the ZD group was decreased, there was no difference between the C and PF animals. Liver zinc levels were affected by the level of zinc in the diet and not by depressed food intake. Cossack (1984; 1986) also reported similar liver zinc values for pair-fed and control animals.

An interaction between hGH and zinc was observed for the zinc content of the tibia. In the C rats, tibial zinc was significantly higher in the hGH treated animals compared to the shams but this increase did not occur in the PF and ZD groups. Ignoring the interaction, tibial zinc declined in both the ZD and PF animals with a more substantial decline for the ZD groups. This result would be in agreement with earlier reports of Cossack (1984; 1986). Similar results have been shown for the femur as well (Oner et al., 1984; Hambidge et al., 1986; Bolze et al., 1987).

Plasma and bone tend to show a more marked reduction in zinc content in the zinc deficient state compared to the liver (Hambidge et al., 1986; Park et al., 1986). When the values for plasma, liver, and tibial zinc in the ZD groups are compared, plasma and tibial zinc did show greater decreases in zinc content in response to the lack of dietary zinc compared to the liver. Plasma and tibial zinc would appear to be more sensitive indicators of zinc status for the rat.

Liver iron and copper and tibial copper were analyzed since these minerals interact with zinc. Prasad et al. (1969) found an inverse relationship between zinc supplementation and iron content of liver, bone, and other tissues. Wallwork et al. (1981) did not find a significant effect of dietary zinc on iron and copper in the liver and femur although values for both minerals tended to be lower as dietary zinc increased. In the present study, the lowest mineral values were consistently

in the C group although not always significant. Results for the PF and ZD groups were variable. Liver copper was highest in the PF animals but tibial copper was highest in the ZD animals. The interaction between hGH and zinc for liver iron was the result of significantly higher iron concentrations in the ZD sham group and in the PF hGH treated group. The interaction for tibial copper occurred because of the high copper content in the ZD animals who received hGH. While the interactions were significant, there was no consistent effect for hGH treatment on mineral content in these tissues when the means were individually compared. These results support a mineral interaction with high dietary zinc decreasing iron and copper concentrations.

PLASMA INSULIN-LIKE GROWTH FACTOR I

Plasma IGF-I was significantly lower in the PF and ZD groups compared to the C group. Numerically lower plasma IGF-I levels were found in the ZD group compared to the PF group but the difference did not reach statistical significance (LSD = 87.6 at $p < 0.05$). This would indicate that IGF-I declined because of decreased food intake only with no decline attributable to a lack of zinc in the diet. These results are contradictory to the work of Oner et al. (1984) and Cossack (1984; 1986; 1988) who all found a small but separate effect of zinc on IGF-I. In a study by Bolze et al. (1987), somatomedin activity in the ZD rats was 75 percent of PF levels but not statistically significant at the end of

depletion. Somatomedin activity in the PF animals normalized within 48 hours while recovery of somatomedin activity in the ZD rats did not occur until Day 8 which suggested an additional effect of zinc on somatomedin activity. Therefore, this decline in IGF-I may be a biologically important result of the zinc deficiency although it was not statistically significant.

A direct comparison of the values in this study with those of earlier work is difficult. Oner et al. (1984) measured extracted serum IGF-I, Bolze et al. (1987) measured somatomedin activity, and Cossack (1984; 1986; 1988) measured unextracted IGF-I. The values in all studies were expressed as U/ml in reference to a pool of normal rat serum or plasma designated to contain 1 U/ml. In this study, IGF-I was extracted from its binding proteins using acid:ethanol and was expressed in ng/ml in relation to human IGF-I standards. Acid extraction yields results which are two to three times greater than untreated plasma. Therefore, we can only compare the trend in values among the various studies. In this study, IGF-I was highest in the C animals, intermediate in the PF, and lowest in the ZD group. The effect of lowered food intake was much larger than that for the decreased dietary zinc.

