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# The effect of total parenteral nutrition (TPN) on zinc (Zn) retention in the tissue of rats

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**FLORIDA INTERNATIONAL UNIVERSITY**

**Miami, Florida**

**THE EFFECT OF TOTAL PARENTERAL NUTRITION (TPN) ON  
ZINC (Zn) RETENTION IN THE TISSUES OF RATS**

**A thesis submitted in partial fulfillment of the  
requirements for the degree of**

**MASTER OF SCIENCE**

**IN**

**NUTRITION**

**by**

**Martha M. Chisholm**

**1995**

To: Judith A. Blucker, Ph.D  
Acting Dean, College of Health

This thesis, written by Martha M. Chisholm, and entitled The Effect of Total Parenteral Nutrition (TPN) on Zinc (Zn) Retention in the Tissues of Rats, having been approved in respect to style and intellectual content, is referred to you for judgement.

We have read this thesis and recommend that it be approved.

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Florida International University, 1995

I dedicate this thesis first to my Lord, Almighty God, without Whom I, nor the rest of this story would have been possible; and to my parents, whose love, understanding, and support helped me complete this work.

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**ABSTRACT OF THE THESIS**  
**THE EFFECT OF TOTAL PARENTERAL NUTRITION (TPN) ON**  
**ZINC (Zn) RETENTION IN THE TISSUES OF RATS**

by

**Martha M. Chisholm**

**Florida International University, 1995**

**Miami, Florida**

**Professor Evelyn B. Enrione, Major Professor**

Zn is regularly added to TPN solutions, however, requirements are poorly understood. This research investigated whether or not the route of Zn intake (oral vs parenteral) changes Zn concentration in rat tissues. Twenty-four male Fischer-344 rats were equally and randomly assigned to one of four groups (n=6): control (CON), baseline (BS), orally fed (OF), and intravenously fed (IV). After fifteen days of feeding, organs and sera were collected and analyzed for Zn by atomic absorption spectrophotometry. Of the nine tissues analyzed, the Zn concentrations in the liver, kidney, and lung of the IV rats were significantly higher ( $p < 0.01$ ) than those of the CON, BS, and OF rats. Results indicated that rats fed intravenously with Zn displayed increased Zn concentrations in liver, kidney and lung tissues, and that the concentration of Zn in the serum may not reflect tissue Zn levels. This suggests that the route of Zn intake affects tissue Zn concentration.

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## Introduction

Zinc (Zn) nutriture is recognized as being important not only for the healthy population but also for the acutely and chronically ill patient experiencing and/or recovering from a variety of medical conditions, especially those necessitating total parenteral nutrition (TPN) (1,2,3,4,5,6,7,8,9,10,11,12). Although trace elements, including Zn, are regularly added to TPN solutions, requirements are poorly understood. This is due to the lack of sufficient animal and human data which would support specific recommendations for Zn requirements in TPN solutions, and to the wide range of medical diseases which alter Zn requirements and require TPN (2).

Despite the 1979 guidelines set forth by the American Medical Association on parenteral trace element administration (13), suboptimal body Zn nutriture continues to be a risk in patients receiving TPN for long periods of time. In the United States, studies which evaluate the effect of TPN on trace element retention in body tissues have not been completed. Few foreign studies have been conducted to investigate changes in tissue Zn nutriture during TPN; these have been limited by availability of tissues for analyses (14), or have used a variety of Zn dosages unrelated to the daily Zn requirement for rats (15,16). It was apparent that studies were needed to evaluate the effects of intravenous Zn

administration on tissue Zn retention, and to compare these results to the effect which normal oral Zn intake has on tissue Zn homeostasis. Previously, it was not clear how the oral Zn requirement for rats related to the intravenously administered requirement nor how this might change tissue Zn concentration in specific organs. Therefore, this study was developed to determine if the route of Zn intake (oral vs parenteral) changes Zn retention in rat tissues.

## Review of Literature

### Zn Nutriture with TPN Solutions

#### **Historical Review**

Zn deficiency was not diagnosed during the initial years of parenteral nutrition because infusions could only be administered for a few days (17,18,19). In the early 1960's and 1970's, technical problems with hyperosmolar parenteral nutrition solutions continued (17), preventing long-term administration of the solutions. This preceded administration of solutions through deep venous catheters (17,20,21,22), which allowed longer parenteral nutrition infusions without complications. There was no immediate concern about Zn deficiency in patients, although reference to possible risk of Zn deficiency was made (18,23).

Originally, the nitrogen components of the solution were protein hydrolysates which were naturally contaminated with trace elements (23,24). This contamination made Zn deficiency relatively rare for early TPN patients (24). However, these solutions contained protein hydrolysates and dextrose that were heat sterilized (2). This heat sterilization process formed sugar-amino acid complexes (2). These complexes bound Zn and formed metal-sugar-amino acid complexes that resulted in excessive loss of Zn in the urine and increased Zn deficiency risk due to the inability to retain Zn

appropriately in the body (2).

In the mid to late 1970's, the protein source in TPN solutions changed from protein hydrolysates to crystalline amino acids, containing only L-amino acids. Because the solutions no longer required heat sterilization (2,17), the metal-sugar-amino acid complexes did not form and thus urinary Zn loss was prevented (2). Conversely, it is documented that infants fed parenterally lose Zn predominately through the urine (rather than through the stool, as in orally-fed infants) (2,12,24,25).

The use of crystalline amino acids without Zn supplementation was an important factor in producing Zn deficiency in adult patients receiving TPN. Highly purified intravenous solutions, with minimal Zn contamination, were related to an increased risk of Zn deficiency, as was the concurrent altered plasma concentration of certain amino acids. The Zn-binding amino acid, histidine, increased the risk of renal Zn ultrafilterability, and along with cysteine, promoted increased urinary excretion of Zn in animals and humans (25,26,27). In 1977, Freeman and Taylor showed how the administration of histidine, either orally or intravenously, significantly increased (three to six times) urinary excretion of Zn in rats, when compared to controls (27). However, no clinical evidence of Zn deficiency was found after histidine was administered for 43 days (27).

Nevertheless, by the late 1970's, because of the change to crystalline amino acids, and the experiences with Zn deficiency, it became apparent that formal recommendations were needed for Zn supplementation in TPN (12,13).

### **Zn Supplementation Recommendations**

In 1979, the Nutrition Advisory Group of the Department of Foods and Nutrition, American Medical Association (AMA) directed an expert panel to set guidelines for essential trace element preparations for parenteral use (13). The AMA expert panel modified the essential human dietary Zn requirements (attained via balance studies, intestinal absorption and excretory route studies), in order to develop guidelines for intravenous Zn intake (13). They considered that the daily dietary oral requirement for Zn was 10.1-11.5 mg/day, the intestinal absorption factor was 10-40% (factors such as phytate, fiber, calcium, and protein content of a meal could affect Zn absorption), and the absolute daily oral requirement for Zn was 2.5 mg/day when absorption losses were eliminated (13).

Suggested daily intravenous Zn intake was 2.5-4.0 mg for the stable adult and an additional 2.0 mg for adults in acute catabolic states. For a stable adult with intestinal losses, an additional 12.2 mg of Zn/liter of small-bowel fluid lost was recommended, or 17.1 mg of Zn/kg of stool or ileostomy losses. The panel stressed the need for frequent monitoring

of blood levels in these patients to provide proper dosage (13).

In 1988, the Subcommittee on Pediatric Parenteral Nutrient Requirements from the Committee on Clinical Practice Issues of The American Society for Clinical Nutrition (ASCN) published an update and extension of the 1979 statement by the AMA expert panel (12). The Committee of the ASCN stressed the need to add Zn to TPN solutions for pediatric patients, even for supplemental or short-term (less than four weeks) parenteral support, since deficiency symptoms could develop quickly if Zn status was poor or when there were significant Zn losses (12). Daily intravenous recommendations for the pediatric population were: 1) 400  $\mu\text{g}/\text{kg}$  for premature infants weighing less than 1500 grams and up to 3 kg, 2) 250  $\mu\text{g}/\text{kg}$  for full term infants less than 3 months old, and 3) 100  $\mu\text{g}/\text{kg}$  for full term infants older than 3 months and 50  $\mu\text{g}/\text{kg}$  (maximum 5000  $\mu\text{g}/\text{day}$ ) for children up to 5 years old (12,13). After 5 years of age, the recommendations of intravenous Zn supplementation for adults applied (13).

However, even with these recommendations, Zn deficiency and/or negative Zn balance continued to be documented in adults and children (1,8,9,10,11,12,28). A simple diagnostic test such as serum Zn concentration was not reflective of body tissue Zn status (14,29,30,31,35). Therefore, there was a need to investigate the effects of parenteral Zn



administration and subsequent Zn concentration in body tissues in order to add to the data about the Zn requirement necessary to maintain Zn balance and homeostasis during intravenous nutrition support.

#### Tissue Retention of Zn with TPN Administration

Only one study from Finland evaluated tissue retention of Zn from Zn administered intravenously during TPN in humans (14). Twenty-four malnourished surgical patients received one of three different parenteral nutrition regimens, consisting of total kilocalories predominantly as glucose, lipid or a high amino acid (3 g AA/kg/day) solution. Liver and muscle were biopsied for Zn and serum was collected (14).

The Zn concentration decreased in the liver and serum regardless of the nutrition regimen (14). No changes in Zn concentration were seen in muscle. Daily urinary Zn excretion increased in eight patients, and the amount of daily intravenous Zn the patients received was approximately that excreted in their urine (14). Tulikoura and Vuori concluded that daily supplementation of 1.9 mg of Zn in the parenteral solution was inadequate, and that at least 5 mg of Zn daily was necessary in their patients, if not more, and that these recommendations were in accordance with the 1979 AMA guidelines (13,14).

Tulikoura and Vuori's human study had several factors

which may have affected their results. First, the parenteral solutions they used were not proportionately correct in reference to recommended macronutrient distribution. Kilocalorie intake was not equivalent among the groups and protein intake was either too low or too high (14). High protein intake has been previously documented to affect Zn metabolism (32,33). Also since it was a human study, limitations were expected on the variety of tissues available for Zn analysis.

Although one human study demonstrated changes in tissue Zn homeostasis, most of the information regarding tissue retention of intravenously administered Zn has come from animal studies, specifically the rat. Several foreign studies investigated the effect of Zn in TPN solutions on Zn retention in rat tissues.

Poriadkov et al. studied rats that received complete intravenous nutrition for fifteen days and found growth of the liver during parenteral nutrition followed by an increase in Zn content in whole liver tissue (34). Yokoi et al. studied six week-old, Sprague-Dawley male rats (160-180 g) that were orally fed a trace element-deficient diet for two weeks (15). There were three groups nourished for one week as follows: Group A - TPN without trace element supplementation (approximately 11  $\mu\text{g}/\text{kg}$  body weight/day); Group B - TPN supplemented with trace elements including zinc (approximately

1312  $\mu\text{g}/\text{kg}$  body weight/day); and Group C - an oral diet without trace elements (14.2 mg Zn/kg diet). A control group (Group D) was fed an oral diet for three weeks supplemented with trace elements (55.3 mg Zn/kg diet). The Zn concentration in the plasma and liver of Group B was higher than that of Group C (15). Heart, kidney, testis, muscle, and tibia Zn concentrations remained constant between these two groups (15). In general, plasma and tissue Zn concentrations decreased during TPN without Zn, and this effect was prevented by Zn supplementation in the TPN solution (15).

Matsuda et al. studied four groups of five-week old, Sprague-Dawley male rats (180-210 g) (16). All were allowed an acclimation period of one week prior to undergoing surgical placement of a central catheter into the superior vena cava. Treatments consisted of either an oral diet or TPN for one week as follows: Group A - a Control group fed a synthetic normal diet (approximately 34.43 mg Zn/kg diet); Group B - received TPN without trace element supplementation; Group C - had infused TPN supplemented with trace elements including Zn (approximately 101  $\mu\text{g}/\text{kg}$  body weight/day); and Group D - received TPN supplemented with three times the dose of Zn (approximately 302  $\mu\text{g}/\text{kg}$  body weight/day). Groups B, C, and D received lower daily intravenous Zn infusions when compared to that received in a standard oral rat diet. The Zn concentration in the brain, heart, kidney, tibia, plasma, and

whole blood decreased in Group B compared to control Group A (16). The higher the supplemental Zn received by Groups B, C, and D respectively, the higher the Zn concentration in the tissues (16). This was seen most significantly in the plasma Zn concentration, where Groups B, C, and D were different from each other and Groups B and C had significantly lower Zn concentration compared to control Group A (16). Also for kidney, the Zn concentration of control Group A was higher than for Groups B, C, and D (16). The authors found no difference in Zn concentration in liver, femoral muscle, testis, and spleen among the groups (16). However, since the Zn concentrations fell in plasma and kidney, it was not conclusive as to whether the Zn infusion in the TPN solution of Groups C or D was adequate or inadequate to maintain tissue Zn homeostasis. However, it was evident that dosage of intravenous Zn plays a major role in certain tissue Zn concentrations.

Yamato investigated the concentration of Zn in the serum and organs of male Wistar rats (200 to 250 g) during TPN, while they received solutions containing different concentrations of Zn (35). The rats were divided into four groups. Group A rats were fed orally a diet containing 60 mg/kg of Zn. Groups B, C, and D received TPN for one week. Zinc from Zn sulfate was infused as follows: Group B received TPN without added trace elements; Group C received

approximately 80  $\mu\text{g}$  Zn/kg body weight/day; Group D received approximately 4440  $\mu\text{g}$  Zn/kg body weight/day. In Group D, the author found the Zn concentration increased in the liver, kidney, small intestine, serum, muscle, and thigh bone, (as well as in pancreas, skin, brain, and cecum) compared to control Group A (35). In Groups B and C, the Zn concentration decreased in the small intestine (it also decreased in the serum in Group B), compared to control Group A; however, the Zn concentration increased in their muscle and thigh bone (reasons for this increase were not discussed in Yamato's study) (35). The Zn concentrations in all other tissues in Groups B and C were not significantly different from those in Group A (35). Yamato has shown that about 80  $\mu\text{g}$ /kg body weight/day of intravenous Zn could maintain normal Zn tissue concentrations in rats not losing gastrointestinal fluids, and that 180  $\mu\text{g}$ /kg body weight/day of intravenous Zn was necessary to maintain positive Zn balance in rats with abnormal pancreatic and bile loss (35). However, Matsuda et al. (16) have shown that intravenous Zn infusions of 101 and 302  $\mu\text{g}$  Zn/kg body weight/day produced lower plasma and kidney Zn concentrations compared to controls consuming an oral diet (even when considering that oral diet contained three times the dietary Zn requirement for rats). Therefore, Yamato's results were not conclusive as to whether the Zn infusion in the TPN solution of Group C (80  $\mu\text{g}$  Zn/kg body weight/day) was

adequate or inadequate to maintain tissue Zn homeostasis, although it seemed that 4440  $\mu\text{g Zn/kg/day}$  was excessive and disrupted tissue Zn balance (35).

Matsuda et al. investigated the effects of intravenous injection of a trace element supplement on zinc concentrations in tissue and plasma of rats (mean weight 240 g) (36). After a one week acclimation period, eating a normal diet, the rats were treated for seven days by intravenous injection of a trace element preparation containing Zn as follows: Group 1 - approximately 78  $\mu\text{g Zn/kg body weight/day}$ ; Group 2 - approximately 784  $\mu\text{g Zn/kg body weight/day}$ ; Group 3 - approximately 2352  $\mu\text{g Zn/kg body weight/day}$ ; and Group 4 - approximately 7840  $\mu\text{g Zn/kg body weight/day}$ . The authors found that neither 78  $\mu\text{g Zn/kg/day}$  nor 784  $\mu\text{g Zn/kg/day}$  affected the zinc concentrations in tissues, except a significantly higher plasma Zn concentration with 784  $\mu\text{g Zn/kg/day}$  compared to controls and those receiving the lesser amount (36). After receiving the larger dose of 2352  $\mu\text{g Zn/kg/day}$ , Zn concentrations in the liver, kidney, tibia and plasma of the rats increased significantly compared to controls (36). With the largest dose of 7840  $\mu\text{g Zn/kg/day}$ , all the rats died, and Zn concentrations in their tissues increased tremendously compared to controls (36). The authors suggested that Zn concentrations in tissues and plasma were appropriately maintained, compared to controls, with doses up

to approximately 784  $\mu\text{g}$  Zn/kg/day, but that doses higher than that would likely unbalance Zn homeostasis (36). This data also supported the importance of intravenous Zn dosage on tissue Zn concentrations and homeostasis, although difference of a single one-time daily administration dose versus a 24-hour continuous administration dose in TPN needed to be considered.

