DIETARY CHROMIUM AND COPPER EFFECTS ON PARAMETERS RELATED TO CARDIOVASCULAR DISEASE RISK IN THE BHE/cdb RAT

By

RUTH ANN GIVENS

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Thesis Approved:

Barbara J. Stoecker
Thesis Advisor

Andrea B. Argiratt

Kathryn S. Kemp

Thomas C. Collins
Dean of the Graduate College

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CHAPTER I

RESEARCH PROBLEM

Introduction to Topic

Elevated serum uric acid concentrations, or hyperuricemia, can be caused by impaired renal uric acid excretion or enhanced renal tubular uric acid reabsorption (Voet and Voet 1990). Hyperuricemia is associated with the incidence of obesity, diabetes mellitus, hyperlipidemia, hypertension, and atherosclerosis (Wyngaarden and Kelley 1976). Several studies have found hyperuricemia to independently predict ischemic heart disease and total mortality (Freedman et al. 1995).

Facchini and associates (1991) suggested that insulin resistance decreases renal uric acid clearance which increases serum uric acid concentrations. Hyperuricemia is also related to certain stages of glucose intolerance. Herman and Goldbourt (1982) reported that serum uric acid concentrations were elevated in Impaired Glucose Tolerance (IGT), then began to fall and continued to decrease with the manifestation and progression of Non-Insulin Dependent Diabetes Mellitus (NIDDM).

Insulin seems to inhibit uric acid secretion or enhance uric acid reabsorption at the renal tubular level (Galvan et al. 1995). In addition, glucose competitively inhibits uric acid reabsorption in the proximal tubules of the kidneys in hyperglycemic and glycosuric patients (Herman et al. 1976).

Hyperinsulinemia is a state of abnormally elevated insulin levels in the blood (Zimmet 1993). Hyperinsulinemia is most commonly associated with the insulin resistant state, a fundamental aspect of IGT (Perry et al. 1995). Hyperinsulinemia does not continue throughout the natural progression of NIDDM, though. Once overt diabetes has developed and fasting glucose levels exceed 120 mg/dL, the pancreas decompensates by reducing the secretion of insulin in response to the continued hyperglycemia (DeFronzo 1988). Insulin resistance, characterized by hyperinsulinemia, is now thought to be the underlying metabolic abnormality connecting obesity, hypertension, hyperlipidemia, and glucose intolerance (Vuorinen-Markkola and Yki-Jarvinen 1994).

Chromium is a trace element that appears to increase the activity or efficiency of insulin (Anderson et al. 1991) which improves glucose tolerance. Anderson and colleagues (1991) found that consuming diets low in chromium produced detrimental effects on glucose tolerance, insulin, and glucagon levels in hyperglycemic subjects. Doisy and associates (1976) reported IGT to be one of the first physiological signs of chromium deficiency.

In humans, chromium deficiency has resulted in glucose and lipid abnormalities, neuropathy, and encephalopathy (Anderson 1987). In animals deficient in chromium, impaired growth, decreased longevity, aortic plaques, corneal lesions, and decreased fertility have all been observed (Anderson 1987). In our laboratory, deficient chromium nutriture resulted in elevated serum uric acid concentrations (Stoecker et al. 1996). Chromium deficiency has been hypothesized as a significant risk factor for cardiovascular disease (Schroeder 1968). Because serum lipid levels are affected by glucose tolerance

and insulin sensitivity, adequate chromium nutriture may help maintain normal serum lipids (Riales and Albrink 1981).

Three patients dependent upon total parenteral nutrition for long periods of time were documented as having chromium deficiency symptoms including weight loss, peripheral neuropathy, glucose intolerance, and an increased caloric requirement for weight maintenance (Brown et al. 1986, Freund et al. 1979, Jeejeebhoy et al. 1977). Chromium supplementation generally reversed these observed deficiency symptoms (Anderson 1987, Jeejeebhoy et al. 1977), but the effects of supplemental chromium depend on the degree of deficiency (Mertz et al. 1965) and the degree of glucose intolerance (Anderson et al. 1991). Metabolic stress may intensify chromium deficiency symptoms and increase its essentiality in selected subjects (Nielsen 1988, Stoecker 1995).

Anderson and collegues (1991) found that more than 90% of the self-selected diets in the United States provided less than 50 μg of chromium, the minimum Estimated Safe and Adequate Daily Dietary Intake (ESADDI) suggested by the National Academy of Sciences (Anderson et al. 1991, Food and Nutrition Board 1989).

Copper is a mineral that supports the integrity of the cardiovascular system through elastin and collagen biosynthesis (Davis and Mertz 1987). Many studies have also shown strong effects of copper on the metabolism of lipids and cholesterol (Davis and Mertz 1987). Copper has also been shown to affect immune function, the regulation of glucose metabolism, and play many other physiological roles in the body (Davis and Mertz 1987).

Copper deficiency results in anemia, alterations in iron metabolism, skeletal changes, hair and skin changes, neurologic disease and cardiovascular disorders (O'Dell 1990). Klevay (1980) observed elevated serum uric acid concentrations in response to copper deficiency. Investigators have documented an impairment of glucose metabolism in copper deficient animals and humans (Davis and Mertz 1987). Fields and associates (1983b) injected both insulin and copper separately into streptozotocin-induced diabetic rats to study the affects of copper on glucose utilization. Copper alone did significantly improve IGT, as did insulin; but when insulin and copper were injected together, a stable complex was formed which synergistically increased peripheral insulin binding and decreased insulin degradation (Fields et al. 1983b). In another study, Klevay and associates (1986) found IGT to be the chief manifestation of copper deficiency in two men. Copper sulfate supplementation returned the glucose tolerance to normal. Davis and Mertz (1987) reported that true copper deficiency results in an elevation of serum cholesterol. Allen and Klevay (1980) reported a significant increase of low density lipoprotein cholesterol (LDL), total cholesterol, and triglyceride levels and a significant decrease in high density lipoprotein cholesterol (HDL) in serum of copper deficient rats.

Pennington and colleagues (1986) reported that diets of infants, children, adolescents, adult men and women, and elderly men and women in the United States were all low in copper when compared to their respective ESADDI recommendations.

The BHE/cdb rat is an excellent animal model for studying IGT. This strain is bred to reliably manifest age-related abnormal glucose tolerance and glomerulosclerosis without the usual complications of obesity and hydronephrosis (Berdanier 1991). By 300

days of age, 75% of this strain develops abnormal glucose tolerance, fasting lipemia, premature renal disease, and a fatty liver (Berdanier 1991). With the presence of IGT, the BHE/cdb rat should exhibit hyperinsulinemia and hyperuricemia.

Significance of the Problem

Hyperuricemia and hyperinsulinemia are present in many related physiological and metabolic abnormalities often associated with cardiovascular disease and diabetes mellitus (Facchini et al. 1991, Zimmet 1993). Chromium and copper dietary intakes were found to be low in self-selected diets in the United States (Anderson et al. 1991, Pennington et al. 1986).

Chromium has the potential to prevent or delay the deterioration of glucose tolerance that ultimately develops into NIDDM (Mertz 1993) by potentiating the action of insulin. If self-selected diets are too low in chromium, this benefit will not be observed. In addition, chromium could help lower the resultant hyperinsulinemia associated with the insulin resistant state if chromium potentiates the action of insulin on glucose uptake by peripheral tissues. Lower insulin levels in the blood would also decrease the enhanced tubular reabsorption of uric acid associated with hyperinsulinemia (Galvan et al. 1995). Lower serum levels of both uric acid and insulin could decrease risk of cardiovascular disease.

Klevay (1980) suggested that a metabolic imbalance in regard to zinc and copper is a major factor in the etiology of ischemic heart disease. In one study, copper deficient rats exhibited hyperuricemia, hypercholesterolemia and increased mortality (Klevay 1980). If copper nutriture is adequate, the risk of cardiovascular disease from hyperuricemia and

dyslipidemia could decrease. Copper deficiency adversely affects glucose metabolism which is fundamental to the metabolic abnormalities of diabetes (Davis and Mertz 1987). Copper and insulin together increased peripheral insulin binding (Fields et al. 1983b) which would lessen hyperinsulinemia. Sufficient copper, then, should improve glucose tolerance which could delay the onset of NIDDM. The interaction between deficient copper and chromium should even further exacerbate the expected metabolic changes leading to increased risk of cardiovascular disease and NIDDM.

Objectives

The objectives of this research were to investigate the effects of chromium and copper on serum uric acid concentrations and other physiological parameters in the BHE/cdb rat, an animal model for abnormal glucose tolerance.

The objectives of this study were to:

1. Determine the effect of dietary chromium on selected factors related to cardiovascular disease risk in the BHE/cdb rat.
2. Determine the effect of dietary copper on selected factors related to cardiovascular disease risk in the BHE/cdb rat.
3. Determine the interactive effect of dietary chromium and copper on selected factors related to cardiovascular disease risk in the BHE/cdb rat.

Null Hypotheses

The following hypotheses were developed for this study:

1. There will be no statistically significant effect of dietary chromium on selected factors related to cardiovascular disease risk in the BHE/cdb rat.
2. There will be no statistically significant effect of dietary copper on selected factors related to cardiovascular disease risk in the BHE/cdb rat.
3. There will be no statistically significant interactions between dietary chromium and copper effecting selected factors related to cardiovascular disease risk in the BHE/cdb rat.

Limitations

This study is subject to several limitations which must be identified. Data from animal studies cannot be extrapolated directly to humans. This particular animal study evaluated only the effects of adequate and inadequate intakes of chromium and copper; thus, supplementation effects of these minerals were not studied. In addition, chromium research is subject to particular environmental contamination issues which increase the difficulty of establishing a true mineral deficiency.

Format of Thesis

The tables and bibliographic citations in this thesis follow the guide for authors for the Journal of Nutrition.

CHAPTER II

REVIEW OF THE LITERATURE

This chapter includes a literature review of hyperuricemia, hyperinsulinemia, chromium, and copper and their roles in glucose and lipid metabolism. A discussion of the BHE/cdb rat as an animal model for Impaired Glucose Tolerance (IGT) is also included.

Hyperuricemia

Uric acid is a circulating end product of purine metabolism (Frohlich 1993). Hyperuricemia is associated with impaired uric acid excretion, and thus, elevated serum uric acid concentrations (Voet & Voet 1990); however, not all hyperuricemia indicates the condition of gout.

Elevated serum uric acid concentrations are encountered regularly in situations such as hypertension, reduced high density lipoprotein (HDL) cholesterol, hyperinsulinemia, reduced physical activity, increased body mass index, and increased alcohol intake. These factors as a group are commonly referred to as the Obesity-Insulin Resistance Syndrome (Lee et al. 1995). This syndrome of related physiological abnormalities is often found in coronary heart disease patients (Facchini et al. 1991). Several of these conditions are also characteristic of non-insulin dependent diabetes mellitus (NIDDM) (Zimmet 1993).

Hyperuricemia, or elevated serum uric acid concentration, is genetically determined, but also influenced by many environmental factors (Tuomilehto et al. 1988). Serum uric acid concentrations ≥ 7.0 mg/100 mL (0.42 mmol/L) in adult males and ≥ 6.0 mg/100 mL (0.36 mmol/L) in adult females generally indicate hyperuricemia (Wyngaarden and Kelley 1976).

Incidence

The prevalence of hyperuricemia seems to follow the incidence of obesity, diabetes mellitus, hyperlipidemia, hypertension, and atherosclerosis and coronary heart disease (Wyngaarden and Kelley 1976). In the Normative Aging Study involving middle to older age men, serum uric acid levels were positively and significantly associated with an increase in body mass index, abdomen to hip ratio, post-carbohydrate insulin level, and alcohol intake (Lee et al. 1995). Serum uric acid was negatively related to age and physical activity level, while total body adiposity and abdomen to hip ratio were strongly related to high serum uric acid after all other factors were removed (Lee et al. 1995).

In a study of healthy individuals with no history of disease, insulin resistance and serum uric acid concentrations were significantly related (Facchini et al. 1991). This positive relationship remained significant when age, sex, body mass index, and abdominal obesity were considered. As individuals became more insulin resistant, renal uric acid clearance decreased and serum uric acid concentrations increased. This study suggested that insulin resistance decreases renal uric acid clearance, which subsequently increases serum uric acid concentrations (Facchini et al. 1991).

A recent study of pregnant women by Kaaja et al. (1995) also supports a connection between hypertension and the incidence of hyperuricemia. This study found elevated serum uric acid concentrations, 18% lower HDL cholesterol, 65% higher triglyceride levels and 100% higher insulin levels in hypertensive pregnant women as compared to normotensive pregnant controls (Kaaja et al. 1995).

Fructose seems to have a hyperuricemic effect in certain individuals. Henry et al. (1991) suggest that those individuals who are predisposed to this hyperuricemia include hyperinsulinemic men. Stirpe and associates (1970) summarized reports of fructose administration as causing serum uric acid elevations in both normal children and children with congenital fructose intolerance. On the other hand, intravenous fructose had no effect on serum uric acid concentrations in young men, but an obvious and prolonged rise in serum uric acid resulted from fructose administration to patients with gout and children of patients with gout.

Hyperuricemia not only accompanies different risk factors for cardiovascular disease, but seems to only associate with certain stages of glucose intolerance. Modan et al. (1987) found elevated serum uric acid levels only in the presence of hyperinsulinemia in 1016 Jewish men and women participating in the Israel Study of Glucose Intolerance, Obesity and Hypertension. The elevated serum uric acid concentrations were found in normal glucose tolerant, impaired glucose tolerant, and newly diagnosed diabetic patients. As diabetes progressed, though, serum uric acid levels decreased significantly (Modan et al. 1987). A similar study also done in Israel with 10,000 men confirmed these findings (Herman et al. 1976). Subjects who did not have symptoms of diabetes at first, but

developed symptoms in the next three to five years were termed "prediabetic." These prediabetics exhibited the highest serum uric acid concentrations. The abnormal glucose tolerant had intermediate, but still higher than normal serum uric acid concentrations while the subjects diagnosed with Type II diabetes exhibited the lowest serum uric acid levels. This trend seemed to continue with the development of the disease (Herman et al. 1976). Herman and Goldbourt again reported in 1982 that serum uric acid concentrations were high before the onset of diabetes, began to fall, and continued to decrease with the increasing duration of the disease.

In the biracial population of Fiji, both the indigenous Melanesians and the immigrated Asians exhibited first elevated and later decreased serum uric acid concentrations as glucose intolerance and diabetes progressed despite differences in the diagnostic criteria for these two disease conditions (Tuomilehto et al. 1988). Using the World Health Organization classifications for IGT and diabetes mellitus (WHO 1980), the highest plasma uric acid levels were found in men with impaired glucose tolerance in both racial groups. Type II diabetic men had the lowest plasma uric acid concentrations in both populations. Hyperuricemia occurred in 27% of the Melanesian men and women and 22% in Asian men and 11% in Asian women (Tuomilehto et al. 1988).

Pathophysiology

The exact mechanism for the development of elevated serum uric acid concentrations is not known, although many suggestions have been made. Hyperuricemia can be caused by either an overproduction of uric acid due to an increased breakdown of nucleic acids through purine metabolism or by decreased excretion due to increased

retention of uric acid in the kidneys indicative of kidney disease (Tuomilehto et al. 1988). Both possibilities will be reviewed here.

Herman and Goldbourt (1982) recognized that renal uric acid excretion is enhanced by hyperglycemia. They postulated that hyperuricemia could be due to an error in carbohydrate metabolism. An interference in glycolysis would divert metabolic flow through the hexose monophosphate pathway which would result in uric acid production. Additionally, an increase in fatty acid oxidation would inhibit pyruvate dehydrogenase and phosphofructokinase resulting in uric acid formation (Herman and Goldbourt 1982).

In a study of 14 males, insulin resistance and serum triglyceride levels were positively and significantly correlated with serum uric acid, independent of obesity, age, and sex factors (Vuorinen-Markkola and Yki-Jarvinen 1994). These researchers proposed a mechanism to explain this connection. Elevated serum uric acid may originate from increased turnover and degradation of AMP, ADP, or ATP, or dinucleotides such as NADP or NADPH, since uric acid is a purine base. In patients with hypertriglyceridemia, lipolysis is enhanced and resistant to the action of insulin. The proportion of free fatty acids (FFA) released from triglycerides during lipolysis accelerates ATP turnover which may then increase serum uric acid (Vuorinen-Markkola and Yki-Jarvinen 1994).