Because very few authors expressed IGF-I as ng/ml, it was difficult to determine whether values obtained in this study were normal for the rat. One study by Thissen et al. (1990) reported serum IGF-I values of 713 ± 53 ng/ml for normal

female rats while another study (Thissen et al., 1991) reported values of 1186 ± 123 ng/ml. They extracted the binding proteins using a filtration column. In general, IGF-I values tend to exhibit large variations. Acid extraction, which was the method of analysis in this study, may leave residual binding proteins which can interfere with some RIA's (Daughaday and Rotwein, 1989). This is particularly true for rat plasma which has higher insulin-like growth factor binding protein (IGFBP) concentrations. Two human controls with known IGF-I were extracted and assayed along with the rat samples. Results were consistent between assay runs and fell within the specified range of concentrations. However, it is possible that the acid extraction did not remove all IGFBP's in our rat plasma and the values given here are lower than true values.

EFFECT OF HUMAN GROWTH HORMONE

Treatment with hGH failed to improve indices of growth or alter plasma IGF-I in the zinc deficient rats. One concern is the absorption of the injected hGH. We used a commercial kit designed to assay hGH in human serum or plasma. Any matrix differences between human plasma and rat plasma may have made it difficult to detect the hGH. There was some non-specific binding when rat plasma was incubated alone with the labelled GH. Mean values for the hGH was 1.0 ng/ml in the sham animals and 2.7 ng/ml in the hGH treated animals. Some cross reactivity occurred since the shams, who received no hGH, failed to show a concentration of 0 ng for the assay. It was

assumed that the injected hGH was absorbed into the rat circulation since only the hGH treated animals had values above 1 ng/ml.

We failed to show a response to hGH not only in the ZD group but also in the C group who should have shown an increase in circulating IGF-I in response to the hGH injection. We chose the dose, 20 μg per 100 g body weight, based on the work of Prasad et al. (1969). Thissen et al. (1990) required 400 μg of recombinant GH to increase serum IGF-I two-fold in normal protein-deprived rats. Other studies have used equally large doses. It would appear that the lack of response in the present study is the result of the insufficient dose of GH.

Because of lowered food intake, protein energy malnutrition accompanies zinc deficiency in the rat. In humans, as well, zinc deficiency often occurs with protein energy malnutrition (Golden and Golden, 1979). Nutritional variables may be equally as important as GH in regulating IGF-I levels (Furlanetto, 1990). Protein and/or energy restriction has been shown to depress IGF-I levels in rats (Phillips and Young, 1976; Price et al., 1979; Prewitt et al., 1982) and in humans (Isley et al., 1983; Clemmons et al., 1985). In these nutritional insults, the mechanism(s) for the depression of IGF-I have not been a simple decline in circulating GH levels. Fasting appears to decrease GH binding in the liver because of reduced numbers of GH receptors (Maes et al., 1983; 1984a;

1984b). A post-receptor defect appears to occur with protein deprivation which blunts an animal's response to exogenous GH (Maes et al., 1988; Maiter et al., 1988). Thissen et al. (1991) also suggested that protein deprivation causes resistance to IGF-I at the organ level and reduces synthesis of IGFBP-3. Normalization of circulating IGF-I failed to promote growth in protein deprived rats. It is increasingly apparent that nutritional sufficiency is essential for IGF-I to promote growth (Thissen et al., 1990). A zinc deficient rat is not in a state of nutritional sufficiency. If these events were occurring in the zinc deficient animal as a result of its lowered food intake, it may also be difficult to detect the animal's response to exogenous GH. Alternatively, the zinc effect on IGF-I may be mediated through one of these other mechanisms.

SUMMARY

The present study confirmed the reports of previous investigators who showed that IGF-I (or SM-C or somatomedin activity) declines in zinc deficient rats. While IGF-I levels were not significantly different between the ZD and PF animals, a marked decline was apparent in the ZD groups. Together with earlier work, it is probable that zinc has an effect, separate from decreased food intake, on circulating IGF-I levels.

