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SUPPLEMENTAL CHROMIUM FOR STRESSED BABY PIGS: EFFECTS ON PERFORMANCE, ZINC AND COPPER RETENTION AND THE IMMUNOLOGIC RESPONSE OF CELLULAR COMPONENTS OF PERIPHERAL BLOOD

By

Víctor Siberio Torres

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ABSTRACT

SUPPLEMENTAL CHROMIUM FOR STRESSED BABY PIGS: EFFECTS ON PERFORMANCE, ZINC AND COPPER RETENTION AND THE IMMUNOLOGIC RESPONSE OF CELLULAR COMPONENTS OF PERIPHERAL BLOOD

By

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Two experiments were conducted to determine the effects of dietary supplementation of Cr to weanling pigs. Performance, Cu and Zn retention, immunologic response and the Cr x Cu interaction were evaluated. In the first experiment 12 pigs were used in a 15-day digestibility trial. A basal diet was supplemented with: 0 (T1), 0.50 (T2), or 1.0 (T3) ppm Cr. Feces and urine were collected every 24h for 10d and duplicate samples were digested with nitric and perchloric acids for Cu and Zn determination. Numerically pigs in T1 had a greater retention of Cu than pigs in T2 and T3. Likewise, pigs on T3 had a greater retention of Zn compared to T1 and T2. Both these differences were not significant ($P>0.10$). Pigs on T2 and T3 had 1 and 2d of negative Cu balance respectively. In contrast pigs in T1 had a positive Cu balance. Dietary Cu needs of the stressed pig may not be met by 22 ppm. No negative balances were observed for Zn. In the second experiment 96 pigs were used in two, 35-day trials. Phase 1 and phase 2 basal diets were supplemented with either 0 ppm Cr and Cu (T1 basal), 0.5 ppm Cr, 0 ppm Cu (T2), 0.5 ppm Cr and 250 ppm Cu (T3), or 0 ppm Cr, 250 ppm Cu (T4). Pigs were assigned to 3 blocks by weight in a 2 x 2 factorial arrangement of treatments and housed in 0.61 m^2 stainless steel pens (0.15 m^2/pig). The floor space provided to the pigs ranged from 6 to 59% less than the recommendations during the course of the experiment.

Phase 1 diet was fed for 14d before switching to phase 2. Blood was collected d0 and weekly thereafter to evaluate lymphocyte function, phagocytic activity (PA) and microbicidal ability (MA) of the neutrophils. Lymphocytes and neutrophils were separated by Ficoll-Hypaque density gradient technique. Aliquot of lymphocytes was incubated with the mitogen ConA, PWM and PHA and [^{3}H]thymidine to evaluate the blastogenic response. Opsonized baker yeast and neutrophils were incubated in a shaking bath at 37°C for one hour to evaluate the PA and MA. Although T2 had a greater numerical ADG, ADF and a lower F/G than the other treatments, these differences were not significant (P>.10). There were no interactions. No significant differences were found in PA, MA, or in the response of cells to mitogen stimulations. However a reduction in the subjectively-determined incidence of diarrhea was observed in both trials for pigs receiving the Cr diets. In summary, dietary Cr supplementation as provided by high Cr yeast had no apparent effect on either performance, on Cu or Zn retention, Cr and Cu interaction or the immune response of baby pigs.

Dedicated with love to

my first grade teacher, Rosaura Pérez who taught me to love school

And to

the Isabela, P.R. Agricultural Extension Agent, José E. Abreu who showed

me to love agriculture and was my model for my career

And to

my family especially Mame, Can, Diana, Lurdes and Daniel

but very special

to the grace of God.

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INTRODUCTION

An important goal of the swine industry is to increase the number of pigs weaned per sow per year. To achieve this goal, many swine producers have decreased the weaning age. On the other hand, the immaturity of the immune and digestive systems of young pigs are of major concerns in pork production (Gatnau et al., 1993). Passive antibody-mediated immunity derived from colostral immunoglobulin are very low at this age (Speer et al. 1959, Miller et al. 1962, Porter, 1976), and according to Haye and Kornegay (1979) the decreased physiological maturity from stress of weaning at an early age lowers antibody synthesis.

Early-weaned pigs usually suffer a significant setback at weaning (post-weaning lag) because of a change to a dry diet and considerable stress (Fowler and Gill, 1989). Stress represents the reaction of the body to stimuli that disturbs its normal physiological equilibrium or homeostasis, often with detrimental effects (Khansari et al., 1990). A number of social and environmental factors stress the animal and alter its resistance to disease. Excess heat or cold, crowding, mixing, weaning, limit-feeding, shipping, noise and restraint are stressors that are often associated with intensive animal production and have been shown to influence immune function in various species (Kelley, 1985). There is ample evidence that both physical and psychological distress can suppress immune function in animals leading to an increased incidence of infections disease (Roth, 1992).

Weaning is certainly a stressful event for domestic animals. Piglets are usually separated from the sow, handled extensively, regrouped with unfamiliar pigs, and shifted from a liquid to a solid diet. Weaning at 2,3 or 4 weeks of age has been shown to decrease the in vivo and in vitro response of porcine lymphocytes to phytohemagglutinin (Blecha et al., 1983). This is considered to be a measure of the pig's ability to mount a cell-mediated immune response. The stress of being removed from the dam is believed to raise the blood concentration of cortisol which has an immune suppressive effect at physiological (Westly and Kelley, 1984) and at high concentration (Jefferies, 1991) by inhibiting the synthesis of several cytokines and cell surface molecules required for immune function (Auphan et al., 1995). If an animal is immunosuppressed due to stress, such as a preexisting viral infection, immunotoxicants or nutritional factors, the nonspecific defense mechanisms may not be functioning optimally. In addition, the specific immune response may be slow to develop or inadequate, which can result in clinical disease due to an infectious agent that would otherwise be controlled by a non-impaired immune system (Roth, 1992).

Studies with humans and mice have indicated that various stressors due to infection, strenuous exercise, or trauma may have marked effects on glucose metabolism and increased losses of chromium (Cr) in urine (Borel et al., 1984; Pekarek et al., 1975). Chromium is a natural constituent of living matter and an essential trace element for both plants and animals (Anderson, 1981; Squibb and Snow, 1993). Early data suggests that the predominant physiological role of Cr may be as an integral component of the Glucose Tolerance Factor (GTF) which is necessary to potentiate the action of insulin (Anderson, 1981). Thus, metabolic processes that are regulated by insulin including carbohydrate, lipid and protein

metabolism may be influenced by Cr (Anderson ,1981). In stressed cattle, the urinary excretion of Zn and Cu may also increase (Orr et al., 1990). When these minerals are supplemented, the animals performance may improve (Nockels, 1990, 1995). Pull (1990) in a review of effects of trace minerals on immunity concluded that deficiencies of Zn, Fe, Cu, or Se results in a lowered resistance to disease.

In research with mice, Schrauzer et al. (1986) suggested that supplemental Cr may prevent stress induced urinary losses of Cu, Zn, Mn and Fe. Research by Chang and Mowat (1992) showed a marked increase in weight gain and feed efficiency of calves fed supplemental organic Cr from a high-Cr yeast during the first 28 days after arrival at the feedlot. No effect was noted on performance during the later growing period, but a marked reduction in serum cortisol and an increase in total immunoglobulin were detected. In the same study, Cr supplementation also reduced morbidity. Primary antibody response to human red blood cell was higher for Cr treatments. Steele and Rosebrough (1981) found that inorganic Cr (chromium chloride) increased the 21-day rate of gain in turkey poults. Using chromium picolinate as the source of Cr supplementation in pigs from 30 to 100 kg of body weight has been shown to increase the percentage of muscle by 7% and longissimus dorsi muscle area by 18% (Page et al., 1993). Schroeder et al. (1965) also reported that supplemental inorganic Cr improved growth rates of mice and rats. Mertz and Roginski (1969) also found that Cr supplementation reduced mortality in stressed rats subjected to additional stress of acute hemorrhage. Schroeder et al. (1965) reported that Cr reduced mortality from an epidemic of pneumonia with female rats. Studies in rats and monkeys confirmed that Cr deficiency decreases glucose tolerance. In addition, severe Cr deficiency

leads to decreased growth, shortened life span, elevated blood cholesterol, aortic atherosclerosis and decreases sperm count (Guthrie, 1982). A synthetic glucose tolerance factor containing Cr potentiates insulin activity in pigs and this glucose tolerance factor is biologically active in pigs (Steele et al., 1977).

There is no recommendation for Cr in swine diets (NRC, 1988) and most swine diets are composed of ingredients from plant origin which are usually low in Cr. Drinking water in the United States also supplies only minute amounts of Cr in the diet (Durpur, 1962). So it is possible that conventional diets for pigs may be moderately deficient in Cr. We therefore decided to study the effect of supplemental organic Cr on the weanling pigs.

Our alternate hypothesis was that stressed baby pigs at weaning and during nursery period supplemented with organic chromium in the diet will respond more positively to immunological challenges, grow faster, and more efficiently and excrete less zinc and copper compared to those receiving a non Cr supplemented diet. The general objective of this research project was to demonstrate the effectiveness of organic chromium supplementation (High Cr Yeast) to baby pigs during stress periods at weaning and in the nursery and to evaluate if there was an interaction between Cr and Cu supplementation. The specific objectives of this research were:

1. To determine the average daily gain, feed intake and feed efficiency of pigs supplemented with 0, and 0.50 ppm of Cr in the diet, using two levels of Cu (25 and 250 ppm) without antibiotic supplementation.

2. To evaluate the immune response measured by the ability of lymphocytes to proliferate when stimulated by mitogen.

3. To evaluate the ability of neutrophilic polymorphonuclear cell to phagocytize and kill invading organisms.

4. To determine the effect of Cr on the excretion of zinc and copper.

LITERATURE REVIEW

Historical and General Characteristics

The name chromium came from the Greek word "Chromos", meaning color, and was given to this metal due to the pronounced and varied colorations of chromium compounds. The red color of ruby gemstones and green color of emerald gemstones are due to small amounts of chromium. This metal is a transition chemical element of Group VIB of the periodic table (Cotton and Wilkinson, 1980; Greenwood and Earnshaw, 1986). It is a hard steel metal that takes a high polish and is used in alloys to increase strength and corrosion resistance. This element was discovered in 1797 by the French chemist Louis-Nicolas Vauquelin and isolated as the metal a year later (Squibb and Snow, 1993).

Chromium is a relatively abundant element. It is the twenty first most abundant element in the earth crust, but occurs in a much greater concentration in the earth core and mantle. The free metal is never found in nature. In natural deposits, it is always combined with other elements especially oxygen. Most ores consist of the mineral chromite which is the most important mineral and the ideal formula is $FeCr_2O_4$ (Encyclopedia Britannica, 1995).

Chromium exist in a range of valence states from -2 to +6. The most common oxidation states are +6, +3, and +2, although a few compounds of +5, +4 and +1 oxidation states are known. In biologicals materials only Cr (III) and Cr (VI) poses the stability to exist for extended periods of time. Chromium (VI) can form various species with the most important being chromate (CrO_4^{2-}) and dichromate ($Cr_2O_7^{2-}$). This two ions form the basis

for a series of industrially important salts. In addition chromates and dichromate are both potent oxidizing agents that can be relatively toxic to living organisms. Chromium (III) is the most abundant form of the element in nature. It is also the most stable species in acid solution and the most stable oxidation state in biological materials. Trivalent chromium compounds such as chloride, oxide and sulfate are generally insoluble in water, on the other hand chromium chloride hexahydrate and the acetate and nitrate are readily soluble. It readily forms octahedral complexes with oxygen, nitrogen and sulfur (Kumpulainen, 1988). In biological fluids, chromium forms organic complexes with many ions such as oxalate, amino acids, proteins, (Standeven and Werrerhahn, 1989; Kortenkamp et al., 1991), nucleic acids (Cupo and Wetterhahw, 1985; Snow and Xu, 1991; Snow, 1994) and small molecules such as glutathione (Anderson, 1987; Snow 1994). Chromium (V) species can be observed experimentally and may play an important role in the toxicity of chromium, however this species does not form stable biological compounds. Chromium (IV) is highly reactive and has not been observed directly in experimental systems, but exists as an intermediate in the reduction of chromium (V) (Klein et al., 1992 ; DeFlora et al., 1990).

The mineral chromium is an important element for humans for several reasons. First, Cr is used as a raw material by industry and is for example, a constituent of stainless steel. Second, it may exist in toxic concentrations in the work place and industrial waste waters (Kumpulainen, 1988). Third, Cr is considered to be an essential trace element for man, animals (Anderson, 1981) and has beneficial effects in plants (Huffman and Allaway, 1973).

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On a global basis, about 10 million tons of chromite ore are used annually. Of this, approximately 76% is used for metallurgical purposes, 13 % for refractory uses and 11% for chemical applications (Squibb, 1993). A small amount is used in medicine and research. One of the primary uses of Cr in medicine and biomedical research is the utilization of radioactive ^{51}Cr as a means of tracing specific cell populations (Kumpulainen, 1988). Because the chromate anion is readily accumulated and retained by living cells, cells can be labeled with ^{51}Cr in vivo and their half life or distribution within the body followed by gamma ray detection or autoradiography. By labeling red blood cells, the total blood volume, cell turnover, splenic function and gastric bleeding can be measured. White blood cells also have been labeled to study the distribution and function of these cells in vivo. Laboratory techniques have been developed that provide a means of measuring immune cells functions in vitro by measuring the release of Cr from pre-labeled target cells (Squibb, 1993). During the last few years, scientists have been studying Cr as a supplement for food producing animals, mainly due to its possible potential for serving as an immunomodulator and improven os performance.

Chromium as an Essential Nutrient

An essential element as defined by Cotzias (1967) has the following characteristics: 1) it is present in all healthy tissues of all living beings 2) its concentration from one animal to the next is fairly constant 3) its withdrawal from the body induces reproducible the same structural and physiological abnormalities regardless of the species studied 4) its addition either prevents or reverses these abnormalities 5) the abnormalities induced by deficiency are

always accompanied by pertinent, specific biochemical changes 6) these biochemical changes can be prevented or cured when the deficiency is prevented or cured. According to Mertz (1969) an element is essential if its deficiency results in reproducible impairment of a function from optimal to suboptimal.

Chromium deficiency has been induced by feeding rations low in available Cr in animals (Mertz and Schwarz, 1959; Doisy, 1963; Schroeder, 1966; Davidson and Parker, 1974; Seaborn et al., 1994) and with deficient diets in humans (Anderson et al., 1991). The discovery of Cr as an essential element goes back to 1959. In an experiment with rats, Schwartz and Mertz (1959) reported that with a diet based on Torula yeast, the animals presented an impaired intravenous glucose tolerance. When these researchers supplemented the diet with small amounts of brewers yeast, the abnormality was corrected. They suggested that to maintain normal glucose metabolism in animals fed Torula yeast, a dietary agent present in brewers yeast was needed. They called this hypothetical dietary agent Glucose Tolerance Factor (GTF). This potentiating effect of yeast on hypoglycemic animals was reported 20 years before, but the phenomenon was attributed to the effects of a high content of vitamins in yeast (Glaser and Halpern, 1939). The active ingredient responsible for the metabolic effects of GTF was later identified as trivalent Cr (Schwarz and Mertz, 1959).

The partial purification of GTF yielded a substance with a molecular weight between 300 and 500 dalton, which was water soluble and heat stable (Mertz and Roginski, 1971). Toepfer et al. (1977) further purified and analyzed the structure of GTF and described it as a complex of two molecules of nicotinic acid, a tri-peptide and Cr(III) (Toepfer et al,.1977).

The predominant amino acids are glycine, cysteine and glutamic acid, the components of glutathione (Mertz et al., 1978). Mixtures of synthetic compounds with a similar composition proved to be nearly identical to the described physical and chemical characteristics (Toepfer et al. 1977). These synthetic compounds were highly active in the in vitro systems, and acutely improve glucose and blood lipid concentrations in genetically obese diabetic mice in which simple Cr compounds such as chromium chloride were ineffective (Tuman et al., 1978). Various groups of researchers agree with this description of glucose tolerance factor (Mirsky et al., 1980; Yamamoto et al., 1989.) However, Haylock et al. (1983) does not consider glucose tolerance factor from brewers yeast a Cr complex, and he described it as a cobalt rather than a Cr complex. During the last decade an interesting and controversial Cr compound has been isolated and characterized. This substance called low molecular weight Cr ligand (LMWCr) was isolated from human urine, mouse and rabbit liver (Yamamoto et al., 1987), plasma, urine and feces (Yamamoto et al., 1981), dog liver (Wada et al., 1983) and bovine colostrum (Yamamoto et al., 1984). It has a molecular weight of 1500 daltons, an its composition is believed to be Cr, aspartic acid, glutamic acid, glycine and cysteine, but not nicotinic acid (Vincent, 1993; Yamamoto et al., 1989) although some other substance was present. Because the compound was seen initially after injection of hexavalent Cr, they thought that the function of the compound was detoxification. Later research has shown that the compounds exhibit some biological activity on glucose oxidation by isolated fats cell (Yamamoto et al., 1989) similar to GTF. So they concluded that LMCr plays an essential role in both the detoxification and nutrition according to physiological demands in mammals.