Israel et al. (1983) postulated that hyperuricemia was induced by sucrose or fructose. Fructose is rapidly phosphorylated resulting in sudden decreases in hepatic ATP and serum inorganic phosphorous concentrations which causes a breakdown of preformed nucleotides to uric acid (Henry et al. 1991). An increase in activity in the hexose

monophosphate shunt could also result in the production of the substrate necessary for purine synthesis (Israel et al. 1983).

Kock et al. (1994) reported that during ischemia, there is a calcium dependent enzymatic degradation of xanthine oxidoreductase from the NAD+ consuming dehydrogenase form to an oxidase form, which directly utilizes molecular oxygen as an electron acceptor in the oxidation of hypoxanthine via xanthine to uric acid. The xanthine concentrations measured in serum significantly increased in samples of myocardial infarction patients. Therefore, the metabolic activity of xanthine oxidoreductase in myocardial infarction was supported by this research study.

In 1980, Klevay found that copper deficiency demonstrated by hypercholesterolemia in male rats did increase the concentration of serum uric acid. Klevay (1980) discussed two possible mechanisms for hyperuricemia in the presence of copper deficiency. He suggested that perhaps when copper nutriture is adequate, xanthine oxidase, which contains molybdenum, is partially inhibited, thus lowering the level of uric acid produced. Molybdenum is an antagonist of copper and has been shown to hyperactivate xanthine oxidase and the production of uric acid when ingested in excessive amounts (Davis and Mertz 1987). Klevay (1980) also suggested that copper deficiency inhibits the action of uricase, an enzyme which contains copper in pigs.

In 1973, Diamond and Paolino published a report suggesting for the first time that renal tubular reabsorption of uric acid occurs in part at a site in the renal tubule distal to the site for uric acid secretion. Thus, a portion of the secreted uric acid is reabsorbed at

this site. Their work is now commonly accepted (Tietz 1994). This suggestion explained, in part, the renal handling mechanisms of uric acid.

Tietz (1994) describes four steps in the renal handling of uric acid. First, the kidney filters virtually all of the plasma uric acid entering the glomerulus. Secondly, about 98% to 100% of the filtered uric acid is reabsorbed in the proximal convoluted tubule. Next, 5 to 10% is secreted in the distal portion of the proximal tubuli. Finally, a further reabsorption of secreted uric acid takes place at the distal post-secretory site described above.

Insulin increases proximal sodium reabsorption. Researchers in Italy suggested that in conditions of hyperinsulinemia where more sodium is reabsorbed at sites of the nephron proximal to the distal tubule, more uric acid could be reabsorbed as well (Cappuccio et al. 1993). An activation of a sodium-hydrogen exchange in the proximal renal tubular cells would promote a increase in sodium reabsorption, and also an increase in anion reabsorption, including uric acid. This would lead to elevated serum uric acid concentrations, or hyperuricemia, and reduced urinary uric acid levels.

Insulin inhibits uric acid secretion or enhances uric acid reabsorption at the tubular level (Galvan et al. 1995). Acute hyperinsulinemia also decreases the excretion and clearance rate of sodium. Galvan et al. (1995) postulated that hyperinsulinemia could exert a constant antinatriuretic pressure which would lead to high blood pressure and reduce uric acid excretion leading to hyperuricemia.

Serum uric acid levels are frequently elevated in patients with hypertension or ischemic heart disease (Freedman et al. 1995). Freedman and colleagues suggested that

uric acid is involved in platelet adhesiveness and aggregation, or it may be secondary to preexisting disease, which they felt could indicate early renal involvement with cardiovascular disease. Newland (1975) also indicated that hyperuricemia might play a role in heart disease through thrombosis. Newland (1975) stated that hyperuricemia increases the stability of platelet aggregates, the initial event in thrombosis, and thus would increase the incidence of thrombosis and arterial disease in hyperuricemic individuals.

Glucose seems to competitively inhibit uric acid reabsorption in the proximal tubules of the kidney in patients exhibiting hyperglycemia and glycosuria (Herman et al. 1976). This decreased uric acid reabsorption would eventually result in increased uric acid excretion and decreased serum uric acid concentrations. This mechanism could help explain the decreasing serum uric acid levels seen in the progression of uncontrolled diabetes (Herman et al. 1976).

Tuomilehto et al. (1988) found plasma creatinine to be the strongest predictor of plasma uric acid concentrations. This finding suggests a strong renal involvement in the balance of plasma uric acid (Tuomilehto et al. 1988).

Risk Factor for Cardiovascular Disease and Diabetes Mellitus

Numerous studies indicate that some populations with NIDDM share several common casual factors with cardiovascular disease and in particular with coronary heart disease (Perry et al. 1995). Hyperuricemia is one of the common conditions of these two disease states (Perry et al. 1995). Several studies have found hyperuricemia to be an independent predictor of ischemic heart disease and total mortality. Others have concluded that the apparent connection is due to other related factors such as

hypertension, obesity, and hyperlipidemia (Freedman et al. 1995). Regardless of disagreements, the relationship of hyperuricemia with other causal factors for cardiovascular disease and diabetes mellitus has received scant attention even though obvious relationships exist (Zimmet 1993).

Hyperinsulinemia

Hyperinsulinemia is a state of abnormally elevated insulin in the blood for a given plasma glucose concentration (Zimmet 1993). Hyperinsulinemia, then, can occur in the presence of normoglycemia or hyperglycemia (Zimmet 1993). Hyperinsulinemia is most commonly associated with the terms "insulin resistant" or "insulin insensitive" which indicate a resistance to insulin mediated glucose uptake (Perry et al. 1995). The resistance to the effect of insulin is a fundamental aspect of NIDDM and obesity (Zimmet 1993). In fact, insulin resistance, characterized by hyperinsulinemia, is commonly thought to be the underlying metabolic abnormality explaining the association between obesity, hypertension, hyperlipidemia, and glucose intolerance, all components of the Obesity-Insulin Resistance Syndrome (Lee et al. 1995, Vuorinen-Markkola and Yki-Jarvinen 1994).

Incidence

Hyperinsulinemia is a characteristic feature of populations with a high prevalence of NIDDM, such as Pima Indians, other American Indians, Micronesian Nauruans, Mexican Americans, Australian Aborigines, Hispanics, Asian Indians, and African Americans (Zimmet 1993). In the Strong Heart Study examining American Indians on

tribal rolls from Arizona, Oklahoma, and North and South Dakota, the majority of those studied were found to be insulin-resistant determined by high fasting plasma insulin levels (Howard et al. 1995). Over 60% of the Arizona Indians exhibited NIDDM. Diabetes was more prevalent in women in all three locations with 40% showing symptoms in both Oklahoma and the Dakotas. Over one third of the men in Oklahoma and North and South Dakota had NIDDM. Thirteen to 20% of the subjects at all three locations exhibited IGT (Welty et al. 1995). Obesity was very common in subjects from all three areas with the majority of body fat deposited in upper-body regions as determined by waist to hip ratios. Obesity is of concern in American Indians for its apparent affect on the development of both cardiovascular disease and diabetes mellitus (Howard et al. 1995).

Modan et al. (1987), using a representative population in Israel, found IGT patients exhibited significantly increased insulin sums (the sum of 60 and 120 minute post glucose load insulin levels) as compared to normal glucose tolerant individuals. Newly diagnosed Type II diabetics showed significantly lower insulin sums as compared to IGT individuals.

Hyperinsulinemia is common among hypertensive individuals, also (Cappuccio et al. 1993). In a study of pregnancy-induced hypertension, insulin levels were two times higher among hypertensive pregnant women than among the controls (Kaaja et al. 1995).

Perry et al. (1995) state that insulin resistance antedates NIDDM. As NIDDM progresses, many changes in glucose tolerance occur (Zimmet 1993). The presence of hypoinsulinemia or hyperinsulinemia, thus, depends on the actual stage of the disease. Hyperinsulinemia observed during IGT is a result of increased insulin secretion by the

pancreas (DeFronzo 1988). Once the fasting glucose level exceeds 120 mg/dL, indicating the presence of diabetes-like symptoms, the pancreas decompensates by progressively decreasing insulin secretion even though hyperglycemia persists (DeFronzo 1988). This inverted U-shaped curve relating insulin secretion to the fasting plasma glucose concentration has been termed the "Starling curve of the pancreas" (DeFronzo 1988). An individual at the transition point of IGT to NIDDM will exhibit hyperinsulinemia whereas a person with NIDDM studied at the stage when pancreatic decompensation has taken place will show hypoinsulinemia (Zimmet 1993).

Pathophysiology

A number of studies have concluded that a defect in insulin sensitivity is present in NIDDM, hypertriglyceridemia, essential hypertension, and obesity (Galvan et al. 1995). If target tissues are insensitive to the action of insulin, more insulin will be produced to attempt to appropriately handle a glucose load. This increase in insulin then produces hyperinsulinemia. Several of the postulated mechanisms for the development and expression of hyperinsulinemia are reviewed here.

The possible causes of insulin resistance include an abnormal β-cell secretory product, circulating insulin antagonists, or a target-tissue defect in insulin action. The latter condition is considered to be the most plausible cause of insulin resistance (DeFronzo 1988).

Zimmet (1993) reports that insulin resistance seems to be the cause of glucose intolerance in the majority of adult, nonobese patients with NIDDM and in obese subjects. A reduced number of insulin receptors could cause this insulin resistance and subsequent

glucose intolerance and hyperinsulinemia (Zimmet 1993). Others believe both binding and postbinding defects cause insulin resistance in NIDDM (DeFronzo 1988). Individuals with IGT or very mild diabetes seem to experience diminished insulin binding and then exhibit hyperinsulinemia. Postbinding defects, on the other hand, have been observed in fully developed NIDDM patients. These include diminished tyrosine kinase activity, decreased glucose transport, impaired glycogen synthase activity, and reduced pyruvate dehydrogenase stimulation (De Fronzo 1988).

Considerable debate continues over which occurs first, hyperinsulinemia or insulin resistance (Zimmet 1993). It has been postulated that centripetal obesity stimulates the increase of insulin levels (Lee et al. 1995). Zimmet (1993) suggests that in obese NIDDM subjects, hyperinsulinemia may be the primary defect and insulin resistance the result. On the other hand, perhaps tissue insensitivity to insulin is the primary defect and hyperinsulinemia is the resultant response in nonobese NIDDM subjects.

Either way, insulin resistance in the peripheral tissues causes a rise in plasma glucose which prompts the pancreas to increase circulating insulin concentrations. This, in turn, furthers the tissue insensitivity to insulin by causing a down regulation of the insulin receptors. Plasma glucose continues to rise, causing the apex of the "Starling curve of the pancreas" (DeFronzo 1988). At this point, the β-cell decompensates, insulin secretion decreases, and hyperglycemia intensifies (Zimmet 1993).

Hyperinsulinemia and Lipid Metabolism

Insulin resistance enhances lipolysis (Zimmet 1993); thus, hyperinsulinemia occurs

along with dyslipidemia. Insulin resistance also impairs the ability of insulin to normally suppress very low density lipoprotein (VLDL) secretion (Vuorinen-Markkola and Yki-Jarvinen 1993). Thus, excessive lipolysis which frees extra FFA that incorporate into VLDL and increased VLDL secretion both contribute to hypertriglyceridemia (Yki-Jarvinen and Taskinen 1988). The resultant dyslipidemia presents with decreased HDL cholesterol and increased VLDL cholesterol, which are both risk factors for development of cardiovascular disease (Perry et al. 1995).

Circulating FFA concentration is suppressed quickly by insulin in normal subjects. However, individuals with NIDDM have a reduced capacity to suppress FFA's thereby increasing hepatic glucose output and contributing to hyperglycemia in these subjects (Reaven 1988). This could further exacerbate hyperinsulinemia and insulin resistance (Reaven 1991).

Risk Factor for Cardiovascular Disease and Diabetes Mellitus

The Obesity-Insulin Resistance Syndrome is considered to be a rather strong risk factor for cardiovascular disease (Lee et al. 1995). Central to this syndrome of connected abnormalities are hyperinsulinemia, reduced physical activity, hypertension, reduced HDL cholesterol, increased body mass index, and increased alcohol intake (Lee et al. 1995). Reaven (1988) coined the term "Syndrome X" to describe a cluster of symptoms very similar to those of the Obesity-Insulin Resistance Syndrome. Syndrome X, which increases risk of coronary artery disease, may be caused by insulin resistance (Foster 1989). Many studies also report an association between atherosclerotic cardiovascular disease and NIDDM, stating that hyperinsulinemia and insulin resistance are common

factors to both conditions (DeFronzo and Ferrannini 1991). Additionally, the major cause of mortality in NIDDM is coronary artery disease (Zimmet 1993). Heart disease is twice as common in patients with diabetes (Wingard et al. 1995).

Through its effects on lipid metabolism, hyperinsulinemia could initiate the deposition of adipose tissue in the upper-body region, another well documented risk factor for NIDDM, hypertension, and cardiovascular disease (Kaplan 1989). Hyperinsulinemia causes increased lipolysis which, in turn, increases hepatic triglyceride production and the resultant hypertriglyceridemia. This dyslipidemia could accelerate the development of atherogenesis (Zimmet 1993). Lee et al. (1995) indicate that hyperinsulinemia stimulates the sympathetic nervous system and suppresses adrenomedullary activity, resulting in lower HDL cholesterol levels and higher blood pressure, both risk factors for coronary heart disease. Additionally, high abdomen to hip measurement ratios, another risk factor for heart disease, are positively associated with fasting and postcarbohydrate insulin levels (Lee et al. 1995).

In the presence of hyperinsulinemia, there seems to be a high risk of coronary heart disease, long before NIDDM develops (Zimmet 1993, Reaven 1988). In the follow-up study of the Paris Prospective Study, two-hour post-load plasma insulin independently predicted deaths from coronary heart disease in subjects without NIDDM (Zimmet 1993). Thus, subjects who are destined to develop NIDDM are at increased risk of developing coronary heart disease many years before manifestation of diabetes, apparently because of their baseline insulin concentrations (Zimmet 1993).

Perry et al. (1995) in the British Regional Heart Study found that the risk factors that predict cardiovascular disease also predict NIDDM. Those cardiovascular risk factors linked with insulin resistance, such as serum triglyceride, HDL cholesterol, and uric acid concentrations, predicted the development of NIDDM over a decade before onset of the disease, even after adjustments were made for body mass index, hypertension, and presence of coronary heart disease (Perry et al. 1995)

Chromium

Schwarz and Mertz (1959) first demonstrated that chromium was required to maintain normal glucose tolerance in rats. Chromium is now identified primarily with its role in potentiating insulin action (Mertz 1993).

Trivalent chromium was identified as the active component of a compound termed glucose tolerance factor (GTF) that effectively restored glucose tolerance upon consumption (Anderson 1987). Chromium deficiency has been identified in total parenteral nutrition patients (Brown et al. 1986, Freund et al. 1979, Jeejeebhoy et al. 1977). In addition, poor chromium nutriture contributes to the progressive impairment of glucose tolerance (Mertz 1993). The nutritional requirement in humans for chromium is apparently increased by nutritional or physiologic stress (Nielsen 1988).

Dietary Intake

In the United States, reported dietary intake of chromium has varied widely through the years from 200-290 μg/day in 1969 to 28 μg/day in 1985 (Anderson and Kozlovsky 1985). Dietary chromium intakes reported before 1980, though, should be

questioned. Many were flawed by contamination and analytical problems (Nielsen 1994). Increased knowledge of contamination issues and improved instrumentation in chromium research is credited for the marked decrease in chromium values, rather than a dramatic decrease in actual dietary intake (Anderson 1987).