The hypothesis behind the present study was that zinc deficiency decreased circulating growth hormone which, in

turn, depressed circulating IGF-I levels with resultant subnormal linear growth. We have neither proved nor disproved this hypothesis since the hGH treatment was inadequate and the lack of effect is due to the low dose of hGH. The dose may be another problem concerning the studies of Prasad et al. (1969) and Oner et al. (1984) who used 20 μg and 40 μg of bGH respectively and failed to show an effect in zinc deficiency. If large doses of GH are needed for a response to occur in the rat, a question arises concerning the biological importance of both the dose and the response. Future research should explore other possible mechanisms.

The original somatomedin hypothesis, which stated that circulating IGF-I influenced growth in an endocrine manner, has been expanded to include autocrine and paracrine IGF-I and activities. It is possible that the decline in circulating IGF-I does not play a significant role in the retarded growth accompanying zinc deficiency. Thus, it is important to determine the relevance of this decline in endocrine IGF-I in zinc deficiency. One method is to treat zinc deficient rats with IGF-I. Larger quantities of IGF-I are becoming available for research purposes because of recombinant technology.

Events that take place at the level of the liver mediate the effects of protein and/or energy malnutrition on IGF-I. Instead of lower levels of GH in circulation resulting in the decline in circulating IGF-I with zinc deficiency, this decline in IGF-I levels may be mediated through a mechanism at

the level of the liver. Growth hormone resistance or a decline in GH receptors may occur which would both decrease IGF-I synthesis. Circulating IGF-I is also dependent upon the availability of IGF binding proteins, especially IGFBP-3. The lack of available zinc may be inhibiting synthesis of the binding proteins. Future research should focus on these possible mechanisms and whether any are influenced by the level of zinc in the diet.

Zinc may also influence IGF-I at the level of the tissue, ie. autocrine or paracrine activities. Schlecter et al. (1986a) developed a method whereby rats are cannulated and substances, such as GH or IGF-I, are infused directly into the limb. A similar procedure could be done in zinc deficient rats and the local effects of GH and IGF-I studied. This method is useful since the opposite leg of the same animal can act as a control. Alternatively, the development of an in vitro system, such as that of Scheven et al. (1991), would be worthwhile for isolating the specific effects of zinc at the local tissue level.

The applicability of these results to humans is unknown. One concern regarding the extrapolation of results to humans is the severity of the zinc deficiency. Most studies, including the present one, produced a severe deficiency in the animals. Zinc deficiency in humans is usually marginal or mild with a severe one occurring under unusual circumstances. A more appropriate approach would be to induce a mild

deficiency in the rat and to determine its effect on IGF-I. Another problem is the lack of studies in this area. Ghavami-Maibodi et al. (1983) found that somatomedin-C increased after oral zinc supplementation in short children but Payne-Robinson et al. (1991) found no significant association of zinc with IGF-I in young malnourished children when they controlled for the malnutrition. No other studies have investigated the effect of zinc on IGF-I in humans; therefore, studies using a human model of zinc deficiency would help to clarify the relationship between zinc and IGF-I.

CONCLUSION

In the present study, zinc deficiency in the rat reduced food intake with a cyclical pattern of intake developing. Tissue zinc content was significantly reduced and showed a greater effect of dietary zinc compared to the decreased food intake. Plasma and tibia zinc showed a greater response to the zinc deficiency compared to liver zinc. Tissue iron and copper showed a competitive interaction with zinc and was lower in the animals receiving the 85 ppm zinc diet. There was no consistent effect of lowered food intake on tissue copper and iron. Zinc deficiency also decreased plasma alkaline phosphatase.

Growth retardation also accompanied the zinc deficiency. Body weight gain was significantly reduced by the lowered food intake but showed no separate effect for dietary zinc. Tail length showed a small but separate effect of the zinc

deficiency in addition to the larger effect of decreased food intake. Both tibia and liver weight were reduced by the dietary zinc deficiency and the lowered food intake. The effect for zinc was the smaller of the two effects. Tibial epiphyseal cartilage width was greater in the zinc deficient animals compared to the pair fed animals.

The present study also showed a possible effect of zinc on free IGF-I. While not significant, IGF-I was lower in the zinc deficient group compared to the pair fed group. Lowered food intake caused the more substantial decrease in IGF-I.