Of all Cr compounds, only trivalent species exhibits biological activity, and it appears that the most effective form of Cr is as GTF. The main physiological action of Cr appear to be related to all insulin dependent systems in which Cr deficiency should result in diminished responsiveness to the hormone especially relative to carbohydrate metabolism. It also exerts an effect on lipid and protein metabolism and on certain enzyme systems (Mertz, 1969; Giri et al., 1990). Although research had shown that this is the main action of Cr, additional potential sites of action can not ruled out. The mechanisms by which this occurs is not well known, however, and the identity of the GTF is still controversial. Chromium (III) by participating in the complex GTF appears to facilitate or initiate the formation of the disulfide linkages between the intra-chain disulfide of insulin and the mitochondrial membrane sulfhydryl groups (Christian et al., 1963; Mertz, 1967; 1993) so it may act as a cofactor with insulin, or it may act independently at the membrane level in the production of peripheral insulin receptors (Anderson, 1987) . Vincent (1994) disagrees with this theory. Indeed he postulates that GTF seems to play no role in glucose metabolism in normal cells and acts only as a dietary agent providing a readily accessible source of Cr. He also said that GTF could be deleterious to insulin action in insulin dependent cells if present in sufficient concentrations. Vincent postulates that the active form of Cr in insulin dependent cells is the LMWCr complex and has a post receptor role inside these cells instead of a receptor or cell membrane role and that the role of glucose tolerance factor is as a nutrient supplying Cr to the Cr deficiency species in the cell (Vincent, 1994). Evans (1989) also postulated a post receptor action.

Regardless of where Cr has its effect, it appears that Cr is required for normal glucose tolerance in animals and humans. After Schwartz and Mertz demonstrated that Cr was necessary to maintain a normal glucose tolerance in rats, other researchers reported similar findings. Glinsmann et al. (1966) reported an improved glucose tolerance by Cr supplementation in human subjects. In patients receiving total parenteral nutrition, glucose intolerance was improved after Cr supplementation (Brown et al., 1986; Freund et al., 1979). Davidson and Blackwell (1968) reported that the impairment of glucose tolerance of monkeys eating a Cr free diet was restored to normal by Cr supplementation. Anderson et al. (1991) fed 17 middle-aged human volunteers a low Cr (<0.4 μmol/d) diet and then 4 μmol of Cr or placebo was given. He found no change in control subjects who entered the study with a normal glucose tolerance test, but the glucose tolerance test of subjects with impaired glucose tolerance deteriorated further during the placebo period and significantly improved again during Cr supplementation.

Other signs of Cr deficiency include elevated serum cholesterol and triglyceride concentrations, peripheral neuropathy and metabolic encephalopathy. Experimental animals maintained on a Cr deficient diet had impaired growth, decreased survival, fasting hyperglycemia and glycosuria, elevated serum cholesterol concentrations, and sclerotic plaques in the aorta (Schroeder, 1969). Stress tolerance was also significantly lower in animals with Cr deficiency than in Cr-supplemented animals (Merts and Roginsky, 1969). Seaborn (1994) also reported lower cortisol concentration in guinea pigs supplemented with Cr compared with those fed a Cr deficient diet (<0.06 μg/g).

Jeejeebhoy et al., (1977) reported that in addition to glucose intolerance, human subjects receiving a Cr deficient diet show serious metabolic abnormalities such as impaired growth, peripheral neuropathy, negative nitrogen balance, and decreased respiratory quotient. Lack of Cr has been reported to reduce growth in malnourished children (Gurson and Saner, 1973). The role played by Cr in these processes is also poorly understood (Squib, 1994). According to Schroeder (1966) Cr deficiency represented a significant risk factor for cardiovascular disease.

Chromium may also have an effect on lipid metabolism. Schroeder et al. (1962) showed that Cr supplemented to rats receiving a low Cr diet caused a decrease in serum cholesterol. He also reported that serum cholesterol and fasting serum glucose concentration were depressed by brown sugar (high in Cr) or chromium (III), but a diet with white sugar (without Cr) caused a relative increase in serum cholesterol and glucose concentration (Shroeder, 1969). In a study with human subjects with elevated serum cholesterol, Cr supplementation resulted in a 6% reduction of total cholesterol compared to 6% increase in the control group (Wang et al., 1989). Li and Stoecker (1986) reported a reduction in lipid concentration in the liver in obese mice when supplemented with Cr in the diet. Doisy et al. (1976) observed that in addition to the reduction in plasma glucose and insulin concentration, some human subjects with impaired glucose tolerance also respond to GTF supplementation with a significance reduction in fasting serum cholesterol. Other studies have shown that Cr supplementation decreases serum triglycerides and total cholesterol and increases high density lipoprotein cholesterol (Riales and Albrink, 1981; Mossop, 1983; Page et al. 1993). Chromium also appeared to inhibit the development of aortic plaques. With a Cr deficient

diet, rats that died of natural causes had a higher incidence of aortic plaques. Schroeder et al. (1970) found a lower aortic Cr concentration in people who died from arteriosclerotic heart disease compared with people who had died from traffic accidents.

Insulin besides playing a role in carbohydrate and lipids metabolism also stimulate the accumulation of amino acids in many tissues and enhances the incorporation of amino acids into protein (Flelig and Begman, 1995). So if Cr is a cofactor for insulin, it also probably plays a role in protein metabolism. Indeed, Mertz and Roginski (1969) reported a stimulation in growth of rats when Cr was supplemented to rats eating a low Cr diet as compared with un-supplemented control. They found that the increase in body weight was associated with a proportional increase in tissue protein which was not due to water retention or fat deposit. In an experiment with guinea pigs, Seaborn (1994) reported larger liver, epididymal fat pad, testes and kidney weights of Cr supplemented animals compared with a diet deficient in Cr suggesting that Cr is involved in both protein synthesis and glucose incorporation into fatty acids. However, the detrimental effect of a Cr deficiency on growth did not affect hydroxyproline and creatine excretion, and they concluded that the increase in body weight in Cr supplemented animals was associated more with increased body fat and water in glycogen than with increased protein synthesis. Page et al. (1993) reported that growing finishing pigs with Cr supplementation in the diet had an increase in longissimus muscle area that could be related to an increase in protein synthesis. They also found that Cr affected nuclear protein and RNA synthesis. Wenk et al. (1995) found similar results. However, there were no statistical differences compared to the control.

In protein synthesis, Cr probably facilitates the insulin stimulated incorporation of amino acids into protein (Hambridge, 1974). Roginski and Mertz (1969) investigated the effects of Cr in amino acid transport. They demonstrated in heart tissue that rats receiving a low Cr-low protein diet have an impaired capacity to incorporate several amino acids into protein. This incorporation was enhanced by Cr (III) supplementation. The amino acid affected were α amino isobutyric acid, an amino acid analogue, glycine, serine and methionine. When insulin was injected, the amino acid analog uptake by heart tissue of Cr supplemented rats was greater as compared to unsupplemented rats.

Chromium also exerts an effect on several enzyme systems (Mertz, 1969). Horecker et al. (1939) reported that Cr stimulated oxygen consumption in a succinic-cytochrome c dehydrogenase system. Depending on the concentration Cr can also activate or inhibit DNA and RNA polymerase (Snow, 1994)

Chromium will also inhibit certain enzyme reactions, such as those that involve bacterial urease, and β glucosidase, when added to the media in excessive amounts. The stimulations and depressing effects of Cr appears to be related to the concentration of Cr in the medium (Mertz, 1969).

If Cr is accepted as an essential nutrient, not as a drug, its apparent effect, like that of any other essential nutrient, depends on the nutritional status of the test subjects. It improves an impaired function or restores it to normal, if that impairment developed because of a Cr deficiency (Mertz, 1993).

Sources of Nutritional Chromium

Compared to other trace minerals, the information about Cr concentration in foods and feed or the form in which it exists in these commodities is very limited. Also, many of these results may be high for Cr due to contamination during sampling and sample preparation, as well as to improper analysis (Kumpulainen, 1988). Although, its seems that Cr exists naturally at relatively low concentrations in plants and animals, mainly because Cr (III) does not penetrate easily through cell membranes (Giri et al., 1990).

The best known source of Cr is brewers yeast. This is also the most characterized form of Cr in feed and is discussed elsewhere in this thesis. Plants are also a source of Cr. Both Cr (VI) and Cr (III) are transported across root tips (Huffman and Allaway, 1973), and both have been isolated from plant products (Lyon et al., 1969; Skeffington et al., 1973). The form in which Cr is present in plants and its availability have been studied by Starich and Blincoe (1983) and Huffman and Allaway (1973). They reported that both Cr (VI) and Cr (III) are absorbed from the soil by plants. Most Cr (VI) is reduced to Cr (III) and then is assembled into a medium molecular weight complex different from the GTF present in yeast. This complex is highly stable in conditions that would be encountered in food and feed preparation. While this complex has not been characterized, it may contain a mixture of the oxidation states of Cr. Similar results have been reported by Schroeder et al. (1962) for oak leaves and corn oil. They showed that this form of Cr complex is more available than other inorganic forms of Cr.

The Cr concentration of cereals, pulses, vegetables, fruits (fresh and dried) condiments and spices, edible- flower and meats have been also determined (Guthrie, 1975; Kumpulainen

and Koivistoinen, 1977; Giri et al., 1990; Wilplinger et al., 1995). The mean Cr value for the analyzed cereals was 0.55 ppm, with wheat having the highest concentration with a value of 0.74 ppm (Giri et al., 1990). Previous reports found similar concentrations (Guthrie, 1975; Carry and Olson, 1975; Kumpulainen and Koivistoinen, 1977; Kumpulainen, 1978) for whole grains. Schroeder (1971) reported a reduction by more than 70% of Cr in refined white flour. Also, white sugar retained only a small fraction of the original Cr content of raw sugar. Between pulses, the highest Cr content was reported in broad bean with 0.50 ppm. Soya beans, a common ingredient in animal feed preparations, have a concentration of 0.27 ppm. Figs have 0.97 ppm of Cr, which is the richest form of Cr in fresh fruits. Dried fruits and black pepper contain the highest Cr concentration varying between 1.20 and 1.50 ppm (Giri et al., 1990). This value is lower that previous reports of 3.90 ppm (Guthrie, 1975). Most other foods contain less than 0.10 ppm. For ready-to eat cured meats, a mean concentration of 0.60 ppm has been reported (Kirkpatrick and Coffin, 1975). Most sea foods are poor sources of Cr, whereas liver, kidney, spleen and beef contain relatively high amounts.

Metabolism

The metabolism of Cr is quite different from that of other trace elements because of the strict dependence on the chemical form in which the element is present (Saner, 1980). The majority of studies related to Cr metabolism involve the intestinal absorption, transportation, distribution to the tissues, mode of action and excretion. There is very little information about digestion. The rat had been used as the primary animal model.

Several factors can affect the metabolism of Cr. Some of them are valency, inorganic vs. organic sources, health status and interaction with other minerals including Zn, Fe and Mn ant other components of the food.

Digestion

Chromium in feed and food can be found as: Cr (III), Cr (VI) (Starich and Blincoe, 1983), GTF (Anderson and Mertz, 1977) low molecular weight Cr compound in milk (Yamamoto et al., 1988), and medium weight Cr complex (Starich and Blincoe, 1983). When hexavalent Cr is exposed to gastric acid it is reduced to trivalent Cr (Donalson and Barreras, 1966). In the small intestine, due to the neutral pH, the majority of Cr compounds precipitate and turn into an insoluble substance with characteristics different fron the original compound, and later appear in the feces. According to Saner (1980) and Evans (1993) small amounts can be converted to GTF, and this transformation process is age related. Starich and Blincoe (1983) studied the stability of brewers yeast GTF and medium weight chromium complex in the gastrointestinal tract. They found that both are stable in the alimentary canal.

Absorption

It is not clear what mechanism is involved in the absorption of Cr. In vitro investigations using rat intestine suggest that Cr (III) may enter into the mucosal cell by a process of either simple or facilitated diffusion (Mertz and Roginski, 1971). Based on animal studies, its appear that the small intestine is the site of absorption. Chen et al. (1973) performed some in vivo and in vitro studies and indicated that Cr absorption occurs mainly

in the jejunum, followed by some absorption in the ileum and the duodenum. Several factors have been shown to affect the absorption of Cr. One of these factors is the valence state of ingested Cr. Inorganic Cr in its trivalent state has been shown to be less absorbable than hexavalent Cr. Chromium (VI) is able to cross cell membranes using existing anion (PO_4 and SO_4) transport systems, while Cr (III) with its positive charge does not readily enter cells. The greater toxicity of Cr (VI) versus Cr (III) in biological system is in large part due to this difference in cellular uptake (Snow, 1994). In an experiment with rats, the absorption of trivalent Cr was around 2-3 %, of an oral dose (Mertz et al., 1965) compared to 3-6% for hexavalent Cr (Mackenzie et al., 1959). In an experiment with normal humans, Donaldson and Barreras (1966) found that the absorption of Cr was 0.1 to1.2% and 0.2 to 4.4% when administered as an oral dose for trivalent and hexavalent forms respectively. Similar results have been reported by Witmer et al. (1989, 1991). Both Cr (VI) and Cr (III) bind to components present in gastric juice. In addition, acidic gastric juice possess the capacity to reduce Cr (VI) to Cr (III). In vitro studies in which Cr was exposed to acidic gastric secretions showed similar results. The importance of the interaction of Cr with gastric juice on intestinal absorption has been demonstrated in experiments comparing intragastric versus intraduodenal administration of Cr. Absorption of Cr (VI) increased 6 to19 % when doses were administered directly in the intestine, while on the other hand absorption of Cr (III) remained basically the same. These researchers concluded that the acidic gastric juice reduced hexavalent Cr to the trivalent state which is poorly absorbed (Donaldson and Barreras, 1966; Doisy et al., 1968). These results were confirmed in an experiment with rats. Thus, hexavalent Cr was found to be poorly absorbed when administered orally, however

when it was delivered directly to the intestine, the absorption was greater than trivalent Cr. The form in which Cr is administered is another factor that can also affect its absorption. Although inorganic Cr is poorly absorbed, complex molecules of organic Cr are absorbed in larger quantities. Casey and Hambridge (1980) reported that the amount of absorbed brewers yeast Cr glucose tolerance factor is 10 to 25% of an oral dose. Starich and Blincoe (1983) studied the absorption of medium weight Cr complex present in plants and reported a 30% uptake. Robles et al. (1981) reported similar results. Other anions present in feed may also affect Cr absorption. In vivo and in vitro studies in rats demonstrated that oxalate significantly increased and phytate significantly decreased intestinal Cr absorption (Chen et al., 1973). Using everted jejunum segments, Mertz and Roginski (1971) found that complexes of Cr with several amino acids were absorbed more readily than inorganic Cr. Substances such as glutamate and penicillamine will increase Cr absorption (Chen, 1973).

The absorption of Cr can be affected by other nutrients in the gastrointestinal tract. According to Mertz and Roginski (1971) some minerals such as iron, manganese, titanium and calcium may depress the absorption of Cr. Chromium and Fe may share a single gastrointestinal transport mechanism. In iron deficient animals, more Cr is absorbed from $CrCl_3$ than in iron supplemented controls, because Fe depressed Cr binding to transferrin (Hopkins and Schwarz, 1964). Transferrin forms a complex with Mn as well as Cr (Hurley and Keen, 1987). Manganese deficiency in the guinea pig induces a diabetes mellitus-like syndrome characterized by decreased utilization of glucose (Everson and Shrader, 1968). This may reflect an interaction between Cr and Mn as a deficiency of Cr decreases glucose tolerance (Mertz, 1967). Another interesting phenomenon is the apparent interaction between

Cr and Zn. Whole body contents of an oral dose of ^{51}Cr were greater in Zn-deficient than in Zn-supplemented rats (Hahn and Evans, 1975). In an experiment with rats it appears that Cr and Zn share a common metabolic pathway in the intestine. Both Zn and Cr eluted in the same low-molecular weight fraction when mucosal supernatant extracts were separated by gel filtration, suggesting that in the intestine a similar ligand binds both metals (Borel and Anderson, 1984).

Health status, can also affect Cr absorption. The intestinal absorption of Cr(III) in man is from 0.5 to 1.0% (Doisy et al., 1971) but in insulin requiring diabetes during the first 24 hours after a single oral dose of Cr, absorption increase two to four times more chromium compared to normal subjects (Doisy et al., 1971).

Transport

The transport of Cr appears to be different for the organic and inorganic forms of Cr (Doisy et al., 1976). After inorganic Cr is absorbed from the gut, it is transported in the blood, bound to the β globulin fraction of serum protein, especially to the protein transferrin (Hopkins and Schwartz, 1964; Yamamoto et al., 1984). This is the fraction that also binds iron, but only 30% is usually saturated with iron, so the remainder can provide transport for Cr. The affinity of transferrin for Cr and Fe is very similar, so two metals can compete for binding sites. Sargent et al. (1979) reported that transferrin has two binding sites, and the affinity for iron of these binding sites varied. At a low Fe concentration, iron binds to site A and Cr to site B, but at high Fe concentration, both metals, compete for binding mainly for site B. When Cr is administered in high concentrations, it can also bind nonspecifically to

other plasma protein fractions (Hopkins and Schwarz, 1964) but with a higher affinity for albumin (Tongeren and Major, 1966; Doisy et al., 1971; Yamamoto et al., 1983) and partly to low molecular weight Cr compound (Yamamoto et al., 1983). Hexavalent Cr can also penetrates the red blood cell membrane and bind to the globulin fraction of hemoglobin (Hopkins, 1965). In contrast, trivalent Cr cannot cross the erythrocyte membrane and do not migrate into the red blood cells (Snow, 1993).