More than 90% of people studied in the United States and several other countries regularly consume diets providing less than the minimum Estimated Safe and Adequate Daily Dietary Intake (ESADDI) of chromium of 50 μg/day recommended by the National Academy of Sciences (Anderson et al. 1991, Food and Nutrition Board 1989). Numerous studies have found no adverse consequences as the result of this low chromium intake (Nielsen 1994). In 1985, Anderson and Kozlovsky measured chromium intake of 10 men and 22 women with self-selected diets. Average chromium intake was 33 μg/day in 2300 kilocalories for the men, indicating about 14 μg/1000 kilocalories. The women exhibited slightly higher intakes of 25 μg/day in 1600 kilocalories averaging about 16 μg/1000 kilocalories (Anderson and Kozlovsky 1985). These ranges of average chromium intake in the United States are approximately half of the lower limit of the ESADDI which is 50-200 μg/day for adults (Food and Nutrition Board 1989). Even institutionally planned diets with well-balanced menus did not provide adequate chromium based on the ESADDI (Anderson et al. 1992). Perhaps, then, the current ESADDI for chromium which was originally adopted in 1980 when measurement techniques needed improvement, might be higher than needed by most healthy adults (Stoecker 1995). In contrast, marginal dietary intake of chromium over many years can lead to a depletion of chromium in the human

body which could detrimentally affect at-risk populations such as the elderly and the glucose intolerant (Offenbacher and Pi-Sunyer 1980).

A study examining chromium content in breast milk found that infants consuming human milk received only an average of 0.18 μg/L (Anderson et al. 1993). Apparently, breast milk chromium concentrations are not significantly altered with increased maternal dietary intake of chromium (Casey and Hambidge 1984). Thus, when an infant consumes an average of 715 mL/day of breast milk, the child would receive less than 2% of the minimum ESADDI of 10 μg/day for infants less than 6 months old (Anderson et al. 1993). Once again, researchers wonder if ESADDI values need to be redefined to represent actual intake (Anderson et al. 1993).

Dietary sources of chromium are scattered. Apparently, processing techniques remove natural chromium in the case of refined sugar, and at other times add chromium as in processed meats (Kumpulainen 1992). Significant amounts of chromium may be added to the food supply through use of stainless steel cookware in food preparation and processing. Chromium leaches out of the stainless steel in an acid environment present in many food products (Offenbacher and Pi-Sunyer 1983). Anderson et al. (1992) indicates that the best sources of chromium include processed meats, whole grain products, and spices in contrast with poor sources such as dairy products, fruits, and vegetables. Brewer's yeast has traditionally been considered the best source of chromium, but Iraqi barley may be an even richer source (Mahdi 1995). In Anderson and Kozlovsky's study (1985), dietary chromium intake significantly and positively correlated with potassium, fat,

saturated fat, sodium, oleic acid, phosphorous, vitamin B_6, protein, carbohydrate, and copper intake.

Absorption

Although little is known about the specific mechanisms involved in chromium absorption, chromium has been observed in the urine within two hours of ingestion, indicating rather rapid absorption and excretion rates (Anderson 1987). Increased urinary chromium losses seem to indicate an increase in chromium mobilization and utilization. Apparently, chromium is rapidly lost in the urine rather than reabsorbed after it has responded to a glucose load or an elevated insulin response (Anderson et al. 1990).

Intestinal absorption of trivalent chromium and inorganic chromium compounds is low. In contrast, hexavalent chromium absorption may be several fold higher than trivalent chromium (Anderson 1987, Stoecker 1995). Absorption of chromium has been reported in ranges from <0.5% to 2-3% (Stoecker 1995). Absorption of chromium is inversely related to dietary intake when ingested diets contain less than 40 μg/day, indicating a highly efficient method of maintaining sufficient chromium stores (Anderson and Kozlovsky 1985). Urinary chromium excretion can be used as a fairly accurate estimation of the amount of chromium absorbed since absorbed chromium is primarily excreted in the urine (Anderson and Kozlovsky 1985). Anderson and Kozlovsky (1985) found that when dietary chromium intake was 10 μg/day, 2% was excreted in urine indicating that around 2% was absorbed. When 40 μg/day of chromium was ingested, 0.4% to 0.5% was excreted in the urine and thus assumed to represent absorption (Anderson and Kozlovsky 1985). Subjects with sufficient chromium stores lose more

chromium in the urine in response to a glucose load than do subjects with depleted chromium stores. Urinary chromium losses may depend upon the glucose tolerance of subjects and whether any glucose intolerance is a chromium related deficiency (Anderson et al. 1991).

Chromium is best absorbed in the midsection of the small intestine in the rat, followed by the ileum and duodenum. In addition, rats who have been deprived of food absorb chromium faster than do fed rats (Chen et al. 1973).

Absorption of chromium is also altered by mineral status. In zinc deficient rats, whole-body and intestinal mucosa chromium absorption were increased. When oral zinc was administered, though, the increase in intestinal mucosa chromium absorption was decreased (Hahn and Evans 1975). These investigators also found that chromium inhibited ^{65}Zn absorption in zinc deficient rats and decreased the intestinal mucosa content of this isotope, suggesting that chromium and zinc may share a common pathway in metabolism (Hahn and Evans 1975). Iron and chromium may also share a common mechanism. In one study, iron deficient animals absorbed more chromium than iron supplemented controls. When iron was administered orally to the deficient animals, chromium absorption was inhibited (Hopkins and Schwarz 1964).

Chromium absorption is also altered by dietary components and drugs. Chen et al. (1973) observed that oxalate increased and phytate decreased trivalent chromium transport in the rat intestine. Dietary carbohydrate sources affect chromium absorption and retention. Starch appeared to increase chromium absorption in obese and lean mice as compared to sucrose, glucose, and fructose as dietary carbohydrate sources (Seaborn and

Stoecker 1989). Dietary starch also increased tissue chromium concentrations in this study (Seaborn and Stoecker 1989). Anderson et al. (1990) supported these findings by indicating that human diets habitually high in simple sugars lead to elevated urinary chromium losses as compared with diets high in complex carbohydrates. Even over-the-counter (OTC) drugs affect chromium absorption. In rats, aspirin increased ^{51}Cr absorption from $^{51}CrCl_3$ while Maalox decreased absorption of this isotope (Davis et al. 1995). Seaborn and Stoecker (1990) investigated the effects of antacids on tissue accumulation and urinary excretion of ^{51}Cr. These researchers found that antacids have a negative effect on chromium absorption from chromium chloride. In addition, Kamath and associates (1996) determined that indomethacin, which blocks prostaglandin synthesis, enhances ^{51}Cr absorption. In contrast, 16,16-dimethylprostaglandin E_2 decreased ^{51}Cr absorption in this study (Kamath et al. 1997). Indomethacin is an OTC nonsteroidal antiinflammatory drug commonly used for the treatment of arthritis.

Distribution

Once absorbed, trivalent chromium combines with the α-globulin portion of the plasma and is transported or distributed to the tissues while bound to transferrin (Anderson 1987). Chromium is widely distributed in the body with no known accumulations or concentrations in any specific tissues or organs (Anderson 1987). Chromium concentrations have been reported to be greater in samples of human aortas from areas of the world where atherosclerosis is rare as compared to aorta samples from areas where the disease is prevalent (Newman et al. 1978). Caution should be used, though, in considering data obtained when chromium sampling and analysis methods were

subject to numerous contamination problems. Various tissues seem to retain chromium much longer than the plasma does, suggesting no equilibrium exists between tissue stores and circulating chromium (Mertz 1993). Therefore, plasma or serum chromium levels may not accurately indicate chromium body stores and nutritional status.

There is presently no reliable method for accurately assessing the chromium status of an individual (Mertz 1993). Urinary chromium reflects recent intakes. Hair concentrations have not been unequivacally shown to be an acceptable method of determining an individual's chromium status (Anderson et al. 1985). As indicated above, serum chromium concentrations are not in equilibrium with body stores, and thus, cannot be utilized either (Mertz 1993). The only method currently available to measure chromium status is to analyze an individual's response to chromium supplementation, which cannot be used as a clinical screening tool (Anderson et al. 1985).

Chromium Deficiency

Impaired glucose tolerance is one of the first physiological signs of chromium deficiency (Doisy et al. 1976). In the human, glucose and lipid metabolism abnormalities, neuropathy, and encephalopathy have subsequently occurred. In animal models, impaired growth, decreased longevity, aortic plaques, corneal lesions, and decreased fertility and sperm count have all been observed manifestations of chromium deficiency (Anderson 1987).

In 1977, a patient who had been receiving only total parenteral nutrition for three years experienced weight loss, peripheral neuropathy, glucose intolerance, and an increased caloric requirement for weight maintenance. Measured respiratory quotient

indicated the use of fat as the major energy source even though daily infusions of glucose and insulin were being received (Jeejeebhoy et al. 1977). After an infusion of 250 μg chromium as chromium chloride was given for two weeks, plasma glucose returned to within the normal range after only three days indicating a possible chromium deficiency in the patient. Increases in urinary excretion of chromium with continued infusions eventually leveled suggesting a resaturation of chromium stores after 7 days of supplementation (Jeejeebhoy et al. 1977). Two subsequent examples of chromium responsive glucose intolerance have been documented in patients also sustained with total parenteral nutrition (Brown et al. 1986, Freund et al. 1979, Mertz 1993).

In the general population consuming regular diets with varied chromium content, chromium deficiencies are not acute or simply observed (Nielsen 1988). In addition, there is not a reliable method to diagnose chromium deficiency at this time (Mertz 1993). As a result, some scientists consider chromium to be nutritionally insignificant (Nielsen 1988). Other investigators, though, have shown chromium to be nutritionally significant if the organism is under some form of stress that enhances its need for chromium (Nielsen 1988). Metabolic stress may intensify chromium deficiency symptoms and increase chromium's essentiality in selected subjects (Nielsen 1988). Other stresses including trauma, infection, surgery, intense heat or cold, and even exercise alter glucose metabolism and affect chromium metabolism (Nielsen 1988).

Chromium and Glucose Metabolism

Nearly 40 years ago, a new dietary compound termed the glucose tolerance factor (GTF) was identified by Schwarz and Mertz (1957). This biologically active compound

which is very richly found in Brewer's yeast (Mertz 1975), has been postulated to contain nicotinic acid, glutathione, and chromium (Toepfer, et al. 1977). Further research has been unsuccessful at confirming the actual components of GTF. Mertz, Roginski, and Schwarz (1961) studied the effect of GTF with trivalent chromium on glucose uptake by peripheral tissues using rat epididymal fat in vitro. These investigators demonstrated that very small amounts of chromium enhanced the conversion of glucose to fat by 70 to 95% (Mertz et al. 1961). In later studies, GTF appeared to facilitate the binding of insulin to receptor sites of sensitive tissues (Mertz 1975). In laboratory testing, GTF in Brewer's yeast acutely improved glucose and blood lipid concentrations within minutes in genetically diabetic mice (Tuman et al. 1978). As a result of these conclusions, GTF with trivalent chromium has been proposed to be necessary for the maintenance of normal carbohydrate metabolism (Tuman et al. 1978).

Govindaraju et al. (1989) has since concluded that chromium assembles insulin and the receptor units in cell membranes, furthering the support for the beneficiary role of trivalent chromium in glucose metabolism. Using a GTF mixture formed in his laboratory, a researcher in Israel observed significantly decreased blood glucose and free fatty acid levels in streptozotocin-induced diabetic rats within two hours of injection (Mirsky 1993). In another study, both diabetic and non-diabetic elderly patients supplemented with a chromium rich brewer's yeast mixture exhibited significantly improved glucose tolerance and decreased insulin output (Offenbacher and PiSunyer 1980). These results further support the assumption that trivalent chromium within the biologically active molecule

GTF may be essential for optimal insulin function and glucose disposal in mammals (Offenbacher and PiSunyer 1980).

Many studies have chosen to isolate trivalent chromium outside of any GTF compound, and study it as a sole contributor to glucose homeostasis. Rats fed a low chromium diet in a strictly controlled environment designed to decrease chromium contamination showed uniform and severe impairment of glucose tolerance in addition to depressed growth indicating a chromium deficiency (Mertz et al. 1965). When supplemented with 5 ppm hexa-aquo chromium trichloride, these deficiency trends seemed to reverse. These results supported the conclusion that chromium enhances the effects of insulin by facilitating the initial reaction of insulin with the insulin receptor sites on membranes in the peripheral tissues (Mertz et al. 1965). In another study, rats fed a moderately chromium deficient diet exhibited hyperglycemia, glycosuria, depressed growth, and early mortality once again supporting the essentiality of chromium in animals (Schroeder 1966).

In humans, chromium deficiency has been observed in three patients dependent on long term total parenteral nutrition (Brown et al. 1986, Freund et al. 1979, Jeejeebhoy et al. 1977). All three patients experienced weight loss, glucose intolerance, and increased exogenous insulin requirements, with one even experiencing peripheral neuropathy. Chromium supplementation reversed these diabetic like symptoms (Brown et al. 1986, Freund et al. 1979, Jeejeebhoy et al. 1977). Chromium enhances the effects of insulin to lower serum glucose (Mertz et al. 1965). Thus, insulin must be present in order for chromium to potentiate its action.

Chromium seems to only restore deficient functions to normal, indicating that it only has a physiological effect, not a pharmocological effect (Mertz et al. 1965). Thus, the effect of supplemental chromium depends on the degree of deficiency (Mertz et al. 1965). Anderson et al. (1991) found that hyperglycemic subjects who consumed diets low in chromium (<50 μg/day) experienced detrimental effects on their glucose tolerance and insulin and glucagon levels. Thus, the effect of supplemental chromium also depends on the degree of glucose intolerance (Anderson et al. 1991). Mertz (1993) recently reviewed 15 controlled studies of trivalent chromium supplementation in subjects with varied states of glucose tolerance. In 12 of the 15 selected studies, chromium supplementation improved the efficiency of insulin. Those studies with impaired glucose tolerant subjects reported greater responses to chromium supplementation (Mertz 1993). In this review, Mertz (1993) lists two hypotheses to summarize the role of chromium in glucose metabolism. First, marginal chromium status contributes to the progressive impairment of glucose tolerance with age. Secondly, marginal chromium nutriture may increase the risk of diabetes and possibly coronary heart disease (Mertz 1993).

Chromium and Lipid Metabolism

Schroeder (1968) hypothesized over twenty-five years ago that chromium deficiency was a significant risk factor for cardiovascular disease. Atherosclerosis has been shown to be related to abnormalities in both glucose and lipid metabolism (Newman et al. 1978). In addition, atherosclerosis is the primary cause of death in patients suffering from diabetes mellitus (Zimmet 1993). Since normal serum lipids depend upon normal

glucose tolerance and normal insulin sensitivity, adequate chromium nutriture may help maintain normal serum lipids (Riales and Albrink 1981).

In areas of the world where atherosclerosis is mild or virtually absent, samples of human aortas were found to contain more chromium than did samples from coronary artery disease-prone areas (Schroeder 1968). Newman and associates (1978) also determined that chromium might be an important factor in coronary artery disease. These investigators measured serum cholesterol, triacylglycerols, and chromium concentrations in subjects referred for selective coronary arteriography (Newman et al. 1978). The appearance of coronary artery disease was found to be significantly related to reduced serum chromium concentrations and elevated serum triacylglycerol concentrations.

Lee and Reasner (1994) investigated the effects of supplemented chromium picolinate on the lipid profile of Hispanics with NIDDM. Supplemented chromium picolinate was associated with an 17% average reduction in plasma triglyceride levels, which would indicate a possible, subsequent reduction in atherosclerotic disease risk. No differences were found in LDL-cholesterol or HDL-cholesterol levels (Lee and Reasner 1994). In this study, Lee and Reasner (1994) assumed the patients were chromium deficient since all but two had undetectable plasma chromium levels (<0.2 μg/L.) using the flameless atomic absorption spectrometry method. According to Offenbacher et al. (1996), though, average chromium concentrations in serum or plasma are near the detection limits of currently available instruments, including the atomic absorption spectrophotometer; thus, these parameters do not appear to be good indicators of chromium status.