As demonstrated here, both the lowered zinc intake and the lowered food intake contribute to the effects seen with zinc deficiency; however, the effects are not equally distributed. It is the zinc intake which has the most significant effect on mineral content in the various tissues. Growth parameters, however, show a greater effect for the lowered food intake with a relatively small effect attributable to zinc. Plasma IGF-I also shows a greater response to the lowered food intake compared to the lack of zinc.

APPENDIX 1. DETERMINATION OF ZINC IN THE DIET

1. Dry the sample in a drying oven at 56°C overnight.
2. Grind the sample to a fine powder.
3. Weigh 0.5 g of sample and place in a digestion tube.
4. Add concentrated nitric and perchloric acids in a ratio of 5:1 (approximately 10 ml).
5. Place the tube in the digestion unit and digest the sample at 180-200°C until the brown fume disappears.
6. Transfer the remaining solution to a 50 ml volumetric flask and bring to volume with distilled deionized water.
7. Analyze the zinc content with flame atomic absorption spectrophotometry using a Perkin-Elmer spectrophotometer (Model 3100, Perkin-Elmer (Canada) Ltd., Montreal, PQ).

APPENDIX 2. TIBIAL CARTILAGE EPIPHYSEAL WIDTH

1. Dissect the tibia free from the surrounding soft tissue and bone. Care must be taken to keep the epiphysis intact.
2. Split the tibia with a sharp razor in the mid-sagittal plane.
3. Soak the tibial halves in water for one-half hour.
4. Immerse the the tibial halves in acetone for one hour.
5. Soak the tibial halves again in water for one-half hour.
6. While the tibial halves are soaking, prepare the 2% (w/v) silver nitrate solution and the 10% (w/v) sodium thiosulfate solution.
7. Soak the tibial halves in the 2% silver nitrate solution for 1-2½ min. Immediately rinse with water.
8. Place the tibial halves in water and expose them to a strong light source until the calcified portions appear dark brown. The cartilage section should remain clear and gelatinous in appearance.
9. Immerse the tibial halves in the 10% sodium thiosulfate solution for 25-30 sec.
10. Wash the tibial halves several times with water and then soak them in water for one-half hour.
11. Measure the width of the cartilage under the high power of a microscope fitted with a micrometer. Make 8-10 readings across the cartilage section and average the readings to give the width of cartilage band.

APPENDIX 3. ACID EXTRACTION OF IGF-I BINDING PROTEINS

1. EDTA plasma samples are thawed at room temperature.
2. Pipet 10 µl of sample or control into a 1.5 ml microeppendorf centrifuge tube. Add 40 µl of a hydrochloric acid:ethanol mixture[1] and thoroughly mix with a votex.
3. Incubate at room temperature for 30 min.
4. Centrifuge at 3500 rpm for 30 min in a refrigerated centrifuge. A white precipitate will form.
5. Pipet 10 µl of the supernatant (containing the free IGF-I) into a 10x75 mm disposable plastic (polystyrene or polypropylene) culture tube. Add 1.4 ml of phosphate buffer containing 300 mg Tris base/l[2] and thoroughly mix with a vortex. At this point, the samples can be stored at 4°C for one week. The supernatant fraction (100 µl) contains 1 µl sevenfold diluted plasma.
6. For the radioimmunoassay, pipet 100 µl of the supernatant fraction into another 10x75 disposable plastic culture tube. Follow the procedures as outlined in the RIA.

[1] For the hydrochloric acid:ethanol mixture (1:7, v/v), combine:
21.6 ml concentrated hydrochloric acid
103.4 ml distilled deionized water
875 ml absolute ethanol.

[2] For the phosphate buffer, add 300 mg of Trizma base (Sigma, St. Louis, MO) to 1 L of phophate buffered saline (pH 7.6)(Sigma, St. Louis, MO).