Organic Cr seems to be transported as GTF-bound Cr (Doisy, 1976). If this theory is correct, then a mechanism of absorption for GTF as is, should be present, or a mechanism to reassembly it after absorption from its components.

All forms of Cr have a rapid blood clearance rate, whereas various other organs retain Cr much longer. These findings suggest that there is no equilibrium between tissue stores and circulating Cr concentrations, and thus, concentrations of plasma Cr do not reflect the body Cr state (Saner, 1980).

Tissue Distribution

Tissue distribution and elimination of absorbed Cr depends upon the valence state of the Cr as it enters the blood stream and the reducing capacity of the serum. As stated above Cr (III) binds to serum proteins such as transferrin and albumin, then is distributed primarily to liver, kidney and spleen. Liver is the most important organ in which Cr is metabolized (Yamamoto et al., 1989). Chromium (VI) rapidly crosses red blood cell membranes by the sulfate transport system (Sugillama, 1992) and accumulates in the blood. Trapping of Cr (VI) in red blood cells occurs through an intracellular reduction of Cr (VI) to Cr (V) and Cr (IV)

species and then to stable Cr (III) (Snow, 1994). These reduction is performed mainly by ascorbic acid, riboflavin, glutathione, and the flavoenzymes cytochrome P-450 reductase and glutathione reductase (Seaborn et al., 1994; Sugillama, 1994), with subsequent binding of the Cr to hemoglobin. Species differences in Cr blood concentration occur and may be due, in part to differences in ability of Cr to bind to hemoglobin molecule. The presently accepted normal concentration of Cr in serum and plasma is 0.1 to 0.2 ng/ml (Versieck and Cornelis, 1980; Versieck, 1985).

Chromium concentration in other tissues is 10 to100 fold higher than in the blood (Borel and Anderson, 1984). In humans and maybe also in animals, the concentration of Cr varies with the geographic location. In general, in areas of the world with a high incidence of maturity-onset diabetes and atherosclerosis, the tissue concentration is low (Borel and Anderson, 1984). Factors associated with age also affect tissue Cr concentration in humans and rats. Hopkins (1965) injected radioactive Cr into young, growing, and mature rats and measured the radioactivity. He found that the spleen, kidney, testis, and epididymis retained higher concentrations of Cr compared to heart, pancreas, lung and brain. Yamamoto et al. (1988) using low molecular weight Cr found similar results. Young growing rats retained greater amounts of Cr in the bones, whereas mature animals showed higher retention in the spleen, kidney, testis and epididymis. In humans, Cr concentration in lung, aorta, heart and spleen decreases considerably within the first few months of life, while the liver and kidney retain neonatal concentrations until after ten years of age (Schroeder et al., 1962). The lungs are the only tissue in which subsequent increase in Cr concentration is universally observed, probably because of contamination in air (Borel and Anderson, 1984). Chromium

concentration in the hair also decreases with age. Infants have a concentration of 0.90 ppm compared to 0.44 ppm for children of three years old. (Hambidge, 1972; Gurson et al., 1975).

The liver and the spleen of the infant which are the long term body pools of Cr (Lim and Kusubov, 1983; Yamamoto et al., 1989) have been shown to contain 8 and 15 ng/g dry weight respectively (Vouri and Kumpulainen, 1987). In adult human livers, Lievens et al. (1977) and Bailey et al. (1983) reported a range of 2 to10 ng/g higher levels for both organs. Other tissues contain less than 5 ng/g. The Cr concentration in human milk was demonstrated to be approximately 0.4ng/ml (Kumpulainen et al., 1983). The accepted concentration of Cr in the urine is 0.11ng/ml (Kumpulainen et al., 1983).

At the subcellular level, the nuclear fraction contained high amounts of Cr (Edwards et al., 1961), specially the nucleoprotein fraction (Wacker and Vallee, 1959; Okada et al., 1989). When the source of Cr is as glucose tolerance factor, the highest concentration appeared in the liver tissue, followed by uterus, kidney and bone (Mertz et al., 1971).

Chromium at Cellular Level and Metabolic Conversion

Although the most accepted theory on how Cr acts at the cellular level is the direct interaction of Cr with insulin, the exact mechanism has not yet been elucidated. Protocols to demonstrate the site or mode of Cr, have been performed by measuring the biological activity of rat isolated adipose tissue. Biological activity refers to the ability of cells to stimulate production of CO_2 from glucose as a function of insulin concentration. In an experiment using epididymal adipose tissue from Cr deficient and Cr supplemented rats in cell cultures, Mertz et al. (1961) reported that the glucose removal rate in the Cr supplemented rats was

significantly higher than the low Cr group. These studies showed that epididymal adipose tissue from Cr supplemented rats had a glucose uptake identical with that obtained from Cr deficient rats in the absence of added insulin. However, a significant difference in glucose uptake appeared with the addition of insulin to the incubation medium. The increase in glucose uptake by insulin was much greater in the adipose tissue obtained from Cr supplemented rats. The effects of Cr on glucose uptake takes place in the presence of small amounts of insulin. Insulin alone was ineffective, and small amounts of Cr were required for the demonstration of an insulin effect. Similarly, the addition of Cr in vitro significantly increased the effect of insulin on the incorporation of glucose carbon into fat and the oxidation of glucose to CO_2. It appears that Cr is more effective when glucose is the substrate, because when acetate, whose transport is insulin independent, was used the effect was less pronounced (Mertz, 1965).

Much greater glycogen formation from glucose after insulin administration was found in Cr supplemented rats than in deficient controls (Roginski and Mertz, 1969). The Cr supplemented rats also showed higher glycogen concentrations in their tissues than did low Cr controls. These results demonstrated that Cr deficient rats were less responsive to insulin than Cr supplemented rats (Roginski and Mertz, 1969). Farkas and Roberson (1965) and Farkas (1967) investigated the action of Cr on glucose metabolism on the rat lens. In this case, the action of Cr without insulin was ineffective but in combination was effective. Mertz and Roginski (1963) studied the role of inorganic Cr on the transport mechanism of glucose to the cell, and the possible interdependence of insulin and Cr. They used d-galactose, a sugar that depends on insulin for transport, and it is not metabolized by peripheral tissue. In

this experiment, the transport of d-galactose in Cr deficient rats increased slightly but when they added Cr the uptake of d-galactose was greatly enhanced. They interpreted these results to means that Cr plays an important role in sugar transport across the cell membrane acting as a cofactor for the action of insulin. Saggerson et al. (1976) reported that Cr in general stimulates glucose uptake and lipogenesis by rat adipose tissue when incubated in vitro. They considered that this insulin like effect was due to an alteration of plasma membrane structure that would facilitate sugar transport, but would not mimic insulin through the insulin stimulated second messenger process. Steele and Rosebrough (1981) reported that dietary inorganic Cr accelerates the rate of glucose uptake by poult liver incubated in vitro, but the effect was not necessarily due to a synergism with insulin.

Glucose tolerance factor has qualitatively the same effect on glucose metabolism as Cr, but the effect is greater than any inorganic Cr compound (Mertz, 1969). A significant increase of glucose oxidation by the addition of GTF was also observed only in the presence of insulin (Roginski et al., 1970; Evans et al., 1973). Using different forms of Cr, Evans and Bowman (1992) demonstrated that both glucose and leucine uptake are greatest when skeletal muscle is cultured in the presence of Cr picolinate compared to Cr chloride, Cr nicotinate and a control. Significant uptake of glucose and the amino acid by cultured skeletal muscle cells occurred only in the presence of insulin and uptake was greater when the cells had been pre-cultured in a medium containing Cr picolinate. In cells with Cr picolinate, both the quantity of insulin initially bound to receptor and insulin internalization rate were markedly elevated. They conclude that Cr picolinate may affect the action of insulin through an effect on the rate

of insulin internalization, which by an unidentified mechanism regulates the synthesis and or insertion of insulin receptors into the plasma membrane.

During the past few years, Yamamoto et al.(1983, 1987) and Okada et al. (1989) have been studying the action of Cr at the intracellular level. They injected Cr intraperitonealy or intravenouslyin rats and followed its metabolism. They found that Cr is incorporated into two chemical species. The first was described as a high molecule weight protein which contained 5 to6 atoms of Cr per molecule and has a molecular weight of 70 KD. This protein is induced in regenerating liver by the administration of Cr to partially hepatectomized rats. Chromium in this form can bind to nucleolar chromatin resulting in significant stimulation of RNA synthesis (Okada et al., 1989). These observations suggest a regulatory role of the protein-bound Cr in nucleic acid synthesis. Products of high amount of Cr consisting of 70% RNA and 30% protein was reported early in beef liver (Wacker and Valle, 1959). The second product has been described as a low molecular weight chromium protein. It has a molecular weight of 1500 D and consists of Cr, aspartic acid, glutamic acid, glycine, cysteine and an undetermined substance. This protein exists as Cr-free apo-low molecular weight compound in various organs of many species and quickly binds invaded Cr to form stable Cr-bound low molecular weight Cr (Yamamoto et al.,1983). When Cr reaches organs especially the liver and kidney, it is incorporated into this protein. Yamamoto et al. (1983) measured the toxicity of LMWCr and found a lower toxicity, higher rates of urinary excretion and renal clearance, and lower rates of renal tubular reabsorption and retention in kidney and liver. Its appear that this protein has a role in detoxification and also a physiological role in Cr metabolism. They also measured the biological activity in vitro by measuring the effects of LMWCr from cow

colostrum on [U^{14}C] glucose conversion to $^{14}CO_2$ and [3^{3}H]glucose incorporation into lipids in the presence or absence of insulin. They found no effect of M-LMWCr when added to the incubation medium alone. On the contrary, when insulin was added to the incubation mixture, the glucose oxidation to about 10% more than the value produced by insulin alone at a Cr content of 0.15 ng/ml and to about 20-25% at 1.5 and 15ng/ml. Milk low molecular weight Cr also caused an increase in the rate of incorporation of glucose into lipids of 16% alone and 22% combined with insulin.

Excretion

Chromium absorbed from an oral dose appears to be excreted mainly by the kidneys with small amounts lost in hair, perspiration, and bile (Collins, 1961; Doisy et al. 1971; Mertz ,1993). Collings et al. (1961) injected intravenous radioactive Cr (III) and Cr (VI) into anesthetized dogs and followed the excretion pattern. Four hours later they measured the urinary excretion of Cr and found a concentration of 25% for trivalent and 9% for hexavalent Cr. Biliary excretion was less than 0.5%. Four days later the excretion values in urine, bile and feces were 50%, 0.5% and 3.7% for trivalent and 20%, 0.5% and 3.7% for hexavalent Cr respectively. Urinary elimination also predominates for Cr (VI) compounds, however biliary excretion may play a somewhat larger role, particularly following exposure via inhalation (Squib and Snow, 1993). The exact mechanism of Cr metabolism by the kidney is not known. The urinary excretion rate of Cr after an intravenous injection is quite rapid during the first eight hours, which represents the rapid clearance of injected Cr from the blood into the urine. Chromium excreted in the urine is derived from the dialyzable fraction of the

serum Cr, which includes glucose tolerance factor. The daily urinary excretion of Cr is approximately 3 to 50 μg/24 hours (Davidson and Secrest, 1972; Wolf et al., 1974). Schroeder (1968) demonstrated that glucose loading caused an increased Cr excretion in the urine during the first 2 hour after loading. Whole body elimination of a single intravenous dose of Cr (III) in rats occurs in 3 phases with half life values of 0.5, 5.9 and 83.4 days. The valence can also affect Cr excretion. Studies in rats indicate that Cr (VI) compounds are more rapidly excreted than Cr (III). The half life for Cr (VI) was 22 days compared to 92 days for Cr (III) (Squib and Snow, 1993).

The elimination of Cr from body tissues has been determined in the rat following a single subcutaneous dose of Cr (VI). Lung ,kidney, brain, heart and testes elimination curves consisted of two phases, with terminal half life values of 20.9, 10.5, 9.6, 13.9 and 12.5 days, respectively. The biologic half life of Cr in blood after reaching a peak at 5 day was 13.9 days. Liver tissue elimination of Cr occurred in three phases with half life values of 2.4 hour, 2.2 days , and 15.7 days (Squib, 1993). It appears that Cr is excreted in the form of LMWCr protein (Yamamoto et al.,1988; Okada et al., 1983). Some Cr is reabsorbed from the filtrate in the renal tubuli. Estimates of renal tubular reabsorption of filtered Cr have ranged from 63% in dogs (Collins, 1961) to 80-97% in man (Davidson and Parker, 1974; Rabinowitz et al. 1980).

Stress caused by trauma, infection, surgery, intense heat or cold all elevate secretions of hormones, and either directly or indirectly affect Cr excretion (Mertz and Roginski, 1969; Nielsen, 1988). Urinary Cr excretion and plasma cortisol were increased in runners

(Anderson et al.,1982) and traumatized patients 42 hours following hospital admission (Borel and Anderson 1984).

Chromium present in feces is mainly dietary non absorbable Cr but minimal amounts of absorbed Cr may be excreted by the gastrointestinal tract. Hopkins (1965) injected an intravenous dose of Cr to rats and 8 hours later sacrificed them to determine the Cr present in their intestinal feces. He found that nearly 1% of the Cr present was from endogenous origin. Starich and Blincoe (1983) and Borel and Anderson (1984) reported similar results.

Toxicity and carcinogenicity of chromium compounds

Toxicity

As with other elements, Cr has a role as an essential nutrient but can also be a potent toxic and carcinogenic agent. Its relative toxicity is dependent on the concentration, properties, the ability of the compound to enter the cell, the intracellular redox metabolism of the compound and the reactivity of the compound or its metabolites (Squib, 1993). High concentrations of Cr compounds have been found to be toxic in cell free systems, in bacteria and non-mammals eukaryote, in mammalian cells in culture, in plants, animals and humans (Costa et al., 1984; Leonard and Lauwerys, 1980). Very high doses of Cr may be lethal or produce severe systemic toxicity. In humans, Cr poisoning is limited to accidental ingestion of chromic acid or chromate (Borel and Anderson, 1984). According to Langard (1980) damage to the kidney, liver, nervous system and blood are the major causes of death. Chromium exposure in working areas can cause allergies and skin ulceration, perforation of nasal septum, and bronchial asthma (Langard, 1980).

Of all the Cr valences, Cr (VI) is the most toxic. The acute toxicity is poorly understood (Witmer et al., 1994). However, the mechanisms of toxicity of Cr (VI) in cells is thought to involve the reduction of Cr (VI) to Cr (III) by intracellular reducing agents such as ascorbate, glutathione, and microsomal enzymes. Chromium (III) then binds to nitrogen and sulfhydryl groups and is known to react with amino acids, proteins and nucleic acids. Thus Cr (III) although the most stable form of Cr available in environment can bind to DNA and produce effects on DNA replication in cell free systems but it does not readily enter into cells and therefore is less toxic than Cr (VI) in vivo. Likewise the bioavailability of Cr compounds may depend on the route of uptake. Soluble Cr (VI) is taken up by all types of cells via cellular anion transport channels, thus these compounds are bioavailable and toxic to bacteria and lower eukaryote as well as to mammalian cells in culture and in vivo. Insoluble and sparingly soluble Cr compounds are not bioavailable or toxic unless they are first solubilized. In contrast, many types of mammalian cells, including human lung cells, can take up insoluble Cr compounds by phagocytosis into membrane bound vacuoles (Snow and Squib, 1993). The inside of the vacuole is acidic, thus once the metal particles are taken up, they are slowly dissolved and the metal ions are released inside the cell where they can reach the DNA and other constituents. When tested, insoluble Cr compounds have often given a positive genotoxic response in mammalian cells, both in vivo and in vitro. Organic Cr compound has a very low toxicity compared with inorganic (Saner, 1980).

Carcinogenicity

Metal compounds, as a class, are among the best documented of all human carcinogens, but their mechanism are not fully understood (Snow, 1994). Chromium has been the most extensively investigated metal with respect to genetic and related effects (DeFlora, 1989). The carcinogenicity of Cr compounds also appears to result from the attack on DNA by Cr (III) formed in situ by reduction of Cr (VI) (Witmer et al., 1994). In cell free systems, but not in whole cells, Cr (III) can interact directly with DNA to produce DNA cross links, strand breaks and other conformational changes (Bianchi et al., 1983). Chromium (VI) does not directly interact with DNA except at very high concentrations unless incubation occurs in the presence of either biological or chemical reducing agents. However, within a cell or in the presence of glutathione, hydrogen peroxide or other cellular reductants, the reduced Cr(V) or Cr (III) can also interact with DNA and proteins to form stable Cr (III) complexes. Once formed, these complexes are slow to exchange and intracellular Cr (III) complexes are poorly repaired. Chromium (III) compounds also alter the kinetics and fidelity of DNA replication (Snow, 1994).

In bacterial and non-mammalian systems Cr (III) compounds are not as biologically active as Cr (VI) in whole systems. Chromium induces DNA damage as measured by DNA strand breaks, SOS induction (an inducible response to DNA damage in bacterial cells) and differential microbicide of cells that are deficient in DNA repair mechanisms. Chromium (VI) also induces various types of DNA mutations in bacteria and higher organisms as frame shifts and base pair substitutions (Snow, 1993).