In a study of 23 middle aged men, ingestion of a trivalent chromium solution for twelve weeks resulted in a significant increase in HDL-cholesterol and a decrease in body weight, serum insulin and serum glucose levels as compared to controls (Riales and Albrink 1981). The largest improvements in HDL-cholesterol and insulin and glucose were found in those subjects exhibiting baseline hyperinsulinemia, but normal glucose tolerance (Riales and Albrink 1981).

Vinson and Bose (1984) separated 23 subjects into groups of normal, hyperglycemic, insulin dependent diabetes mellitus (IDDM), and NIDDM patients. Each subject took a high potency organic chromium yeast tablet for six months. After two months of chromium supplementation, all groups showed improvements in percent glycosylated hemoglobin, serum glucose, HDL-cholesterol, LDL-cholesterol, and triglycerides. After six months of supplementation, though, only the hyperglycemic group continued to exhibit improved blood glucose control, lowered serum lipids, and thus, a decreased risk of coronary heart disease (Vinson and Bose 1984).

A long term total parenteral nutrition patient experiencing chromium deficiency symptoms exhibited very high plasma free fatty acids and a low respiratory quotient, suggesting that fat was being used as a major energy source (Jeejeebhoy et al. 1977). After chromium infusion in the parenteral solution, plasma free fatty acids decreased to a level only slightly higher than the normal postprandial range. A subsequent rise in the respiratory quotient indicated a return of normal utilization of glucose for energy (Jeejeebhoy et al. 1977).

In elderly subjects presumed to be rather chromium deficient, serum cholesterol and total lipid levels were significantly improved in both diabetic and non-diabetic groups receiving a chromium rich supplement of Brewer's yeast compared to those who received a chromium poor supplement source of torula yeast (Offenbacher and PiSunyer 1980). Reduced serum triglyceride levels were only observed in subjects having cholesterol levels >300 mg/dL (Offenbacher and PiSunyer 1980).

In animal studies, Mirsky (1993) found a decrease in blood glucose and free fatty acids in diabetic rats injected with a GTF preparation extracted from yeast extract powder, in the absence of exogenous insulin. The decrease in free fatty acids was observed within thirty minutes and lasted 24 hours (Mirsky 1993). Abraham and associates (1991) observed a marked reduction in aortic weight and cholesterol content and the percentage of aortic intimal surface covered by plaque in rabbits injected with potassium chromate and chromium chloride while consuming a cholesterol-enriched diet. In another study with rats, chromium picolinate, a lipophilic complex, produced a significant increase in membrane fluidity. This chromium complex also increased insulin internalization in cultured rat skeletal muscle cells accompanied by a marked increase in the uptake of glucose (Evans and Bowman 1992).

Copper

Studies have reported copper to be inadequate in diets of Americans of all ages (Pennington et al. 1986). Low dietary copper intakes are reflected in subnormal blood copper concentrations in all animals studied (Davis and Mertz 1987). Copper status is currently measured by serum copper, ceruloplasmin, and erythrocyte superoxide

dismutase. All three indicators decline with frank copper deficiency but none appear to be sensitive to marginal copper status (Turnland 1988)

In addition, many dietary components interact with copper and alter copper status. The bioavailability of copper is influenced by the dietary levels of copper, zinc and molybdenum, ascorbic acid, carbohydrates including fructose, glucose and starch, fiber, phytate, some drugs, and the presence of iron deficiency (Turnland 1988).

Copper serves as an extracellular scavanger for oxygen radicals to protect cell membranes (Linder 1991). Copper may facilitate iron transport (Linder 1991) and assist in the oxidation of iron (Turnland 1988). Copper plays a vital role in the cross linking of collagen, skeletal mineralization, and myelin formation (Turnland 1988). Copper is required by all cells for oxidative phosphorylation (Linder 1991). Copper also has physiological roles in the integrity of the cardiovascular system, immune function, regulation of glucose metabolism, and lipid and cholesterol metabolism (Davis and Mertz 1987). Copper is an essential component of many enzymes, including but not limited to cytochrome oxidase, lysyloxidase, superoxide dismutase, and ceruloplasmin (Turnland 1988).

Dietary Intake

In 1989, the National Academy of Sciences readjusted the ESADDI of copper for adults to 1.5 to 3 mg/day from 2 to 3 mg/day to maintain copper balance with a margin of safety (Food and Nutrition Board 1989). Based on the ESADDI values for each age group for copper, Pennington et al. (1986) determined that usual diets of infants, children, teenagers, adult women and men, and elderly women and men were all low in copper.

The average intake for adult women was 0.93 mg/day and 1.24 mg/day for adult men (Pennington et al. 1986).

Balance studies have indicated an adult male requires 1.3 mg/day of copper (Turnland 1988). Klevay and associates (1980) found the copper requirement for an adult male to be 1.55 mg/day including an estimated surface loss of 0.25 mg/day. Many conventional diets did not meet this requirement when tested (Klevay et al. 1980). Turnland (1988) has suggested the dietary recommendations be reevaluated for copper.

Reportedly, the amount of copper required for balance is directly related to the amount of dietary zinc consumed (Sandstead 1995). In one study, zinc accounted for nearly 30% of the variance in the copper requirement (Sandstead 1982) while protein accounted for 3% of the variance (Sandstead 1982) Abnormalities have been seen with zinc to copper ratios greater than 16 (Sandstead 1995). Apparently, persons who habitually consume diets high in zinc and low in protein have an increased risk of becoming copper deficient. Requirements for copper are lowest when protein intake is high and zinc intake is low (Sandstead 1982).

The content of copper in foods is influenced by many factors. Measured values of copper in any food item will reflect its origin and its production, handling, and preparation conditions (Davis and Mertz 1987). In some areas of the world, copper contamination from use of fungicides and fertilizers made with copper compounds will increase copper concentrations in plants and animals grown there (Davis and Mertz 1987). Foods rich in bioavailable copper include liver, oysters, peanut butter, nuts, legumes, mushrooms, and whole grains. Meat and dairy products are low in copper (Sandstead 1995). Drinking

water contains highly variable concentrations of copper influenced by the interaction of the water's acidity with the piping system (Food and Nutrition Board 1989).

Absorption

It is believed that intestinal absorption of copper is regulated by the nutritional status of the individual, the chemical form of the element, and the interactions with other dietary factors that affect bioavailability (Davis and Mertz 1987). Copper seems to be absorbed in all segments of the gastrointestinal tract with the small intestine being the major site of absorption (Davis and Mertz 1987). Some minimal absorption occurs in the stomach in man (Linder 1991).

Metallothionein is believed to be a copper binding protein (Prasad 1993) that is involved in the regulation of intestinal absorption of copper (Davis and Mertz 1987). Cunnane et al. (1985) observed higher absorption rates of copper in the presence of a copper deficiency as compared to adequate copper nutriture in the rat.

Low concentrations of dietary copper are predominantly transported by a saturable mechanism utilizing active transport. Conversely, high concentrations use an unsaturable mechanism with simple diffusion (Davis and Mertz 1987).

There are powerful exogenous factors that also seem to regulate copper absorption. The presence of other minerals and dietary components can strongly affect the biological availability of copper. In addition, the chemical form in which copper is ingested also influences its bioavailability. Neutral or anionic copper complexes were better utilized in one study than equivalent amounts of copper sulfate (Davis and Mertz 1987).

Dietary intake of several minerals and organic substances may affect copper absorption. Phytate from dietary fiber may impair the bioavailability of copper (Sandstead 1982). Picolinic acid may facilitate copper absorption when it forms complexes with copper (Sandstead 1982). Fructose exacerbates the severity of copper deficiency symptoms (Fields et al. 1983c, Fields et al. 1984, Henry et al. 1991) while replacement with cornstarch reduces or prevents copper deficiency symptoms (Fields et al. 1983c, Fields et al. 1984, Turnland 1988). Ascorbic acid depresses copper bioavailability (Davis and Mertz 1987). Hunt et al. (1970) found that adding ascorbic acid to the purified diets of chicks increased the severity of copper deficiency. Van Campen and Gross (1968) found less severe copper deficiency symptoms in rats fed ascorbic acid, but still found substantial evidence that ascorbic acid can depress the intestinal absorption of copper. Turnland (1988) reported that dietary levels of ascorbic acid up to 600 mg did not affect serum copper in young men. The popularity of vitamin C supplementation, though, could provide even higher levels of ascorbic acid which could negatively impact copper status (Davis and Mertz 1987).

Copper bioavailability is impaired in the presence of excessive zinc (Turnland 1988). It is believed that copper and zinc compete for binding sites on metallothionein in the intestinal mucosal cells (Sandstead 1982). Copper deficiency symptoms and abnormalities are associated with a zinc to copper ratio greater than 16. Most Americans maintain a zinc to copper ratio from 5 to 14 (Sandstead 1995). In addition, interactions between copper and cadmium, iron, molybdenum, and calcium have all been documented (Davis and Mertz 1987).

Distribution

Evans and LeBlanc (1976) suggested that an intestinal binding protein in rats regulated the passage of absorbed copper through the intestinal walls into the blood. Albumin, which binds only 10% of serum copper, is now believed by some to be the most important carrier protein for newly absorbed copper in the bloodstream (Cartwright and Wintrobe 1964a, Gordon et al. 1987). Albumin and some other amino acids may provide copper for uptake by the liver and other tissues contributing to systemic copper transport (Gordon et al. 1987). Contrastingly, Darwish et al. (1984) found that histidine competed with albumin for copper binding, which resulted in copper being transported as a free ion. Additionally, an albumin-deficient rat injected with copper intraperitoneally transported copper to the liver at a comparable rate to a rat with normal albumin levels (Suzuki et al. 1989). Lau and Sarkar (1971) believed that a ternary coordination complex between human serum albumin, copper (II) and L-histidine is responsible for the transport of copper.

Regardless of which transport protein is used, the liver accumulates orally ingested or intravenously injected copper. Consequently, liver parenchymal cell plasma membranes might contain a specific transport protein for copper (II) and related trace elements (Darwish et al. 1984). Copper uptake by the liver and the kidneys has been postulated to be regulated by the synthesis of metallothionein in the tissues (Suzuki et al. 1989). In their study, Suzuki and associates (1989) observed that copper taken up by the liver was bound to metallothionein suggesting that liver uptake of copper depends on the induction of metallothionein synthesis. From the liver, copper enters the bile where some is used for

production of internally required liver proteins and much is incorporated into ceruloplasmin for resecretion into the blood (Linder 1991). Ceruloplasmin is an α-globulin that binds more than 90% of copper in blood plasma (Cartwright and Wintrobe 1964a, Suzuki et al. 1989) and delivers it via specific receptors to other target tissues (Linder 1991). It is currently unknown how copper reaches cells bathed by fluids not directly derived from blood plasma like brain and placenta cells (Linder 1991). Ultimately, copper must be distributed to all cells in order for them to carry out oxidative phosphorylation and other essential functions (Linder 1991).

The biliary system is the major pathway for the excretion of copper, excreting 80% (Cartwright and Wintrobe 1964a). Approximately 16% of copper is excreted directly into the bowel and 4% is lost through the urinary system (Cartwright and Wintrobe 1964a).

Total body copper appears to vary with the species, age, and copper status of the individual (Davis and Mertz 1987). Relatively constant copper tissue concentrations indicate sufficient dietary intake and effective homeostatic control of copper (Food and Nutrition Board 1989). Normal blood levels of copper in healthy animals range between 0.5 to 1.5 μg/mL with most values lying between 0.8 and 1.2 μg/mL (Davis and Mertz 1987).

During gestation, the concentration of copper in the human fetus increases greatly with half of the total fetal copper accumulating in the liver. It is believed that these hepatic reserves are designed to protect the infant from copper deficiency in early life (Food and Nutrition Board 1989). Women who are pregnant or taking oral

contraceptives exhibit greatly elevated serum copper levels, apparently from the effects of estrogen (Davis and Mertz 1987).

Copper Deficiency

Copper deficiency in animals results in symptoms of hypocupremia, depletion of tissue copper, anemia, alterations in iron metabolism, skeletal changes, hair and skin changes, neurologic disease and cardiovascular disorders (Cartwright and Wintrobe 1964b). Other biological parameters affected by copper deprivation include glucose tolerance, connective tissue function, cholesterol and lipid formation, transport or metabolism, brain development, fertility, and immunity (Linder 1991). Klevay (1980) observed elevated serum uric acid concentrations in response to copper deficiency in male rats.

In 1979, Mason reported that copper deficiency symptoms in humans had only been observed in infants. In accordance with his observations, dietary copper deficiency is not currently known to occur in adults under normal conditions (Food and Nutrition Board 1989). Hypocupremia as a result of copper depletion has been noted in patients on total parenteral nutrition support and in Menke's steely hair disease (Food and Nutrition Board 1989), an inherited disease of impaired copper utilization which afflicts newborn infants (Mason 1979).

Hypocupremia, indicating a copper deficient state, can occur from a defect in apoceruloplasmin synthesis, low dietary intake of copper, low dietary intake of protein, decreased absorption of copper, and a loss of ceruloplasmin through urine or bowel excretion (Cartwright and Wintrobe 1964b). Hypocupremia has been observed in iron

deficiency, hypoproteinemia, kwashiorkor, tropical sprue, nontropical sprue, and nephrotic syndrome. The presence of hypocupremia in these abnormalities is probably not due to inadequate dietary copper intake (Mason 1979).

As dietary copper deprivation progresses, tissue concentrations of copper fall with time and the activity of copper dependent enzymes decrease (Linder 1991). Copper concentrations in body and tissues decline with deficiency, but they are not reliable or valid as the sole criteria of deficiency (Davis and Mertz 1987). Circulating copper is subject to many non-nutritional factors both hormonal and infectious, in addition to the dietary interactions affecting copper absorption (Davis and Mertz 1987) making it a rather variable index of copper status.

In animal studies, copper deficiency symptoms have been observed with a variety of altered factors. Streptozotocin-induced diabetic rats showed significant increased oxidant stress indicated by alterations of antioxidant enzyme activities when fed diets deficient in copper (McDermott et al. 1994). This study suggested that the observed increase in renal and liver copper concentrations were caused by increased intestinal absorption in the presence of diabetes mellitus (McDermott et al. 1994).

Fructose has been shown to aggravate copper deficiency manifestations in laboratory animals (Fields et al. 1984, Fields et al. 1984, Henry et al. 1991) when used as the sole carbohydrate source in the diet. Female rats, though, appear to be protected from the lethal consequences of copper deficiency and dietary fructose (Fields et al. 1992, Henry et al. 1991). In a study with male rats fed a high fructose and high fat diet deficient in copper, detrimental effects on growth, tissue trace element concentrations, energy

metabolism, and substrate availability for nucleotide synthesis were observed (Wapnir and Devas 1995). A synergistic effect was observed with the three dietary factors of high fructose, high fat, and deficient copper (Wapnir and Devas 1995).

Fields and associates (1992) studied whether differences in copper and iron status between male and female rats can be detected during the development of copper deficiency. Male and female rats were fed either copper deficient or adequate diets with fructose for 31 days. Fields et al. (1992) found that copper-deficient males experienced a reduction in body weight, increase in heart and liver sizes, and a decrease in pancreas size compared to the females who did not experience any change in organ size. In addition, the copper deficient males were anemic and their livers showed the presence of free radicals, while the females exhibited none of these symptoms (Fields et al. 1992).

Using pigs which have gastrointestinal tracts much like humans, Schoenemann et al. (1990) contrastingly found no differences in copper deficiency manifestations in either males or females between sucrose and cornstarch diets. Anemia and cardiac enlargement developed in rats fed copper deficient diets with sucrose or fructose, but not with starch as the carbohydrate source (Fields et al. 1984). Others have also noted such striking reductions or prevention of sudden death from copper deficiency in rats fed starch as compared to sucrose or fructose (Redman et al. 1988). Fructose apparently forms a complex with copper that has different metabolic properties (Reiser et al. 1985). Schoenemann et al. (1990) suggested that starch spared the dietary copper requirement of rats by an unknown mechanism.

In humans, fructose ingestion in a copper deficient diet reduced cuprozinc superoxide dismutase activity of erythrocytes as compared to starch ingestion. Fructose had no effect on serum ceruloplasmin activity or serum copper concentrations (Reiser et al. 1985).