APPENDIX 4. FAT EXTRACTION OF THE TIBIA

1. Dissect the tibia free of the surrounding tissue and bone.
2. With a fine-pointed needle, make a hole in both ends of the bone to facilitate extraction. Care must be taken to prevent the bone from splitting.
3. Soak the bone overnight in methanol.
4. Using the Soxelet System for fat extraction, place several bones in each column of the system. Label the bones by tying different colored thread around the bone. If available, india ink with a fine tip pen can also be used.
5. Extract the fat with petroleum ether for approximately 6 hours.
6. Remove the bones from the extraction apparatus and air dry in the fume hood overnight.

APPENDIX 5. DETERMINATION OF TRACE ELEMENTS

1. Place the tibia or liver in a plastic vial and freeze-dry to a constant weight (48 hours).
2. Grind the liver into a powder. The tibia can remain whole.
3. Place 250-350 mg of ground liver (or whole tibia) in a 25 ml Erlenmeyer flask. Add 4 ml (3 ml for the tibia) of nitric acid. Cover and pre-digest at room temperature overnight (12 hours).
4. Set the hot plate (Thermolyne 2200, Thermolyne Corp., Dubuque, IA) at 100°C and preheat for one-half hour.

5. Place the samples on the hot plate and cover with a glass plate. Because of the uneven heat distribution, the samples should be rotated 2 or 3 times during the digestion to ensure even heating.
6. Heat the samples for 4-6 hours until all particulate matter is digested. The digested solution should be a pale yellow.
7. Remove from the heat and allow to cool to room temperature.
8. Transfer the digested samples to 25 ml volumetric flasks. Rinse the inside of the Erlenmeyer flasks several times with distilled deionized water to ensure complete transfer of the acid digest. Bring to volume with distilled deionized water.
9. Dilute the samples to the appropriate volumes with distilled deionized water.
10. Analyze mineral concentrations with flame atomic absorption spectrophotometry using a Perkin-Elmer spectrophotometer (Model 3100, Perkin-Elmer (Canada) Ltd., Montreal, PQ).

APPENDIX 6. STATISTICAL METHODS

ANOVA: analysis of variance followed by the least significant difference (t test) for all parameters.

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PROC GLM;

CLASS REP ZINC GHORM;

MODEL variable(s) = ZINC GHORM ZINC*GHORM/SS3;

MEANS ZINC GHORM ZINC*GHORM/LSD;

PROC MEANS MEAN STDERR N;

BY ANIMAL;

VAR variable(s);
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APPENDIX 7. SUMMARY OF SIGNIFICANT STATISTICS

Each table summarizes the ANOVA for the dependent variables tested using the model: VARIABLE = ZINC GHORM ZINC*GHORM.

SOURCE	DF	MEAN SQUARE	F VALUE	PR > F
DEPENDENT VARIABLE: cumulative food intake				
ZINC	2	145583.300	694.03	0.0001
GHORM	1	118.073	0.56	0.4558
ZINC*GHORM	2	27.893	0.13	0.8757
ERROR	65	209.766		
MODEL	R^2 = 0.9553			

SOURCE	DF	MEAN SQUARE	F VALUE	PR > F
DEPENDENT VARIABLE: cumulative weight gain				
ZINC	2	92919.268	580.56	0.0001
GHORM	1	322.978	2.08	0.1540
ZINC*GHORM	2	34.800	0.22	0.8052
ERROR	65	160.050		
MODEL	R^2 = 0.9471			

SOURCE	DF	MEAN SQUARE	F VALUE	PR > F
DEPENDENT VARIABLE: cumulative tail length				
ZINC	2	6413.387	412.52	0.0001
GHORM	1	21.004	1.35	0.2494
ZINC*GHORM	2	1.799	0.12	0.8909
ERROR	65	15.547		
MODEL	R^2 = 0.9271			

SOURCE	DF	MEAN SQUARE	F VALUE	PR > F
DEPENDENT VARIABLE: liver weight				
ZINC	2	108.865	313.52	0.0001
GHORM	1	0.235	0.68	0.4142
ZINC*GHORM	2	0.047	0.14	0.8738
ERROR	65	0.347		
MODEL	R^2 = 0.9077			