In summary, differences found in various rat studies evaluating the effects of intravenous Zn infusions on tissue Zn retention were: 1) control rats which underwent a surgical procedure to receive a central venous catheter had an added variable that may have affected Zn metabolism and homeostasis, 2) control rats often received oral diets with higher Zn content than that required, 3) results were not conclusive as to the quantity of intravenous Zn which maintained tissue Zn homeostasis, and 4) intravenous Zn infusion was not always evaluated in the context of TPN. Therefore, collection of additional data was indicated to evaluate how Zn in TPN affects body tissue Zn status compared to Zn consumed from a standard oral diet.

## Methodology

### Animal Management and Experimental Design

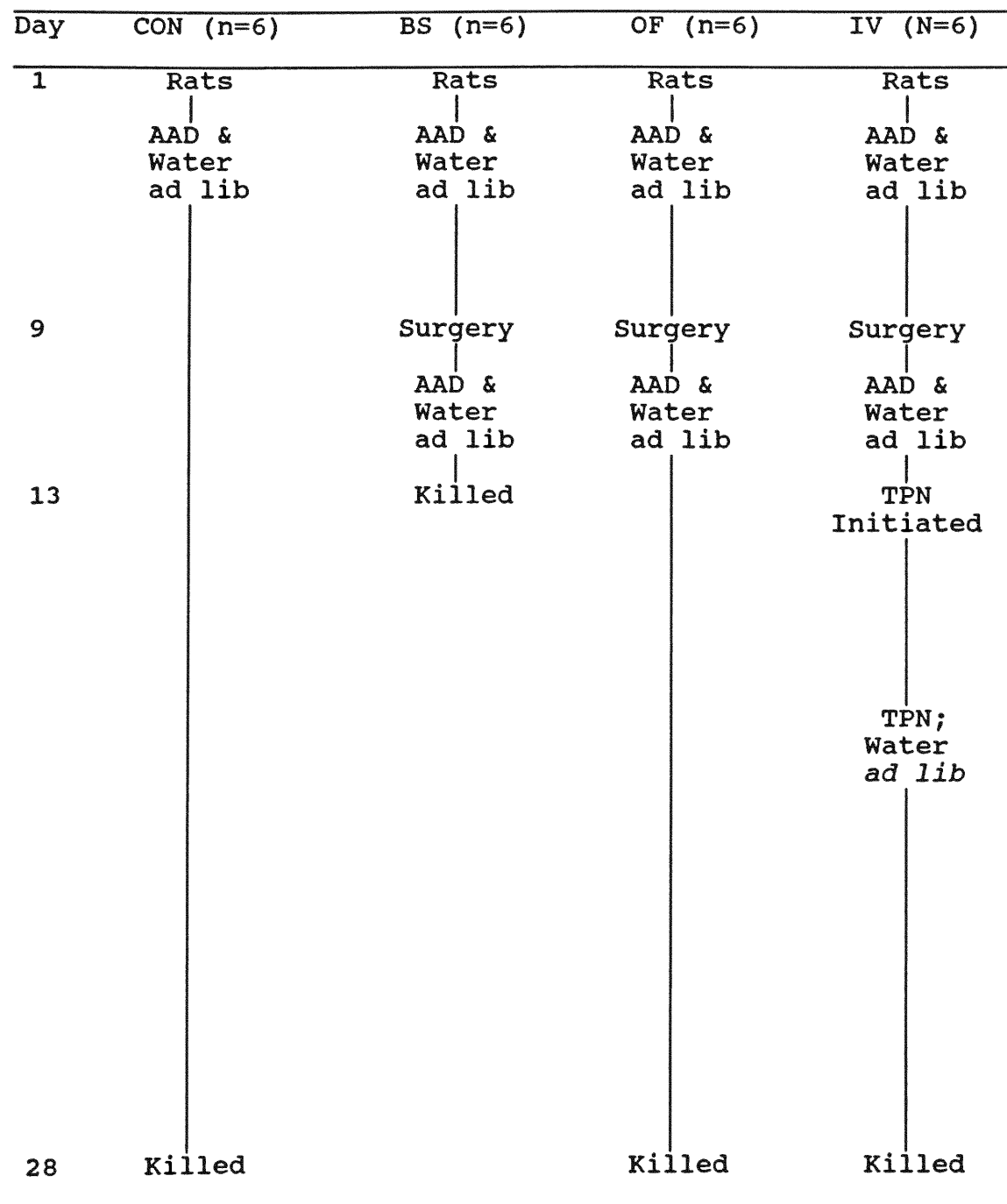
Twenty-four male Fischer-344 rats (Harlan Sprague-Dawley, Indianapolis, IN) weighing 140 to 160 grams (g) were housed individually in stainless steel metabolic cages containing wire-mesh bottoms, in an environmentally controlled room with 12-hour light and dark cycles. During the experimental period, the temperature ranged from 18°C to 26°C and the relative humidity ranged from 40% to 70%.

The animals had free access to an amino acid diet (AAD) and distilled and deionized (DDI) (Mega-Pure, Barnstead Co., Dubuque, IA) water during an acclimation period of eight days (Figure 1). Food consumption and body weights were recorded every three days. Rats were equally and randomly assigned to one of four groups (n=6): a control group (CON), baseline group (BS), an intravenously fed group (IV), and an orally fed group (OF).

On day nine of the experiment, all rats (except the CON group) were anesthetized, and had a catheter surgically placed into the superior vena cava. During a recovery period of four days, all the rats were allowed *ad libitum* access to the AAD and DDI water. On day thirteen of the experiment, the rats in the BS group were anesthetized, exsanguinated and eviscerated in order to determine baseline tissue values.



Figure 1. Experimental Design of Animal Management and Nutritional Protocol



Total parenteral nutrition was initiated in the IV rats, while the CON and OF rats continued to consume the AAD. All rats continued to have *ad libitum* access to DDI water. The rats were fed for 15 additional days with food intake and body weights recorded every third day for the entire experimental period. Feces and urine were also collected at that time. On day twenty-eight of the experiment, all rats were killed in a manner similar to that of the BS group.

The rats were cared for according to the Guide for the Care and Use of Laboratory Animals (37) and the experiment was approved by the Institutional Animal Care and Use Committee of Florida International University.

### Surgical Catheter Placement

A catheter was surgically placed into the superior vena cava of all rats (except the CON group) by aseptic technique, according to the methods of Enrione et al. and Popp et al. (Appendix A) (38,39). The rats were anesthetized with an intraperitoneal injection of sodium pentobarbital (30 mg/kg body weight). After cleansing the ventral neck, mid-scapular and suprascapular areas, a 3 cm incision was extended from above the suprasternal notch up the ventral neck of the rat. The right external jugular was dissected free and a small incision was made in it. The beveled end of a silicone rubber catheter (Silastic, Dow Corning Co., Midland, MI) was threaded

2 cm down the external jugular and into the superior vena cava. The catheter was secured in place and irrigated with heparinized saline. The suprascapular area was recleansed and a 2 cm incision was made. A 13-gauge needle was tunneled subcutaneously from a dorsal incision through to the ventral neck incision. The catheter was then threaded through the needle and out the dorsal incision, and the needle was removed. The catheter was then passed through a stainless steel spring cable, secured with sutures and autoclips, and the cable with the catheter was passed through a ventilation hole of a metabolic cage top, and connected to a swivel on the cage top. The rats were weighed with the cage top and then placed in the metabolic cage.

Catheter patency was maintained with daily flushes of 0.5 mL heparinized saline (10 U/mL). At the end of surgery, 0.1 mL of the antibiotic cefazolin sodium USP 500 mg (Marsam Pharm., Inc., Cherry Hill, NJ) was administered to four of the rats in the IV group.

#### **Diet and TPN Solution Preparation**

The AAD, which was consumed orally, and the TPN solution were formulated based on the nutritional needs for the growth and development of a normal rat (Appendix B: Tables B1-B4) (40,41). Both diets contained a mixture of all the amino acids, carbohydrates (Appendix B: Table B1,B2), vitamins, and

minerals required for rats (Appendix B: Tables B3-1, B3-2, and B4). Fluoride was not added to the diets since no growth or other benefit has previously been found from addition of fluoride to rat diets (13,42).

Fat requirements for the rats also were provided (Appendix C: C1,C2). In the AAD, the soybean oil and glycerin were added in similar caloric ratios as provided in the commercially-available, intravenous fat emulsion used in the TPN solution. The small amount of egg yolk phospholipids found in the intravenous fat emulsion was not present in the AAD, however, it has previously been suggested that this component contains no essential nutrients and can be considered unnecessary in the oral diet (41).

Zinc was added as zinc sulfate (52.87 mg) to provide respectively a final concentration of 12 mg Zn/Kg diet and 4.16 mg Zn/Kg TPN solution (12 mg Zn/2.886 Kg TPN solution).

Both the AAD and the TPN solution had similar kilocaloric distribution. The AAD contained 3.84 kcal/g with 22.2% from the amino acids, 68.6% from dextrose, and 9.2% from fat. Nitrogen content of the AAD was 33.5 mg/g, or approximately 8.72 mg of N/kcal.

The TPN solution formulation is that described by Miyata et al. (Appendix B) (42). The solution was infused at 1.7 mL/hour/day, and supplied 1.18 kcal/g or 1.32 kcal/mL (specific gravity was 1.115 g/mL). Approximately 21.7% of the

kilocalories were from the amino acids, 67% from dextrose, and 11.3% from fat. Nitrogen content of the TPN solution was 10.08 mg/g, or approximately 8.54 mg of N/kcal.

Daily Zn intake was calculated by multiplying the Zn concentration of the diet or TPN solution by the respective Zn intake or infusion and dividing by three, during each three-day collection period.

#### **Preparation of the Amino Acid Diet (AAD)**

The amino acid mixture was combined with the vitamins, choline-chloride, and minerals and this was mixed with the dextrose (Appendix C: C1). The soybean oil and glycerin were added to the other ingredients, and the completed mixture was stored at 4°C.

#### **Preparation of the TPN Solution**

The amino acid mixture was combined with the dextrose, slowly heated, and then cooled (Appendix C: C2). The minerals and vitamins were mixed with the other ingredients, the solution was filtered through a 0.22 micron filter (MSI, Westborough, MA), and was stored at 4°C. One day prior to infusion, the solution was passed again through a 0.22 micron filter, then stored at 4°C. Immediately prior to infusion, the lipids and fat-soluble vitamins were added (Appendix C: C2).

## Collection of Samples

### BS Group:

On day 13 of the experiment, the BS rats were killed. After exsanguination, the blood was placed in labeled polystyrene tubes, coagulated and centrifuged. The separated serum was pipetted into polystyrene tubes and frozen at  $-20^{\circ}\text{C}$ . The liver, kidney, lung, heart, gastrocnemius muscle (left leg), testicle, and femur bone (left leg) were dissected free, rinsed with DDI water, blotted dry and weighed. After dissection of the small intestine, residuals in the lumen were removed, the tissue was rinsed with DDI water and blotted dry. All tissues were placed in polystyrene freezer bags and frozen at  $-20^{\circ}\text{C}$ . All feces and urine were frozen at  $-20^{\circ}\text{C}$ .

Zn balance was determined by the following formula: Zn balance = Zn intake - (fecal Zn + urine Zn).

### CON, IV, and OF Groups:

On day 28 of the experiment, rats in the CON, IV, and OF groups were killed as previously described and femurs, gastrocnemius muscles, viscera, and sera were collected by the same method described for the BS group and frozen at  $-20^{\circ}\text{C}$ .

The consumption of AAD, infusion of TPN, and collections of feces and urine were recorded every third day.

## Preparation of Glassware for Sample Digestion

Prior to use, all glassware was soaked in a detergent

solution, and rinsed with deionized water. The glassware was then resoaked in a 20% nitric acid solution prepared with DDI water, rinsed again with DDI water, and then rinsed with a 0.001 M EDTA solution. A final rinsing with DDI water completed the preparation of the glassware for sample digestion (Appendix D).

### **Digestion of Samples**

In preparation for digestion, wet tissue samples, and feces for each rat were weighed and placed in Erlenmeyer flasks. Two-mL urine samples also were collected. National Institute of Standards and Technology bovine liver (Standard Reference Material {SRM} 1577b, NIST, Gaithersburg, MD) served as quality control samples for tissues and feces (except bone and sera). Two samples of non-fat milk powder (SRM 1549, NIST, Gaithersburg, MD) were the designated quality controls for the femur bone, and bovine serum (SRM 1598, NIST, Gaithersburg, MD) was the quality control for the serum samples. Two blanks, containing only digestion reagents, were also prepared for each sample set.

A protocol by Hill et al. (43) was applied for the dry ashing (430°C) and wet digestion of all tissues (except sera), urine and feces (Appendix D). An entire set of Erlenmeyer flasks containing tissue or feces was placed into muffle furnaces for dry ashing, prior to continuing with the wet

digestion protocol using nitric acid and hydrogen peroxide (Appendix D) (43). Samples were then transferred quantitatively and brought to a final volume of 5 mL, poured into polystyrene storage-tubes (washed with EDTA), and refrigerated at 4°C.

The urine was prepared for digestion by addition of hydrogen chloride (HCl) and then digested according to the protocol by Hill et al. (Appendix D) (43). Samples were transferred quantitatively and brought to a final volume of 2.5 to 5 mL.

Sera were prepared for direct assay by AAS, and stored in polystyrene tubes at -20°C. There was no serum sample for one rat in the CON, BS, and IV groups.

### **Analysis of Samples**

Samples were prepared for AAS aspiration by appropriate dilution with 0.1 M HCl (or diluted with DDI water for serum), to bring the Zn concentration of the samples within the range of the standard. A flame atomic absorption spectrophotometer (Perkin Elmer Model 5000, Perkin Elmer, Norwalk, CT) was used to analyze the Zn concentration in the samples (Appendix D). The spectrophotometer was stabilized after positioning of the Zn lamp, setting the resonant wavelength, and igniting the flame. The instrument was brought to equilibrium with a 0.1 M HCl solution and calibrated using the appropriate Zn



standards that were prepared to cover the linear absorbance range (0.25 ppm to 2.00 ppm) of the trace element. Zn standards were aspirated at the beginning of the sample aspirations, and then every 5-10 samples to ensure appropriate calibration.

Standard reference materials and blanks were aspirated with their corresponding sample set. Triplicate sample aspirations completed the sample analysis for Zn concentration by AAS (Appendix D). The blanks analyzed with the sample sets were negligible in their Zn concentrations, but were still subtracted from the sample Zn concentration readings for accuracy of total Zn content. Weights or volumes and dilutions of the tissues, feces, urine, and sera were used with their respective AAS readings to attain final Zn concentration of the respective sample.

### **Statistical Analysis**

Statistical analyses were completed to determine significant differences among dietary groups for each dietary treatment. Means for whole body weight, energy intake, total Zn intake, total Zn output, Zn balance, and tissue Zn concentrations were analyzed by a one-way analysis of variance (ANOVA) ( $p < 0.05$ ). All values are expressed as means  $\pm$  standard deviation (SD).

## Results

### Digestion Standards

Standards for bovine liver, serum, and nonfat milk powder were analyzed by the AAS. Certified values were  $127 \pm 16 \mu\text{g Zn/g}$  in bovine liver,  $0.89 \pm 0.06 \mu\text{g Zn/g}$  in bovine serum,  $46.1 \pm 2.2 \mu\text{g Zn/g}$  in nonfat milk powder, and analyzed values were  $119.6 \pm 5.1 \mu\text{g Zn/g}$ ,  $0.89 \pm 0.0 \mu\text{g Zn/g}$ , and  $44.25 \pm 0.05 \mu\text{g Zn/g}$  respectively. Only one (of two) bovine liver standards, analyzed with a feces set, was lower than the certified range at  $27.2 \mu\text{g Zn/g}$ .