In mammals and mammalian cells, soluble and insoluble Cr salts are toxic in vivo and to mammalian cells in culture. All forms of Cr induce some degree of chromosomal damage, DNA damage or mutagenesis. Both Cr (VI) and Cr (III) compounds bind to DNA in vivo (Cupo and Wetterhahw, 1985) but only Cr (VI) compounds induce DNA strand breaks, DNA-DNA cross links (Cupo and Wetterhahw, 1985; Sugiyama, 1986) and chromosomal aberration (Newbold et al., 1979). Human fibroblast cells are especially sensitive to mutagenesis by all forms of Cr (Squib and Snow, 1993). All forms of Cr also induce genotoxicity in vivo and both insoluble and weakly soluble Cr including Cr oxide have been found to be carcinogenic to rodents. Chromium (VI) compounds induce tumors in rodents at the site of injection. Most Cr (VI) compounds, regardless of solubility induce morphological transformation in cultured rodent cells (DiPaolo et al., 1979; Bianchi et al.,1983). Higher concentration of Cr (III) may be needed to achieve the same biological effectiveness as Cr (VI). Soluble and insoluble Cr (II) and Cr (III) compounds are usually negative in in vivo carcinogenicity assays. Soluble Cr (VI) compounds are taken up rapidly by cells via the sulfate transport system and reduced intracellularly to Cr (III) which is considered to be an ultimate intracellular carcinogen. The reduction process itself may also contribute to the carcinogenicity of chromate (Norseth, 1986). Chromium (III) ions cannot permeate the cell membrane but insoluble Cr (III) particles may be taken up by phagocytosis and then dissolve intra cellularly (Elias et al,.1986).

There are conflicting reports concerning the mutagenicity of Cr (III) compounds. Chromium (III) compounds were found nonmutagenic in a number of studies (IARC, 1980., Bianchi et al., 1983, Sugiyama et al., 1986. , Levis and Majorne, 1981, and Levy and

Venitt, 1986), but were mutagenic in V79 cells (Elias et al., 1986.) and also increased the frequency of sister chromatid exchanges in CHO3 cells (Venier et al.,1985). The toxicity and carcinogenicity of organic Cr compound has been less study, however its been assumed that these compounds have a very low toxic effect. Yamamoto et al. (1989) studied the toxigenic effect of MMLWCr and concluded that it is a very nontoxic agent.

Chromium in Food Producing Animals

Chromium as an essential micromineral for mammals is mainly associated with the metabolism of carbohydrates, lipids and proteins, due to its believed effect on potentiating insulin activity. Although a daily recommended allowance of 1 to 4 μmol/day of Cr, based in obligatory losses and bioavailability of Cr in food, is recommended for humans (Mertz, 1993), the exact requirement has not been established. For food producing animals there are no recommendations from the National Research Council.

During the past few years, the importance of Cr as a supplement for food producing animals has been studied. The effects of different concentrations and forms of dietary Cr supplementation had been reported in dairy cattle (Burton et al., 1993; Kegley et al., 1995; Chang and Mowat, 1992; Subiyatno et al., 1994; Wright et al., 1994), beef cattle (Burton et al., 1994; Moonsie-Shaeger, 1993; Mowat et al., 1993; Smith et al., 1994), swine (Evock et al., 1993; Lindeman et al., 1993, 1995; Boleman et al. 1995; Page et al., 1993; Wang et al., 1995; Word et al, 1995; Harris et al., 1995; Chung et al. 1995; Wenk et al. 1995), poultry (Anderson et al. 1989; Steele et al., 1981; Jensen et al., 1978; Steele and Rosebrough, 1981) lambs (Kitchalong et al., 1993, 1995; Forbes et al. 1995) and fish (Bureau et al., 1995).

Variables including performance, carcass merit, and reproduction have been evaluated, with varying results.

It appears that Cr supplementation is more effective during periods of heavy stress. Several experiment have been conducted to evaluate the effect of Cr supplementation in stressed animals. In dairy cows, 0.5 ppm of supplemental Cr during late gestation and early lactation, two severe stress periods, increased milk production during the first 16 weeks of lactation by 11% in primiparous cows but had no effect in multiparous cows (Subityatmo et al., 1995). Others reported metabolic aspects to be improved by Cr supplementation such as gluconeogenesis and normalization in glucose, insulin and glucagon kinetics (Chang and Mowat, 1994). During the early lactation period, cows supplemented with Cr, also have a higher humoral and cell mediated immune response compared to nonsupplemented animals (Burton et al., 1993). In beef cattle, Chang et al. (1994) reported an improvement of 16% on rate of daily gain and 19% on feed efficiency in feeder calves during the initial 28 days in the feedlot. Moonsei-Shager and Mowat (1993) found similar results. However, in an earlier study, Chang (1993) found no effect and other researchers have also reported no effect (Lindell et al., 1994; Kegley et al. 1995). Another aspect that has been evaluated in feeder calves is the effect of Cr on diseases and immune response. An improvement in humoral and cell mediated immunity (Chang and Mowat, 1992; Kegley et al., 1995) and a decrease in morbidity (Moonsie-Shaeger and Mowat, 1993; Lindell et al., 1994) have been reported. In lambs, growth rate, feed intake and glucose kinetics were not affected by Cr picolinate supplementation (Forbes et al. 1995). In swine, Page et al. (1993) found that supplemental Cr, in the form of Cr picolinate increased loin eye area and percent muscling and decreased

back fat in growing-finishing pigs. Chromium chloride had no effect. Similar results have been reported by Lindeman et al. (1993), Mooney and Cromwell (1993) and Chung et al. (1995). Wenk et al. (1995) also reported an improvement on carcass characteristics and an increase in average daily gain and decrease in feed gain ratio but only when the source of Cr was Cr chloride and found no significant effect with either high Cr yeast or Cr picolinate [$(C_7H_5NO_2)Cr$]. Ward et al. (1995) reported that in growing-finishing swine the differences between treatments for metabolites, hormones and growth and carcass characteristics were minimal. Wang et al. (1995) conducted two trials, using adequate and a low protein feed supplemented with 200 ppb of Cr picolinate. He reported that feeding Cr picolinate to growing finishing pigs did not affect growth and back fat thickness, but larger loin areas were noted. Conflicting results in nitrogen utilization were reported. In one trial, nitrogen retention was increased but no effect was found in a second trial. Harris et al. (1995) concluded that Cr picolinate had no effect on growth performance or carcass characteristics in pigs. Lindemann et al. (1995) reported that total pigs born, live pigs born and pigs at 21 days were not affected by Cr supplementation, however in a previous experiment he found that 200 ppb organic Cr fed through growing and gestation in gilts increased number of pigs born alive and 21 day litter weight (Lindeman, 1994). In poultry Cr (III) increased the rate of conversion of glucose to acetyl CoA in turkey (Steele and Rosebrough, 1981) and five ppm of Cr (III) improved the egg interior quality of laying hens (Jensen et al 1978). In fish, Ward et al. (1995) found no effect on final body weight and nitrogen and energy retention of rainbow trout supplemented with Cr. He concluded that Cr had no effect on the parameters of economic importance in this type of fish.

Why does Cr supplementation produce positive effects in some trials but have no effect in other trials? The key points appear to be stress, Cr status of the animal and Cr concentration of the feed. When the animal is marginally deficient, or the amount of Cr in the feed is low, or a combination of both and the animal is under heavy stress, a response to Cr supplementation may be possible. However, when none of these factors are present, the animals will not respond to Cr supplementation

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MATERIALS AND METHOD

Experiment One

Animals, Feed, and Experimental Period.

This experiment was conducted to evaluate the effect of supplemental Cr on the retention of copper and zinc in stressed baby pigs. This study was approved by the All-University Committee on Animal Use and Care of Michigan State University (AUF number: 12/94-221-00).

Twelve weanling (21± 1 day), crossbred (♀ Yorkshire-Landrace x ♂ Hampshire) castrated male pigs with an average weight of 6.02±0.65 kg BW, from four different litters were used. At day-one of age, pigs were identified by ear notch, and at day-ten they were castrated. The pigs were not vaccinated and no creep feed was provided during the lactation period.

A whey-corn-soybean meal basal starter diet (Table 1) was prepared and supplemented with either 0.5 or 1 ppm of organic Cr. This basal diet was formulated to meet the National Research Council (NRC, 1988) requirements for pigs of this age (Appendix). The source of organic Cr was a high Cr yeast product (Cofactor III), kindly provided by Alltech Co. (Nicholasville, KY) with a concentration of 1000 ppm of Cr. The actual concentration of Cr was confirmed by inductively coupled plasma general mineral analysis procedure (Faires, 1982) before the product was mixed with the basal diet (Table 2). The basal diet did not contain any antibiotics or supplemental copper. Forty six or twenty three grams of the high Cr yeast was mixed with 0.45 kilograms of the basal diet and then with 45

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kilograms of feed for 25 minutes in a feed mixer, to prepare a diet with either 1.0 or 0.50 ppm supplemental Cr. The final Cr concentration of the diet by analysis was 1.54, 2.12, and 2.61 ppm for T1, T2, and T3 respectively.

At weaning, pigs were weighed and assigned to three treatments in a completely randomized block design (Gill, 1978). Treatments consisted of 0 (basal, T1), 0.50 (T2), and 1 ppm (T3) of supplemental Cr. The experiment had a duration of 15 days; first five days were used to adapt pigs to diet and environment. During this time the pigs were housed individually in a 0.84 m x 0.43 m elevated stainless steel with screened floor cages. Basal diet and deionized water were provided ad libitum. The housing temperature was maintained at 30°C. During day six, the animals were individually weighed and transferred to 0.61 x 0.79 m metabolic stainless steel cage for total collection of feces and urine.

Feeding the Animals and Sample Collection.

To avoid contamination of feces with feed, the pigs were removed three times daily (9:00 AM, 3:00 PM, and 9:00 PM) from the metabolic cages for about 30 minutes and transferred to the adaptive period cages to be fed (Yokoyama et al., 1982). A rubber pad was laid on the floor and an aluminum pan was placed under each cage to collect any feces or urine voided during the feeding period. By closely watching the pigs during the feeding periods, no losses were incurred in feces and urine. Before moving the pigs back to their cages, their snouts were cleaned with a wet (deionized water) paper towel. The meals at day 6 and day 16 were mixed with 50 mg of brilliant blue dye (F.D. & C Blue No. 1)

Table 1. Ingredient composition of the basal diet [a].

INGREDIENTS	GRAMS/KILOGRAM
CORN, DENT YELLOW	558.50
SOYBEAN MEAL DEHULL	200.00
WHEY DRIED	150.00
FISH MEAL, MENHADEN	50.00
CALCIUM CARBONATE	10.00
MONO-DICAL PHOSPHATE	15.00
MSU SWINE VIT-TM-PMX [b]	7.50
SELENIUM E PREMIX [c]	5.00
WHITE SALT	2.50
L-LYSINE	1.50
TOTAL	1000.00

[a] On as-fed basis

[b] Provided the following per kilogram of the complete diet: Vitamin A, 4950 IU; Vitamin D3, 990 IU; Vitamin K, 3.3 mg; Riboflavin, 4.95 mg ; Pantothenic acid, 19.80 mg; Niacin, 26.4 mg; Choline, 168 mg; Vitamin B12, 30 mcg; Manganese, 56.1 mg; Iron, 89 mg; Copper, 14.85 mg; Iodine, .75 mg; Zinc 112 mg.

[c] Provided the following per kilogram of the complete diet: Selenium, 0.3ppm, and Vitamin E,16.5 IU.

Table 2. Mineral analysis of the high Cr Yeast and the Basal diet.

MINERAL PPM	BASAL DIET	HIGH CR YEAST
ALUMINUM	90.90	20.50
ANTIMONY	< 5.00	< 5.00
ARSENIC	<2.50	< 2.50
BARIUM	2.79	1.86
BORON	11.60	< 5.00
CADMIUM	<0.50	< 0.50
CALCIUM	5690.00	1410.00
CHROMIUM	1.54	988.00
COBALT	<0.50	< 0.50
COPPER	22.20	21.90
IRON	166.00	66.40
LEAD	<2.50	< 2.50
MAGNESIUM	1690.00	1610.00
MANGANESE	66.10	11.40
MERCURY	<10.00	< 10.00
MOLYBDENUM	1.59	1.38
PHOSPHORUS	6200.00	12200.00
POTASSIUM	8040.00	16600.00
SELENIUM	<10.00	< 10.00
SODIUM	830.00	957.00
THALLIUM	<12.50	< 12.50
ZINC	111.00	57.20

(Lutwak and Burton, 1964; Marlett et al., 1981). This dye colored the feces and allowed the collection of feces voided during the experimental period by visual examination. Deionized water was provided ad libitum. Feed consumption and refusals were recorded daily. A sample of the feed offered and the total of the refusals were collected into plastic bags and stored at -20°C until analyzed. Feces and urine were collected every 24 hours for ten days. Feces were collected separately on a fine plastic screen under the expanded metal floor of the stainless steel metabolism cages in which pigs were housed. They were collected into plastic bags (Nasco Whirl-Pak) weighed and stored at -20°C until analyzed (Ilori et al., 1984). Urine was allowed to pass through the screen onto a stainless steel collecting tray and collected untreated into plastic containers. The total urine volume was recorded and a sample of 100 ml was retained and stored in plastic conical test tubes with screw caps (Sarsdet, W. Germany) at -20°C until analyzed.

Analytical procedures

Feces were oven dried at 65°C in aluminum pans for three days, and then ground in a waring blender (Model 1120, New Hartford, CT). Feed and refused feed were ground through a 1-mm screen using a Wiley mill. Duplicate samples of approximately one gram of feed, 0.50 gram of feces and 17 grams of urine were weighed into 250 Phillip beaker. Twenty ml of nitric acid (14M) and four ml of perchloric acid (10M) were added to the samples and digested under a perchloric hood until approximately one ml of volume remained in the flask (Analytical Methods Committee, 1960). The flask was removed from the hood and the interior sides were washed with deionized water. When the flask was cool, deionized water

was added to attain a net weight of 50 grams. When necessary, the samples were further diluted. The concentration of Cu and Zn in the samples were determined by flame atomic absorption spectrophotometry (model II 951, Instrumentation Laboratory, Wilmington, MA). The final concentration of minerals was calculated using the following formula:

$$PPM = [(D)(FWF-FW)(C)] \div (SW)$$

PPM = concentration of mineral in part per million

D = final dilution

FWF = final weight of the flask

FW = initial weight of the flask

C = concentration of the mineral (ppm)

SW = sample weight.

Intake and fecal and urinary mineral excretion daily and for the experimental period was calculated and adjusted as mg per kilogram of body weight (Kimmel et al.,1988; Lei et al., 1993).

Statistical Analysis

The experimental design was a complete randomized block with four replications. Animals were blocked by litter, and individual pig was the experimental unit. The data set consisted of Cu and Zn values for each day and the average for the period. Data were analyzed using the ANOVA procedure of Statistical Analysis System (SAS, 1988). The general linear model used was:

$$Y_{ij} = \mu + B_j + T_i + E_{ij}$$

Y_{ij} = the individual value

μ = grand mean

B_j = effect of block

T_i = effect of treatment

E_{ij} = residual error

Differences was considered significant at the level of $P< 0.05$ and the Scheffe method was used for mean comparison.

Experiment Two

In the second experiment two similar trials were conducted (May-June and September-October, 1995) to evaluate the effect of supplemental Cr on performance, cell mediated in vitro immune response of lymphocytes and the phagocytic activity and microbicidal ability of the neutrophilic polymorphonuclear leucocyte (NPL) of peripheral blood. This study was approved by All-University Committee on Animal Use and Care of Michigan State University (AUF number: 12/94-221-00).

Animals and experimental period

Forty-eight (24 castrated males and 24 females) weanling (21±1day), crossbred (♀ Yorkshire-Landrace x ♂ Hampshire) pigs with an average weight of 7.28± 0.40 kg, from nine different litters, were used in each trial. At one day of age, pigs were identified by ear notch and the standard management practices were performed, including castration at ten days of

age. The pigs were not vaccinated and no creep feed was provided during the lactation period.

A whey-corn-soybean meal (phase 1) and a corn-soybean meal (phase 2) basal diets (Table 3) were prepared and supplemented with either, 0 ppm Cr and Cu (T1 basal), 0.5 ppm Cr, 0 Cu (T2), 0.50 ppm Cr and 250 ppm Cu (T3), or 0 ppm Cr, 250 ppm Cu (T4). The basal diets were formulated to meet the National Research Council (NRC, 1988) requirements for pigs of this age (Appendix). The source of organic Cr was high Cr yeast product (Cofactor III) with a concentration of 1000 ppm of Cr, which was kindly provided by Alltech CO. (Nicholasville, KY). The source of Cu was feed grade copper sulfate. The basal diet did not contain any antibiotics or supplemental Cu. Thirty five grams of the high Cr yeast, or forty two grams of copper sulfate, or thirty five grams of the high Cr yeast and forty two grams of copper sulfate or forty two grams of copper sulfate were mixed with 0.45 kilograms of the basal diet and then with 67.73 kilograms of feed for 25 minutes in a feed mixer to make the diets with 0.5 ppm supplemental Cr, 0.50 ppm supplemental Cr and 250 ppm Cu and 250 ppm supplemental Cu. The final concentration of Cr and Cu for T1, T2, T3, and T4 are presented in Table 4.