SOURCE	DF	MEAN SQUARE	F VALUE	PR > F
DEPENDENT VARIABLE: tibia weight				
ZINC	2	0.036	290.01	0.0001
GHORM	1	0.000	0.10	0.7515
ZINC*GHORM	2	0.000	0.26	0.7722
ERROR	61	0.000		
MODEL	R^2 = 0.9059			

SOURCE	DF	MEAN SQUARE	F VALUE	PR > F
DEPENDENT VARIABLE: cartilage epiphyseal width				
ZINC	2	290106.368	70.44	0.0001
GHORM	1	488.791	0.29	0.5946
ZINC*GHORM	2	6149.797	3.60	0.0331
ERROR	63	1708.797		
MODEL	R^2 = 0.8483			

SOURCE	DF	MEAN SQUARE	F VALUE	PR > F
DEPENDENT VARIABLE: plasma zinc				
ZINC	2	9.176	130.68	0.0001
GHORM	1	0.444	6.32	0.0151
ZINC*GHORM	2	0.073	1.03	0.3630
ERROR	51	0.070		
MODEL	R^2 = 0.8400			

SOURCE	DF	MEAN SQUARE	F VALUE	PR > F
DEPENDENT VARIABLE: alkaline phosphatase				
ZINC	2	41730.047	68.60	0.0001
GHORM	1	221.250	0.36	0.5491
ZINC*GHORM	2	273.294	0.45	0.6406
ERROR	52	608.347		
MODEL	R^2 = 0.7351			

SOURCE	DF	MEAN SQUARE	F VALUE	PR > F
DEPENDENT VARIABLE: insulin-like growth factor I				
ZINC	2	2544976.243	120.10	0.0001
GHORM	1	3340.191	0.16	0.6927
ZINC*GHORM	2	5570.130	0.26	0.7697
ERROR	61	21190.604		
MODEL	R^2 = 0.7977			

SOURCE	DF	MEAN SQUARE	F VALUE	PR > F
DEPENDENT VARIABLE: human growth hormone				
ZINC	2	4.267	2.42	0.1028
GHORM	1	27.592	15.68	0.0003
ZINC*GHORM	2	4.670	2.65	0.0841
ERROR	36	1.760		
MODEL	R^2 = 0.4178			

SOURCE	DF	MEAN SQUARE	F VALUE	PR > F
DEPENDENT VARIABLE: liver zinc				
ZINC	2	1211.396	8.02	0.0008
GHORM	1	3.981	0.03	0.8715
ZINC*GHORM	2	37.636	0.25	0.7802
ERROR	64	151.049		
MODEL	R^2 = 0.2055			

SOURCE	DF	MEAN SQUARE	F VALUE	PR > F
DEPENDENT VARIABLE: liver copper				
ZINC	2	91.646	16.12	0.0001
GHORM	1	0.769	0.14	0.7143
ZINC*GHORM	2	7.924	1.39	0.2557
ERROR	62	5.683		
MODEL	R^2 = 0.3580			

SOURCE	DF	MEAN SQUARE	F VALUE	PR > F
DEPENDENT VARIABLE: liver iron				
ZINC	2	105826.253	8.08	0.0008
GHORM	1	11897.102	0.91	0.3443
ZINC*GHORM	2	63184.573	4.83	0.0113
ERROR	61	13094.290		
MODEL	R^2 = 0.3159			

SOURCE	DF	MEAN SQUARE	F VALUE	PR > F
DEPENDENT VARIABLE: tibia zinc				
ZINC	2	208582.288	350.19	0.0001
GHORM	1	1930.743	3.24	0.0764
ZINC*GHORM	2	3690.974	6.20	0.0034
ERROR	65	595.629		
MODEL	R^2 = 0.9168			

SOURCE	DF	MEAN SQUARE	F VALUE	PR > F
DEPENDENT VARIABLE: tibia copper				
ZINC	2	12.177	11.92	0.0001
GHORM	1	0.948	0.93	0.3390
ZINC*GHORM	2	4.160	4.07	0.0216
ERROR	64	1.021		
MODEL	R^2 = 0.3455			

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