### Body Weights

Rats gained an average of three g per day (Table 1) in the first nine days. There was no significant difference in the pre-surgical body weights (day 9) of the rats in the four groups (Table 1). Body weights of the CON group from the pre-surgical weight period to the feeding weight period (day 13) was significantly higher ( $p < 0.01$ ) than the BS, IV, and OF groups. The CON, IV and OF groups continued the respective diet treatments (days 14-28), and the body weights did not differ from the feeding weight to final weight (day 28).

### Energy Intake

Energy intake during the acclimation period (days 1-9)

**TABLE 1.  
MEAN BODY WEIGHTS OF FISCHER-344 RATS FED EITHER ORALLY OR INTRAVENOUSLY**

RAT GROUP	n	PRE-SURGERY WEIGHT (gm) (DAY 9)	FEEDING WEIGHT (gm) (DAY 13)	FINAL WEIGHT (gm) (DAY 28)
CON	6	166.4 ± 8.3#	172.1 ± 8.6*	204.5 ± 9.8
BS	6	174.9 ± 5.7	145.5 ± 4.3	_____
IV	6	171.3 ± 9.7	144.7 ± 10.6	174.0 ± 12.4
OF	6	173.6 ± 4.9	142.8 ± 6.0	182.3 ± 3.4

# Mean ± SD

\* (p<0.01)

did not differ significantly among the CON, BS, IV and OF groups (Table 2). In the postoperative recovery period (days 10-13), the rats in the CON group consumed significantly more food ( $p < 0.01$ ) than the rats in the BS, IV and OF groups. The rats in the CON, IV, and OF groups continued the respective treatments (days 14-28), and no significant difference in energy intake among the groups occurred.

### Daily Zn Intake

Daily Zn intake during the acclimation period (days 1-9) was not significantly different among the groups (Table 3). While recovering from surgery (days 10-13), the CON rats had a higher ( $p < 0.01$ ) Zn intake than did the BS, IV, and OF rats. During the treatment period (days 14-28), the daily Zn intake by the IV rats was significantly higher ( $p < 0.01$ ) than that of the CON and OF rats. The IV rats received approximately 1000  $\mu\text{g Zn/kg/day}$ , whereas the CON and the OF rats consumed approximately 800  $\mu\text{g Zn/kg/day}$ .

### Zn Balance During Specific Feeding Periods

Although feces and urine were collected every three days from the recovery period (day 10), only two sets were analyzed by AAS. The total Zn intake for Collection #3 (days 17-19) of the experiment showed there was an approximately 20% higher ( $p < 0.01$ ) intake of Zn by the IV group compared to the CON and

**TABLE 2. MEAN ENERGY INTAKE (IN KILOCALORIES PER DAY) OF FISCHER-344 RATS FED EITHER ORALLY OR INTRAVENOUSLY**

RAT GROUP	n	ACCLIMATION PERIOD (DAY 1-9)	RECOVERY PERIOD (DAY 10-13)	TREATMENT PERIOD (DAY 14-28)
CON	6	45.3 ± 3.8#	44.9 ± 4.1*	44.9 ± 2.9
BS	6	45.1 ± 3.2	13.1 ± 7.5	—
IV	6	44.2 ± 5.9	18.4 ± 8.9	49.5 ± 3.7
OF	6	47.1 ± 4.8	13.6 ± 5.8	46.5 ± 4.9

# Mean ± SD  
\* (p<0.01)

TABLE 3.

MEAN DAILY ZINC INTAKE ( $\mu\text{g}/\text{DAY}$ ) OF FISCHER-344 RATS FED  
EITHER ORALLY OR INTRAVENOUSLY<sup>a</sup>

RAT	ACCLIMATION PERIOD ( $\mu\text{g}/\text{DAY}$ ) (DAYS 1-9)	RECOVERY PERIOD ( $\mu\text{g}/\text{DAY}$ ) (DAYS 10-13)	TREATMENT PERIOD ( $\mu\text{g}/\text{DAY}$ ) (DAYS 14-28)
CON	6    141.5 $\pm$ 12.0 <sup>#</sup>	140.1 $\pm$ 12.9*	140.3 $\pm$ 8.9
BS	6    140.9 $\pm$ 10.1	41.0 $\pm$ 23.5	_____
IV	6    138.1 $\pm$ 18.6	57.6 $\pm$ 27.7	174.5 $\pm$ 13.0*
OF	6    147.2 $\pm$ 14.9	42.5 $\pm$ 18.2	145.3 $\pm$ 15.2

<sup>#</sup> Mean  $\pm$  SD

\* (p<0.01)

&  $\frac{\text{Diet Intake} \times \text{Zn Concentration}}{3} = \text{Daily Zn Intake } (\mu\text{g}/\text{day})$

OF groups (Table 4). However, the total Zn intake for Collection #6 (days 26-28) did not differ significantly among the groups.

Total Zn output via urine and feces for Collection #3 and Collection #6 showed no significant difference among the CON, OF and IV groups, and Zn balance for Collection #3 and Collection #6 did not differ among the groups.

### **Tissues**

Zinc concentration in the liver (Figure 2), kidney (Figure 3) and lung (Figure 4) was significantly higher ( $p < 0.01$ ,  $p < 0.01$ , and  $p < 0.01$  respectively) in rats of the IV group compared to those in the CON, BS, and OF groups. Small intestine Zn concentration in the rats of the IV group was significantly higher ( $p < 0.03$ ) than that of the CON and BS groups, but did not differ from that of the OF rats (Figure 5). Zinc concentration in the small intestine of the OF rats also was not significantly different when compared to that of the CON and BS rats (Figure 5).

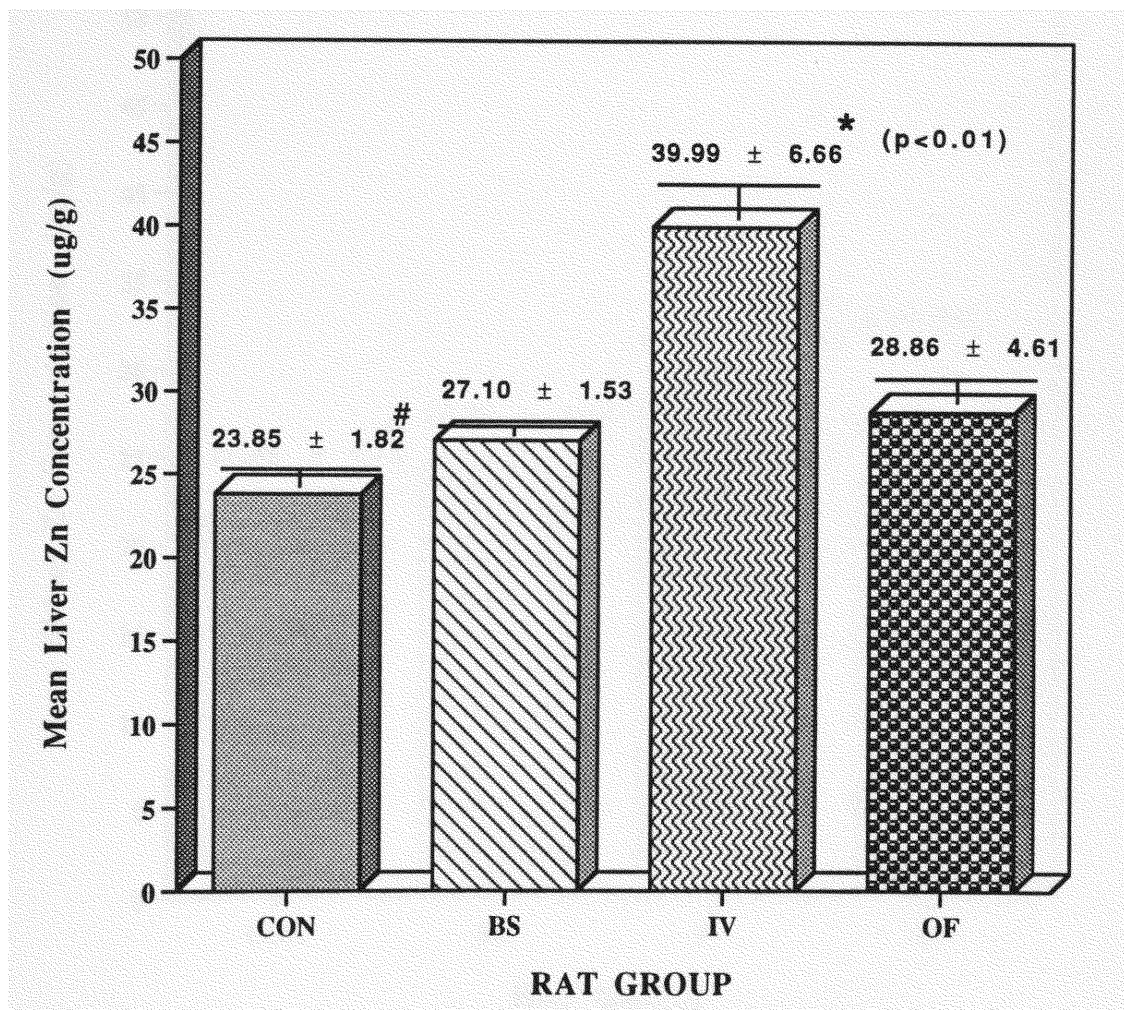
Zinc concentrations in serum (Figure 6), heart (Figure 7), muscle (Figure 8), testicle (Figure 9), and bone (Figure 10) were not significantly different among the four groups.

**TABLE 4.  
MEAN ZINC BALANCE OF FISCHER-344 RATS FED EITHER ORALLY OR INTRAVENOUSLY**

RAT	COLLECTION #3 (DAYS 17-19)				COLLECTION #6 (DAYS 26-28)			
	GROUP	n	INTAKE	OUTPUT ( $\mu$ g)	BALANCE	INTAKE	OUTPUT ( $\mu$ g)	BALANCE
CON	6	406 $\pm$ 33#	278 $\pm$ 265	128 $\pm$ 280	423 $\pm$ 62	208 $\pm$ 97	215 $\pm$ 68	
BS	6							
IV	6	511 $\pm$ 43*	143 $\pm$ 116	368 $\pm$ 99	487 $\pm$ 106	184 $\pm$ 86	304 $\pm$ 135	
OF	6	430 $\pm$ 55	151 $\pm$ 86	279 $\pm$ 57	439 $\pm$ 47	115 $\pm$ 73	324 $\pm$ 87	

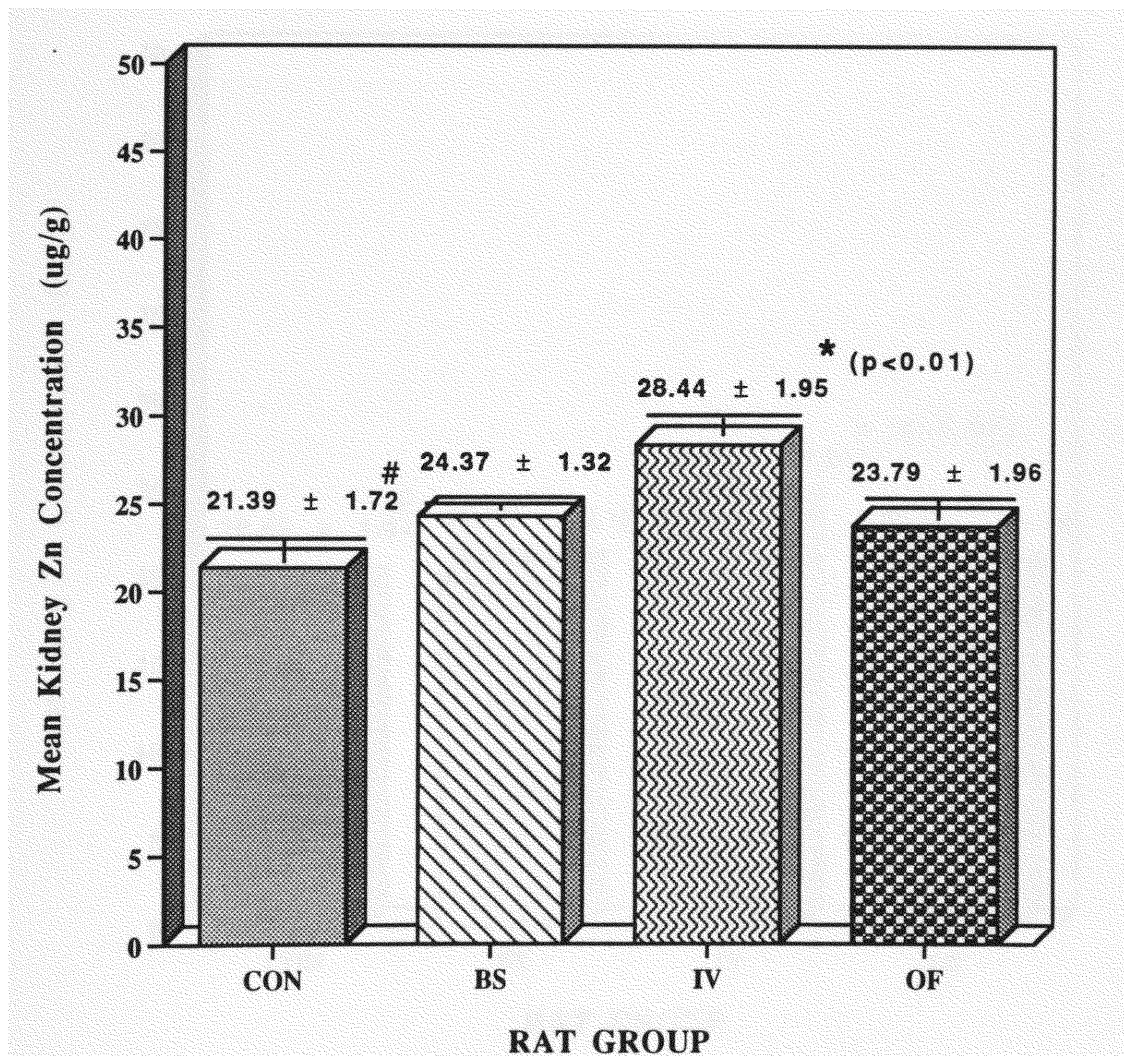
# Mean  $\pm$  SD  
\* (p<0.01)





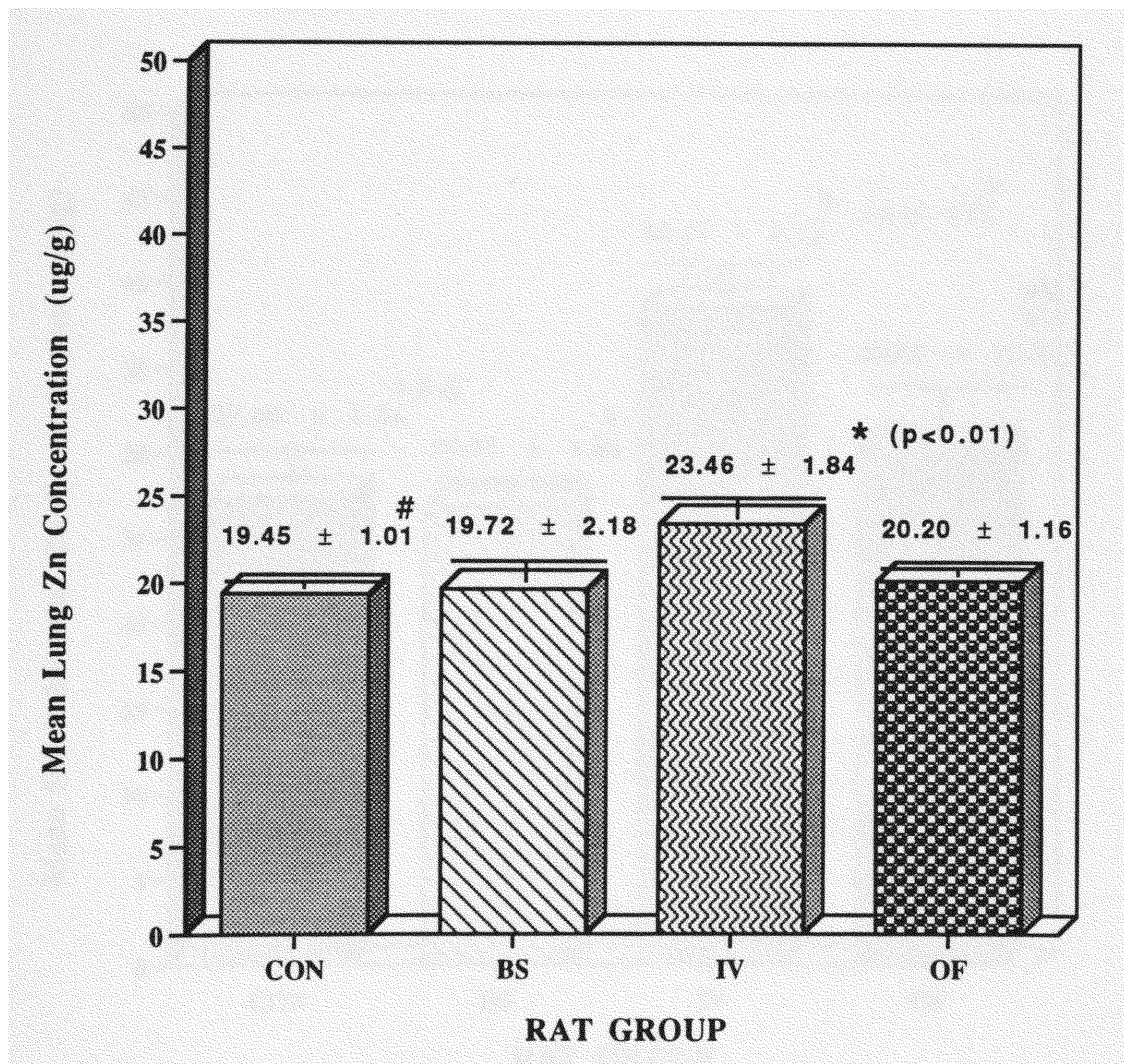
**Figure 2. Zinc concentration in liver of Fischer-344 rats fed either orally (CON, BS, OF) or intravenously (IV).**