In rats fed a copper deficient diet, beer consumption increased longevity nearly six fold, lowered plasma cholesterol, lessened cardiac enlargement, and raised liver copper concentrations. Beer also seemed to increase absorption and biological half-life of oral radiocopper (Klevay and Moore 1990).

Considerable evidence exists that female animals are protected from severe copper deficiency symptoms (Fields et al. 1987, Fields et al. 1992, Lynch and Klevay 1994). Female rats also remain immunologically normal when copper deficient as compared to males (Bala et al. 1991). The copper deficiency effects on immune response in male rats were more pronounced in the preweanling rat, suggesting that the developing neonatal immune system is more susceptible to the effects of copper deficiency (Bala et al. 1991). Contrastingly, Lynch and Klevay (1994) found that female mice experienced a more extreme form of copper deficiency than did males. Their animal models exhibited reactions resembling those of humans with acute myocardial infarction: the male animals died suddenly while the females died with thrombosis when deficient in copper (Lynch and Klevay 1994).

Fields and associates (1987) have reported their observations of male and female rats fed a copper deficient diet containing fructose with equal deficiencies of copper. The male rats were anemic, had hypertrophied hearts and died. In contrast, the females were

not anemic, did not show heart hypertrophy and pathology and did survive (Fields et al. 1987). These researchers attributed this female protection to the presence of endogenous estrogens and support the possibility that testosterone in the male may play a role in the severity of copper deficiency observed (Fields et al. 1987).

Copper and Glucose Metabolism

Over 60 years ago, Keil and Nelson (1934) reported abnormal glucose metabolism in rats deficient in copper. Recently, investigators have shown an impairment of glucose metabolism in copper deficient rats and humans (Davis and Mertz 1987).

In adipocytes isolated from the epididymal fat pad of young rats, copper and zinc stimulated glucose transport and increased specific insulin binding in vitro, with copper producing a greater effect than zinc (Fields et al. 1983a). In this study, the increased insulin binding was attributed to an increase in the number of receptor sites (Fields et al. 1983a). In streptozotocin-induced diabetic rats, insulin and copper seemed to form a stable complex which increased insulin binding and/or decreased insulin degradation. The effect was more significant than any seen from insulin or copper alone (Fields et al. 1983b). In male rats fed a copper deficient diet, the insulin response was delayed 30 minutes after glucose injection as compared to copper adequate rats. Thus, it appeared likely that copper deficiency interferes with normal glucose utilization (Hassel et al. 1983).

Glycosylated hemoglobin reflects the degree of hyperglycemia, or the concentration of glucose in plasma over the last two or three months in humans. Klevay (1982) tested the hypothesis that the concentration of glycosylated hemoglobin would

increase in copper deficient rats. The rats fed a copper deficient diet, exhibited an 18% to 67% increase in glycosylated hemoglobin concentrations (Klevay 1982).

Few studies have investigated copper deficiency and glucose metabolism in humans (Smith et al. 1991). In case study reports of two men, Klevay et al. (1986) found that impairment of glucose tolerance was the chief manifestation of deficient copper nutriture. Upon repletion with copper sulfate, glucose tolerance returned to normal in these men (Klevay et al. 1986).

Smith and associates (1991) studied the effects of pregnancy and glucose loading on plasma copper levels. As pregnancy progressed, plasma copper and ceruloplasmin activity increased. Pregnant women at 28-34 weeks of gestation exhibited half the glucose disappearance rate that non-pregnant women and women at 13-17 weeks of gestation experienced. Insulin disappearance from the peak to 180 minutes was 71.5% for non-pregnant women, 62.5% for early pregnant women, and 39.3% for late pregnant women. Plasma copper responses were similar, though, for all three groups suggesting that no association exists between plasma copper and serum glucose or plasma insulin responses in pregnancy (Smith et al. 1991).

Some investigators have proposed specific biochemical connections for copper and glucose metabolism. Johnson and Nordlie (1977) found that physiologic concentrations of copper inhibited hydrolysis of glucose-6-phosphatase in vitro. They suggested this effect would decrease the release of glucose into the blood (Johnson and Nordlie 1977). Others have shown that copper promotes glucose incorporation into fatty acid and glycerol moieties of adipose cell glycerides (Saggerson et al. 1976), stimulates insulin binding

(Fields et al. 1983c), and increases lipogenesis (Fields et al. 1983b), and glucose oxidation (Fields et al. 1983c, Smith et al. 1991).

Copper and Lipid Metabolism

Nearly 50 anatomical, chemical, and physiological similarities have been identified in people with ischemic heart disease and animals with copper deficiency (Klevay and Moore 1990). Hypercholesteremia is related strongly to the development of coronary heart disease and is now considered the major risk factor for development of coronary heart disease (Sempos et al. 1989). Many studies have reported strong effects of copper deficiency in experimental animals on the metabolism of lipids, cholesterol, and on peroxidation (Davis and Mertz 1987). Klevay (1975) hypothesized that coronary heart disease is characterized by an imbalance in the ratio of dietary zinc to copper. Hermann et al. (1993) found that elderly subjects with low copper intakes exhibited elevated plasma lipids and plasma triglycerides.

Davis and Mertz (1987) reported that true copper deficiency results in an elevation of serum cholesterol. Allen and Klevay (1980) found a marked elevation in plasma cholesterol and triglyceride concentrations in the copper deficient rat. Copper deficiency produced a significant increase in LDL-cholesterol and a significant decrease in HDL-cholesterol in these rats (Allen and Klevay 1980). Lefevre et al. (1986) found that rats fed copper deficient diets developed elevated plasma cholesterol along with an increase in HDL-cholesterol and LDL-cholesterol. Lefevre et al. (1986) confirmed through separation techniques that the increase in HDL-cholesterol seen in their copper deficient rats occurred in conjuction with an increase in apolipoprotein E-rich HDL-cholesterol.

They also found that hepatic membranes from the copper-deficient group bound significantly fewer lipoproteins than the control group. This decreased binding activity was also negatively correlated with the proportion of HDL-cholesterol enriched with apolipoprotein E (Lefevre et al. 1986).

In another study, high intakes of zinc in relation to copper induced copper deficiency which in turn decreased apolipoprotein A-I and HDL cholesterol and injured the myocardium (Sandstead 1995). Koo and associates (1990) suggested that hypercholesteremia found in copper deficient rats may be due to possible defects in the intravascular metabolism of cholesterol after its release from the liver. Copper deficiency decreases lipoprotein lipase activity which may, in turn, explain the hypertriglyceridemia and hypercholesterolemia associated with copper depletion (Koo et al. 1988, Lau and Klevay 1982). Croswell and Lei (1985) postulated from the results of their study that copper deficiency may alter the structure of apolipoprotein E or the function of lipoprotein receptors which would impair lipoprotein-receptor binding and result in hypercholesterolemia.

Cunnane et al. (1985) studied the effects of copper intake on lipid essential fatty acids in rats. Rats with a low intake of copper exhibited decreased palmitic and oleic acids and increased stearic and docosahexaenoic acids in plasma, liver, and heart phospholipids as compared to controls. Copper was then suggested to significantly affect tissue lipid composition of essential fatty acids (Cunnane et al. 1985).

In dietary studies, rats fed copper deficient diets with fructose or sucrose exhibited many cardiac abnormalities (Redman et al. 1988). Ventricular hypertrophy, mild to severe

myocardial inflammation, degeneration, and fibrosis were found in the hearts of these copper deficient animals (Redman et al. 1988). Ingestion of beer lowered plasma cholesterol and lessened cardiac enlargement in rats fed a diet deficient in copper. The mechanism by which beer produced these benefits is unknown, but perhaps attributable to a the mixture of chemicals and compounds found in beer. The authors did not attribute these effects to alcohol, chromium, or copper levels in beer (Klevay and Moore 1990).

Salonen et al. (1991) found elevated serum copper independently predicted ischemic heart disease in a study of men in eastern Finland. Copper has been shown to promote the oxidation of LDL-cholesterol in vitro and in vivo which increases its atherogenicity. Consequently, raised copper levels in the blood could be a risk factor for cardiovascular disease as well as ischemic heart disease (Salonen et al. 1991).

BHE/cdb rat as a Diabetic Model

The BHE/cdb substrain of rats was formed in 1975 from the parent strain of rats named BHE for the U.S.D.A. Bureau of Home Economics (Berdanier 1991). The BHE/cdb rat was bred to reliably manifest age-related abnormal glucose tolerance and glomerulosclerosis without the complications of obesity and hydronephrosis (Berdanier 1991). The BHE/cdb rat strain is maintained as a closed colony that reliably produces diabetes-like symptoms in 75% of the population (Berdanier 1991).

The BHE/cdb rat is a desirable animal model for the study of IGT. The BHE/cdb rat is not prone to obesity, which removes a common confounding factor of animal models with diabetes (Berdanier 1991). The BHE/cdb rat develops abnormal glucose tolerance by

300 days of age, exhibits fasting lipemia, premature renal disease, and a fatty liver (Berdanier 1991).

Mild fasting hyperglycemia and abnormal glucose tolerance can be enhanced in the BHE rat by feeding a diet rich in fat and sucrose (Park et al. 1986). In a study using male weanling BHE rats, starch feeding produced less glucose through gluconeogenesis. Those fed sucrose, though, synthesized more glucose exacerbating hyperglycemia and abnormal glucose tolerance (Park et al. 1986). In addition, age, sucrose feeding, and coconut oil feeding all help potentiate hyperlipemia, gluconeogenesis, and decreased efficiency of ATP synthesis (Mathews et al. 1995). A point mutation was found in the mitochondrial DNA gene that encodes a portion of F1F0ATPase, an enzyme that catalyzes ATP synthesis (Mathews et al. 1995). Mathews and associates believe this mutation is powerful enough to decrease ATP synthesis efficiency (Mathews et al. 1995), and thus affect oxidative phosphorylation.

CHAPTER III

MATERIALS AND METHODS

Research Design

This study was designed as a 2x2 factorial experiment consisting of four diet treatment groups with dietary chromium and copper as variables. Animals were randomly assigned into each of the four experimental groups.

Animals

Forty male weanling BHE/cdb rats (Univ. of Georgia, Athens, GA) were housed according to USDA and NIH guidelines for laboratory animals. To help control mineral contamination, the rats were housed in an isolated laboratory in plastic cages with plastic gratings. The temperature and humidity controlled laboratory was maintained by personnel from Laboratory Animal Resources in the College of Veterinary Medicine at Oklahoma State University. The rats had free access to experimental diets and deionized water for the course of the study. Deionized water was changed three times a week to maintain freshness. Ceramic feed cups and glass water containers were used to reduce possible mineral contamination.

Treatments

The weanling rats immediately began their assigned experimental diet after arriving at our laboratories. The initial experimental diets were prepared by guidelines of the

American Institute of Nutrition (AIN-93G) diet for rats (Reeves et al. 1993), formulated for growth and adaptation (see appendix A). The four diet combinations randomly assigned were:

1) Adequate chromium and adequate copper (+Cr+Cu)
2) Adequate chromium and deficient copper (+Cr-Cu)
3) Deficient chromium and adequate copper (-Cr+Cu)
4) Deficient chromium and deficient copper (-Cr-Cu)

The mineral mix of the AIN-93G diet was altered only according to the experimental design groups listed above. Diets with adequate chromium contained 0.1466 g chromium chloride/kg mineral mix. Diets with adequate copper contained 0.30 g cupric carbonate/kg mineral mix. Diets with deficient chromium contained 0 g chromium chloride/kg mineral mix. Diets with deficient copper contained 0.03 g cupric carbonate/kg mineral mix. The vitamin mix was altered to contain five times the recommended level of Vitamin E, or 0.6 g/kg diet mix, to minimize oxidative damage (Kullen and Berdanier 1992). After weights had plateaued at thirteen weeks, the diet was changed to a high fat diet (see appendix B) with the same factorial design of mineral depletion. This high fat diet was then given ad libitum through the end of the study. Experimental diet and deionized water were available for free access through the end of the study except as specified by the experimental protocol.

Preparation of Diets

Semi-purified diets and mineral mixes were prepared in our laboratory after assaying diet components for trace mineral content. The mineral mixes were prepared from reagent grade or ultrapure chemicals using the lot number found to be lowest in chromium. Different lots have been found to vary greatly in trace mineral concentration. Diet and mineral mix ingredients were weighed in advance. Ingredients for the mineral mix were mixed in a burundum-fortified porcelain jar in a roller type mill for 24 hours. Each batch of diet was mixed for 40 minutes to provide consistency in diet preparation and composition (see Appendix C). Diet was refrigerated and protected from light to prevent the breakdown of light sensitive vitamins.

Experimental Protocol

Throughout the study, animals were weighed weekly. During the third, tenth, and sixteenth weeks, a sample of twenty-four animals, six from each group, were caged in metabolic cages for urine collection, and food was withheld for six hours to create a fasting situation. Urine glucose test strips (Clinistix, Bayer Corporation, Diagnostics Division, Elkhart, IN) were used to test the animals for glucose intolerance. During the twelfth, eighteenth, and twenty-first experimental weeks, baseline blood samples were taken from the tail, and each rat was given a 1g/kg body weight glucose load in a 50% glucose solution for an oral glucose tolerance test. Two hours later, another blood sample was carefully taken from a cut to the end of the tail to avoid hemolysis. Baseline and two hour blood drops were analyzed immediately on a β-glucose analyzer (HemoCue Inc., Mission Viejo, CA) to investigate the state of impaired glucose tolerance (IGT) and

possible mineral depletion in the animals. For the first glucose tolerance test, the glucose difference was determined by the difference between the baseline glucose and the 2 hour glucose.

After twenty-one weeks on the experimental diets, the first set of animals was necropsied. Four animals, one from each experimental group, were necropsied each day for a total of ten days. The animals were caged in metabolic cages and food was withheld for 12 hours overnight. The animals had free access to deionized water. Urine samples were collected during the 12 hour fast and frozen for chemical analysis. The animals were anesthetized with ketamine HCl (60 mg) and xylazine (6 mg) and lean body mass was measured using the EmScan SA-2 small animal body composition analyzer. The animals were then exsanguinated. Blood samples were collected by cardiac puncture at necropsy using two separate syringes for plasma and serum collection. Sodium citrate was used as the anti-coagulating agent for plasma collection. Blood collected for plasma samples was centrifuged and separated as soon as possible. Blood for serum samples was chilled and allowed to clot for at least thirty minutes, then centrifuged and separated. Plasma and serum were both stored at -20°C until assayed. Tissues were removed, weighted, and stored at -20°C.

Weight gain was expressed as the difference between final weight and initial weight. Fat mass was determined by obtaining the difference between lean body mass and final weight. Tissue percent body weights were determine for liver, spleen, testes, heart, and thymus by dividing the tissue weight by the final body weight of the animal and multiplying by 100 to obtain a percentage.

Biochemical Analysis

Serum and Urinary Uric Acid

Serum uric acid was determined on the COBAS FARA clinical analyzer system with a Roche reagent (Roche Diagnostic Systems, Inc., Branchburg, NJ) using a modification of the Fossati method (Fossati et al. 1980). Fossati and associates (1980) used peroxidase to couple the hydrogen peroxide formed by the uricase reaction that oxidizes uric acid to allantoin. The modification uses N-ethyl-N-(2-hydroxy-3-sulfopropyl)-m-toluidine (TOOS), which forms a chromogen with 4-aminoantipyrine in the presence of hydrogen peroxide and peroxidase. The absorbance of the chromogen is measured at 550 nm, which is proportional to the concentration of uric acid. Non-hemolyzed serum was used as the specimen. Uric acid in urine was also measured using this procedure on the COBAS FARA chemical analyzer system. Total urine uric acid was express as mg/12 hours and obtained by the following formula:

Total urine uric acid = (urine volume x 0.01) x urine uric acid

Glucose

Serum glucose concentration was determined on the COBAS FARA clinical analyzer system with a Roche reagent (Roche Diagnostic Systems, Inc., Branchburg, NJ) using the procedure described by Barthelmai and Czok (1962). This procedure is an enzymatic approach using hexokinase coupled with glucose-6-phosphate dehydrogenase

to catalyze two reactions which ultimately yield 6-phosphogluconate and NADH (Barthelmai and Czok 1962). When spectrophotometrically measured at 340 nm, the increase in NADH concentration is directly proportional to the glucose concentration in the sample. Non-hemolyzed serum was used as the specimen.