At weaning pigs were weighed and randomly assigned to the treatments. Each treatment was replicated three times. The animals were housed in 0.83m x 0.73m elevated stainless steel screened floor pen, four animals per pen (two males and two females) providing an average floor space of 0.15 m^2/pig. The Pork Industry Handbook (Fritsche and Muehling, 1987) recommends a floor space allowance of 0.16 to 0.23 m^2 for pigs from 6.8 to 13.6 kg and 0.28 m^2 to 0.37 m^2 for pigs from 13.6 to 27.2 kg. The provided floor space

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Table 3. Ingredient composition of the basal diet experiment 2.

INGREDIENT G/KM	PHASE 1	PHASE 2
CORN	558.50	658.50
SOYBEAN MEAL	200.00	300.00
DRIED WHEY	150.00	----
FISH MEAL	50.00	----
CALCIUM CARBONATE	10.00	10.00
MONO-DICAL PHOSPHATE	15.00	15.00
MSU SWINE VIT-TM PMX[B]	7.50	7.50
SELENIUM E PREMIX[C]	5.00	5.00
WHITE SALT	2.50	2.50
L-LYSINE	1.50	1.50
TOTAL	1000.00	1000.00

[a] On as-fed basis

[b] Provided the following per kilogram of the complete diet: Vitamin A, 4950 IU; Vitamin D3, 990 IU; Vitamin K, 3.3 mg; Riboflavin, 4.95 mg ; Pantothenic acid, 19.80 mg; Niacin, 26.4 mg; Choline, 168 mg; Vitamin B12, 30 mcg ; Manganese, 56.1 mg; Iron, 89 mg; Copper, 14.85 mg; Iodine, .75 mg; Zinc 112 mg.

[c] Provided the following per kilogram of the complete diet: 0.3ppm Se and 16.5 IU Vitamin E.

Table 4. Chromium and copper concentration in the experimental feed.

	Trial 1				Trial 2			
	Phase 1		Phase 2		Phase 1		Phase 2	
	Cr	Cu	Cr	Cu	Cr	Cu	Cr	Cu
T1	1.58	23.00	1.70	26.20	1.40	18.02	1.60	20.30
T2	2.16	28.05	2.20	19.08	2.05	23.00	2.12	18.04
T3	2.25	263.00	2.35	258	2.12	268	2.23	248
T4	1.45	265	1.35	270	1.38	258	1.60	261

ranged from 6 to 59 % less than these recommendations over the course of the experiment. This was the source of stress which followed the stress related to weaning. Experimental housing temperature was maintained at 28 to 30°C for fourteen days then was gradually reduced to 20°C. Each pen had two nipple waterer and a three-hole plastic feeder. Feed and tap water were provided ad libitum. In the second experiment a dose of 200 μg of Cr in 5 ml of deionized water was administered orally by gavage to the pigs in T2 and T3 during the first four days of the experiment. Pigs in T1 and T4 received 5 ml of deionized water. Daily observation was performed to assess the health of the animals.

The experiment had a duration of 35 days. Phase one diet was fed for fourteen days before switching to phase two. Animals were weighed weekly. Records of feed offered and refusals were recorded weekly. Average daily gain, average daily feed consumption and feed gain ratio were calculated by week and accumulative.

Blood Sample Collection

Twelve pigs from three different litters were blocked by litter and assigned at random to the treatments. These animals were bled early in the morning (7:00 AM) at day 0 and weekly thereafter by vena cava venipuncture into 10 ml sterile evacuated tubes containing 143 USP units of sodium heparin (Vacutainer, Becton Dickson, Rutherford, N.J.) for cell isolation. An additional blood sample was collected into 5 ml sterile tubes without preservative for serum collection. The blood samples were held at room temperature for subsequent immunoassay later the same day. Fletcher et al. (1987) had shown, that phytohemagglutinin assays lose significant activity by holding samples overnight.

Immunological Test

Mitogen Preparation and Storage

Stock solutions of mitogen were prepared and stored at -70°C. Two hundred and fifty mg of concanavalin A (ConA), a broad T-cell mitogen, 100 mg of phytohemagglutinin (PHA), a potent T-cell mitogen, 40 mg of pokeweed mitogen (PWM), a broad T and B-cell mitogen and 10 mg of **E. coli** lipopolysaccharide (LPS) a potent B cell mitogen (Sigma Chemical Co: St. Louis, MO) were dissolved separately into 100 ml of Rosewell Park Memorial Institute (RPMI) 1640 culture media (Gibco Laboratories, Grand Island NY) containing 6 μl/ml of fungizone and 0.32 μl/ml of gentamicin sulfate in addition to 1 mmol of L-glutamine, sodium bicarbonate and 10% of fetal bovine heat inactivated serum. The mixture was sterile filtrated with a 0.22 μm filter (Millipore Corp., Bedford, Ma) and stored

in one ml aliquot making a stock solution of 2.5 mg/ml, 1mg/ml, 0.4 mg/ml and 1 mg/ ml of ConA, PHA, PWM and LPS respectively.

Before the study, various concentrations of the respective mitogens were incubated with isolated pig lymphocytes to determine concentrations for optimum response. A final well concentration of 20 μg/ml, 16 μg/ml, 16 μg/ml and 10 μg/ml gave the maximal response for ConA, PHA, PWM and LPS respectively. Mitogen solutions were thawed immediately before use and working solutions of 275 μg/ml (0.3 cc stock solution of ConA + 2.43 cc RPMI), 176 μg/ml (0.40 cc stock solution of PHA + 1.87 cc RPMI), 176 μg/ml (1 cc stock solution + 1.27 cc RPMI) for ConA, PHA, PWM and LPS were prepared. Unused portions of both stock solutions and working solutions were discarded (Barta, 1984).

Yeast Cell Solution Preparation

Dried baker's yeast (Fleischmanns Yeast Inc., Oakland, Ca.) was used as target cells to measure the neutrophilic phagocytic activity and microbicidal ability in this study . Fresh yeast solution was prepared as needed. A half gram of dried yeast was dissolved in 200 ml of 0.9% saline and stirred for 20 minutes on a magnetic mixer. The yeast cell number and viability were determined, using a Neubauer Hemocytometer under a light microscope and adjusted with 0.9% saline to 2 x 10^6 cell per ml (Simpson et al., 1979)

Cell Isolation

The peripheral blood lymphocytes (PBL) and the neutrophilic polymorphonuclear leukocytes (NPL) were separated from the blood by differential centrifugation (Boyum,

1968) using the Ficoll-Hypaque density gradient technique. Sterile procedure under a Bioquest Biological Cabinet was used throughout the entire procedure.

Peripheral Blood Lymphocytes

Blood was diluted 1:2 in RPMI 1640 culture media in a 45 ml centrifuge test tubes (Beckton Dickinson, Rutherford, NJ) and mixed very gentle by inversion. Seven ml of diluted blood was layered carefully over 3 ml of cell separation media (Histopaque 1077, Sigma Chemical Co: St. Louis, MO, 1.077 g/ml density gradient) in a 17 x 100 mm sterile plastic test tube with cap (Falcon, Williams Ca.) using a ten ml sterile pipet and then centrifuged at 400 xg for 30 minutes with a table centrifuge (HN-S Centrifuge, International Equipment) at 18-20°C. After centrifugation the lymphocyte-rich layer was transferred from the buffy interface into another sterile 17x100 mm sterile plastic tube with a sterile Pasteur pipette, diluted in 10ml sterile EDTA phosphate buffered saline (EPS) solution (18g NaCl, 7.16g EDTA-Na3, 2.16g KH_2PO_4, 972.68g deionized water, pH 7.4) and centrifuge at 275 xg for 6 min. The supernatant was discarded and the cell pellet was examined for red blood cell (RBC) contamination. When RBC was present the pellet was suspended in 10 ml of Tris ammonium chloride (TAC)(7.5g NH_4Cl, 1.0 g Tris-HCl and 991.5 g deionized water, pH 7.4) and incubated at 37°C in a water bath for 12 min to lyse the RBC. After RBC decontamination, the cell suspension was centrifuged at 150 xg for 8 min. The supernatant was discarded and the cell pellet was washed with phosphate buffered saline (PBS, 8g NaCl, 0.2g KCl, 1.15g NaH_2PO_4 and 990.45 g deionized water, pH 7.4) 2x; the first followed by a centrifugation at 225xg for 6 min and the second followed by centrifugation at 275xg for 8 min. Washed

mononuclear cells were suspended in 2 ml of RPMI,1640 media and counted in a Neubauer hemocytometer under light microscope. The viability of the cells was assessed by trypan blue exclusion (Sigma Chemical Co: St. Louis, MO). The cell suspension was adjusted to $2x10^6$ viable cell per ml (Hussain, 1981).

Neutrophilic Polymorphonuclear Leukocyte (NPL) Cell Isolation

The blood samples were diluted 1:1 with RPMI 1640 medium. For each sample 7 ml of the blood medium mixture was layered into each of two 17x100 sterile plastic tubes with caps containing 4 ml of cell-separating media and centrifuged at 18° to 20°C for 30 minutes at a relative centrifugal force of 400xg. After centrifugation the plasma and cell separation media layers were withdraw very carefully with a 10ml syringe with a long blunt needle. The RBC-NPL granulocyte pellet was suspended in 10 ml of TAC and incubated at 37°C in a water bath for 15 min to lyse the RBC and then centrifuged at 300xg for 6 min. After centrifugation the supernatant was carefully removed and discarded. The pellet was washed with PBS buffer and centrifuged twice at 250xg for 10 minutes at 18° to 20°C. The RBC lysis step was repeated until the cell pellet was clean and slight greenish in color. The NPL cells were counted on a Neubauer Hemocytometer and the viability was assessed by trypan blue exclusion. The cell count was adjusted to 2×10^6 cells/ml in RPMI 1640 media.

Blastogenesis Assay

A cell suspension of PBL (100 μl) from each pig containing $2x10^5$ cells (Hussain et al., 1981) was dispensed into 12 wells of a round-bottom 96-well micro titer cell culture plate

(Corning Glass Works, Corning, NJ). The cells in the first three wells of each row were unstimulated. To stimulate lymphocyte proliferation in the other nine wells a volume of 10 µl of Concanavalin A (Con A) with a concentration of 330 µg/ml, phytohemagglutinin (PHA) 176 µg/ml and pokeweed mitogen (PWM) 176 µg/ml were dispensed to each of three samples. This made a final concentration of 20, 16 and 16 µg/ml of ConA, PHA and PWM respectively. The plates were then covered with Mylar sealers (Dynatech Laboratories Inc. Chantilly, Va). Cultures were then incubated in a humidified incubator with 5% CO_2 at 37° C for 4 days.

A total of 1 µCi of [^{3}H] thymidine (specific activity 6.7 Ci/mmole, NEN Research Products, Boston, Mass) in 20 µl volume was added to each well 18 to 22 hours before termination of incubation period. After the incubation period the cell cultures were harvested (Micro cell harvester, Straton Lier Norway) onto paper filter pads using TCA and deionized water. Each well formed a disc in the filter pad and each disc was transferred to plastic scintillation vials. A volume of 100 µl of soluene 350 (Packard Instrument Co., Downer's Grove, Ill) was added to each vial to solubilize the cells. The vials were laid undisturbed for 30 minutes and after that 4.5 ml of High Flash Point Cocktail Safety Solvent (Liquiflour, New England Nuclear, Boston, MA) were added and the vials capped and labeled properly. The incorporation of [^{3}H] thymidine into newly synthesized DNA of stimulated and non stimulated cells were determined with a liquid scintillation counter, (TM Analytic Inc., Elk Grove Village, Ill) three five minutes rounds per sample, and the results were recorded as counts per minute (CPM). Stimulations indices (SI) were calculated as follows:

$$SI = RSC \div RUC$$

SI = Stimulation index

RSC = average counts per minute in stimulated cells

RUC = average counts per minutes in unstimulated cells

Phagocytic Activity and Microbicidal Ability

To evaluate the ability of neutrophilic polymorphonuclear leukocyte (NPL) cells to phagocytize and kill invading organisms the methods described by Simpson et al. (1979) was used. Dried baker's yeast was used as target cells. Equals volume (200 µl) of yeast suspension , NPL cell, autologous blood plasma, and RPMI 1640 were dispensed into tubes with caps and incubated at 37°C in a shaking water bath for 60 min. After the incubation period, 1 ml of 0.01% methylene blue was added to the mixture and was centrifuged at 400xg for 10 min. The supernatant was discarded and the cell pellet re-suspended in 20µl of RPMI 1640 and observed under a light microscope with a Neubauer hemocytometer. The number of PMN cells containing 2 or more yeast cells per 100 cells were counted to quantify the phagocytic activity. The microbicidal ability of NPL cells was determined by counting the number of NPL cells containing 2 or more dead yeast particles per 100 NPL cells.

Statistical Analysis

The experiment design was a randomized complete block with three repetition in a 2 x 2 factorial arrangement of treatments. Animals were blocked by weight and litter. In the performance evaluation, the pen was the experimental unit and in the immunological evaluation individual animal was the experimental unit. The data set for performance

consisted of average daily gain, average daily feed consumption and feed gain ratio values for each week period and accumulative. For the immunological test the data set was stimulation index for ConA, PHA, PWM and LPS and the values for phagocytic activity and microbicidal ability. The day 0 values for SI and phagocytic activity and microbicidal ability were used as covariate. Differences was considered significant at the level of $P< 0.05$. For mean comparison the Scheffe test was used. The general linear model used for performance evaluation was:

$$Y_{ij} = \mu + B_j + T_i + E_{ij}$$

Y_{ij} = the individual value

μ = grand mean

B_j = effect of block

T_i = effect of treatment

E_{ij} = residual error

The general linear model used for the immunological test evaluation was:

$$Y_{ij} = \mu + B_j + T_i + C_{ij} + E_{ij}$$

Y_{ij} = the individual value

μ = grand mean

B_j = effect of block

T_i = effect of treatment

C_{ij} = effect of covariate

E_{ij} = residual error

All statistical analysis were performed in a Personal Computer using the General Linear Model procedure of Statistical Analysis System (SAS, 1988).

RESULTS

Experiment one

This experiment was a digestibility trial to determine the effect of dietary chromium supplementation on copper and zinc retention. It had a 15 day duration. The first five days were the adaptive period, and the last ten days were the collection period. The first two days were very stressful for the pigs. Pigs were highly agitated, jumping all the time trying to escape, and barely ate the feed provided. Indeed they also lost some weight. On average the pigs lost about 122 g/d during the first 2 days. The data related to the animals weight during the whole period is presented on Table 5. The initial average weight was 6.02 kg. At day 4 they totally recovered and started to gain weight again. At day 5 they were back to the initial weight. Based in average daily gain pigs eating the Cr supplemented diet (T2, T3) recovered faster than those on the unsupplemented diet. All four pigs receiving the unsupplemented diet and three in the 0.50 ppm of Cr developed diarrhea by the d8. In contrast, pigs receiving 1.00 ppm supplemental Cr did not develop any diarrhea.

Effects of Cr supplementation on Cu retention.

The effect of Cr supplementation on Cu consumption, excretion and retention are presented on Tables 6, 7, and 8. On average the amount of Cu consumed expressed as mg/kg BW increased as the number of days in experiment progressed.

Table 5. Weight(kg) and performance of pigs, experiment one.

Day	T1[e]	T2[f]	T3[g]
Day 1 [ab]	6.07	6.16	5.83
Day 3	5.67	5.69	5.60
Day 4	5.89	6.06	5.88
Day 5 [c]	5.99	6.35	6.02
Day 12	6.93	7.27	7.17
Day 16 [d]	7.85	8.51	8.45
Performance			
ADG[h] g/d	186	216	243
ADFI[i] g/d	265	304	323
F/G[j]	1.42	1.41	1.33

[a] Weaning day

[b] Beginning of experimental period

[c] Beginning of collection period

[d] Last day of collection

[e] Basal diet

[f] Basal diet with 0.50 ppm supplemental Cr

[g] Basal diet with 1 ppm supplemental Cr

[h] Average daily gain

[i]Average daily feed intake

[j]Feed gain ratio

Table 6. Copper consumption in baby pigs supplemented with dietary organic Cr.

	T1 [a]		T2 [b]		T3 [c]	
Day	Total	mg/kg BW	Total	mg/kg BW	Total	mg/kg BW
Day 1	3.26	0.55	4.16	0.65	4.09	0.68
Day 2	4.64	0.79	4.99	0.80	5.36	0.89
Day 3	4.98	0.83	6.03	0.96	5.11	0.87
Day 4	5.08	0.85	4.96	0.82	5.05	0.84
Day 5	4.86	0.81	4.45	0.75	5.47	0.91
Day 6	5.11	0.73	5.52	0.77	6.29	0.88
Day 7	5.81	0.83	6.09	0.84	6.71	0.93
Day 8	6.39	0.90	6.75	0.93	6.86	0.97
Day 9	6.59	0.92	6.69	0.92	8.32	1.17
Day 10	7.64	0.93	8.12	0.99	8.71	1.28
$\bar{x}$	5.33	0.81	5.69	0.84	6.24	0.94

[a] Basal diet

[b] Basal diet with 0.50 ppm supplemental Cr

[c] Basal diet with 1 ppm supplemental Cr

The data is an average of 4 pigs for treatment

Total is expressed as mg of Cu

Table 7. Copper excretion in baby pigs supplemented with dietary organic Cr.