# Means (n=6) ± SD



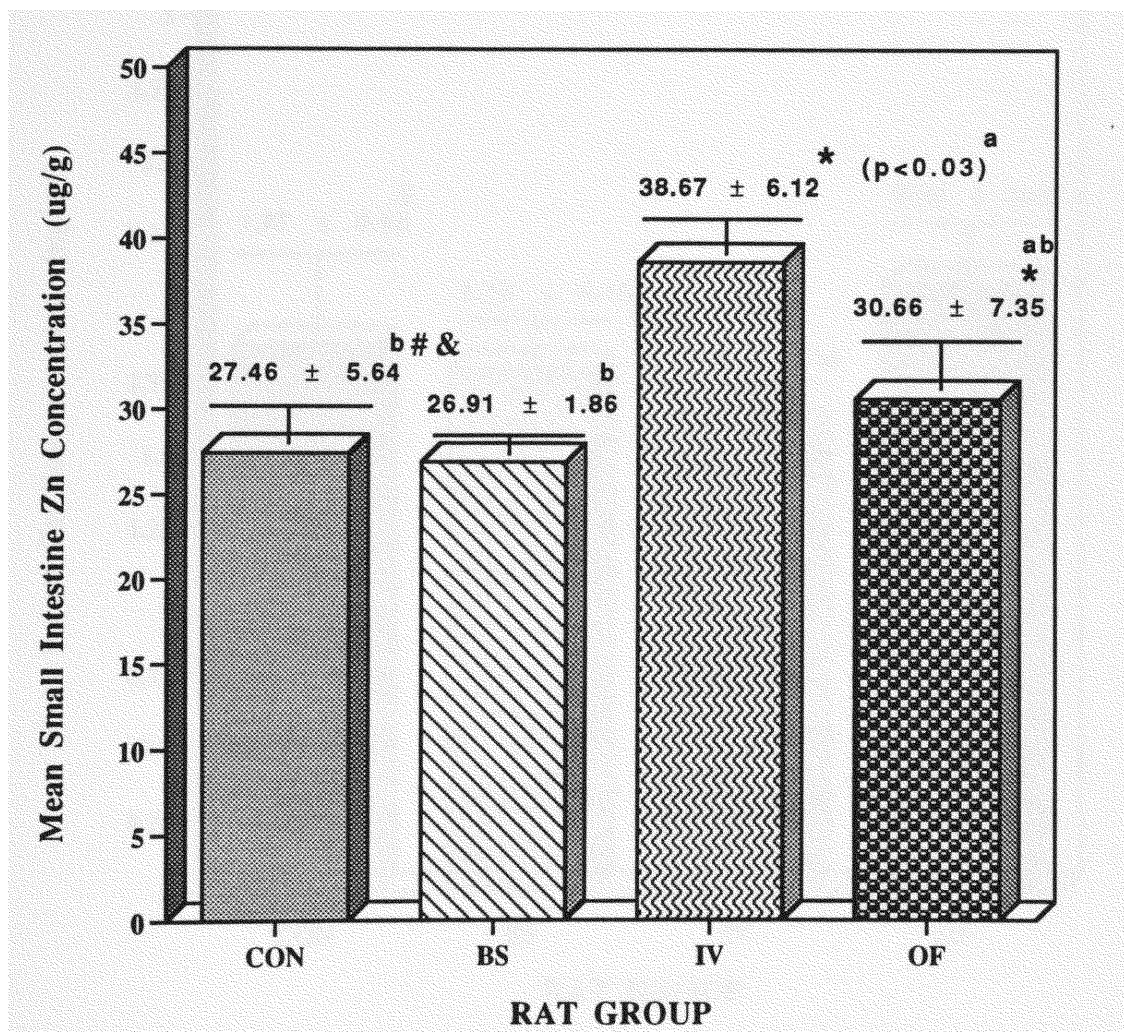
**Figure 3. Zinc concentration in kidney of Fischer-344 rats fed either orally (CON, BS, OF) or intravenously (IV).**

# Means (n=6) ± SD

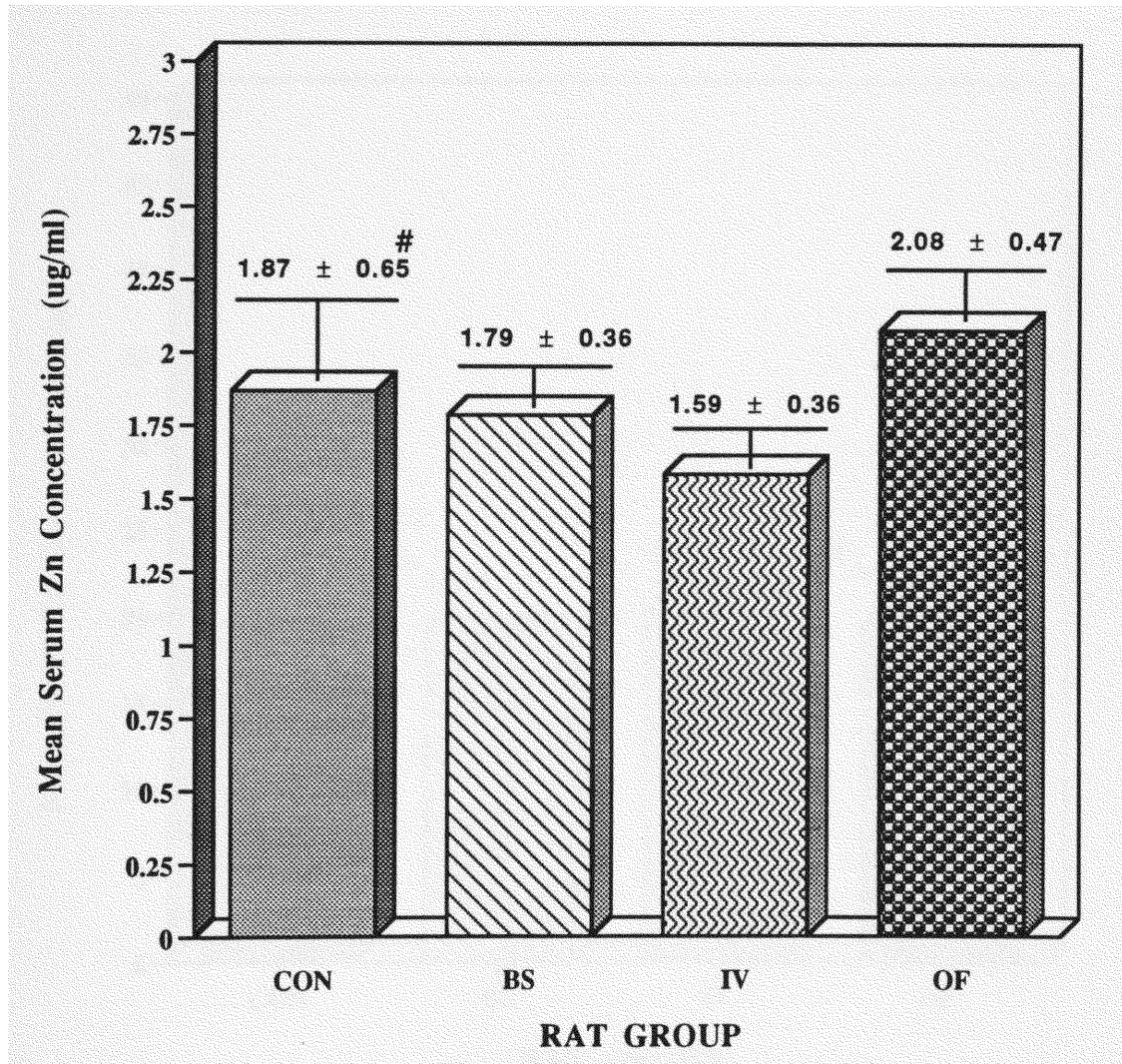


**Figure 4. Zinc concentration in lung of Fischer-344 rats fed either orally (CON, BS, OF) or intravenously (IV).**

# Means (n=6) ± SD

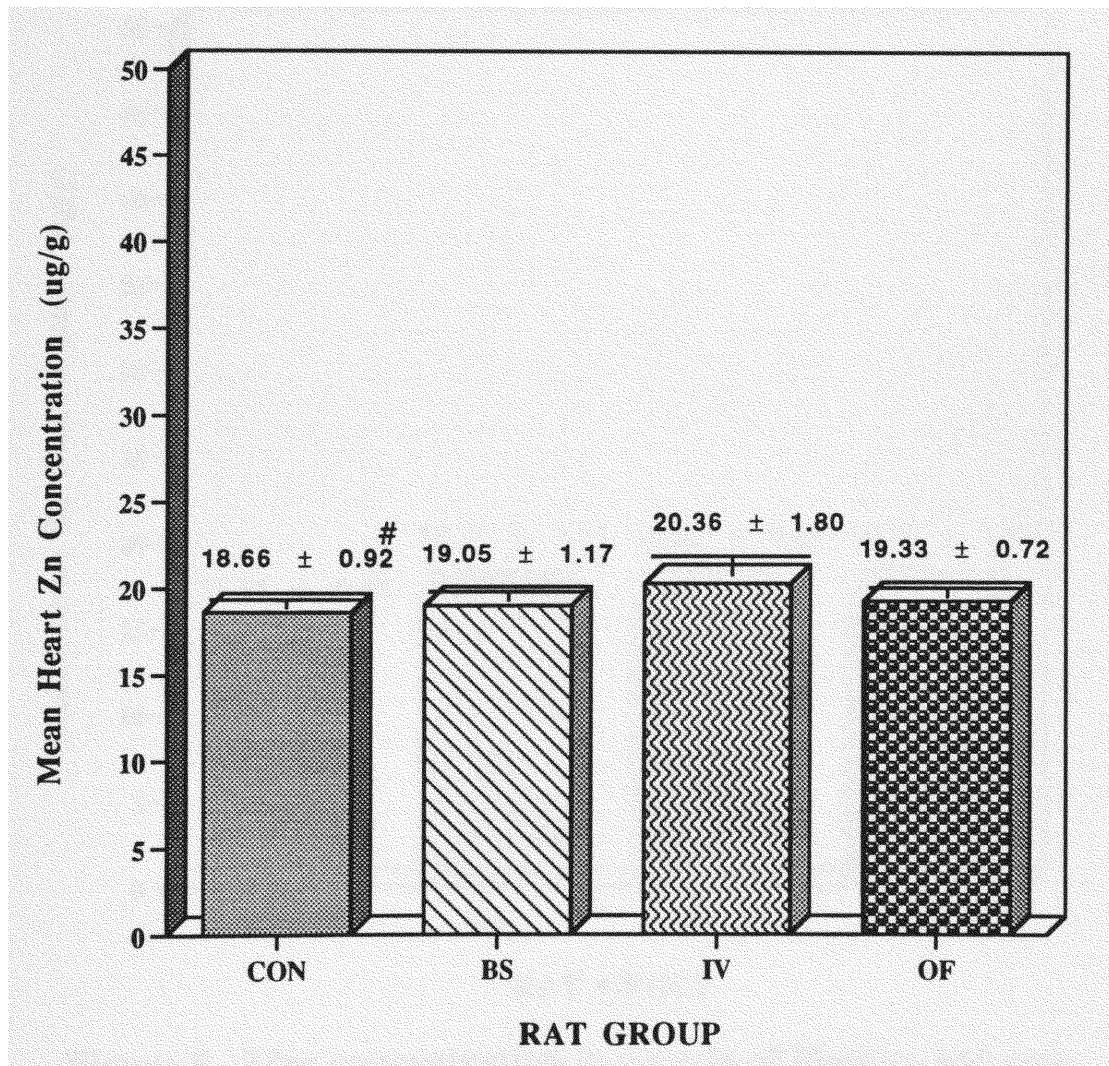


**Figure 5. Zinc concentration in small intestine of Fischer-344 rats fed either orally (CON, BS, OF) or intravenously (IV). # Means (n=6) ± SD & Means having different superscripts (a,b) are significantly different**



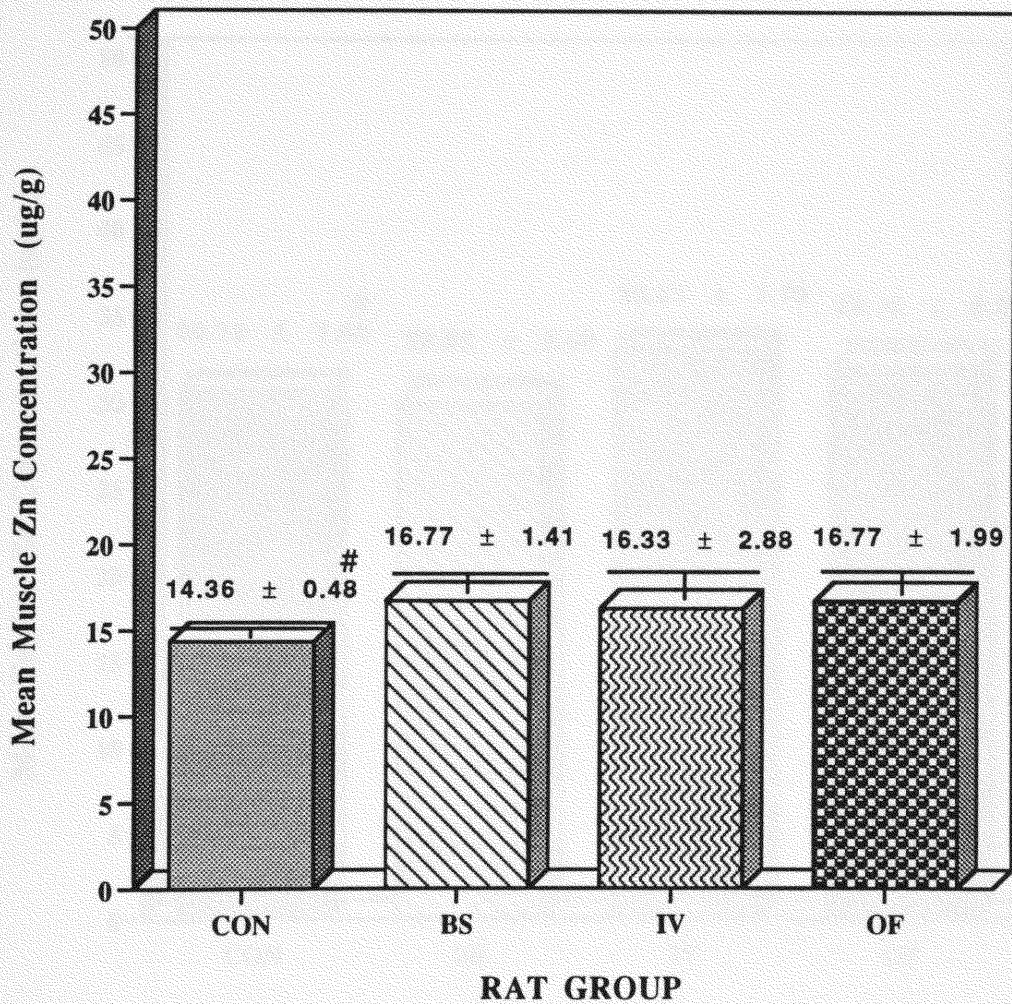
**Figure 6. Zinc concentration in serum of Fischer-344 rats fed either orally (CON, BS, OF) or intravenously (IV).  
# Means (n=6) ± SD**