Insulin

Serum insulin concentrations were measured with a double radioimmunoassay antibody technique using a rat insulin RIA kit (Linco Research, Inc. St. Louis, MO) based on a procedure described by Morgan and Lazarow (1963). Insulin determination is based on the ability of a limited quantity of antibody to bind a fixed amount of radiolabeled antigen. The Linco RIA kit utilizes an antibody made specifically against rat insulin. The amount of radioactivity in tubes was counted using the Packard Cobra II automatic gamma counter (Packard Instrument Co., Meriden, CT) with a data reduction system. Non-hemolyzed serum was used as the specimen. Insulin to glucose ratio, an indicator of insulin sensitivity, was determined by dividing insulin by glucose for each animal.

Xanthine Oxidase

Xanthine oxidase (xanthine:oxygen oxidoreductase, EC 1.1.3.22) is an iron-molybdenum flavoprotein that is assumed to be the rate-limiting step in purine metabolism (Fried and Fried 1974). Xanthine oxidase activity was measured using the methods of Singh et al. (1987). This procedure involves the stoichiometric oxidation of 2,2'-azino-di(3-ethyl-benzthiozoline-6-sulphonate) (ABTS) in the presence of peroxidase and hydrogen peroxide to form a chromogen. The oxidized ABTS, which is proportional to the xanthine oxidase activity, was measured at 410nm on the Beckman DU-64

spectrophotometer with non-hemolyzed serum samples. Repeating samples were performed for each animal to provide a mean result. The serum specimen was stored at -20°C after collection and manually assayed within 5 days (appendix D).

Serum Creatinine and Urinary Creatinine

Determination of serum creatinine was performed on the COBAS FARA clinical analyzer system with a Roche reagent (Roche Diagnostic Systems, Inc., Branchburg, NJ). Using a kinetic modification of the original Jaffe reaction (Larsen 1972), the sample is added to an alkaline picrate solution, and absorbance is read at 500 nm after a 10 second time interval to minimize non-creatinine interferants. The absorbance measured is then proportional to the creatinine concentration in the sample. Non-hemolyzed serum and urine samples were used as the specimens in this procedure. Total urine creatinine was expressed as mg/12 hour and determined by the following formula:

Total urine creatinine = (urine volume x 0.01) x urine creatinine

Blood Urea Nitrogen

Determination of Blood Urea Nitrogen (BUN) was performed on the COBAS FARA clinical analyzer system with a Roche reagent (Roche Diagnostic Systems, Inc., Somerville, NJ). This test is performed as a kinetic assay in which urea in the sample is hydrolyzed by urease to ammonia and carbon dioxide. Glutamate dehydrogenase then converts ammonia and α-ketoglutarate to glutamate and water. Concurrently, reduced nicotinamide adenine dinucleotide (NADH) is oxidized to nicotinamide adenine dinucleotide (NAD). Two moles of NADH are oxidized for each mole of urea present.

The initial rate of decrease in absorbance at 340 nm is proportional to the urea concentration in the sample. This procedure used non-hemolyzed serum as the specimen.

Non-esterified Fatty Acids and β-hydroxybutyrate

In uncontrolled diabetes mellitus, low insulin levels lead to increased lipolysis and decreased re-esterification, thereby increasing serum non-esterified fatty acids (NEFA). These NEFA are oxidized in the liver and result in increased hepatic ketone production and acetoacetate accumulation in the blood. There are three different types of ketone bodies present in the blood: β-hydroxybutyrate, acetoacetate, and acetone. The measurement of β-hydroxybutyrate is one method of detection and estimation of ketone bodies in serum (Tietz 1994).

The COBAS FARA clinical analyzer system was used to determine β-hydroxybutyrate in non-hemolyzed serum with an enzyme kit (Sigma Diagnostics, Catalog No. 310-A, St. Louis, MO). In the analysis of β-hydroxybutyrate, ketones are oxidized by 3-hydroxybutyrate dehydrogenase in the presence of NAD. This reaction forms acetoacetate, NADH and H^+. NADH absorbs light at 340 nm. Thus, the increase in absorbance due to the reaction just described is directly proportional to the β-hydroxybutyrate concentrations in the sample.

NEFA concentrations in serum were determined on the COBAS FARA clinical analyzer system using the NEFA-C kit (WAKO Chemicals USA Inc., Richmond, VA). Acyl CoA from the fatty acids, 4-aminoantipyrene, 3-methyl-N-ethyl-N-(β-hydroxyethyl)-aniline and peroxidase are oxidized and react to form peroxide, which then forms a purple

colored adduct, which can be measured colorimetrically 550 nm. The amount of NEFA in the serum sample was determined from the optical density measured at 550 nm.

Serum Triglyceride

Serum triglyceride concentrations were colorimetrically determined on the COBAS FARA clinical analyzer system with an enzyme kit (Sigma Diagnostics, Catalog No. 339-20, St. Louis, MO) using a non-hemolyzed serum specimen. Initially, lipase catalyzes the triglycerides into glycerol and fatty acids. Glycerokinase then catalyzes the first of three reactions which ultimately produce quinoneimine dye with the addition of peroxidase. The absorbance of quinoneimine dye is then measured at 540 nm and is directly proportional to the triglyceride concentration in the sample.

Total Cholesterol

Serum total cholesterol concentrations were determined using the COBAS FARA clinical analyzer system with a Roche reagent (Roche Diagnostic Systems, Inc., Branchburg, NJ). Using a method formulated from a procedure described by Allain et al. (1974), cholesterol ester hydrolase is added to the sample to release cholesterol from its esters. This free cholesterol is then oxidized by cholesterol oxidase, which produces hydrogen peroxide. When hydrogen peroxide is combined with 4-aminoantipyrine and phenol, a chromophore is formed. The absorbance of this chromophore can be read at 500 nm and is directly proportional to the cholesterol concentration in the sample. Non-hemolyzed serum was used as the specimen in this procedure.

Fructosamine

Fructosamine is a glycated protein assayed to indicate average glucose status over a time frame of one to three weeks. The COBAS FARA clinical analyzer system was used to determine levels of fructosamine in non-hemolyzed serum samples using the RoTAG Fructosamine Assay kit (Roche Diagnostic Systems, Inc., Branchburg, NJ). This colorimetric test relies on the ability of ketoamines to reduce nitroblue tetrazolium at alkaline pH. After a ten minute delay to eliminate endogenous substance interference, the formation rate of formazan is read at 550 nm. This rate is then directly proportional to the fructosamine concentration in the sample.

Serum Magnesium

Serum magnesium was determined on the COBAS FARA clinical analyzer using a Roche reagent (Roche Diagnostic Systems, Inc., Branchburg, NJ) This reagent kit utilizes the methods of Ferguson et al. (1964) by using chlorophosphonazo III as a sensitive dye to determine magnesium spectrophotometrically. The Roche reagent then adds EDTA to prevent calcium interference. Chlorophosphonazo III binds to magnesium, causing an absorbance decrease at 550 nm and an absorbance increase at 675 nm. The delta absorbances at both wavelengths are proportional to the magnesium concentration in the serum sample.

Statistical Analysis

Data obtained from this study were analyzed as a 2x2 factorial experiment using the Statistical Analysis System (SAS Institute Inc., Cary, NC, 1989) version 6.08. The general linear models procedure was used for analysis of variance and least squares means determination. Pearson correlation coefficients were also generated by using SAS. All data were expressed as means ± SEM. Results were considered significant at $p \leq 0.05$.

CHAPTER IV

RESULTS AND DISCUSSION

Three oral glucose tolerance tests were performed during the study. Forty rats were included in the first and third tests while only 26 were included in the second glucose tolerance test. A significant interaction existed between the dietary factors of chromium and copper in the baseline glucose results of the first test (Table 1). Copper had a significant effect upon glucose within the diet groups inadequate in chromium (Table 1). Glucose was lowest in the -Cr-Cu group and highest in the -Cr+Cu group. Overall, the first glucose tolerance test exhibited the highest blood glucose on average, possibly indicating a more impaired state of glucose tolerance early in the study than later (Table 1). The BHE/cdb rat is considered to be genetically predisposed to non-insulin dependent diabetes mellitus (NIDDM), and was expected to have the characteristics of age-related hyperglycemia (Berdanier 1991). Chromium deficiency has produced elevated blood glucose in past studies (Li and Stoecker 1986). In humans, Anderson et al. (1991) states that consuming diets low in chromium leads to detrimental effects on glucose tolerance in hyperglycemic subjects. In the present study, depleted copper decreased glucose within the depleted chromium groups in the first glucose tolerance test. This contradicts the findings by Hassel et al. (1983) of significantly increased plasma glucose levels in copper-deficient rats.

Urine creatinine was measured on the urine collected during the fasting period of the three glucose tolerance tests and converted to total urine creatinine for each test. During the second glucose tolerance test, copper had a significant effect upon total urine creatinine (Table 2). The experimental groups with deficient copper exhibited significantly higher total urine creatinine than those groups with adequate copper (Table 2). This finding possibly indicates less efficient kidney function in the copper deficient groups, as urine creatinine can be used to assess the kidney's ability to clear waste products from the body (Johnson 1989). Increased total urine creatinine can also indicate the increased breakdown of muscle tissue (Johnson 1989). The copper deficient groups with increased total urine creatinine could actually be experiencing tissue breakdown rather than renal insufficiency. Total urinary creatinine from glucose tolerance test 1 positively correlated with body weight from the twelfth week during which the test was performed ($r=0.37$, $p<0.02$, Table 3). Creatinine excretion can indicate the breakdown of lean muscle tissue as muscle creatine is converted to creatinine daily (Johnson 1989). Thus, urinary creatinine is an indicator of muscle mass. Mineral depletion could contribute to this lean muscle mass loss (Croswell and Lei 1985, Hassel et al. 1983). Lean body mass was only measured at necropsy. More frequent body composition tests could have shown a relationship between urine creatinine and lean body mass during the growth period of these animals. These correlations could then help disclose the possibility of muscle mass wasting due to mineral depletion in these animals.

The fact that 2 hour glucose from tests 1, 2, and 3, and glucose difference were not significantly different among the treatment groups could indicate that these animals

never physiologically reached a state of impaired glucose tolerance (IGT) and were not sufficiently mineral depleted. Perhaps an earlier introduction of the high fat diet could have helped induce IGT in these animals. More frequent glucose tolerance tests could have more accurately depicted the pattern of glucose tolerance in these animals. Total urine creatinine from glucose tolerance tests 1 and 3 also was not significantly different among the treatment groups, again indicating the possibility that these animals were not sufficiently mineral depleted to cause an early IGT state and subsequent kidney insufficiency found in the pathophysiology of diabetes.

Upon necropsy, one animal showed signs of severe illness and was deleted from the database. Thus, necropsy parameters were available from only 39 rats. In addition, fructosamine, glucose, blood urea nitrogen (BUN), and non-esterified fatty acids (NEFA) were deleted for nine animals due to hemolysis of the sample. Xanthine oxidase was also deleted for four animals due to erratic results.

Initial and final body weights were obtained from all forty rats and analyzed for the 39 remaining in the database. From these weight measurements, weight gain was determined. There were no significant differences in initial body weight among any of the groups (Table 4). This data was collected before any dietary treatment was begun; thus, these results were desired to avoid experimental error.

Lean body mass was measured on the 26 animals which could physically fit in the measurement device. After the sick animal was deleted, 25 animals remained from which lean body mass was determined. Lean body mass and lean body mass as percent body weight and fat mass and fat mass as percent body weight were not significantly different

among the treatment groups (Table 5). Mooney and Cromwell (1995) examined the effects of dietary chromium picolinate supplementation on body composition of pigs. They found that chromium picolinate significantly increased the percentage of muscle mass and tended to decrease percentage of fat mass (Mooney and Cromwell 1995). In humans, though, Hallmark et al. (1996) found no significant difference in body weight, percent body fat, lean body mass, and skinfold thicknesses with chromium supplementation and resistance training. In addition, Lukaski and associates (1996) found increases in strength, mesomorphy, fat-free mass, and muscle mass with resistance training in men independent of the chromium supplementation.

Fat mass positively correlated with insulin ($r=0.54$, $p=0.0052$, Table 3) as did final body weight ($r=0.54$, $p=0.0004$, Table 3). Kaplan (1989) explains that obesity, or increased fat mass, is associated with peripheral insulin resistance. Hyperinsulinemia then develops in an attempt to maintain euglycemia. Body weight increases with increased fat mass, and would logically also correlate with insulin if fat mass was a significant portion of whole body weight.

Tissue weights were expressed as percent body weight for 39 animals. On this basis, chromium had a significant effect on liver, kidney, and spleen (Table 6). Animals with deficient chromium diets exhibited significantly higher percent body weights of these tissues than animals with adequate chromium diets (Table 6). Li and Stoecker (1986) also found signficantly heavier livers in chromium deficient, genetically obese mice. In addition, total hepatic lipid of the obese mice was significantly greater in the chromium deficient groups, indicating abnormal lipid accumulation and fatty livers in animals with

chromium depletion (Li and Stoecker 1986). Upon necropsy, the animals' livers in the present study appeared enlarged and possibly fatty in composition which could be a result of mineral depletion or their genetic predisposition to diabetes. Seaborn and associates (1994), though, reported increased liver and kidney weights in chromium supplemented guinea pigs, contradicting the results of the present study. These researchers suggested that the increased liver and kidney tissues in chromium supplemented animals were due to the possible involvement of chromium in protein synthesis (Seaborn et al. 1994). The increased weight of these tissues was lean mass rather than fatty in composition (Seaborn et al. 1994). In addition, these guinea pigs were chronically depleted of ascorbic acid (Seaborn et al. 1994), which could help explain the differences seen in our results and theirs.

Final body weight and weight gain were not significantly different among the treatment groups in this study (Table 4). In a few past studies, chromium has had a significant effect on weight gain. Seaborn et al. (1994) emphasized the essentiality of chromium for animal growth. In their study, chromium deficient guinea pigs exhibited significantly decreased body weight when compared to chromium adequate animals. Copper deficiency has produced significantly decreased weight gain and mean body weight in past studies (Croswell and Lei 1985, Hassel et al. 1983). Fields et al. (1983c), though, found no significant effect of copper deficiency on growth rate. Perhaps with different sampling times throughout the growth period of the animals, the mineral effects of chromium and copper would have significantly affected weight gain. Fat mass and lean body mass were also not significantly different among treatment groups (Table 5). As

mentioned above, a different sampling design with more frequent body composition tests during the growth period of the animals might find a signficant effect of chromium and copper. Testes, heart, and thymus as percent body weight were not significantly different among the treatment groups (Table 6). In several animals, the thymus was possibly atrophied from advanced age and maturity and sometimes hidden beneath layers of fatty tissue, making identification and complete removal nearly impossible. Thus, this tissue was not available from enough animals in a complete and accurate form to lend itself to significance.

Serum insulin was not significantly different among the experimental groups, but adequate chromium tended to increase insulin and depleted chromium tended to decrease insulin (Table 7). This finding contradicts what has been seen in the literature (Li and Stoecker 1986) with decreased serum insulin in the presence of adequate chromium. A decrease in insulin with chromium depletion could indicate an effect of mineral depletion upon insulin release in the BHE/cdb rat. In chromium deficient animals, insulin sensitivity is decreased (Mertz et al. 1965), which can cause hyperinsulinemia as the pancreas compensates for hyperglycemia (DeFronzo 1988). Deficient copper has been shown to increase serum insulin (Fields et al. 1983b) which also was not seen in this study (Table 7). Insulin positively correlated with serum uric acid ($r=0.55$, $p=0.0003$, Table 8), which supports the hypothesis that insulin inhibits uric acid secretion or enhances uric acid reabsorption at the renal tubular level (Galvan et al. 1995). This positive correlation was observed in the Normative Aging Study by Lee et al. (1995).