	T1 [a]		T2 [b]		T3 [c]	
Day	Total	mg/kg BW	Total	mg/kg BW	Total	mg/kg BW
Day 1	**3.05**	**0.51**	**2.98**	**0.46**	**2.39**	**0.39**
Day 2	3.83	0.63	3.52	0.56	3.57	0.61
Day 3	**2.90**	**0.48**	**3.79**	**0.60**	**4.70**	**0.78**
Day 4	3.07	0.51	4.78	0.78	5.11	0.85
Day 5	**4.39**	**0.74**	**3.63**	**0.61**	**4.19**	**0.70**
Day 6	4.64	0.68	4.21	0.60	5.48	0.76
Day 7	**4.01**	**0.57**	**4.75**	**0.67**	**8.40**	**1.18**
Day 8	4.40	0.62	5.16	0.71	6.20	0.86
Day 9	4.58	0.65	5.22	0.72	5.30	0.75
Day 10	4.90	0.57	6.36	1.19	8.09	1.18
$\bar{x}$	**3.88**	**0.60**	**4.65**	**0.69**	**5.37**	**0.81**

[a] Basal diet

[b] Basal diet with 0.50 ppm supplemental Cr

[c] Basal diet with 1 ppm supplemental Cr

The data is an average of 4 pigs for treatment

Total is expressed as mg of Cu

Table 8. Copper retention in baby pigs supplemented with dietary organic Cr.

	T1 [a]		T2 [b]		T3 [c]	
Day	Total	mg/kg BW	Total	mg/kg BW	Total	mg/kg BW
Day 1	**0.21**	**0.04**	**1.18**	**0.20**	**1.69**	**0.29**
Day 2	**0.81**	**0.16**	**1.47**	**0.24**	**1.79**	**0.28**
Day 3	**2.08**	**0.35**	**2.24**	**0.36**	**0.41**	**0.09**
Day 4	**2.01**	**0.34**	**0.18**	**0.04**	**-0.06**	**-0.01**
Day 5	**0.46**	**0.06**	**0.82**	**0.14**	**1.28**	**0.21**
Day 6	**0.47**	**0.05**	**1.31**	**0.17**	**0.82**	**0.11**
Day 7	**1.80**	**0.26**	**1.34**	**0.18**	**-1.69**	**-0.26**
Day 8	**1.98**	**0.28**	**1.59**	**0.21**	**0.66**	**0.10**
Day 9	**2.01**	**0.27**	**1.47**	**0.21**	**3.02**	**0.42**
Day 10	**3.28**	**0.37**	**3.80**	**-0.20**	**3.55**	**0.10**
$\bar{x}$	**1.45**	**0.22**	**1.42**	**0.15**	**1.17**	**0.13**

[a] Basal diet

[b] Basal diet with 0.50 ppm supplemental Cr

[c] Basal diet with 1 ppm supplemental Cr

The data is an average of 4 pigs for treatment

Total is expressed as mg of Cu

In general, pigs receiving no supplemented Cr (T1) had a lower Cu consumption compared to those receiving the supplemented diets (T2, T3). Pigs receiving 1 ppm supplemented Cr (T3) had the higher Cu consumption during the whole period followed by those consuming 0.5 ppm supplemented Cr. In general, Cu consumption in T3 was from 5 to 38 % higher compared to T1 and from 2 to 29 % higher compared to T2 during the period. Pigs in T2 had a Cu consumption from 0 to 18 % higher than T1. Pigs in T3 consumed 16 and 12 % more Cu compared to T1 and T2, respectively. Treatment 2 had a 4% higher consumption compared to T1. Although T3 had a higher consumption than either T1 or T2 the differences were not statistically significant ($P>0.10$). The excretion of Cu was variable from day to day. However, on average pigs in T3 had also higher Cu excretion compared to T1 and T2. Pigs in T3 excreted 35 and 17 % more Cu compared to T1 and T2 respectively. Pigs in T2 excrete 15 % more Cu than T1. There were no significant differences due to treatment except for d3 when T3 and T2 had a significantly higher excretion of Cu compared to T1 ($P<0.05$). Copper retention showed the opposite results. Pigs on the unsupplemented diet had a higher retention of Cu compared to pigs on supplemented Cr. On the ten day average pigs in T1 had 7 and 5 % higher Cu retention than pigs in T3 and T2 respectively. Treatment 3 had the lower Cu retention. Pigs in T3 and T2 had two and one day negative Cu balance. In contrast pigs in T1 had a positive copper balance. However differences were not statistically significant ($P>0.10$).

Effects of Cr supplementation on Zn retention.

The effect of Cr supplementation on Zn consumption, excretion and retention are presented on Tables 9, 10 and 11. In general the amount of Zn consumption expressed as mg/kg BW increase as the number of day in experiment progress. In general pigs receiving supplemental Cr (T2, T3) had a higher Zn consumption compared to those receiving the unsupplemented diet (T1). Pigs receiving 1 ppm supplemented Cr (T3) had the higher Zn consumption during the whole period followed by those consuming 0.5 ppm supplemented Cr. In general Zn consumption in T3 was from 8 to 36 % higher compared to T1 and from 0 to 26 % compared to T2 along the period. Pigs in T2 had a Zn consumption from 2 to 42 % higher than T1. On average pigs in T3 consumed 24 and 10 % more Zn compared to T1 and T2 respectively. Treatment 2 had a 12% higher consumption compared to T1. Although T3 had a higher consumption than either T1 or T2 there were no statistically significant differences due to treatments ($P>0.10$). The excretion of Zn was also variable from day to day. However, on average pigs in T3 had higher Zn excretion compared to T1 and T2. Pigs in T3 excreted 42 and 11 % more Cu compared to T1 and T2 respectively. Pigs in T2 excreted 27 % more Zn than T1. Although there was a big difference in excretion between T3 and T1 it was not a treatment but a block effect. Retention of Zn was also variable from day to day. On average pigs in T3 had the higher retention of Zn, followed by T1 and T2. On average pigs in T3 had 8 and 9 % higher Zn retention than pigs in T1 and T2 respectively. Treatment 2 had the lower Zn retention. Treatment 1 had 1% more Zn retention than T2. All treatments had a positive balance of Zn. The differences were not statistically significant.

Table 9. Zinc consumption in baby pigs supplemented with dietary organic Cr.

	T1 [a]		T2 [b]		T3 [c]	
Day	Total	mg/kg BW	Total	mg/kg BW	Total	mg/kg BW
Day 1	**25.32**	**4.29**	**38.70**	**6.08**	**34.43**	**5.71**
Day 2	**35.37**	**5.98**	**49.69**	**7.90**	**47.50**	**7.96**
Day 3	**38.63**	**6.47**	**47.44**	**7.69**	**45.29**	**7.68**
Day 4	**40.75**	**6.80**	**41.77**	**6.93**	**44.25**	**7.36**
Day 5	**38.29**	**6.35**	**38.41**	**6.47**	**47.20**	**7.86**
Day 6	**42.45**	**6.09**	**48.16**	**6.73**	**54.03**	**7.52**
Day 7	**46.97**	**6.65**	**53.18**	**7.36**	**57.37**	**7.93**
Day 8	**52.36**	**7.38**	**58.10**	**7.98**	**59.91**	**8.46**
Day 9	**55.04**	**7.66**	**58.55**	**8.06**	**71.43**	**10.04**
Day 10	**56.44**	**8.04**	**63.18**	**8.65**	**77.95**	**10.94**
$\bar{x}$	**43.16**	**6.57**	**49.72**	**7.38**	**53.94**	**8.15**

[a] Basal diet

[b] Basal diet with 0.50 ppm supplemental Cr

[c] Basal diet with 1 ppm supplemental Cr

The data is an average of 4 pigs for treatment

Total is expressed as mg of Zn

Table 10. Zinc excretion in baby pigs supplemented with dietary organic Cr.

	T1 [a]		T2 [b]		T3 [c]	
Day	Total	mg/kg BW	Total	mg/kg BW	Total	mg/kg BW
Day 1	**15.01**	**2.48**	**15.65**	**2.34**	**13.74**	**2.20**
Day 2	20.49	3.37	17.58	2.78	22.54	3.82
Day 3	**14.61**	**2.44**	**20.59**	**3.25**	**30.02**	**4.97**
Day 4	16.93	2.79	26.57	4.36	30.57	5.08
Day 5	**25.11**	**4.24**	**22.05**	**3.71**	**25.16**	**4.24**
Day 6	24.91	3.57	22.45	3.18	27.95	3.92
Day 7	**22.42**	**3.16**	**33.21**	**4.68**	**42.79**	**6.02**
Day 8	22.96	3.24	32.56	4.52	32.02	4.47
Day 9	24.17	3.44	30.49	4.19	26.14	3.69
Day 10	18.08	2.58	48.30	6.85	42.70	6.01
$\bar{x}$	20.47	3.13	26.95	3.99	29.36	4.44

[a] Basal diet

[b] Basal diet with 0.50 ppm supplemental Cr

[c] Basal diet with 1 ppm supplemental Cr

The data is an average of 4 pigs for treatment

Total is expressed as mg of Zn

Table 11. Zinc retention in baby pigs supplemented with dietary organic Cr.

	T1 [a]		T2 [b]		T3 [c]	
Day	Total	mg/kg BW	Total	mg/kg BW	Total	mg/kg BW
Day 1	**10.31**	**1.80**	**23.05**	**3.74**	**20.69**	**3.50**
Day 2	14.88	2.61	32.11	5.12	24.96	4.15
Day 3	**24.01**	**4.04**	**26.85**	**4.43**	**15.26**	**2.71**
Day 4	23.82	4.01	15.20	2.57	13.68	2.27
Day 5	13.17	2.11	16.36	2.76	22.03	3.63
Day 6	17.55	2.51	25.71	3.56	26.08	3.60
Day 7	24.56	3.49	19.97	2.68	14.58	1.91
Day 8	29.41	4.14	25.55	3.45	27.90	3.99
Day 9	**30.87**	**4.23**	**28.06**	**3.87**	**45.29**	**6.35**
Day 10	35.94	5.46	36.28	1.80	48.70	4.93
$\bar{x}$	**22.45**	**3.44**	**29.41**	**3.40**	**25.92**	**3.70**

[a] Basal diet

[b] Basal diet with 0.50 ppm supplemental Cr

[c] Basal diet with 1 ppm supplemental Cr

The data is an average of 4 pigs for treatment

Total is expressed as mg of Zn

Experiment Two

This experiment was conducted to evaluate the effects of supplemental dietary chromium, and chromium x copper interaction on performance and immune response of baby pigs reared in a crowded nursery environment. Two trials using forty eight pigs per trial were conducted. In the first trial chromium was supplied only in the diet. In the second trial for the first four days 200 μg of Cr (cofactor III) dissolved in 5 cc of deionized water or 5 cc of deionized water was orally administered daily. Pigs in the control diet and with copper supplementation developed diarrhea by day 5 but not the pigs supplemented Cr. The diarrhea problem lasted a week longer in pigs with supplemented Cu compared with the control. No sign of other diseases or death losses were experienced.

Performance

The main effects of Cr supplementation on average daily gain (ADG), average daily feed intake (ADFI), and feed/gain ratio (FGR) are presented in Tables 11, 12, and 13 respectively. Initial and weekly weight is presented in Table 27, in the appendix. There were no statistically significant effects for Cr or Cr x Cu interactions for any of the response criteria ($P<0.10$); therefore main effects are discussed.

Average daily gain (ADG)

In trial 1 in the first week all treatments had similar ADG, however in the second trial ADG was suppressed in pigs receiving 200 μg of oral Cr but not in pigs without extra Cr or Cr and Cu supplementation. Average daily gain progressively increased during the duration

of the experiment except for week two in trial 1 when pigs gained less than in the first week. On average, for the 35 day period in trial 1, pigs receiving the Cr supplementation (T2) but no Cu and Cr supplementation (T3) had the overall better ADG. These pigs gained 16, 13 and 8% more than T3, T1, and T4 respectively. In the second trial pigs in the control group (T1) had the better ADG but the differences compared to the other treatments were less marked than in the first trial. Pigs in T1 gain 5, 3, and 2 % more than T2, T4 and T3 respectively.

Average daily feed intake (ADFI)

Average daily feed intake progressively increased during the course of the experiment. The ADFI was also greater for pigs on T2 in trial 1 except for week 1 where T4 had a better consumption. In the whole period these pigs consume 16% more feed than T3 and 13% more than T1 and T4, respectively. However in trial 2 pigs in T3 consume more feed than T1, T2 and T4. Again the differences between treatment in this trial were less marked and were less than 5 %.

Feed gain ratio (FGR)

The FGR in trial 1 was better for T2 in the first 14 days, however from 14 to 35 days T4 had the best ratio. With the overall trial T4 had a better numerical FGR and there were no numerical differences between T1, T2 and T3. In the second trial during the first week T2 had the worse FGR and T4 the best, but during the trial T2 recovered and finally had a better FGR than T4 and T3. On average T1 had the best FGR followed by T2. Treatment 4 was the worse. All differences were not statistically significant.

Table 12. Average daily gain in grams

	TRIAL 1				TRIAL 2[e]			
Day	**T1[a]**	**T2[b]**	**T3[c]**	**T4[d]**	**T1**	**T2**	**T3**	**T4**
0-7	**189**	**207**	**187**	**194**	**91**	**59**	**107**	**128**
7-14	142	176	172	148	214	213	236	233
0-14	**164**	**191**	**179**	**169**	**152**	**136**	**172**	**180**
14-21	316	358	280	326	266	285	271	306
0-21	217	249	214	224	190	185	205	222
21-28	468	493	412	476	412	395	380	356
0-28	282	312	266	289	246	238	248	256
28-35	528	622	556	568	578	532	537	485
0-35	333	376	325	347	312	297	306	302

[a] Basal diet 0 ppm Cr, 0 ppm Cu supplementation

[b] Basal diet with 0.50 ppm Cr and 0 ppm Cu supplementation

[c] Basal diet with 0.50 ppm Cr and 250 ppm Cu supplementation

[d] Basal diet with 0 ppm Cr and 250 ppm Cu supplementation

Values per treatment are the average of 12 animals

[e]Oral dose of 200μg Cr

Table 13. Average daily feed intake in grams

	TRIAL 1				TRIAL 2[e]			
Day	T1[a]	T2[b]	T3[c]	T4[d]	T1	T2	T3	T4
0-7	220	241	240	255	154	122	167	173
7-14	312	345	337	309	395	385	454	485
0-14	270	297	293	284	274	254	310	329
14-21	506	612	492	482	506	502	522	576
0-21	353	407	363	353	351	337	381	411
21-28	809	892	715	790	703	699	761	696
0-28	471	533	454	466	439	427	476	482
28-35	1052	1185	1030	1072	1051	1011	1002	954
0-35	591	667	573	591	562	544	581	577

[a] Basal diet 0 ppm Cr, 0 ppm Cu supplementation

[b] Basal diet with 0.50 ppm Cr and 0 ppm Cu supplementation

[c] Basal diet with 0.50 ppm Cr and 250 ppm Cu supplementation

[d] Basal diet with 0 ppm Cr and 250 ppm Cu supplementation

Values per treatment are the average of 12 animals

[e]Oral dose of 200μg Cr

Table 14. Average daily feed gain ratio.

	TRIAL 1				TRIAL 2[e]			
Day	T1[a]	T2[b]	T3[c]	T4[d]	T1	T2	T3	T4
0-7	1.20	1.17	1.31	1.32	1.83	2.45	1.98	1.45
7-14	2.33	1.96	2.00	2.11	1.92	1.81	1.94	2.07
0-14	1.65	1.56	1.67	1.69	1.89	1.87	1.83	1.84
14-21	1.61	1.71	1.86	1.48	1.99	1.80	1.95	1.91
0-21	1.62	1.64	1.69	1.58	1.85	1.82	1.86	1.85
21-28	1.73	1.81	1.74	1.66	1.72	1.80	2.00	1.98
0-28	1.67	1.71	1.71	1.62	1.81	1.81	1.92	1.91
28-35	2.00	1.90	1.87	1.89	1.81	1.90	1.86	1.99
0-35	1.77	1.77	1.76	1.71	1.81	1.84	1.90	1.93

[a] Basal diet 0 ppm Cr, 0 ppm Cu supplementation

[b] Basal diet with 0.50 ppm Cr and 0 ppm Cu supplementation

[c] Basal diet with 0.50 ppm Cr and 250 ppm Cu supplementation

[d] Basal diet with 0 ppm Cr and 250 ppm Cu supplementation

Values per treatment are the average of 12 animals

[e]Oral dose of 200μg Cr

Immunological response

The immunological response of the pig was measured as the ability of lymphocytes isolated from peripheral blood to proliferate when stimulated by mitogens and the phagocytic activity and microbicidal ability of the neutrophils. The proliferative responses for nonstimulated and concanavalin A, phytohemagglutinin, pokeweed mitogen, and lipopolysaccharide stimulated lymphocytes are presented in Tables 24, 25, 26, and 27 of the appendix. The stimulation index calculated as the ratio between mitogen stimulated and non stimulated cells are presented on Tables 15,16, and 17. The stimulation index for day 0 was used as a covariate.

ConA mitogen: Table 15 summarizes the effect of Cr or Cr x Cu effects on Con A mitogen stimulation of lymphocytes. There were no effects of Cr or Cr x Cu interaction between treatments except for day 28 in trial 1 and 2. In trial 1 the animals receiving 250 ppm of supplemental Cu had a significantly ($P<0.02$) better response than pigs receiving Cr, Cr and Cu or no supplement. In the same period there were no differences between T1, T2, and T3. However in trial 2 in the same period pigs receiving Cr and Cu supplementation had a significantly higher response ($P<.04$) than T1, T2 or T3. In both trial there was a tendency for a higher response for pigs with 250 ppm of Cu supplementation alone.