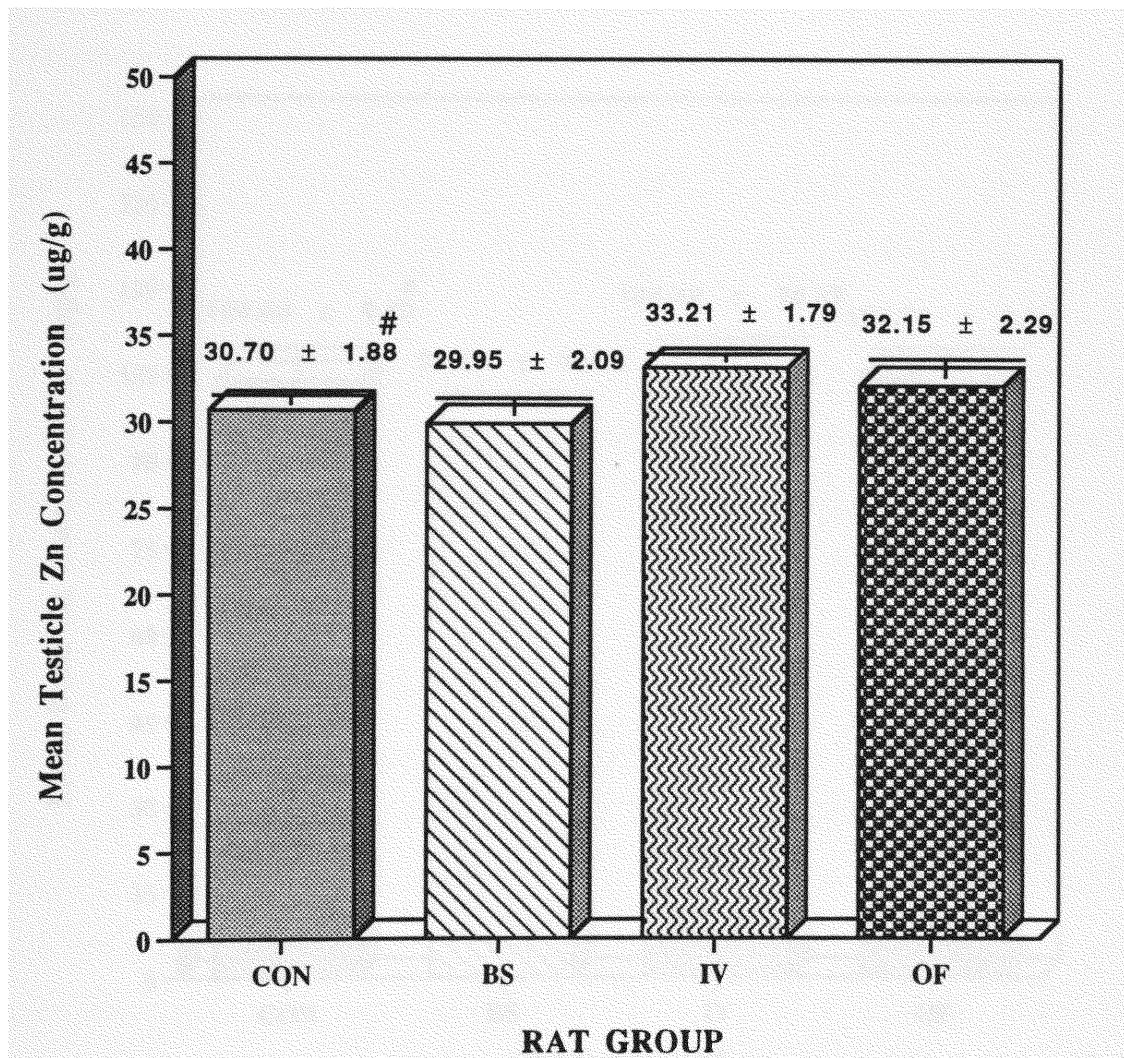
**Figure 7. Zinc concentration in heart of Fischer-344 rats fed either orally (CON, BS, OF) or intravenously (IV).**

**# Means (n=6) ± SD**



**Figure 8. Zinc concentration in muscle of Fischer-344 rats fed either orally (CON, BS, OF) or intravenously (IV).**

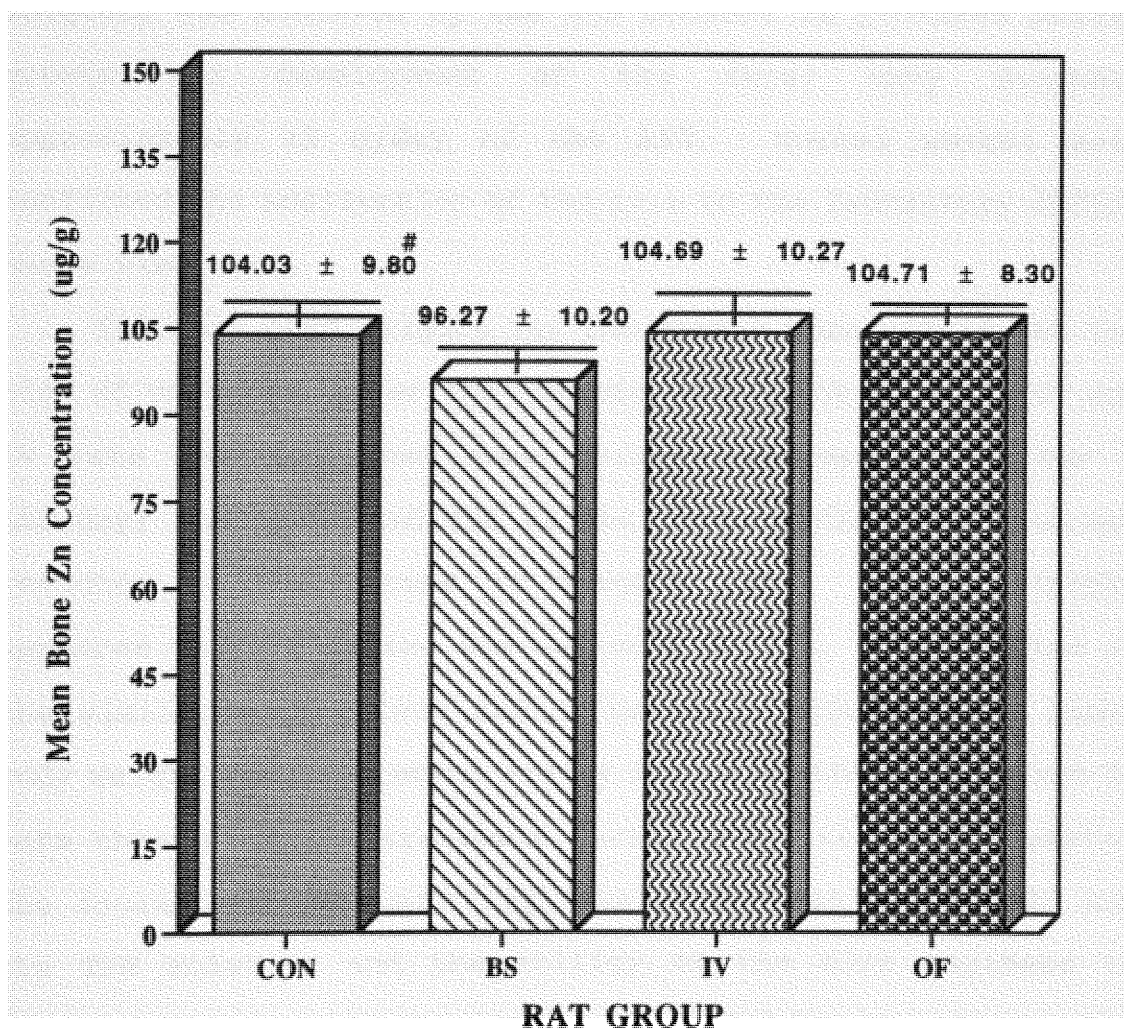
# Means (n=6)  $\pm$  SD



**Figure 9. Zinc concentration in testicle of Fischer-344 rats fed either orally (CON, BS, OF) or intravenously (IV).**

# Means (n=6) ± SD





**Figure 10. Zinc concentration in bone of Fischer-344 rats fed either orally (CON, BS, OF) or intravenously (IV).**

# Means (n=6) ± SD

## Discussion

### Energy Intake and Body Weights

All the rats in this study consumed adequate food during the acclimation period (days 1-9) to achieve normal weight gain (an average gain of three g per day). Nutritional adequacy was achieved by the AAD because it provided normal growth and development of the rats; they consumed approximately 12 g/day of the AAD. Normal weight gain confirmed the acclimation of the rats to the laboratory during those nine days.

The mean body weights for the groups were similar at the time of surgery. After the catheter placement, the BS, OF and IV rats had decreased food intake (approximately four g AAD/day) that preceded the weight loss, reflecting a normal response to the stress of surgery (42). This response appeared uniform during the recovery period, as reflected in the similar weight loss among the three groups. The final body weight and consistent energy intake of the CON, OF and IV rats throughout the treatment period (days 14-28), supported the isocaloric and isonitrogenous properties of the AAD and the TPN solution and the ability of the diet regimens to support growth, development and anabolism in the rats. However, during the treatment period, the OF group showed the greatest food efficiency with an approximately 40 g weight

increase in 15 days, compared to an approximately 30 g weight gain for the CON and IV groups. Though the OF and IV groups recovered from the surgery, the rats in the IV group never developed beyond the pre-surgical state as evidenced by final body weight. Thus the IV rats were exposed to an increased level of stress compared with the OF rats, after surgery.

### Daily Zn Intake

During the acclimation period the daily Zn intake was constant in all the rats. Due to the decrease in food consumption during the post-surgery recovery period, Zn intake decreased in the BS, OF, and IV groups.

At the initiation of the diet-treatment protocol for the OF and IV groups, the main focus was administering a TPN solution that had isocaloric and isonitrogenous properties, similar to an oral diet (41,42). In order to achieve isocaloric and isonitrogenous properties, the TPN solution was adjusted to attain the kilocalories and nitrogen that a rat would receive orally each day, while also considering appropriate daily intravenous infusion of fluids for a rat. It was assumed that trace element concentration would have adjusted accordingly, however this was not the case. Calculations estimate approximately 20% more Zn was received by the IV group (approximately 1000  $\mu\text{g}$  Zn/kg body weight/day for the IV group, versus 800  $\mu\text{g}$  Zn/kg body weight/day for the

OF group).

The greater Zn intake by the IV group may not have been the dominant factor in causing the increased Zn concentration seen in some of the tissues, although it may have played a role. Zinc availability, in combination with the sequestering of Zn in certain tissues as a response to continuous stress (44,45,46,47,48), may have been the dominant factors contributing to increased Zn concentrations in some tissues of the IV rats. The CON rats did not undergo surgery or stress. The OF rats were exposed to the stress of surgery but were not exposed to continuous stress (e.g. continuous IV fluids, flushing of lines, antibiotics, etc.) as were the IV rats.

### Zn Balance

Only two of the six three-day collection periods were analyzed and therefore reflect limited information on the Zn balance of the rats. The similar total Zn output among the groups indicated that the IV rats did not necessarily excrete more Zn than the OF rats even though they received more Zn. Stress factors, retention and/or intravenous availability of the 20% additional Zn received by the IV rats could have resulted in the increased tissue deposition documented. Positive Zn balance in the CON, OF and IV groups confirmed the Zn adequacy of the TPN solution and the oral diet.

The OF and IV groups appeared more efficient in positive

Zn balance than the CON group as evidenced by higher Zn balance values. In the IV group this can be explained by the greater intake and availability of Zn. In both the OF and IV groups, there appeared to be greater efficiency of Zn retention as evidenced by the lower output values. It is possible that after the post-surgery recovery period where the OF and IV rats were exposed to stress and weight loss due to decreased energy and Zn intake, these rats transitioned into an anabolic phase (the OF rats more efficiently), resulting in greater retention of Zn compared to the non-stressed CON rats.

## **Tissues**

### **Liver and Serum:**

The liver is a known site for activity of newly absorbed Zn (45,49,50,51,52). Higher Zn concentration in the liver of the IV rats may be reflective of metallothionein metabolism. Once Zn is circulating in plasma, bound to albumin, it can enter a liver cell as 1) free Zn in a labile pool, or 2) become part of a metalloprotein Zn pool (45). Zinc in the liver cell is generally distributed among metalloproteins (which assist in Zn exchange), organelles, and cell membranes, among other cellular components (44,45). One of the most widely studied metalloproteins is metallothionein (MT), especially its activity in the liver, kidney, and intestine. Metallothionein, a predominantly Zn-containing protein, seems

to play a significant role in Zn homeostasis (45,46,49,50,53,54). Even in the early years of Zn research, it was noted that rats fed a high Zn diet (1000 mg/kg) for 10 days had a significant increase in the Zn concentration of their liver, intestine, and kidney, the Zn likely bound to MT (55). Similar results were found in the tissues of the IV rats which received approximately 1000  $\mu$ g Zn/kg/day intravenously. In later years, Blalock et al. found that rats fed increasing amounts of Zn in their diets had associated increases in total MT in the kidney, but not in liver (56). Other researchers have found increased liver Zn content with increasing dietary Zn supplementation (52,55,57).

In the liver, certain physiological factors, including trauma, stress, and inflammation, as well as the administration of Zn can induce MT (44,45,46,47,48,53,54,58,59). These inducers can promote increased transcription of the MT gene, involving increased synthesis of MT mRNA (44,60). This cycle then boosts Zn accumulation in liver by increasing MT protein which binds Zn, often decreasing serum Zn (44,45,46,48,49,60,61,62,63,64,65). This redistribution of Zn may be a regulating and protective mechanism of the body to assist the liver in having Zn available for increased protein synthesis in response to stress (i.e. to supply Zn for the enzymes required for DNA synthesis) (44,45,47). The BS rats did not show this response

four days after surgery, however, they received no substantial nutrition during that time period. DiSilvestro and Cousins have documented and postulated that serum Zn and hepatic MT Zn may not always present an inverse fluctuation, in response to stress events, and also that examining serum at only one period in the experiment may result in failure to observe a transient decline in serum Zn (48).

The present study documented a significant deposition of Zn in the liver of the IV rats, however, no significant difference in serum Zn concentration was seen among the groups. It is possible that the greater Zn availability and the approximately 20% higher Zn intake by the IV rats may have prevented a drop in serum Zn that could have occurred in response to the stress, inflammation, and/or intravenous Zn infusion.

When in anabolism, after recovering from a catabolic phase, other tissues have been documented to contribute to maintaining serum Zn levels within normal limits, without necessarily affecting the tissue Zn concentrations (29,31). This contributory pool is believed to be located mostly in bone, liver and plasma (29,31). It is also not known to what extent some level of blood sample hemolysis (which can increase serum Zn values) may have affected serum Zn concentrations (14). It is evident that serum Zn is not always reflective of body tissue Zn status (14,29,30,31).

However, one of the goals of this study was to contribute additional information on Zn metabolism to help shed light on better ways to assess Zn status in humans. Some have suggested examination of Zn concentration in the bile (35). Others have suggested measurements of plasma MT concentrations (29) and pancreatic  $\gamma$ -glutamyl hydrolase enzyme activity (66). Nevertheless, it appears that until an accurate, standardized Zn-analysis method is developed, it is imperative to consider the developmental stage of the individual and to measure Zn losses in stool, urine, and fistulas (if applicable) to precisely determine Zn requirements during TPN.