Insulin positively correlated with total cholesterol ($r=0.38$, $p<0.02$, Table 8) which was noted by Li and Stoecker (1986). This correlation is very logical in the relationship between hyperinsulinemia and cardiovascular disease explained by many articles (Reaven 1988, Reaven 1991, Zimmet 1993). Hyperinsulinemia results in a dyslipidemia of decreased high density lipoprotein (HDL) cholesterol and increased very low density lipoprotein (VLDL) synthesis, which ultimately affects total cholesterol (Reaven 1991, Zimmet 1993). Insulin also positively correlated with fat mass ($r=0.54$, $p=0.0052$, Table 3) and final body weight ($r=0.54$, $p=0.0004$, Table 3). Li and Stoecker (1986) also found a positive, though not significant, correlation between insulin and final body weight in obese mice. In 1989, Kaplan explained that obesity, or increased fat mass, is associated with peripheral insulin resistance, which in turn, increases insulin secretion to maintain euglycemia.

Although glucose was not significantly different among the diet groups, glucose did positively correlate with both triglyceride ($r=0.40$, $p<0.03$, Table 8) and non-esterified fatty acids (NEFA) ($r=0.47$, $p=0.009$, Table 8). As hyperglycemia initiates hyperinsulinemia in the insulin resistant individual, lipolysis is enhanced resulting in a rise in NEFA (Reaven 1988, Zimmet 1993), which may be the basis for this correlation. Hyperinsulinemia also results in a dyslipidemia described above that results in elevated serum triglyceride concentration (Reaven 1991, Zimmet 1993).

Fructosamine indicates average glucose status over the past one to three weeks. Therefore, this glycated protein assay assists in obtaining a more accurate estimate of the necropsy glucose since glucose may be falsely elevated by anesthesia. Fructosamine was

not significantly different among the experimental groups in this study (Table 7). Fructosamine did tend to show an interaction effect, though, between chromium and copper (Table 7). Within deficient chromium groups, animals with deficient copper tended to show increased fructosamine as animals with adequate copper tended to exhibit decreased fructosamine (Table 7). Klevay (1982) saw an increase in glycosylated hemoglobin in copper deficient humans, supporting the results shown here. Fructosamine also positively correlated with NEFA ($r=0.44$, $p<0.02$, Table 8). This correlation, again, is explained by the concurrent presence of hyperglycemia and hyperinsulinemia resulting in increased lipolysis and increased NEFA (Reaven 1988).

Serum triglyceride was not significantly affected by dietary chromium or copper, but chromium tended to have an effect upon this parameter (Table 9). The groups with adequate chromium tended to exhibit the highest triglyceride concentrations in comparison to the groups with deficient chromium (Table 9). Vinson and Bose (1984) and Lee and Reasner (1984) both reported decreased triglyceride levels with supplemental chromium in humans with elevated triglyceride concentrations. The present study did not provide chromium in chromium picolinate and high chromium yeast mixtures as these studies did, which could help explain the difference in results. Triglyceride positively correlated with both glucose ($r=0.40$, $p<0.03$, Table 8) and NEFA ($r=0.66$, $p=0.009$, Table 8). These correlations are again well supported and explained by Reaven (1988) and Zimmet (1993). Hyperglycemia exacerbates hyperinsulinemia which, in turn, results in dyslipidemia and increased lipolysis. These changes then elevate serum triglyceride and NEFA, respectively (Reaven 1988, Zimmet 1993).

Serum total cholesterol was significantly affected by dietary copper (Table 9). The groups fed copper depletion diets exhibited decreased total cholesterol, while those with adequate copper diets exhibited increased total cholesterol (Table 9). Allen and Klevay (1980) and Koo et al. (1988) found significantly elevated serum total cholesterol in copper deficient rats, contrasting with our results. Salonen et al. (1991) reported that elevated serum copper concentrations in 1,666 men in the Kuopio Ischaemic Heart Disease Risk Factor Study in Eastern Finland independently predicted ischemic heart disease risk. Salonen et al. (1991) reported that serum copper concentration rose with increasing age and positively, though not signficantly, correlated with serum LDL cholesterol concentration. Salonen and associates (1991) report a high copper content in drinking water that is acidic, running through copper pipes. Thus, this population's diet and environment most likely is copper supplemented, rather than copper adequate as our animals were. Cholesterol positively correlated with insulin ($r=0.38$, $p<0.02$, Table 8), which, again has been intricately explained by many articles addressing hyperinsulinemia and dyslipidemia (Reaven 1988, Reaven 1991, Zimmet 1993). Briefly, hyperinsulinemia results in decreased HDL cholesterol and increased VLDL synthesis, which ultimately affects total cholesterol (Reaven 1991, Zimmet 1993).

NEFA are elevated in the serum of uncontrolled diabetics with low insulin levels and increased lipolysis (Tietz 1994). Chromium had a significant effect upon NEFA in this study (Table 9). Those animals with deficient chromium exhibited significantly lower NEFA than those animals with adequate chromium diets (Table 9). Mirsky (1993) found decreased free fatty acids in diabetic rats injected with a glucose tolerance factor (GTF)

preparation extracted from yeast extract powder in the absence of exogenous insulin. Our findings disagree with the results of the study by Mirsky (1993). The BHE/cdb rat should have a significant amount of insulin circulating at this early stage in their disease state (Berdanier 1991). This hypothesis would make the BHE/cdb rat significantly different than the streptozotocin-induced diabetic rats Mirsky (1993) studied with no insulin production from their damaged beta cells. This strong difference in serum insulin in the rats could help explain the contradictory results we obtained. NEFA did positively correlate with many parameters in this study, though, including glucose ($r=0.47$, $p=0.009$, Table 8), fructosamine ($r=0.44$, $p<0.02$, Table 8), and triglyceride ($r=0.66$, $p=0.0001$, Table 8). These results are well documented and explained by many research studies as being intricately related abnormalities within the "Syndrome X" described by (Reaven 1988). In a state of hyperglycemia, hyperinsulinemia occurs to compensate for the elevated glucose. Hyperinsulinemia then enhances lipolysis which, in turn, raises NEFA. Hyperinsulinemia also results in dyslipidemia by decreasing HDL cholesterol and increasing VLDL synthesis which then elevate serum triglyceride (DeFronzo and Ferrannini 1991, Reaven 1988, Reaven 1991).

Serum magnesium was not significantly different among the treatment groups (Table 9), but serum magnesium did positively correlate with serum uric acid ($r=0.71$, $p=0.0001$, Table 8). An inverse relationship between magnesium nutriture and the incidence of cardiovascular disease has been postulated (Arsenian 1993, Shils 1996). This fact alone, could support the strong correlation seen between serum magnesium and serum uric acid. The relationship may begin, though in the renal tubules. One-third of filtered

magnesium is absorbed in the proximal convoluted tubule, then 50-60% of the remaining filtered magnesium is reabsorbed between the descending limb and the early distal tubule of the kidney (Shils 1996). Ninety-eight to 100% of filtered plasma uric acid is reabsorbed in the proximal convoluted tubule (Tietz 1994). Thus, serum magnesium and uric acid are absorbed and reabsorbed, respectively, at the same time within the renal tubules, perhaps explaining the positive correlation seen in this study.

Glucose, insulin, insulin to glucose ratio, fructosamine, triglyceride, and β-hydroxybutyrate (BOHB) were not statistically different among the treatment groups. These parameters were all expected to be significantly affected by mineral depletion of chromium and copper. Perhaps the sample size was too small to be significantly affected after several animals' data sets were deleted for these parameters because of hemolysis. In addition, the mineral depletion might not have been severe enough to significantly affect these parameters. The BHE/cdb rat may also not have been in a state of physiological IGT, causing these serum parameters not to show significant difference with the mineral combinations used in this study. As Anderson et al. (1991) indicated, the effect of chromium depends on the degree of glucose intolerance.

BUN indicates the presence of azotemia and is an indicator of renal excretory function. Chromium had a significant effect upon BUN (Table 10). Animals with chromium depleted diets exhibited significantly lower BUN than those with adequate chromium diets (Table 10). The +Cr+Cu group exhibited the most elevated BUN, indicating the possible presence of renal insufficiency in this group. Copper, though not statistically significant, tended to have an effect upon BUN. Those animals with deficient

copper tended to have lower BUN than those groups with adequate copper (Table 10). Renal insufficiency is common with nephropathy complications of NIDDM. These complications typically do not occur until more advanced stages of the disease state, though. As the glucose tolerance tests indicated, these BHE/cdb rats might not have even been glucose intolerant, much less uncontrollably diabetic at the time of necropsy.

Serum and total urinary uric acid were also examined at necropsy and found not to be significantly different among the experimental groups (Table 10). Stoecker and associates (1996) observed significantly elevated serum uric acid concentrations when both dietary chromium and supplemented aspirin were low. This study instigated the present study's investigation into the effects of chromium on serum uric acid concentrations. Stoecker and associates (1996) used an additional factor of iron in their study which had a quadratic effect on serum insulin, which is known to significantly affect uric acid metabolism (Galvan et al. 1995). The fact that the present study did not include iron and aspirin as stressors may help explain the lack of significance in the uric acid parameters within the dietary factors of chromium and copper. In addition, Stoecker et al. (1996) used a genetically obese mouse model which could have different metabolic patterns than the non-obese BHE/cdb rat model. Serum uric acid did exhibit a positive correlation with insulin ($r=0.55$, $p=0.0003$, Table 7) which lends support to the hypothesis that insulin inhibits uric acid secretion or enhances uric acid reabsorption at the renal tubular level (Cappuccio et al. 1993, Galvan et al. 1995).

Serum and total urine creatinine, serum and total urine uric acid, and xanthine oxidase were not significantly different within the treatments of dietary chromium and

copper depletion or adequacy. Many of these parameters were expected to be significantly affected by mineral depletion of chromium and copper. Our BHE/cdb rats were not hyperinsulinemic or hyperuricemic, and, quite possibly, not physiologically glucose intolerant either. Considering that depleted copper significantly increased total creatinine during the second glucose tolerance test (Table 2) and that total urine creatinine was positively correlated with weights during the first glucose tolerance test ($r=0.37$, $p<0.02$, Table 3) it is quite possible that mineral depletion most significantly affects the growth period in these animals.

Due to laboratory renovations, analysis of the diets through atomic absorption spectrophotometry is not possible for this report. This spectrophotometer is also needed to accurately determine the state of mineral depletion in the tissues of these animals. Thus, the lack of significance in many parameters within this specific proposal could be elucidated by the complete analysis of all data collected on the BHE/cdb rats depleted in chromium and copper for twenty-one weeks.

TABLE I

Glucose Tolerance Test Results†

Treatment	N	Test 1 Glucose 0 hr. (mg/dL)	Test 1 Glucose 2 hr. (mg/dL)	Test 1 Glucose Difference‡ (mg/dL)	Test 2 Glucose 2 hr. (mg/dL)	Test 3 Glucose 2 hr. (mg/dL)
-Cr-Cu	10	72±2	128±13	56±12	121±9[1]	115±4
-Cr+Cu	10	82±3	133±12	51±14	113±6[1]	120±7
+Cr-Cu	10	78±2	137±13	59±12	132±5[1]	119±3
+Cr+Cu	10	77±2	136±9	59±10	122±6[1]	127±6
Factors				**P Values**		
Cr		0.89	0.61	0.63	0.17	0.28
Cu		0.11	0.90	0.82	0.22	0.25
Cr*Cu		<0.04	0.81	0.82	0.88	0.82

† Values are means ± SEM. Oral glucose tolerance tests were done in experimental weeks 12, 18, and 21.
‡ Values are the difference between 2 hour and 0 hour glucose for test 1.
[1] N=6

TABLE II

Total Urine Creatinine from Glucose Tolerance Tests†

Treatment	**N**	Test 1 **T. U. Creatinine** (mg/12 hr)	Test 2 **T. U. Creatinine** (mg/12 hr)	Test 3 **T. U. Creatinine** (mg/12 hr)
-Cr-Cu	10	5.21±0.59	7.11±0.42[1]	6.80±0.58
-Cr+Cu	10	4.55±0.53	4.91±0.63[1]	6.36±0.50
+Cr-Cu	10	5.62±0.35	6.03±0.74[1]	6.65±0.34
+Cr+Cu	10	5.35±0.40	5.29±0.36[1]	6.42±0.51
Factors			**P Values**	
Cr		0.21	0.54	0.92
Cu		0.34	<0.02	0.50
Cr*Cu		0.69	0.20	0.83

† Values are means ± SEM. Oral glucose tolerance tests were done in experimental weeks 12, 18, and 21.
[1] N=6

TABLE III

Correlation Coefficients (r) Between Blood, Urine, and Body Composition Parameters at Necropsy and Glucose Tolerance Tests

	INSULIN	FINAL WT.	FAT	LBM	T. CREAT 1	T. CREAT 3	WT. 12	WT. 21
Insulin	1.0000	0.5384	0.5410	0.0892	-0.0683	-0.0501	0.4869	0.4363
(p=)		0.0004	0.0052	NS	NS	NS	0.0017	0.0141
Final Weight		1.0000	0.8494	0.7675	0.2493	0.1933	0.8845	0.9603
(p=)			0.0001	0.0001	NS	NS	0.0001	0.0001
Fat Mass			1.0000	0.3136	0.2485	-0.0120	0.7649	0.7479
(p=)				NS	NS	NS	0.0001	0.0001
Lean Body Mass				1.0000	0.6921	0.5081	0.6398	0.7642
(p=)					0.0001	0.0095	0.0006	0.0001
Total U. Creatinine 1					1.0000	0.5161	0.3728	0.3988
(p=)						0.0008	0.0194	0.0263
Total U. Creatinine 3						1.0000	-0.1531	0.1469
(p=)							NS	0.3721
Weight, 12th Week							1.0000	0.9308
(p=)								0.0001
Weight, 21st Week								1.0000
(p=)								

NS = No significance.

TABLE IV

Body Weights and Weight Gain at Necropsy†

Treatment	**N**	**Body Weight (g)**		**Weight Gain (g)**
		Initial[1]	Final[2]	Δ[3]
-Cr-Cu	10	59±3	523±13	464±12
-Cr+Cu	10	52±3	540±23	487±21
+Cr-Cu	10	55±4	552±19	497±17
+Cr+Cu	9	55±3	558±18	503±17
Factors			**P Values**	
Cr		0.77	0.22	0.16
Cu		0.27	0.54	0.38
Cr*Cu		0.31	0.77	0.61

† Values are means ± SEM.
[1] Data were obtained on the day the rats were received.
[2] Data were obtained on the day of autopsy.
[3] Values are the differences between initial and final weights.

TABLE V

Lean Body Mass (LBM) and Fat Mass at Necropsy and as Percent of Final Weight†

Treatment	N	Lean Body Mass (g)	LBM as % of Final Weight	Fat Mass (g)	Fat Mass as % of Final Weight
-Cr-Cu	6	310±8	63±1	185±8	37±1
-Cr+Cu	7	330±13	65±1	174±9	35±1
+Cr-Cu	6	338±11	64±2	198±22	36±2
+Cr+Cu	6	341±11	64±2	194±15	36±2
Factors			**P Values**		
Cr		0.09	0.85	0.26	0.85
Cu		0.30	0.36	0.61	0.36
Cr*Cu		0.46	0.44	0.80	0.44

† Values are means ± SEM.