PHA mitogen: The response of lymphocyte to phytohemagglutinin mitogen stimulation is presented on table 16. There were no significant differences between treatment in both trials. However there was a tendency for pigs in T2 and T4 to have a greater response.

PWM and LPS mitogen. The effect of Cr and Cr x Cu effects on lymphocytes proliferation stimulated by PWM and LPS are presented on Table 16. In trial 1 the mitogen PWM, a "b" cell mitogen was used. There were no effect between treatment except for day 21, where pigs in T3 and T4 had a significantly ($P<.04$) greater response compared to the control group (T1) or pigs receiving only Cr supplementation (T3). In the overall period animals with copper supplementation had the highest response although the differences were not significant. In trial 2 LPS mitogen was used and there were no statistically significative differences. The stimulation index were similar for all treatments.

Phagocytic activity and microbicidal ability of the polymorphonuclear cells.

The phagocytic and microbicidal activities of the polymorphonuclear cells isolated from the peripheral blood are presented on Tables 17 and 18. The phagocytic ability of PMN as measured by the number of neutrophil that phagocytize two or more yeast cells per 100 PMN was un affected by Cr or Cr x Cu supplementation in both trials along the experimental period. However, animals in treatment 4 tended to have a higher activity. The microbicidal activity as measured by the number PMN that had two or more dead yeast cell per 100 PMN was also unaffected. In the first trial all treatment had similar values, however in the second trial those pigs receiving 250 ppm supplemental chromium had a higher microbicidal ability compared to the other treatments.

Table 15. Stimulation index [e] of cells stimulated by concanavalin A.

	TRIAL 1				TRIAL 2[e]			
Day	T1[a]	T2[b]	T3[c]	T4[d]	T1	T2	T3	T4
Day 0	0.92	1.29	0.98	1.27	1.34	1.33	1.48	1.25
Day 7	1.08	1.55	1.59	1.90	1.49	1.43	1.42	1.48
Day 14	1.60	1.09	1.10	1.56	0.74	0.98	0.82	0.98
Day 21	1.15	1.15	0.85	1.00	0.78	0.95	0.68	1.10
Day 28	1.31	1.18	1.05	1.87	0.94	1.22	1.53	1.24
Day 35	1.54	1.69	1.70	1.79	0.80	1.33	0.99	1.53
$\bar{x}$	1.33	1.33	1.25	1.62	.95	1.18	1.09	1.27

[a] Basal diet 0 ppm Cr, 0 ppm Cu supplementation

[b] Basal diet with 0.50 ppm Cr and 0 ppm Cu supplementation

[c] Basal diet with 0.50 ppm Cr and 250 ppm Cu supplementation

[d] Basal diet with 0 ppm Cr and 250 ppm Cu supplementation

e Ratio between thymidine uptake value by stimulated and nonstimulated cells. Values are the $\log_{10}$ of count per minutes.

Values per treatment are the average of 12 animals

[e]Oral dose of 200μg Cr

Table 16. Stimulation index [e] of cells stimulated by phytohemagglutinin.

	TRIAL 1				TRIAL 2[e]			
Day	T1[a]	T2[b]	T3[c]	T4[d]	T1	T2	T3	T4
Day 0	1.12	1.68	1.80	1.64	1.71	1.66	1.74	1.72
Day 7	1.91	1.94	2.08	1.95	1.46	1.39	1.45	1.47
Day 14	1.66	2.03	1.80	1.80	1.55	1.47	1.34	1.32
Day 21	1.60	1.84	1.81	1.92	1.35	1.38	1.38	1.40
Day 28	2.09	1.89	2.01	2.11	1.71	1.62	1.48	1.52
Day 35	1.81	1.86	1.69	1.82	1.42	1.39	1.18	1.48
$\bar{x}$	1.81	1.91	1.88	1.92	1.50	1.45	1.36	1.44

[a] Basal diet 0 ppm Cr, 0 ppm Cu supplementation

[b] Basal diet with 0.50 ppm Cr and 0 ppm Cu supplementation

[c] Basal diet with 0.50 ppm Cr and 250 ppm Cu supplementation

[d] Basal diet with 0 ppm Cr and 250 ppm Cu supplementation

e Ratio between thymidine uptake value by stimulated and nonstimulated cells. Values are the $\log_{10}$ of count per minutes.

Values per treatment are the average of 12 animals

[e]Oral dose of 200μg Cr

Table 17. Stimulation index [e] of cells stimulated by pokeweed mitogen.

	TRIAL 1				TRIAL 2[e]			
Day	T1[a]	T2[b]	T3[c]	T4[d]	T1	T2	T3	T4
Day 0	1.02	1.64	1.43	1.53	1.08	1.33	1.34	1.38
Day 7	1.40	1.61	1.82	1.96	1.21	1.23	1.23	1.27
Day 14	1.58	1.50	1.66	1.85	1.18	1.16	1.07	1.03
Day 21	1.49	1.45	1.78	1.91	1.08	1.21	1.13	1.14
Day 28	1.46	1.56	1.47	1.90	1.33	1.34	1.19	1.25
Day 35	1.50	1.77	1.81	1.96	1.21	1.20	1.24	1.29
$\bar{x}$	1.49	1.58	1.71	1.92	1.20	1.23	1.17	1.20

[a] Basal diet 0 ppm Cr, 0 ppm Cu supplementation

[b] Basal diet with 0.50 ppm Cr and 0 ppm Cu supplementation

[c] Basal diet with 0.50 ppm Cr and 250 ppm Cu supplementation

[d] Basal diet with 0 ppm Cr and 250 ppm Cu supplementation

[e] Ratio between thymidine uptake value by stimulated and nonstimulated cells. Values are the $\log_{10}$ of count per minutes.

[e]Oral dose of 200μg Cr.

Table 18 . Phagocytic activity of the neutrophil.

	TRIAL 1				TRIAL 2[e]			
Day	T1[a]	T2[b]	T3[c]	T4[d]	T1	T2	T3	T4
Day 0	62	60	60	59	48	51	43	59
Day 7	72	70	72	71	53	47	45	57
Day 14	70	68	72	70	59	56	52	61
Day 21	68	69	71	69	70	63	65	75
Day 28	71	73	75	74	68	65	63	70
Day 35	72	71	72	67	75	70	68	73
$\bar{x}$	71	70	72	70	65	60	60	67

[a] Basal diet 0 ppm Cr, 0 ppm Cu supplementation

[b] Basal diet with 0.50 ppm Cr and 0 ppm Cu supplementation

[c] Basal diet with 0.50 ppm Cr and 250 ppm Cu supplementation

[d] Basal diet with 0 ppm Cr and 250 ppm Cu supplementation

Values per treatment are the average of 12 animals

[e]Oral dose of 200μg Cr.

Table 19 . Microbicidal ability of the neutrophil.

	TRIAL 1				TRIAL 2[e]			
Day	**T1[a]**	**T2[b]**	**T3[c]**	**T4[d]**	**T1**	**T2**	**T3**	**T4**
Day 0	**32**	**22**	**29**	**28**	**25**	**19**	**21**	**28**
Day 7	38	30	41	35	28	27	26	25
Day 14	**31**	**40**	**28**	**38**	**26**	**22**	**23**	**36**
Day 21	40	27	35	33	41	27	31	41
Day 28	**39**	**40**	**38**	**41**	**37**	**33**	**35**	**38**
Day 35	43	35	41	36	45	35	37	39
$\bar{x}$	38	34	37	37	35	29	35	36

[a] Basal diet 0 ppm Cr, 0 ppm Cu supplementation

[b] Basal diet with 0.50 ppm Cr and 0 ppm Cu supplementation

[c] Basal diet with 0.50 ppm Cr and 250 ppm Cu supplementation

[d] Basal diet with 0 ppm Cr and 250 ppm Cu supplementation

Values per treatment are the average of 12 animals

[e]Oral dose of 200μg Cr.

DISCUSSION

Experiment one

Weaning is certainly a stressful event for domestic animals. Piglets are separated from the sow, handled extensively, commingled with unfamiliar pigs and shifted from a liquid to a solid diet. In this study pigs were weaned at 21 days and housed individually in pens where they do not see each other. This certainly increased the level of stress. They were highly agitated, jumping all the time trying to escape. When animals are stressed the level of plasma cortisol increases (Rampacek et al., 1984; Becker et al., 1985) and is correlated with suppressed immune function in pigs (Westly and Kelley, 1984). Peripheral concentrations of this adrenal cortical hormone is an acceptable indicator of stress in swine (Strot, 1981). In this study the level of plasma cortisol was not measured, however it has been well documented in pigs that weaning, frustration, penning, mixing and handling will cause a significantly increase in plasma cortisol concentration (Arnone, 1980; Dantzer et al., 1980; Hemsworth et al., 1981; Barnett et al., 1981; Parrot and Misson, 1989; Roth, 1992; Burbolla et al., 1995; Ekkel et al., 1995). All these variables were present in this study. When animals are under stress simultaneously with an increase in cortisol, there is an increase in glucose metabolism. Cortisol acts antagonistically to insulin because it prevents entry of glucose into peripheral tissue to save it for tissues of higher demand, like brain (Burton, 1995). This results in a mobilization of Cr body stores. Once mobilized, Cr is irreversible lost in urine (Mertz, 1993). So if the diet has an inadequate Cr concentration, a deficiency may occur. An adequate balance of nutrients is very important since an excess or deficiency in one component may

influence the availability or requirements for another (Roth, 1992). Studies with humans and mice have indicated that various stressors due to infection, strenuous exercise, or trauma may have marked effects on glucose metabolism and increased loss of Cr in urine (Pekarek et al., 1975; Borel et al., 1984). Adrenocorticosteroid hormones can also affect urinary excretion of Cu. Patients with adrenocorticosteroid insufficiency exhibit significantly lower urinary Cu output than control, and normal patients given adrenocorticotropic hormone decreased their serum concentration and increase their urinary Cu excretion (Davis and Mertz, 1987). Orr et al. (1990) reported that in stressed cattle the excretion of Zn and Cu increase. A direct relationship between plasma cortisol and urinary excretion of Cu and Zn had been reported in humans and cats (Henkin, 1974). In food producing animals when these minerals are supplemented, the animals performance may improve (Nockels, 1990, 1995). In research with mice, Schrauzer et al. (1986) suggested that supplemental Cr may prevent stress induced losses of several microminerals including Cu, Zn, manganese, and iron. The results of this study did not support this theory in pigs. We found that a supplementation of 0.5 or 1 ppm of Cr to a basal diet already containing 1.54 ppm of Cr did not reduce excretion or improve the retention of either Cu or Zn. Indeed, animals without Cr supplementation, although they consumed less Cu per kg of body weight had a greater numerical retention of Cu compared to those animals receiving 0.5 or 1 ppm of supplemental Cr. There was a linear reduction in Cu retention as the level of Cr increased. Besides, pigs in the basal diet had a positive Cu balance during the study, compared to one and two days of negative Cu balance for those pigs receiving 0.5 and 1 ppm of supplemental Cr respectively.

Okonkowo et al. (1979) using a casein-glucose purified diet for 45 day-pigs, concluded that 6 ppm of Cu provides an appropriate base for setting Cu requirements for minimal adequate values of : average daily gain 200 g; hemoglobin 1.5 g/dl; plasma ceruloplasmin activity 0.04 ΔOD_{540}/minute; plasma Cu 100 mg/dl; Cu balance 2 mg/d. Hill et al. (1983) also found that 5 ppm Cu was adequate for producing normal weight and blood and tissue parameters of young pigs fed a casein-glucose diet.

In this study the feed concentration by analysis of Cu and Zn was 22 and 176 ppm respectively. The Cu retention was 1.45, 1.42 and 1.17 mg/day for T1, T2 and T3 respectively. These numbers represent only 27, 25 and 19 % of the copper consumption. The results reported in this study are lower than those reported by Okonkowo et al. (1979). In Okonkowo's study, pigs receiving 6 ppm Cu diet had a retention of 2.87 mg/day representing 49% of consumption.

When animals are under stress there is a substantial increase in Cu and Zn metabolism. There is a substantially increase of ceruloplasmin and metallothionein synthesis designed to cope with oxidant challenge induced by stress, since both these proteins can act as free radical scavengers (Bremmer and Beattie, 1995). Glucocorticoid, cytokines such as the interleukins and tumor necrosis factors, and other stress factors can induce metallothionein synthesis principally in the liver but also in other tissues (Bremmer, 1987). Blalock et al. (1988) found that at low Cu intake levels, Zn is a more potent stimulator of metallothionein gene expression in the small intestine than at higher Cu intake levels. Perhaps a combination of stress and a relatively high Zn concentration compared to Cu, caused an increase in metallothionein synthesis in the intestine, binding and preventing more Cu

absorption. This could be the reason for the lower Cu retention in this study as compared to Okonkowo's findings. Its possible that for stressed baby pigs, receiving a corn soybean diet, 22 ppm of dietary Cu is not sufficient to meet requirement.

In this study Zn retention was not also affected by Cr supplementation. The Zn retention per kilogram of body weight was 3.44. 3.40, and 3.70 mg for T1, T2 and T3 respectively. These number represents 52, 46 and 45% of consumption. These results are in agreement with those reported by Forbes and Yohe (1960), Smith et al. (1960) and O'Dell and Savage (1957), but not with Lei et al. (1993) who reported a negative retention of -46% of consumption.

In summary, 0.50 or 1.00 ppm of dietary chromium supplementation to a diet containing 1.54 ppm of chromium as provided from high chromium yeast had no apparent effect on copper or zinc retention of stressed baby pigs. It is also possible that for stressed baby pigs, receiving a corn soybean diet, 22 ppm of copper is not sufficient to meet requirements.

Experiment two

Performance: Early weaned pigs usually suffer a significant setback at weaning characterized by a growth depression, associated with low voluntary feed intake (post weaning lag), primarily due to a change to a dry diet and the accompanying stress (Fowler and Gill, 1989). Stress represents the reaction of the body to any stimuli, that disturbs its normal physiological equilibrium or homeostasis, often with detrimental effects (Khansari et al., 1990). In order to alleviate this post weaning lag several approaches have been tried including the addition of antibiotics and spray dried porcine plasma to the post weaning diet.

According to Mowat (1994) antibiotics may reduce certain stress symptoms, and the administration of antibiotics to chickens reared in a dirty environment decreased immunologic stress by reducing plasma interleukin-1 levels resulting in improved growth (Klasing and Roura, 1991). Spray dried porcine plasma has been shown to stimulate high feed intake and average daily gain in the first two weeks after weaning (Owen et al., 1995; Zimmermann, 1987; Gatnau and Zimmermann, 1990) reducing the post weaning lag. However, there is concern about the feeding of antibiotics to animals because of the potential hazard of developing of antibiotic resistant pathogens, which may constitute a human health problem. Also, spray dried porcine plasma is a very expensive product to feed for very long. So other promising ways of reducing this post weaning lag should be investigated.

It has been demonstrated that Cr supplementation in beef cattle can increase weight gain and prevent or reduce depression in dry matter intake and in some cases even increase feed intake (Moonsie-Shaeger and Mowat, 1993; Wright et al., 1994). In another study a decreased body temperature by 0.5°C and a lower serum cortisol level in growing steers was reported when their diet was supplemented with dietary high Cr yeast. An increase in the 21 day rate of gain has also been reported in turkey poults when their diet was supplemented with Cr chloride (Steeele and Rosebrough, 1981). In this study, 0.50 ppm of dietary Cr supplementation did not appear to help baby pigs cope with post weaning lag. In the first trial although pigs receiving the supplemental Cr diet had a greater numerical average daily gain during the first 7 days and also for the duration of the trial this differences was not significant compared to the nonsupplemented animals or with the combination of Cr and 250 ppm of Cu. In the second trial when an additional 200μg of Cr was administered as an oral suspension

for four days average daily gain was drastically reduced. When the additional Cr supplementation was stopped the animals recovered. The results of this study is in agreement with the results of van Heughen and Spears (1994) who found that, although Cr supplementation increased ADG and ADFI in comparison to control pigs it was not effective in alleviating a reduction in performance due to stress caused by lipopolysaccharide injection.

The expected ADG, ADFI and F/G ratio for pigs between 5-10 and 10-20 Kg according to NRC (1988) is: ADG 250 g/d and 450 g/d; ADFI 460 g/d and 950 g/d and F/G ratio 1.84 and 2.11 respectively. However, restricted or inadequate floor space allowance will decrease daily gains and feed intake of all classes of pigs, but is more pronounced in weanling than in growing-finishing pigs (Kornegay and Notler, 1984; Petheric, 1983). In this study the provided floor space ranged from 6 to 59% less than Pork Industry Handbook recommendation (1987). The data of this study shows that Cr or Cu supplementation to weanling pigs reared in a crowded nursery environment is ineffective in alleviating the reduction in performance due to a reduced floor space allowance as assessed by ADG and ADFI. Compared to the expected values of NRC these pigs had a 30% and 37% less ADG and ADFI respectively. On the other hand the F/G ratio was 14% better than expected NRC values. Our results are in agreement with Kornegay et al. (1993).