Parenteral Zn has been shown to increase liver MT expression (45,53,60). Richards and Cousins found that after 24 hours of intraperitoneally administered Zn sulfate, Zn-binding-protein-Zn (MT-Zn) increased in the liver and the small intestine, while serum Zn concentration decreased significantly (where as at 8 hours serum Zn had been substantially higher than controls) (50). However, in that study, the authors found that 48 hours after the Zn injection, MT-Zn had decreased in the liver (although not in the small intestine) and the serum Zn was within normal limits (50). Apparently, a single injection of Zn can vary the liver and serum response in rats at different examination times. The IV rats received continuous intravenous infusions of Zn sulfate, which may have promoted an increase in liver Zn concentration.



Huber and Cousins found that liver MT synthesis increased after injection of Zn chloride in rats (67). Simple saline injections have been documented to increase liver MT mRNA (56), which can promote Zn retention. Therefore, a combination of parenteral administration of Zn, possible inflammation, and the general stress of the IV rats receiving continuous TPN as a sole source of nutrition may have promoted increased liver Zn concentration.

Zinc and its role in wound-healing in normal rats was studied by Elias and Chvapil (68). The authors found that after a skin incision wound, and subsequent Zn treatment, Zn concentration increased in the skin wound area and in the liver, but not in the serum. Further investigations indicate (44,45,47,60), that stress inflicted by surgery may have been an additional influence that caused the influx of Zn, into the liver, in the IV rats when compared to controls. The greater Zn availability and possibly the higher Zn intake in the IV rats may have been additional factors causing a significantly higher liver Zn concentration compared to the OF rats, who also underwent surgery.

#### Small Intestine:

Sites of absorption and excretion, such as the small intestine and the kidney, as well as the liver, seem to be the sites of most activity for newly absorbed enteral and parenteral Zn (31,45,50,51,52,53,56,69). Small intestine Zn

concentration was increased in rats by feeding a high-Zn diet (31). Parenteral administration of highly available Zn would be expected to increase Zn concentration in the small intestine of the IV rats, compared to controls. The approximately 20% higher zinc intake by the IV rats may also have played a role.

The reason why the small intestine Zn concentration of the OF rats was not different from the CON and IV rats is unclear. However, during the treatment period, the OF rats apparently must have shared some similar characteristic(s) with both the CON and the IV rats. Studies of rats during Zn depletion-repletion diet regimens have documented increased Zn concentration in the small intestine after rats were repleted with Zn (31,50). During the recovery period, the OF and IV rats entered a catabolic phase with documented weight loss and negligible energy and Zn intake. They were then repleted nutritionally for two weeks, prior to being killed. Therefore, if the OF and IV rats had an increase in MT-Zn in the small intestine due to Zn repletion, it was likely tempered for the OF group (consuming an oral diet similar to the CON group), after entering anabolism, yet this increase may have supported their similarity in small intestine Zn concentration to the IV rats. The IV rats continued to be exposed to stress during the treatment period (e.g. continuous intravenous infusion, flushing of lines, antibiotics, etc.),

where the OF rats did not.

Hempe et al. recently studied male CD-strain/Sprague Dawley rats and found that administration of intraperitoneal Zn sulfate increased MT gene expression in both intestine and liver, and this may serve to regulate protective metabolic changes occurring from high exogenous Zn intake (60). For example, increased MT synthesis in the intestine may decrease Zn absorption (60). Intraperitoneal administration of Zn sulfate and Zn chloride also have increased the synthesis of MT-Zn in the small intestine (50,53). If the IV rats received a substantial amount of available, intravenous Zn, MT gene expression may have promoted higher Zn concentration in their small intestine compared to the CON rats. The BS group did not receive parenteral nutrition and therefore would not be expected to show this response.

Schneeman et al. have observed that bile, pancreatic juice, and intestinal mucosa seem to be sources of endogenous Zn (69). If the IV rats were not secreting as much bile and pancreatic juice as the OF rats who were eating, liver Zn may have been maintained better in the IV rats (especially since liver bile outflow to the intestine is 1.5-2 times greater than pancreatic juice (69)). Alternatively, the OF rats had more opportunity to activate the bile system with enteral feedings, and bile secretions into the intestine may have caused the lack of significant difference in small intestine

Zn concentration between the OF rats and the IV rats. Also, the OF rats probably had a chance to reabsorb intestinal secretions of bile Zn, adding to their overall Zn concentration in the small intestine.

Neither inflammation nor infection were evaluated in the rats of the present study. However, the rats response to general stress and stress mediators, if present, may have influenced intestinal Zn accumulation of the OF and IV rats (70). The BS rats went through surgery but were killed prior to initiation of feeding and probably did not have a chance to experience further possible stresses.

#### Kidney:

The kidney has been mentioned as an active site for newly absorbed Zn (31,45,51,52,56,71,72). This alone may be one reason for significant increased kidney deposition of intravenously administered and available Zn in the IV rats, and in larger quantity than that received by the OF rats. As in the small intestine, studies of rats during Zn depletion-repletion oral regimens have documented that Zn concentration increased in the kidney after Zn repletion (31) and that kidney Zn concentration increased in rats by feeding a high-Zn diet (51,52,55,57,71,72).

Blalock et al. have postulated that the kidney acts as a Zn regulator which can control urinary output of Zn and/or have a role in detoxification, as they observed when increased

Zn-containing MT synthesis occurred in the kidneys of rats consuming adequate or supplemented Zn diets (56). Considering the same can occur, especially in rats fed intravenous Zn, this may explain the higher Zn concentration in the kidneys of the IV group which received approximately 1000  $\mu\text{g}$  Zn/kg/day compared to the CON and OF rats who consumed approximately 800  $\mu\text{g}$  Zn/kg/day.

Increased urinary Zn output was not noticed in the balance study during the two treatment period collections analyzed. As in the study by Blalock et al., enhanced MT production may have occurred in the kidneys of the IV rats which may have been important in the processing of Zn to prevent urinary loss of the trace element and to prevent kidney and urinary tract damage (56).

It has been documented that, unlike the liver, most physiological stress and its mediators do not appear to promote substantial MT induction in the kidney (47).

#### Lung:

In the study by Chvapil et al., the lung was unable to increase Zn content with increasing dietary Zn supplementation (55). Kang et al. also reported that Zn concentrations of lung tissue did not change following diets containing 1.3  $\mu\text{g/g}$ , 55  $\mu\text{g/g}$ , or 550  $\mu\text{g/g}$  of Zn (57). Also Cousins and Lee-Ambrose found that lung MT mRNA levels did not change significantly by increasing dietary Zn intake (52).

It has been shown previously that a substantially high concentration of Zn (30  $\mu\text{g/g}$  wet weight) in an organ was necessary for production of Zn-thionein (53,54). Of the four rat tissues demonstrating significantly elevated Zn concentration, this finding was reflective, for the most part, in the liver and kidney of the IV rats, and the small intestine of the IV and OF rats. The Zn concentration in the lung of the IV rats was well below 30  $\mu\text{g/g}$  wet weight (23.46  $\mu\text{g/g} \pm 1.84$ ), and yet the IV rats showed an appreciably higher lung Zn content. However, it appears that in this population, administration of a substantial amount of intravenously available Zn increased lung Zn concentration significantly in the IV rats.

#### Heart, Muscle, Testes, Bone:

The Zn concentration in heart, muscle, testes, and bone of the rats was not affected by: 1) the stress of surgery, 2) a higher Zn intake by the IV rats, and 3) two different routes of nutritional administration. Similar studies have supported these findings. In a study by Chvapil et al., the heart, muscle, testes, and bone Zn concentration did not substantially increase with increasing dietary Zn supplementation (55). When Zn concentration of water ranged from 2.5 to 40  $\mu\text{g/mL}$ , no changes in the Zn content of heart, leg muscle, testes, and liver were documented (72).

Sherman et al. suggest that in the rat heart, Zn is

mainly present as metalloproteins and metalloenzymes involved with metabolic function and structural responsibilities (32). Larsen and Sandstrom have also documented consistent heart Zn concentration with increasing dietary Zn intake (51).

The Zn concentration in muscle appears to be maintained very consistently (31,51), especially in rats fed Zn-adequate diets (73). Giugliano and Millward documented no differences in rat gastrocnemius muscle Zn concentration after the rats received adequate (55 mg Zn/kg) or severely deficient (0.4 mg Zn/kg) Zn diets fed in Zn-depletion or Zn depletion-repletion diet regimens (31).

Although a study of rats during Zn depletion-repletion diet experiments documented increased Zn concentration in the testes after Zn repletion and that testes Zn concentration increased in rats (as compared to baseline rats) by feeding a high-Zn diet (31), Canton and Cremin studied the effects of Zn depletion and repletion on rat tissue and found Zn concentration maintained in the testes (66). Recently, MT has been located in rat testes and other parts of the male reproductive organs, and Nishimura et al. stated that this "further supports the notion that MT may be physiologically associated with Zn storage and transport..." in the testes (74).

In rats, it appears that Zn continues to deposit in bone when fed both Zn-adequate and high Zn diets (51,73). In a

recent study, Zhou et al. have produced data suggesting there are two Zn pools in the bone skeleton: 1) a quick turn-over pool (10-20 % of total Zn) from which Zn is easily removed when needed to meet soft tissue needs, and 2) a slow turn-over pool from which Zn cannot be used without bone loss and a decrease in growth (73). Giugliano and Millward have data suggesting that Zn is conserved in body tissues during catabolism or is redistributed from bone and released into plasma where it is quickly taken up by other priority tissues, such as muscle, to allow growth (31). This supports consistency in muscle Zn concentration. This may also support why the BS rats never showed any significant difference in tissue Zn concentration compared to the CON rats, even though they were killed immediately after undergoing a catabolic post-surgical recovery period.

Zhou et al. found that with borderline Zn restriction, rats release and use Zn from the bone's quick turn-over pool to maintain Zn homeostasis, and that not until four weeks of Zn restriction is a substantial decrease in rat growth seen (73). Zhou et al. also agreed with King who postulated that changes are not seen in plasma Zn concentrations until body Zn homeostasis cannot be maintained by slowing of growth or reduced Zn excretion (29,73). Since the CON, IV, and OF rats were maintained in positive Zn balance, no need to mobilize bone Zn stores probably arose (except for a possible



utilization of the Zn rapid turn-over pool during the 4-day recovery period for the BS, IV, and OF rats, where there was a trend toward lower bone Zn concentration in the BS rats), hence supporting the consistent bone Zn concentration in all the groups.

### Zinc Toxicity

The question of Zn toxicity arises when addressing higher amounts of parenteral Zn compared to enteral Zn. The Zn intake of the rats in either the OF or IV groups did not come near the level of Zn intakes reported in the literature to have adverse effects on rats. Nevertheless, the importance of avoiding excess intake of Zn enterally is evident (57,75). Even more important would be the concern of avoiding excess intakes of Zn parenterally when no intestinal absorptive factors are present.

Kang et al. divided rats into three groups and gave them *ad libitum* access to a commercially prepared Zn-deficient diet containing 1.3  $\mu\text{g}$  Zn/g diet (57). Group A received just this diet, Group B received the same diet supplemented with Zn carbonate (55  $\mu\text{g}$  Zn/g diet), and Group C received the same diet supplemented with high amounts of Zn (550  $\mu\text{g}$  Zn/g diet) (57). Kang et al. found that the rats in groups B and C had similar weight gain and food efficiency ratios (gram of body weight gain per gram of food consumed), and proposed that a

550  $\mu\text{g}$  Zn/g oral diet was not toxic for the rats (57). However, they found that the liver and kidney Zn concentrations increased with higher dietary Zn intake, but the iron concentrations of those tissues decreased with increasing dietary Zn (57). Yamaguchi et al. have found oral administration of 1000, 10,000 or 100,000  $\mu\text{g}$  Zn/kg/day to weanling rats for 30 days may (especially at the higher doses) inhibit rat bone growth and calcification, and significantly decrease femur bone weight and calcium content (75).

A TPN solution inadvertently providing 23 mg Zn/L daily (instead of 2.3 mg Zn/L), in addition to approximately 3.1 mg Zn/L of mean level Zn contaminant for a total of 25 to 26 mg Zn/L daily, resulted in hyperamylasemia in 6 of 7 human patients (76). None of the patients showed clinical signs of pancreatitis (76).

A death occurred from an inadvertent infusion of 1.7 g of Zn (7.4 g of Zn sulfate) over a 60 hour period in a woman with Crohn's Disease, receiving TPN (77). Symptoms prior to demise included hypotension, pulmonary edema, diarrhea, vomiting, jaundice, oliguria, cardiac arrhythmias, hyperamylasemia without evidence of acute pancreatitis, acute anemia and thrombocytopenia (77).

#### **Tissue Retention of Zn with TPN Administration**

The Russian study by Poriadkov et al. showed results

consistent with this study, where liver Zn concentration increased after receiving complete intravenous nutrition (34).

In the study by Yokoi et al., the daily Zn supplementation in their TPN was similar (approximately 1312  $\mu\text{g}$  Zn/kg body weight/day) to the present study's (approximately 1000  $\mu\text{g}$  Zn/kg body weight/day), and their Zn-deficient diet contained similar Zn concentration (14.2 mg Zn/kg diet) compared to the present study's diet containing the requirement of Zn for rats (12 mg Zn/kg diet) (15). Their results are similar to the present study's in the increase Zn content seen in the liver with the TPN group compared to the diet group, and the consistency of Zn content seen in the heart, testis, muscle, and bone. It is possible that the slightly higher infusion of Zn in their TPN group may have been a factor in the higher plasma Zn concentration documented.

The study by Matsuda et al. is dissimilar to the present study since Zn concentration in the control oral diet was almost three times more than in the Zn-requirement-containing diet in the present study (16). Their Group B received only trace amounts of Zn in the TPN, Group C received only 10 % of the Zn the IV group received in the present study, and Group D received only 31 % of the Zn the IV group received in the present study. Yet muscle and testes showed constant Zn concentration which affirms the Zn concentration stability in

these tissues also seen in the present study. Since the bone is a large mass of tissue, any variation in the bone Zn pool found in the Matsuda et al. study was seen only when Zn intake was substantially low in the TPN. Therefore, in the present study, changes in the bone Zn pool would probably have been likely only with substantially excessive Zn intake.

It is possible the lower Zn infusion in the TPN solution by Matsuda et al. caused no significant change in their rat's liver Zn concentration, but that the higher Zn infusion in the IV rats of the present study did cause a change. Matsuda et al. found varied kidney Zn concentration, depending on the Zn infusion, and this helps support the higher kidney Zn concentration in the IV group of the present study, which received 20% higher Zn intake. The infusion of only 30 % of the Zn the IV rats received in the present study, caused no significant difference in Zn heart concentration between Groups D and A in the Matsuda et al. study. The heart Zn concentration in the present study also showed stability with adequate to higher Zn intake.

In Yamato's study, a high Zn infusion with TPN showed deposition of Zn in key tissues which reflects similar findings in the present study (35). Although in Yamato's study, Group D received 4.5 to 6 times the daily Zn of the present study, Zn did deposit significantly in the liver, small intestine, and kidney compared to controls. Zinc

infusion was so high however, that serum, muscle and bone Zn pools apparently also showed higher Zn concentration compared to controls. Still, unlike the present study, lung Zn concentration did not differ compared to controls.

In another investigation, Matsuda et al. intravenously injected four groups of rats for seven days with a trace element preparation containing Zn (36). The second group of rats received intravenous injections of Zn (784  $\mu\text{g}$  Zn/kg body weight/day) similar to the daily Zn infused in the TPN of the IV rats in the present study (1000  $\mu\text{g}$  Zn/kg body weight/day). However, Matsuda et al. found that this dose of intravenously administered Zn, for the most part, did not affect tissue Zn concentration (except in plasma), as did the higher Zn doses (36). However, rats in the present study were fed or infused Zn-containing diets and TPN for twice as long as ones in the study by Matsuda et al., and also received the trace element in a form containing other nutrients (and over 24 hours daily, rather than a single-dose daily). This may have improved Zn metabolism and tissue availability, possibly having a role in causing its tissue accumulation.

Matsuda et al. also found the highest dose of intravenous Zn (7840  $\mu\text{g}$  Zn/kg body weight/day) toxic and deadly, as it killed all the rats in that group. Those rats received almost eight times the dose of Zn administered to the IV rats in the present study.

In summary, intravenous administration of Zn in TPN can cause elevations in tissue Zn concentrations in rats. In view of these findings, more research is recommended to determine intravenous Zn requirements and/or possible risk of toxicity for long-term Zn administration.

## Summary and Conclusions

The effect of TPN on Zn retention in tissues of Fischer-344 rats was evaluated and compared to rats consuming a Zn-supplemented oral diet. Findings revealed:

1. The BS, OF, and IV rats experienced a 4-day catabolic state during the recovery period after surgical catheter placement. The OF and IV rats then entered a recovery period with documented weight gain. The TPN regimen was able to maintain the IV group rat weights during the treatment period, but with no obvious growth beyond the pre-surgery condition.
2. The IV group received an average of 20% more Zn daily compared to the OF and CON groups ( $p < 0.01$ ), during the treatment period, because the TPN solution was adjusted in order to achieve isocaloric and isonitrogenous properties, similar to an oral diet, and Zn concentration did not adjust accordingly.
3. No significant difference was seen in fecal and urinary Zn output among the groups, although the OF and IV groups appeared to have greater efficiency of Zn retention, as evidenced by lower output values, compared to the CON group.
4. Rats receiving intravenously administered Zn from a TPN solution had significantly higher Zn concentration in

liver, small intestine, kidney, and lung ( $p < 0.01$ ,  $p < 0.03$ ,  $p < 0.01$ ,  $p < 0.01$  respectively). High availability of the intravenous Zn and possibly the higher Zn intake were contributing factors since these organs are known to have high Zn metabolism.

5. Surgical trauma and the continuous TPN protocol were the factors most related to the observed Zn redistribution and concentration in the liver, kidney, lung, and intestine.

Overall, trauma and continuous stress appeared to have greater effect on animal Zn status than did the concentration of Zn in the nutrient source (IV versus oral). Serum Zn is not the best parameter to determine Zn status during stress.

In view of these findings, more research is recommended, especially with more detailed, long-term balance studies, to assist in determining Zn needs in TPN. Future studies to determine TPN needs in rats may include a study which allows rats to reach pre-surgery weight before initiating TPN treatment, and a study to compare bile Zn concentration versus serum Zn concentration as a measure of Zn status.

Measurement of the effect of various dosages of intravenous Zn administration on the rate and level of increased MT mRNA production in the cell, may also assist in determining Zn needs in TPN.



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## APPENDICES

### **Appendix A Surgical Catheter Placement**

1. All surgical instruments, catheters and swivel assemblies were doubly wrapped in autoclave paper and steam sterilized at 125 °C for 20 minutes on the day prior to the surgery.
2. During surgery, all autoclaved instruments and supplies were placed on a 46 x 66 cm disposable impermeable sterile drape (Sterile Drape, ABCO, Milwaukee, WI) by means of aseptic technique (41). Surgical masks and sterile gloves were worn during surgery and when working with materials in the sterile field.
3. The ventral neck, mid-scapular and suprascapular areas were shaved, and the animal was weighed. The rat was restrained in supine position on an animal surgical board and the ventral neck was thoroughly cleansed with povidone iodine, 10% solution (ABCO Solution, ABCO, Milwaukee, WI), using sponges held with a hemostat.
4. A 3 cm midline incision was extended from above the suprasternal notch, up the neck. A surgical drape was placed over the animal with an opening above the incision, and the skin flaps were autoclipped to the drape opening.
5. The surgical site was again cleansed thoroughly with povidone iodine. The right external jugular was dissected free and two, 6 inch pieces, of 4-0 silk suture were placed underneath the vein, about 0.5 cm apart.
6. The 4-0 silk closest to the tail was clamped with a bulldog clamp. Bleeding was prevented by tension put on the vein from the silk closest to the head.
7. A small incision was made into the vein, between the two sutures. The beveled end of a silicone rubber catheter (Silastic, Dow Corning Co., Midland, MI), 0.02 inches in internal diameter and 0.037 inches in outer diameter, was threaded 2 cm down the external jugular, into the superior vena cava. The catheter was tied in place with 4-0 silk sutures.



8. The catheter was irrigated with heparinized saline through a 1.0 ml syringe that had been attached previously to the catheter. Blood was also drawn from the catheter to assure correct catheter position.
9. The drape was folded on itself and the rat was rolled on its side. The syringe and catheter were kept in the sterile fold of the drape.
10. The suprascapular area of the back was cleansed with povidone iodine and a 2 cm incision was made. Another sterile surgical drape was placed over the animal with an opening over the incision and the skin flaps were autoclipped to the drape.
11. A 13-gauge needle was tunneled subcutaneously from the dorsal incision, behind the front leg, through to the ventral neck incision.
12. A bulldog clamp was used to occlude the catheter on the ventral side to prevent an air emboli. The catheter was then threaded through the needle and out the dorsal incision. The needle was removed and the bulldog clamp was moved to the dorsal side.
13. The dorsal drape is again folded and the rat placed supine. The catheter was threaded through a stainless steel, flexible spring cable containing a flange.
14. The catheter was secured with a suture and the ventral incision was closed with auto clips. The flange on the spring cable was sutured with 3-0 silk to the dorsal interscapular fascia. The dorsal incision was closed around the spring cable with autoclips supplemented with sawing a 3-0 silk suture to assure a tight closure.
16. The spring cable with the catheter was passed through a ventilation hole on the cage top and the catheter and cable were attached to the bottom port of a swivel and secured to the cage top.
17. A 3-cc syringe was attached to a 10 cm silicone rubber catheter (0.02 inches in internal diameter and 0.037 inches in outer diameter) which was attached to the top port of the swivel and the cage top.
18. Heparinized saline (10 u/ml) was flushed through the catheter. The incision areas were again cleansed with povidone iodine. The rat was weighed with the cage top and placed in the metabolic cage.

## Appendix B

Table B1. Formulation of the Amino Acid Diet and TPN Solution

Ingredient	Amino Acid Diet # (g/Kg Diet)	TPN Solution ## (g/2886 g Solution)
Amino Acid Mixture *	205.00	205.00
Dextrose	677.42	677.42
Minerals **	74.869	74.869
Vitamins ***	1.770	1.771
Soybean Oil	34.20	-----
Glycerin	6.80	-----
Intralipid 10% @	-----	388.20 ml @@

# Energy content: 3.84 Kcal/g diet

Nitrogen content: 33.5 mg/g diet

## Energy content: 1.18 Kcal/g TPN solution

Nitrogen content: 10.08 mg/g TPN solution

The gravity of the TPN solution is 1.115 g/ml

\* Table 2

\*\* Table 3-1; Table 3-2

\*\*\* Table 4

@ Intralipid 10%, Clintec Nutrition Company, Deerfield, IL, 60015

@@ Added to 2886 g TPN solution

Table B2. Composition of the Amino Acid Mixture in the Amino Acid Diet and TPN Solution

Amino acid	Formulation (g/kg AAD); (g/2886g TPN)	Rat Requirements * g/kg diet
DL-Aspartic Acid	6.36	---
L-Asparagine	6.94	4.00
L-Threonine	8.02	5.00
L-Serine	10.50	**
L-Glutamic Acid	20.00	40.00
L-Glutamine ***	21.86	---
L-Proline	20.24	4.00
L-Glycine	3.60	**
L-Alanine	5.76	**
L-Cysteine	0.82	---
L-Valine	12.78	6.00
DL-Methionine	5.84	
and		
L-Methionine	5.00	6.00
L-Isoleucine	10.56	5.00
L-Leucine	16.94	7.50
L-Phenylalanine	19.80	8.00
L-Histidine	5.46	3.00
L-Lysine	15.02	7.00
L-Tryptophan	2.48	1.50
L-Arginine	7.02	6.00

\* Nutrient Requirements of Laboratory Animals, 3rd ed.,

1978 National Academy of Sciences, Washington, D.C.

\*\* Non-essential amino acids used to meet the total protein requirement

\*\*\* Converted to glutamic acid in vivo

--- Non-essential amino acids not required for the rat

Table B3-1. Mineral Compounds in the Amino Acid Diet and TPN Solution

Mineral Compound	Formulation in Amino Acid Diet (mg/Kg AAD)	Formulation in TPN Solution (mg/2886g TPN)
d-Gluconic Acid Hemicalcium salt (C <sub>6</sub> H <sub>11</sub> O <sub>7</sub> .1/2Ca)	54,867.76	54,867.76
Potassium Phosphate Monobasic;Anhydrous (KH <sub>2</sub> PO <sub>4</sub> )	13,923.27	13,923.27
Sodium Phosphate Monobasic;Monohydrate (NaH <sub>2</sub> PO <sub>4</sub> .H <sub>2</sub> O)	3,688.56	3,688.56
Manganese Chloride (MnCl <sub>2</sub> .4H <sub>2</sub> O)	180.11	180.11
Magnesium Sulfate Anhydrous (MgSO <sub>4</sub> )	1,980.19	1,980.19
Ferric Citrate (FeC <sub>6</sub> H <sub>5</sub> O <sub>7</sub> )	153.51	153.51
Cupric Sulfate Pentahydrate (CuSO <sub>4</sub> .5H <sub>2</sub> O)	19.69	19.69
Zinc Sulfate (ZnSO <sub>4</sub> .7H <sub>2</sub> O)	52.87	52.87
Potassium Iodine (KI)	0.20	0.20
Chromium Potassium Sulfate	2.90	2.90

Dodecahydrate (CrK(SO <sub>4</sub> ) <sub>2</sub> )		
Sodium Selenite (Na <sub>2</sub> SeO <sub>3</sub> )	0.22	0.22

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Table B3-2. Mineral Elements in the Amino Acid Diet and TPN Solution

Mineral Element	Formulation in AAD (mg/Kg AAD)	Formulation in TPN (mg/2886g TPN)	Rat Requirement # (mg/Kg Diet)
Calcium *	5,000.00	5,000.00	5,000.00
Potassium **	4,000.99	4,000.99	3,600.00
Phosphorus ***	4,000.00	4,000.00	4,000.00
Sodium ****	614.80	614.80	500.00
Manganese	50.00	50.00	50.00
Chloride *****	500.00	500.00	500.00
Magnesium	400.00	400.00	400.00
Iron	35.00	35.00	35.00
Copper	5.00	5.00	5.00
Zinc	12.00	12.00	12.00
Chromium	0.30	0.30	0.30
Selenium	0.10	0.10	0.10
Iodine	0.15	0.15	0.15
Fluoride	----	----	1.00
Sulfur *****	535.94	535.94	300.00

# Nutrient Requirement of Laboratory Animals,  
3rd ed., 1978, National Academy of Sciences,

Washington, D. C.

- \* Includes calcium in d-gluconic acid, calcium pantothenate
- \*\* Includes potassium in potassium phosphate, potassium iodine, chromium potassium sulfate
- \*\*\* Includes phosphorus in potassium phosphate, sodium phosphate
- \*\*\*\* Includes sodium in sodium phosphate, sodium selenite
- \*\*\*\*\* Includes chloride in manganese chloride, choline chloride, thiamin hydrochloride, pyridoxine monohydrochloride
- \*\*\*\*\* Includes sulfur in magnesium sulfate, cupric sulfate, zinc sulfate, chromium potassium sulfate

Table B4. Vitamins in the Amino Acid Diet and TPN Solution

Vitamin	Rat Requirement * (mg/Kg Diet)	Amino Acid Diet (mg/Kg AAD)	TPN Solution (mg/2886g TPN)
Thiamin Hydrochloride (C <sub>12</sub> H <sub>17</sub> ON <sub>4</sub> ClSHCl)	4.00	4.00	4.00
d-Biotin (C <sub>10</sub> H <sub>16</sub> N <sub>2</sub> O <sub>3</sub> S)	#	0.20	0.20
Riboflavin (C <sub>17</sub> H <sub>20</sub> N <sub>4</sub> O <sub>6</sub> )	3.00	3.00	3.00
Niacin Niacinamide (C <sub>6</sub> H <sub>6</sub> N <sub>2</sub> O)	20.00	20.00	20.00
Vitamin B <sub>6</sub> Pyridoxine Monohydrochloride (C <sub>8</sub> H <sub>11</sub> NO <sub>3</sub> .Hcl)	6.00	6.00	6.00
Calcium Pantothenate D-Pantothenic Acid Hemicalcium Salt (C <sub>9</sub> H <sub>16</sub> O <sub>5</sub> .1/2Ca)	8.00	8.00	8.00

Folic Acid	1.00	1.00	1.00
Pteroylglutamic Acid (C <sub>19</sub> H <sub>19</sub> N <sub>7</sub> O <sub>6</sub> )			
Vitamin B <sub>12</sub> Cyanocobalamin (C <sub>63</sub> H <sub>88</sub> CoN <sub>14</sub> O <sub>14</sub> P)	0.05	0.05	0.05
Menadione (C <sub>11</sub> H <sub>8</sub> O <sub>2</sub> ) or	0.5	0.5	-----
Phytonadione (C <sub>31</sub> H <sub>46</sub> O <sub>2</sub> )	0.05	-----	0.05 **
Choline Chloride ([2-Hydroxye- thyl]trimethyl- ammonium) (C <sub>5</sub> H <sub>14</sub> ClNO)	1,000.00	1,695.79	1,695.79
Retinol Acetate (C <sub>22</sub> H <sub>32</sub> O <sub>2</sub> )	1.376	1.376	2.752 ***
Cholecalciferol (C <sub>27</sub> H <sub>44</sub> O)	0.025	0.025	0.020 ***
DL- $\alpha$ -tocopheryl Acetate (C <sub>31</sub> H <sub>52</sub> O <sub>3</sub> )	30.00	30.00	-----
DL- $\alpha$ -tocopherol (C <sub>29</sub> H <sub>50</sub> O)	30.00	-----	30.00 ****

# Not required for the rat

\* Nutrient Requirement of Laboratory Animals, 3rd ed., 1978, National Academy of Sciences, Washington, D. C.

\*\* Present in 25  $\mu$ l Aquamephyton, Merck Sharp & Dohme, Div. of Merck & Co. Inc., West Point, PA 19486

\*\*\* Present in 80  $\mu$ l Injacom 100, Roche Vitamins and Fine Chemicals, a div. of Hoffmann-La Roche Inc., Nutley, NJ 07110

\*\*\*\* Present in 113.6  $\mu$ l Rocavit E, Roche Vitamins and Fine Chemicals, a div. of Hoffmann-La Roche Inc., Nutley, NJ 07110. Also included is 1.6 mg DL- $\alpha$ -tocopherol in Injacom 100, Roche Vitamins and Fine Chemicals, a div. of Hoffmann-La Roche Inc., Nutley, NJ 07110

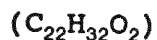
## Appendix C

### C1. Preparation of 1 Kg Amino Acid Diet

1. Weigh the following vitamins and combine together:

Thiamin Hydrochloride ( $C_{12}H_{17}ON_4ClSHCl$ )	4.00 mg
d-Biotin ( $C_{10}H_{16}N_2O_3S$ )	0.20 mg
Riboflavin ( $C_{17}H_{20}N_4O_6$ )	3.00 mg
Niacin Niacinamide ( $C_6H_6N_2O$ )	20.00 mg
Vitamin B <sub>6</sub> Pyridoxine Monohydrochloride ( $C_8H_{11}NO_3.HCl$ )	6.00 mg
Calcium Pantothenate D-Pantothenic Acid Hemicalcium Salt ( $C_9H_{16}O_5.1/2Ca$ )	8.00 mg
Folic Acid Pteroylglutamic Acid ( $C_{19}H_{19}N_7O_6$ )	1.00 mg
Vitamin B <sub>12</sub> Cyanocobalamin ( $C_{63}H_{88}CoN_{14}O_{14}P$ )	0.05 mg
Menadione ( $C_{11}H_8O_2$ )	0.5 mg
Choline Chloride ([2-Hydroxyethyl]trimethyl- ammonium) ( $C_5H_{14}ClNO$ )	1,695.79 mg
Retinol Acetate	1.376 mg





Cholecalciferol 0.025 mg  
(C<sub>27</sub>H<sub>44</sub>O)

DL- $\alpha$ -tocopheryl 30.00 mg  
Acetate  
(C<sub>31</sub>H<sub>52</sub>O<sub>3</sub>)

\* Total weight of vitamin mixture, includes choline chloride, for 1 kg diet is 1769.941 mg.