It was surprising that the animals consuming the diet with 250 ppm of Cu did not exhibit a greater response in ADG and F/G ratio compared to those receiving only 26 ppm of Cu. This lack of beneficial response to supplemental Cu does not agree with a majority of reports in the literature as summarized by Bowland (1990) nor do the results agree with more recent studies by Apgar et al. (1995), Zhou et al. (1994) and Dove (1995) in which positive

growth responses and feed gain ratio improvement were obtained when supplemental Cu was added to the diet. However, not all experiments in which 125 to 250 ppm of supplemental Cu was added to the diet resulted in positive results. Indeed some researchers have reported no effect of Cu supplementation and some have reported a reduction in performance from high levels of Cu supplementation (Wallace et al., 1968; Parris and McDonald, 1968; Livingston and Livingstone, 1968; Gipp et al., 1973; Bradley, 1983).

The exact mechanism by which Cu stimulates growth and feed efficiency in pigs is not fully understood. Some researchers postulate that this effect is primarily due to its bacteriostatic properties (which have microbicidal effects) in the intestinal tract, similar to antibiotics (Hawbaker et al., 1961; Miller, 1991). On the other hand, responses to antibiotics generally are minimal when tested in a clean environment (Cromwell, 1991). In this study pigs were reared in a very clean nursery environment isolated from other pigs. So the lack of a beneficial response in performance compared to other studies may be explained by the cleaner conditions and a more controlled environment than the conventional nursery.

Hemoglobin and hematocrit of pigs in all treatments in this study were within normal ranges found in young pigs. This would suggest that the addition of Cr did not have an effect upon whole blood parameters.

Pigs receiving supplemental Cr seem to have a lower incidence of diarrhea compared to the other treatments. It has been reported that Cr compounds inhibit the growth of microorganisms by interaction between Cr and the microbial DNA and also by the inhibition of DNA polymerase activity (Ogawa et al., 1989). The etiology of the diarrhea in this study was not established, however if it was caused by a bacterial infection then maybe Cr

supplementation inhibited microbial growth in the intestines of those pigs. Perhaps this could explain the lower incidence of diarrhea.

Immunological Response: Several social and environmental factors stress animals and alter their resistance to disease. There is ample evidence that both physical and psychological distress can suppress the immune function in animals leading to an increased incidence of infectious disease (Roth, 1992). Its has been reported that crowding and restraint will stress pigs sufficiently to decrease their immune responsiveness. In a 28 days trial, housing eight pigs (11.5-18.0 kg) per group in pens with 0.13 m^2 of floor space per pig significantly reduced their phytohemagglutinin skin test response as compared with pigs given twice as much space (Yen and Pond, 1987). When young pigs were restrained by placing them in a 13 X 33 cm expanded metal box for two hours per day over a three day period, they had significantly elevated plasma cortisol concentration which correlated with a decrease in the size of their thymus gland and a reduction in the phytohemagglutinin skin test response (Westly and Kelly, 1984). Another report indicated that tethering of sows suppressed antibody synthesis to sheep red blood cell. It also resulted in a reduction in the amount of antigen specific antibodies that were transmitted through the colostrum into the blood of the piglets (Kelley, 1985).

One way to cope with these situations may be to improve the host defense mechanism by boosting the immune system through the administration of immunomodulators. Applications of immunostimulants seems promising as a means for the prevention or treatment of various infections (Araki et al., 1993). In cattle it has been shown that Cr behaves as an immunomodulator (Burton, 1995). Chang and Mowat (1992) reported that

supplemental Cr to beef cattle increased serum immunoglobulin levels during the growing period. In another study, antibody production against human erythrocytes was increased when Cr was supplemented during a stress period to weaned calves (Moonsie-Shaeger and Mowat, 1993), and levels of antibody increased following vaccination (Burton et al., 1994). During early lactation in dairy cows in which their diet was supplemented with chelated Cr, a higher level of antibodies and a better in vitro blastogenesis response was observed when the cows were challenged with a T cell mitogen (Burton et al., 1993). In other species it appears that Cr also has some effect on the immune system. When stressed rats were supplemented with dietary Cr mortality was reduced (Mertz and Roginski, 1969), and Shroeder et al. (1965) reported that Cr supplementation reduced mortality from an epidemic of pneumonia in female rats.

A common way of evaluating the influence of various factors on the immune system is to measure the response of isolated peripheral blood lymphocytes to in vitro mitogen stimulation. In the present study, to determine if Cr had any immunomodulator effect in the cell mediated immune response of weanling pigs, we used phytohemagglutinin and concanavalin A, two potent T cell mitogen and pokeweed mitogen and lipopolysaccharide that are considered B cell mitogen. In this means we could evaluate the components of the intrinsic immune system.

We found that dietary Cr supplementation to pigs reared in a crowded nursery environment was ineffective in enhancing lymphocyte proliferation in the presence of optimal concentration of all mitogen used. In a study with baby pigs, Van Heusken et al. (1994) reports an enhanced lymphocyte proliferation in response to mitogen when Cr was

supplemented in the diet of nonstressed baby pigs, but there was no response in stressed baby pigs. He also found that in vivo cellular immunity was not affected by Cr supplementation. The addition of supplemental Cu, although tended to have a higher response, also failed to influence a significant lymphocyte proliferation response to mitogen stimulation. These results are consistent with the study of Arthington et al. (1995), who found no effect of Cu and Zn supplementation on lymphocyte proliferation when stimulated by a mitogen.

In the first trial of this study there was an increase in mitogen induced lymphocyte proliferation with increasing age for all the mitogen used. Similar results were found by Hoskinson et al. (1990). However, in the second trial the proliferation was higher in the first week and then tended to decrease with increasing age.

Neutrophils play a key role as part of the immune response to microbial infections. Their major function is to rapidly kill bacteria and fungi before they multiply and spread throughout the body. Nutritional deficiencies can affect the ability of neutrophils to phagocytize and kill invading organisms. Sows receiving a selenium or vitamin E deficient diet have a decreased phagocytic and microbicidal ability compared to those receiving a nondeficient diet (Wuryastuti, et al., 1993). Guinea pigs with a deficiency of vitamin C have a reduction in the mobility and phagocytic activity of neutrophils (Ganguly et al., 1976) which was restored upon vitamin C supplementation. In this study we found that Cr or Cu supplementation to a diet already containing 1.54 and 26 ppm of Cr and Cu respectively had no effect on the phagocytic activity and the microbicidal ability of neutrophils of baby pigs. On average all treatments showed an increase in the phagocytic activity and microbicidal ability with increasing age. The measured values of phagocytic activity reported in this study

are higher than those reported by Araki et al. (1994) in pigs of the same age, however a direct comparison of our results with those of Araki et al. are difficult because of different procedures. Our values for phagocytic activity and microbicidal ability are lower than the values reported by Wuryastuty et al. (1993) for sows. These differences may be due to a difference in age. It has been reported that polymorphonuclear neutrophils have a decreased bactericidal abilities in neonates compared with adult animals. In a study with horses, Coignoul et al. (1984) found a significant reduction in myeloperoxidase-hydrogen peroxide-halide activity by blood PMN in foals compared with mares. The myeloperoxidase hydrogen peroxide system is extremely potent in the killing of a wide range of target cells and in the inactivation of biological molecules of microbes. Similarly Hauser et al. (1986) reported that iodination activity was lower in 4 to 5 week old than in 12 to 14 month old calves. In another study blood PMN from calves also have deficient superoxide anion generation, another important route in the neutrophil microbicidal system (Zwahlen et al., 1987).

IMPLICATIONS

The results of this study indicate that dietary chromium supplementation to a diet containing 1.54 ppm of chromium as provided by high chromium yeast had no apparent effect on Cu or Zn retention of stressed baby pigs. It is also possible that for stressed baby pigs receiving a corn-soybean diet 22 ppm of Cu is not sufficient to provide for requirements. This study also showed that Cr supplementation with or without Cu did not decrease the post weaning lag, affect performance or the immune response of baby pigs reared in a crowded nursery environment. However a better numerical ADG, ADFI and F/G ratio was observed in pigs receiving supplemental Cr and a lower incidence of diarrhea was noted.

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APPENDIX

Table 20. Proximal analysis of the basal diet compared to NRC recommendations.

	Phase 1 Feed		Phase 2 Feed	
Nutrient Name	**Diet Composition**	**NRC Recommendation**	**Diet Composition**	**NRC Recommendation**
Dry Matter %	89.82		89.10	
Crude Protein %	19.64	20.00	20.29	18.00
Crude Fat %	2.79		2.63	
`Crude Fiber %	2.04		2.54	
Calcium %	1.11	0.81	0.75	0.70
Phosphorus T %	0.86	0.65	0.69	0.60
Phosphorus A %.	0.60	0.39	0.39	0.32
Ash %	4.22		2.60	
Swine ME Kcal	3215	3245	3267	3252

Table 21. Amino acid composition[a] of the basal diet compared to NRC.

	Phase 1 Feed		Phase 2 Feed	
Amino Acid	**Diet Composition**	**NRC Recommendation**	**Diet Composition**	**NRC Recommendation**
Lysine	**1.26**	**1.15**	**1.22**	**0.95**
Tryptophan	**0.25**	**0.17**	**0.27**	**0.14**
Threonine	**0.85**	**0.68**	**0.81**	**0.56**
Isoleucine	**0.88**	**0.65**	**0.87**	**0.53**
Methionine + Cysteine	**0.65**	**0.59**	**0.73**	**0.48**
Leucine	**1.79**	**0.85**	**1.87**	**0.70**
Histidine	**0.49**	**0.31**	**0.54**	**0.25**
Valine	**1.02**	**0.68**	**1.06**	**0.56**
Phenylalanine + Tyrosine	**0.90**	**0.94**	**1.01**	**0.77**
Arginine	**1.21**	**0.50**	**1.38**	**0.40**

[a]Estimated

Table 22. Vitamin composition[a] of the basal diet compared to NRC.

	Phase 1 Feed		Phase 2 Feed	
Vitamin	Diet Composition	NRC Recommendation	Diet Composition	NRC Recommendation
Vit A 1000 IU	**5.03**	**2.20**	**4.95**	**1.75**
Vit D 1000 IU	0.99	0.22	0.99	0.20
Vit E 1000 IU	**0.03**	**0.02**	**0.03**	**0.01**
Vit K mg	3.41	0.04	3.43	0.05
Riboflavin mg	**10.5**	**3.51**	**6.54**	**3.00**
Panto. Acid mg	32.97	10.02	27.60	9.00
Niacin mg	**48.05**	**15.03**	**48.15**	**12.51**
Choline mg	1424	501	1325	400
Vit B12 μg	**38.70**	**17.54**	**29.70**	**15.00**
Biotin μg	164.60	43.56	142.10	52.63
Folic Acid μg	**453**	**305**	**407**	**294**
Thiamine mg	3.32	1.00	3.37	1.00
Vit B6, mg	5.14	1.50	5.52	1.50

[a]Estimated

Table 23. Mineral composition[a] of the basal diet compared to NRC.

	Phase 1 Feed		Phase 2 Feed	
Mineral	**Diet Composition**	**NRC Recommendation**	**Diet Composition**	**NRC Recommendation**
Sodium %	0.32	0.11	0.11	0.11
Chloride %	0.45	0.09	0.21	0.08
Potassium %	0.81	0.28	0.86	0.26
Magnesium %	0.15	0.04	0.16	0.04
Manganese mg/Kg	72.46	4.01	74.01	3.00
Iron mg/Kg	189.28	100.20	153.13	80.00
Copper mg/Kg	28.34	6.01	23.25	5.00
Iodine mg/Kg	0.83	0.13	0.78	0.14
Zinc mg/Kg	141.86	100.20	141.81	80.00
Selenium mg/Kg	1.16	0.31	1.07	0.25

[a]by analysis

Tab

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Table 24. [^{3}H]Thymidine uptake by unstimulated cells (CPM)[e].

	TRIAL 1				TRIAL 2			
Day	T1[a]	T2[b]	T3[c]	T4[d]	T1	T2	T3	T4
Day 0	3.08	2.60	2.78	2.67	2.57	2.76	2.70	2.82
Day 7	2.71	2.34	2.23	2.39	3.21	3.47	3.34	3.31
Day 14	2.55	2.33	2.61	2.46	3.29	3.77	4.11	4.11
Day 21	2.65	2.63	2.61	2.42	4.02	3.70	3.84	3.63
Day 28	2.27	2.42	2.47	2.42	2.96	3.15	3.37	3.24
Day 35	2.63	2.48	2.75	2.56	3.21	3.50	3.24	3.23

[a] Basal diet 0 ppm Cr, 0 ppm Cu supplementation

[b] Basal diet with 0.50 ppm Cr and 0 ppm Cu supplementation

[c] Basal diet with 0.50 ppm Cr and 250 ppm Cu supplementation

[d] Basal diet with 0 ppm Cr and 250 ppm Cu supplementation

[e] Ratio between thymidine uptake value by stimulated and non stimulated cells. Values are the $\log_{10}$ of count per minutes.

Table 25. [^{3}H]Thymidine uptake by concanavalin A stimulated cells (CPM)[e].

	TRIAL 1				TRIAL 2			
Day	T1[a]	T2[b]	T3[c]	T4[d]	T1	T2	T3	T4
Day 0	2.84	3.31	2.70	3.37	3.45	3.64	3.96	3.51
Day 7	2.95	3.60	3.56	4.54	4.72	4.94	4.69	4.77
Day 14	4.09	2.55	2.88	3.80	2.44	3.54	3.36	4.00
Day 21	3.08	3.06	2.22	2.41	3.05	3.53	2.60	3.98
Day 28	2.97	2.86	2.54	4.50	2.78	3.88	5.16	4.06
Day 35	4.03	4.20	4.66	4.59	2.57	4.64	3.19	4.90

[a] Basal diet 0 ppm Cr, 0 ppm Cu supplementation

[b] Basal diet with 0.50 ppm Cr and 0 ppm Cu supplementation

[c] Basal diet with 0.50 ppm Cr and 250 ppm Cu supplementation

[d] Basal diet with 0 ppm Cr and 250 ppm Cu supplementation

[e] Ratio between thymidine uptake value by stimulated and non stimulated cells. Values are the $\log_{10}$ of count per minutes.

Table 26. [^{3}H]Thymidine uptake by phytohemagglutinin stimulated cells (CPM)[e].

	TRIAL 1				TRIAL 2			
Day	T1[a]	T2[b]	T3[c]	T4[d]	T1	T2	T3	T4
Day 0	3.42	4.31	4.93	4.40	4.38	4.57	4.70	4.85
Day 7	5.16	4.54	4.64	4.68	4.61	4.76	4.78	4.78
Day 14	4.22	4.73	4.67	4.44	5.10	5.44	5.52	5.41
Day 21	4.20	4.83	4.70	4.67	5.28	5.12	5.26	5.10
Day 28	4.74	4.58	4.89	5.10	5.04	5.09	5.02	4.91
Day 35	4.76	4.60	4.64	4.67	4.55	4.88	3.81	4.73

[a] Basal diet 0 ppm Cr, 0 ppm Cu supplementation

[b] Basal diet with 0.50 ppm Cr and 0 ppm Cu supplementation

[c] Basal diet with 0.50 ppm Cr and 250 ppm Cu supplementation

[d] Basal diet with 0 ppm Cr and 250 ppm Cu supplementation

[e] Ratio between thymidine uptake value by stimulated and non stimulated cells. Values are the $\log_{10}$ of count per minutes.

Table 27. [^{3}H]Thymidine uptake by pokeweed stimulated cells (CPM)[e].

	TRIAL 1				TRIAL 2			
Day	T1[a]	T2[b]	T3[c]	T4[d]	T1	T2	T3	T4
Day 0	3.12	4.20	3.88	4.07	2.75	3.67	3.60	3.90
Day 7	3.83	3.74	4.07	4.70	3.85	4.21	4.06	4.09
Day 14	4.04	3.50	4.33	4.54	3.87	4.34	4.37	4.24
Day 21	3.95	3.83	4.64	4.63	4.17	4.50	4.33	4.15
Day 28	3.31	3.77	3.63	4.61	3.92	4.21	4.02	4.04
Day 35	3.94	4.39	4.99	5.02	3.88	4.18	3.99	4.14

[a] Basal diet 0 ppm Cr, 0 ppm Cu supplementation

[b] Basal diet with 0.50 ppm Cr and 0 ppm Cu supplementation

[c] Basal diet with 0.50 ppm Cr and 250 ppm Cu supplementation

[d] Basal diet with 0 ppm Cr and 250 ppm Cu supplementation

[e] Ratio between thymidine uptake value by stimulated and non stimulated cells. Values are the $\log_{10}$ of count per minutes.

Table 28. Total pigs weight.

TRIAL 1					TRIAL 2			
Day	T1[a]	T2[b]	T3[c]	T4[d]	T1	T2	T3	T4
Day 0	7.14	7.87	7.15	7.03	6.29	6.31	6.32	6.28
Day 7	8.28	9.11	8.27	8.20	6.93	6.72	7.07	7.17
Day 14	9.27	10.34	9.47	9.23	8.42	8.21	8.72	8.81
Day 21	11.48	12.85	11.44	11.52	10.28	10.20	10.61	10.95
Day 28	14.76	16.30	14.32	14.85	13.17	12.96	13.27	13.44
Day 35	18.45	20.66	18.22	18.83	17.22	16.69	17.03	16.84

[a] Basal diet 0 ppm Cr, 0 ppm Cu supplementation

[b] Basal diet with 0.50 ppm Cr and 0 ppm Cu supplementation

[c] Basal diet with 0.50 ppm Cr and 250 ppm Cu supplementation

[d] Basal diet with 0 ppm Cr and 250 ppm Cu supplementation