2. Weigh the following minerals and add to vitamin-choline mixture:

d-Gluconic Acid 54,867.76 mg  
Hemicalcium salt  
(C<sub>6</sub>H<sub>11</sub>O<sub>7</sub>.1/2Ca)

Potassium Phosphate 13,923.27 mg  
Monobasic; Anhydrous  
(KH<sub>2</sub>PO<sub>4</sub>)

Sodium Phosphate 3,688.56 mg  
Monobasic; Monohydrate  
(NaH<sub>2</sub>PO<sub>4</sub>.H<sub>2</sub>O)

Manganese Chloride 180.11 mg  
(MnCl<sub>2</sub>.4H<sub>2</sub>O)

Magnesium Sulfate 1,980.19 mg  
Anhydrous  
(MgSO<sub>4</sub>)

Ferric Citrate 153.51 mg  
(FeC<sub>6</sub>H<sub>5</sub>O<sub>7</sub>)

Cupric Sulfate 19.69 mg  
Pentahydrate  
(CuSO<sub>4</sub>.5H<sub>2</sub>O)

Zinc Sulfate 52.87 mg  
(ZnSO<sub>4</sub>.7H<sub>2</sub>O)

Potassium Iodine 0.20 mg  
(KI)

Chromium 2.90 mg  
Potassium Sulfate  
Dodecahydrate

(CrK(SO<sub>4</sub>)<sub>2</sub>)

Sodium Selenite                      0.22 mg  
(Na<sub>2</sub>SeO<sub>3</sub>)

\* Total weight of minerals for 1 Kg diet is 74,869.28 mg.

3. Grind vitamins-mineral mixture thoroughly together and set aside.

4. Weigh the following amino acids and mix together:

DL-Aspartic Acid	6.36 g
L-Asparagine	6.94 g
L-Threonine	8.02 g
L-Serine	10.50 g
L-Glutamic Acid	20.00 g
L-Glutamine	21.86 g
L-Proline	20.24 g
L-Glycine	3.60 g
L-Alanine	5.76 g
L-Cysteine	0.82 g
L-Valine	12.78 g
DL-Methionine	5.84 g
L-Methionine	5.00 g
L-Isoleucine	10.56 g
L-Leucine	16.94 g
L-Phenylalanine	19.80 g
L-Histidine	5.46 g
L-Lysine	15.02 g
L-Tryptophan	2.48 g

L-Arginine

7.02 g

- \* Total weight of amino acids in 1 kg diet is 205 g.
- 5. Weigh 677.42 g dextrose and add it to vitamin-mineral mixture.
- 6. Add amino acid mixture to vitamins-mineral-dextrose mixture.
- 7. Weigh 34.20 g soybean oil and 6.80 g glycerin and add to above mixture and mix thoroughly.
- 8. Keep diet refrigerated at 4 °C. Lasts up to two weeks in refrigerator.

## C2. Procedure For Preparing 2588 ml TPN Solution

1. Weigh the following vitamins and combine together:

Thiamin Hydrochloride                      4.00 mg  
( $C_{12}H_{17}ON_4ClSHCl$ )

d-Biotin    0.20 mg  
( $C_{10}H_{16}N_2O_3S$ )

Riboflavin    3.00 mg  
( $C_{17}H_2ON_4O_6$ )

Niacin    20.00 mg  
Niacinamide  
( $C_6H_6N_2O$ )

Vitamin B<sub>6</sub>    6.00 mg  
Pyridoxine  
Monohydrochloride  
( $C_8H_{11}NO_3 \cdot HCl$ )

Calcium Pantothenate                      8.00 mg  
D-Pantothenic Acid  
Hemicalcium Salt  
( $C_9H_{16}O_5 \cdot 1/2Ca$ )

Folic Acid    1.00 mg  
Pteroylglutamic Acid  
( $C_{19}H_{19}N_7O_6$ )

Vitamin B<sub>12</sub>    0.05 mg  
Cyanocobalamin  
( $C_{63}H_{88}CoN_{14}O_{14}P$ )

Phytonadione    0.05 mg  
( $C_{31}H_{46}O_2$ )

Choline    1,695.79 mg  
Chloride  
([2-Hydroxyethyl]trimethyl-  
ammonium)  
( $C_5H_{14}ClNO$ )

\* This vitamin mixture weighs (includes choline chloride):  
1738.09 mg.

2. Keep the above vitamin-mixture separately.

3. Weigh the following minerals and mix together.

Potassium Phosphate            13,923.27 mg  
Monobasic; Anhydrous  
( $\text{KH}_2\text{PO}_4$ )

Sodium Phosphate                3,688.56 mg  
Monobasic; Monohydrate  
( $\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$ )

Manganese Chloride              180.11 mg  
( $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$ )

Magnesium Sulfate               1,980.19 mg  
Anhydrous  
( $\text{MgSO}_4$ )

Cupric Sulfate                    19.69 mg  
Pentahydrate  
( $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ )

Zinc Sulfate                      52.87 mg  
( $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ )

Potassium Iodine                 0.20 mg  
(KI)

Chromium                          2.90 mg  
Potassium Sulfate  
Dodecahydrate  
( $\text{CrK}(\text{SO}_4)_2$ )

Sodium Selenite                  0.22 mg  
( $\text{Na}_2\text{SeO}_3$ )

\* Total weight of this mineral mixture is 19,848.01 mg.

4. Keep the above mineral mixture separately.

5. Weigh the following amino acids and mix together:

DL-Aspartic Acid                 6.36 g

L-Asparagine                      6.94 g

L-Threonine                        8.02 g

L-Serine                            10.50 g

L-Glutamic Acid	20.00 g
L-Glutamine	21.86 g
L-Proline	20.24 g
L-Glycine	3.60 g
L-Alanine	5.76 g
L-Cysteine	0.82 g
L-Valine	12.78 g
DL-Methionine	5.84 g
L-Methionine	5.00 g
L-Isoleucine	10.56 g
L-Leucine	16.94 g
L-Phenylalanine	19.80 g
L-Histidine	5.46 g
L-Lysine	15.02 g
L-Tryptophan	2.48 g
L-Arginine	7.02 g

- \* Total weight of this amino acid mixture is 205 g.
- 6. Measure 1,600 ml of DDI water into a 3,000 ml beaker.
- 7. Weigh 54,867.76 mg d-gluconic acid and mix with the amino acid mixture. Add the amino-gluconic acid mixture to the DDI water.
- 8. Heat the DDI water mixture on a hot plate while stirring with an automatic stirrer (do not exceed 50 °C and keep mixing time to a minimum).
- 9. Dissolve 153.51 mg ferric citrate in 40 ml DDI water in a separate beaker. Heat the ferric citrate solution (not exceeding 50 °C). Cool the solution when the ferric citrate dissolves completely.
- 10. Add 677.42 g dextrose to the amino-gluconic acid

solution with the hot plate on "low" temperature. When all nutrients are dissolved, cool solution to 22 °C.

11. Add all minerals, and then vitamins, to the solution on the hot plate while stirring. Add DDI water to the total volume of 2588 ml.
12. Filter solution to a sterile plastic container (Corning Glass Works, Corning, NY): 600 ml/container.
13. Pipet 25  $\mu$ l Aquamephyton (vitamin K) (Merck and Co., West Point, PA), to 2588 ml TPN solution.
14. Twenty-four hours before feeding, filter the TPN solution into evacuation containers (Abbott Laboratories, North Chicago, IL).

100 ml for two days  
150 ml for three days

15. Store in refrigerator at 4°C.

16. Immediately prior to infusion, add:

- 1). 388.20 ml Intralipid 10% (Clintec Nutrition Co., Deerfield, IL) to 2588 ml TPN solution.  
(15 ml Intralipid/100 ml TPN )
- 2). 80  $\mu$ l Injacom 100 (vitamin A & vitamin D)  
(Hoffmann-La Roche, Nutley, NJ) to 2588 ml TPN solution.
- 3). 113.6  $\mu$ l Rocavit E (vitamin E) (Hoffmann-La Roche, Nutley, NJ) to 2588 ml TPN solution.

## APPENDIX D

### Zinc Analysis Procedure

#### **Preparation and Digestion of Samples:**

1. All glassware was soaked in a detergent solution overnight and then rinsed with deionized water.
2. Detergent washed glassware was soaked in a 20% nitric acid solution (made from 70% nitric acid and DDI water) overnight. After rinsing with DDI water, the acid-washed glassware was rinsed with 0.001 M EDTA solution and then rinsed with DDI water.
3. Approximately 0.5 gram wet tissue samples were weighed and transferred into a 50 ml Erlenmeyer flask. The exception was weighing of the whole individual kidney, testis, heart, and femur bone used for digestion. The feces samples from the third collection (days 17-19 of experiment) and sixth collection (days 26-28 of experiment) were weighed and transferred into the Erlenmeyer flask.
4. Two 0.5 gram bovine liver samples (SRM 1577b, NIST, Gaithersburg, MD) were weighed for each tissue and feces set (except femur bone and serum) and served as quality controls. Two 0.5 gram samples of non-fat milk powder (SRM 1549) were the designated controls for the femur bone, and bovine serum (SRM 1598) was the control for the serum samples. Two blanks, containing only digestion reagents, were also prepared for each sample set. Fifty-ml beakers were used to cover the Erlenmeyer flasks containing the samples.
5. An entire set of tissue or feces was placed in muffle furnaces randomly. Furnace temperature was increased 50°C/hour to 430°C and held at that temperature for 48 hours.
6. Samples were removed from the furnaces and cooled prior to adding 0.10 ml of DDI water and 0.10 ml of 70% nitric acid to each flask.
7. Hot plates were prepared in a laboratory hood, and a set of sample tissue or feces was placed on them until a temperature of 90°C was reached. The samples were then removed from the hot plates and cooled. After a five-minute cooling, 0.5 ml of 50 % hydrogen peroxide



was then added at 10 - 15 minute intervals. This cycle was repeated four to five times with each sample set, until all black carbon particles were digested.

8. After the last cycle, samples were evaporated to dryness at 90°C, cooled and 2.0 ml of DDI and 0.5 ml of 70 % nitric acid were added to all flasks. Samples were returned to the hot plates and heated at 90°C for 15 minutes to dissolve the residue.
9. After cooling, quantitative transfer of each sample was completed to 5 ml volumetric flasks and adjusted to volume with DDI water. Samples were then transferred to polystyrene storage tubes washed with EDTA and refrigerated at 4°C.
10. The urine was prepared for digestion by addition of 20  $\mu$ l of 36 % HCl. Two-ml samples of the urine were placed in acid-washed borosilicate tubes and evaporated to dryness in heating blocks at 90°C. Then the digestion protocol by Hill et al. (44) was followed, with a total of 1.5 ml of Ultrex nitric acid added.
11. The urine samples were subject to the 15-minute heating cycles at 90°C while adding 0.1 ml of 50 % hydrogen peroxide per cycle. Samples were evaporated to dryness, cooled, and DDI water and HCl were added. Samples were reheated at 90°C for another 15 minutes, cooled, and transferred quantitatively to a final volume of 2.5 to 5.0 ml with 0.1 M HCl.
12. Sera were prepared for direct assay by AAS.

### **Analysis of Samples**

13. A Perkin Elmer Flame Atomic Absorption Spectrophotometer and Zn lamp analyzed the Zn concentration in the samples. Acetylene gas and air were the fill and oxidant for the flame.
14. Zinc standards (SRM 3168) were prepared in a 0.1 M HCl matrix at 0.25 ppm, 0.50 ppm, 1.00 ppm, 1.50 ppm, and 2.00 ppm concentrations. For serum, Zn standards were prepared in a DDI water matrix at 0.25 ppm, 0.50 ppm, and 0.75 ppm concentrations.
15. Samples were prepared for AAS aspiration by appropriate dilution using an automatic diluter. Dilutions were completed with 0.1 M HCl (or with DDI water for the serum). Standard reference materials were aspirated

with their corresponding sample set. Bovine liver standards were diluted 1:20 using 0.1 M HCl. Milk powder standards were diluted 1:10 using 0.1 M HCl. Bovine serum standards were diluted 1:5 with DDI.

16. Liver, small intestine, lung, heart, kidney, muscle and corresponding blanks were aspirated after dilutions of 1:5 with 0.1 M HCl. Serum and serum blanks were aspirated after a 1:5 dilution with DDI, except two of the samples which were diluted 1:10 with DDI. The testicle and blanks were diluted 1:10 with 0.1 M HCL and the bone at 1:40. The feces and feces blanks were diluted from 1:10 to 1:100 with 0.1 M HCl.
17. The spectrophotometer was allowed stabilization time after positioning the lamp, setting the resonant wavelength, and igniting the flame. The parameters set on the spectrophotometer were as follows:

ABS/CONC/EM: CONC	AA/AA-BG/BG: AA
Energy: 58	Lamp MA: 15
Slit Height: 0.7	$\lambda$ Peak: 213.9
Sample Aspirations: Triplicate	
Aspiration Interval: 1.5 seconds	

18. The instrument was brought to equilibrium with the 0.1 M HCl solution and calibrated using the appropriate Zn standard, prepared to cover the linear absorbance range (0.25 ppm to 2.00 ppm) of Zn. This was usually completed with the 1.00 ppm Zn standard (however, calibration with 0.50 ppm Zn standard, in DDI, for serum was used). A Zn standard was then aspirated to ensure appropriate calibration.
19. Triplicate sample aspirations were then completed within 1.5 seconds and reading of the appropriate Zn standard was completed every 5 to 10 samples to ensure the spectrophotometer had not drifted from calibrated range.
20. If drift had occurred, the instrument was again brought to equilibrium with the 0.1 M HCl solution, calibrated if necessary, and the Zn standard was aspirated once again to ensure appropriate calibration. The Zn concentration reading of the samples was determined from the mean concentration of the triplicate aspirations.

**Reagents:**

1. Detergent: Versa-Clean Detergent, Fisher Scientific Co., Pittsburgh, PA
2. EDTA: Fisher Certified, ACS, Fisher Scientific Co., Pittsburgh, PA
3. Nitric Acid: 70% Trace Metal Grade, Fisher Scientific Co., Pittsburgh, PA
4. DDI Water: For washing and reagent preparation; MEGA-PURE, Barnstead Co., Dubuque, IA
5. DDI Water: Added in samples during digestion, except in urine and serum; Reagent Grade, Baxter Healthcare Co., McGaw Park, IL
6. Hydrogen Peroxide: 50%, Certified, Stabilized; Fisher Scientific Co., Pittsburgh, PA
7. Hydrogen Chloride: 36%, Sub-Boiling, Distilled, Sea-Star Chemicals, Vancouver, British Columbia, Canada
8. Ultrex Nitric Acid: 70%, Baker, Phillipsburg, NJ
9. DDI Water: For dilution of samples before analysis; Millipore Corp., Bedford, MA
10. Acetylene: For flame in AAS; Air Products and Chemicals, Inc., Allentown, PA

**Standard Materials:**

1. Bovine Liver: Standard Reference Material 1577b, National Institute of Standards and Technology (NIST), Gaithersburg, MD
2. Non-fat Milk Powder: Standard Reference Material 1549, NIST, Gaithersburg, MD
3. Bovine Serum Standard: Standard Reference Material 1598, NIST, Gaithersburg, MD
4. Zinc Standard: Standard Reference Material 3168, NIST, Gaithersburg, MD

**Instruments:**

1. Electronic Analytical Balance: Model A-250, Denver Instrument Co., Arvado, CO
2. Muffle Furnaces: Type 4800 Furnace, Barnstead Co., Dubuque, IA
3. Hot Plate: Model PC101, Corning Glass Works, Corning, NY
4. Automatic Diluter: Micro Lab M, Hamilton Co., Reno, NV
5. AAS: Perkin Elmer Flame Atomic Absorption Spectrophotometer, Model 5000, Perkin Elmer, Norwalk, CT
6. Zn Lamp: Perkin Elmer, Norwalk, CT