TABLE VI

Liver, Kidney, Spleen, Testes, Heart, and Thymus as Percent Body Weight at Necropsy†

Treatment	N	Liver	Kidney	Spleen	Testes	Heart	Thymus
				% Body Weight			
-Cr-Cu	10	2.70±0.07	0.65±0.01	0.23±0.01	0.65±0.02	0.241±0.005	0.042±0.005
-Cr+Cu	10	2.58±0.05	0.64±0.04	0.21±0.01	0.62±0.02	0.241±0.007	0.051±0.007[1]
+Cr-Cu	10	2.49±0.07	0.57±0.02	0.20±0.01	0.62±0.02	0.235±0.006	0.049±0.007[1]
+Cr+Cu	9	2.51±0.04	0.61±0.01	0.20±0.01	0.61±0.02	0.230±0.006	0.045±0.007[2]
P Values							
Cr		<0.03	<0.04	<0.03	0.33	0.17	0.93
Cu		0.43	0.49	0.38	0.44	0.70	0.65
Cr*Cu		0.24	0.34	0.33	0.71	0.75	0.35

† Values are means ± SEM.
[1] N=9
[2] N=7

TABLE VII

Glucose, Insulin, Insulin to Glucose Ratio, and Fructosamine at Necropsy†

Treatment	N	Glucose (mg/dL)	Insulin (ng/mL)	Insulin : Gluc Ratio	Fructosamine (μmol/L)
				x 10^{-3}	
-Cr-Cu	10	296±14	0.62±0.04	2.119±0.145	128±4
-Cr+Cu	10	302±15	0.69±0.08	2.383±0.297	117±5
+Cr-Cu	10	299±14[2]	0.77±0.08	2.747±0.226[2]	124±4[2]
+Cr+Cu	9	328±15[1]	0.80±0.09	2.395±0.308[1]	131±4[1]
Factors			**P Values**		
Cr		0.32	0.07	0.21	0.28
Cu		0.24	0.47	0.86	0.69
Cr*Cu		0.41	0.81	0.23	0.06

† Values are means ± SEM.
[1] N=8
[2] N=9

TABLE VIII

Correlation Coefficients (r) Between Blood Parameters at Necropsy

	GLUC Glucose	**FRUC** Fructosamine	**TRIG** Triglyceride	**CHOL** Cholesterol	**INSULIN**	**S.URIC** (Serum Uric Acid)	**NEFA** (Non-esterified Fatty Acids)	**S. Mg** (Serum Magnesium)
GLUC	1.0000	0.3596	0.4029	0.3189	-0.2174	-0.0488	0.4689	0.1603
(p=)		0.0510	0.0273	NS	NS	NS	0.0090	NS
FRUC		1.0000	0.2895	0.1951	-0.0436	-0.0741	0.4429	-0.0250
(p=)			NS	NS	NS	NS	0.0143	NS
TRIG			1.0000	0.2972	-0.0514	-0.0203	0.6565	0.1852
(p=)				NS	NS	NS	0.0001	NS
CHOL				1.0000	0.3803	0.1318	0.3127	0.1604
(p=)					0.0169	NS	0.0526	NS
INSULIN					1.0000	0.5453	0.0789	0.2522
(p=)						0.0003	NS	NS
S. URIC						1.0000	-0.1531	0.7108
(p=)							NS	0.0001
NEFA							1.0000	0.0583
(p=)								NS
Mg								1.0000
(p=)								

NS = No significance.

TABLE IX

Triglyceride, Cholesterol, β-hydroxybutyrate (BOHB), Non-esterified Fatty Acids (NEFA), and Serum Magnesium at Necropsy†

Treatment	N	Triglyceride (μmol/L)	Cholesterol (mg/dL)	BOHB (mg/dL)	NEFA (mEq/L)	S. Magnesium (meq/L)
-Cr-Cu	10	115±11	104±7	7.614±0.636	0.743±0.043	2.15±0.11[1]
-Cr+Cu	10	126±11	113±6	8.884±0.722	0.738±0.040	2.15±0.06[2]
+Cr-Cu	10	144±20	101±4	9.389±1.249	0.909±0.066[3]	2.21±0.05[1]
+Cr+Cu	9	153±22	119±6	8.544±0.671	0.956±0.080[2]	2.16±0.10[3]
Factors			**P Values**			
Cr		0.10	0.77	0.41	<0.002	0.67
Cu		0.56	<0.03	0.81	0.71	0.74
Cr*Cu		0.96	0.40	0.23	0.65	0.74

† Values are means ± SEM.
[1] N=8
[2] N=9
[3] N=5

TABLE X

Serum and Total Urine Creatinine, Blood Urea Nitrogen (BUN), Serum and Total Urine Uric Acid and Serum Xanthine Oxidase at Necropsy†

Treatment	**N**	**S. Creatinine** (mg/dL)	**T. U. Creatinine** (mg/12 hr)	**BUN** (mg/dL)	**S. Uric Acid** (mg/dL)	**T. U. Uric Acid** (mg/12 hr)	**Xanthine Ox.** (U/L)
-Cr-Cu	10	0.64±0.02	6.26±0.49	8.4±0.7	1.6±0.3	0.91±0.16	8.70±2.05[1]
-Cr+Cu	10	0.65±0.02	6.10±0.21	9.9±0.5	1.8±0.1	1.06±0.09	5.04±1.41[2]
+Cr-Cu	10	0.65±0.02	6.32±0.65	10.2±0.4[1]	1.8±0.3	0.88±0.09	5.51±1.65[2]
+Cr+Cu	9	0.66±0.02	6.24±0.63	11.1±0.4[2]	1.8±0.3	0.99±0.24	7.44±3.75[3]
Factors				**P Values**			
Cr		0.79	0.84	<0.006	0.83	0.75	0.86
Cu		0.93	0.82	0.10	0.77	0.40	0.71
Cr*Cu		0.70	0.94	0.74	0.67	0.92	0.23

† Values are means ± SEM.
[1] N=9
[2] N=8
[3] N=7

CHAPTER V

SUMMARY, HYPOTHESIS TESTING AND RECOMMENDATIONS

Summary

The purpose of this study was to examine the effects of depletion of dietary chromium or copper on selected parameters related to cardiovascular disease risk in the BHE/cdb rat.

The BHE/cdb rat is genetically predisposed to non-insulin dependent diabetes mellitus (NIDDM) and exhibits age-related hyperglycemia (Berdanier 1991). This study assigned forty male weanling BHE/cdb rats to four experimental diet groups within a 2x2 factorial design with deficient and adequate dietary chromium and copper. Several glucose tolerance tests were performed throughout the study to analyze the state of glucose tolerance and possible mineral depletion in these animals. After thirteen weeks on AIN-93G recommended diets, the animals' weights had plateaued, and they were switched to a high fat diet containing 22% soybean oil to try to exacerbate the effects of mineral depletion upon glucose tolerance. After twenty-one weeks on the experimental diets, the animals were killed. At necropsy, several serum and urine parameters related to cardiovascular disease were analyzed. Analysis of variance, least squares means, and Pearson correlation coefficents were used to determine statistical significance among the treatment groups.

Copper depletion significantly lowered baseline glucose within the chromium depleted groups in the first glucose tolerance test and significantly increased total urine creatinine in the second glucose tolerance test. Total urinary creatinine positively correlated with current body weight during the first glucose tolerance test. At necropsy, chromium depletion significantly increased liver, kidney, and spleen percent body weight. Chromium depletion also significantly decreased non-esterified fatty acids (NEFA) and blood urea nitrogen (BUN). Copper depletion significantly lowered total cholesterol at necropsy.

Several parameters related to cardiovascular disease risk were positively correlated to one another. Triglyceride was positively correlated with glucose and NEFA. Insulin positively correlated with cholesterol, serum uric acid, fat mass, and final weight. NEFA also positively correlated with glucose and fructosamine. Serum magnesium positively correlated with serum uric acid. This set of correlations includes interrelated parameters pertinent to diabetes mellitus and cardiovascular disease. Lee et al. (1995) termed this relationship the Obesity-Insulin Resistance Syndrome, while Reaven (1988) coined the term "Syndrome X" to describe this cluster of symptoms.

Regardless of the term used, several of these physiological abnormalities which are intertwined within diabetes mellitus and cardiovascular disease were significantly present in the BHE/cdb rat within the dietary treatments of depleted and adequate chromium and copper. These results indicate that mineral depletion of chromium and copper may have a significant effect upon cardiovascular disease risk in the BHE/cdb rat model.

Test of Null Hypotheses

The null hypotheses were tested based upon the data obtained in this study:

Ho 1. There will be no statistically significant effect of dietary chromium on selected factors related to cardiovascular disease risk in the BHE/cdb rat.

Chromium depletion significantly increased liver, kidney, and spleen percent body weight at necropsy. In addition, chromium depletion significantly decreased NEFA and BUN at necropsy. Chromium depletion or adequacy did not have an effect upon the remaining parameters analyzed in this study. Hypothesis one was rejected.

Ho 2. There will be no statistically significant effect of dietary copper on selected factors related to cardiovascular disease risk in the BHE/cdb rat.

Copper depletion significantly increased total urine creatinine in the second glucose tolerance test and significantly decreased total cholesterol at necropsy. Copper depletion or adequacy did not significantly affect the remaining factors included in this study, though. Hypothesis two was rejected.

Ho 3. There will be statistically significant interactions between dietary chromium and copper affecting selected factors related to cardiovascular disease risk in the BHE/cdb rat.

A significant interaction did exist in the first glucose tolerance test during the study. Copper depletion significantly lowered baseline glucose within the chromium depleted groups in this test. There were no other significant interactions between dietary chromium and copper in the remaining factors studied. Hypothesis three was rejected.

Recommendations

To confirm the effect of depleted chromium and copper on factors related to cardiovascular disease in the BHE/cdb rat, more studies are recommended. A confirmed state of impaired glucose tolerance (IGT) or hyperinsulinemia should be present to accurately assess the effects of mineral depletion in many of the parameters selected for this study in the BHE/cdb rat. As seen in the results of the glucose tolerance tests, this glucose intolerant state may never have been reached in this study. The BHE/cdb rats were not hyperinsulinemic at necropsy, either. In addition, the high fat diet was only administered during the last eight weeks of the study. With the use of a high fat diet in BHE/cdb rats from weanling age for the duration of a study, IGT and hyperinsulinemia might be obtained more rapidly and cause more significant effects from mineral depletion in the factors selected here. As laboratory facilities become available, other parameters will be measured to fully examine the effects of mineral depletion in the BHE/cdb rat. These parameters include urine hydroxyproline, bone and tissue chromium and copper, plasma fibrinogen, serum corticosterone and ceruloplasmin, and experimental diet concentration of chromium and copper.

The most elevated blood glucose was observed during the first glucose tolerance test during the twelfth week. This observation could indicate that mineral depletion effects are more significant during growth for this animal. Future studies using high fat, mineral depleted diets with documentation of IGT and hyperinsulinemia are strongly recommended within this animal model of NIDDM.

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APPENDICES

APPENDIX A

COMPOSITION OF INITIAL DIET

Ingredient	Amount (g)	Percentage
Casein	200.0	20.00%
Cornstarch	150.0	15.00%
Cellufil	50.0	5.00%
Dextrose	379.5	37.95%
Sucrose	100.0	10.00%
Vitamin Mix[1]	10.0	1.00%
Soybean Oil	70.0	7.00%
Choline	2.5	0.25%
L-Cysteine	3.0	0.30%
Mineral Mix[2]	35.0	3.50%

[1] Vitamin mix formulated to meet the AIN-93G recommendations for growing rats except for a five fold increase in Vitamin E, or 0.6 g/kg diet mix.

[2] Mineral mix formulated to meet the AIN-93G recommendations for growing rats except for inadequate levels of chromium (0 g/kg added to mineral mix) and copper (0.03 g/kg mineral mix) as specified by the experimental design.

APPENDIX B

COMPOSITION OF HIGH-FAT DIET

Ingredient	Amount (g)	Percentage
Casein	140.0	14.00%
Cornstarch	150.0	15.00%
Cellufil	50.0	5.00%
Dextrose	289.5	28.95%
Sucrose	100.0	10.00%
Vitamin Mix[1]	10.0	1.00%
Soybean Oil	220.0	22.00%
Choline	2.5	0.25%
L-Cysteine	3.0	0.30%
Mineral Mix[2]	35.0	3.50%

[1] Vitamin mix formulated to meet the AIN-93G recommendations for growing rats except a five fold increase in Vitamin E, or 0.6 g/kg diet mix.

[2] Mineral mix formulated to meet the AIN-93G recommendations for growing rats except for inadequate levels of chromium (0 g/kg added to mineral mix) and copper (0.03 g/kg mineral mix) as specified by the experimental design.

APPENDIX C

DIET PREPARATION

Instructions for Mixing:

Step 1: Put on clean mineral free gloves
Step 2: In the small mixing bowl, combine the following ingredients one at a time:

1) Vitamin Mix
2) L-Cysteine
3) Choline
4) Casein (≈ 1 cup)
5) Dextrose (≈ 3 cups)
6) Sucrose

Step 3: Mix each ingredient thoroughly as they are added using your hand with mineral-free gloves. (Make sure all small clumps are broken.)
Step 4: Use the large mixing bowl to combine the following ingredients. Again, add each ingredient one at a time and use your hands to combine and break up any clumps.

1) Remainder of Dextrose
2) Remainder of Casein
3) Celufil
4) Cornstarch

Step 5: Add around 2 Tbsp. of oil and mix at a setting of "1" on the large mixer for 10-15 minutes (or until well mixed). Add the remainder of the oil and continue mixing.
Step 6: When the six ingredients in the small mixing bowl have been thoroughly combined by hand, add about 1/2 Tbsp. of oil and mix at low speed (setting of "1") for 5-19 minutes or until well mixed. Next, add the Mineral Mix and stir with plastic spoon. Continue mixing at a setting of "1" until the ingredients are completely mixed.
Step 7: When the ingredients in each mixer appear to be ready, combine all ingredients into the large mixing bowl and set speed at "1.5" and mix for approximately 20 minutes.
Step 8: Place diet in a plastic bag, mark it accordingly and store it in the freezer.

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APPENDIX D

XANTHINE OXIDASE PROCEDURE
(Singh et al., 1987)

Substrate Buffer: Total volume = 100mL
Dissolve 0.1361g hypoxanthine (10mM, FW=136.1g/mole) in 70 mL 25mM NaOH. Add 0.1300g NaN_3 (20mM, FW=65.01 g/mole). Add 30 ML of 66.7 mM KH_2PO_4. This solution should have pH=7.9.

Reagent: Total volume = 1000mL
Dissolve 1.0974g (2mM, FW=548.7g/mole) ABTS (2,2'-azino-di(3-ethyl-benzthiozoline-6-sulphonate) in a little 66.7 mM KH_2PO_4. Add 2500 unites of horseradish peroxidase (POD) (2500 units/L). Dilute to 1L with 66.7 mM KH_2PO_4. This solution is stable for more than 2 months.

Uricase:
10 units/mg in 50% glucose

2M $HClO_4$:
In a 1L volumetric flask, add 220 mL of concentrated $HClO_4$ (9.1M). Dilute to 1L with distilled water.

PROCEDURE

1. Pipet 1 mL substrate buffer + 5uL uricase + 50 uL serum into a centrifuge tube. For blank: 1 mL (66.7mM KH_2PO_4 with 20mM NaN_3) + 5uL uricase + 50uL serum into a centrifuge tube. *Each subject will have a blank.
2. Incubate at 30°C for 10 minutes.
3. Add 1.0 mL reagent solution. Vortex.
4. Immediately add 1.0 mL of 2M $HClO_4$. Vortex.
5. Centrifuge for 5 minutes at 3000 rpm.
6. Read A410nm against blank. Final reaction product is stable for 15 minutes.

Preparation of other reagents:
1. 25mM NaOH (FW=40g/mole): Dissolve 0.100g NaOH in a little distilled water. Dilute to 100mL.
2. 66.7mM KH_2PO_4 (FW=136.1g/mole): Dissolve 9.0779g KH_2PO_4 in a little distilled water. Dilute to 1000 mL.
3. 66.7mM KH_2PO_4 with 20mM NaN_3 (FW=65.01 g/mole): Dissolve 0.1300g NaN_3 in 100 mL 66.7mM KH_2PO_4.

Calculation: Xanthine Oxidase activity (Units/L) = A_{410nm} x 79.9

VITA

Ruth Ann Givens

Candidate for the Degree of

Master of Science

Thesis: DIETARY CHROMIUM AND COPPER EFFECTS ON PARAMETERS RELATED TO CARDIOVASCULAR DISEASE RISK IN THE BHE/cdb RAT

Major Field: Nutritional Sciences

Biographical:

Personal Data: Born in Shattuck, Oklahoma, September 15, 1973, the daughter of Charles and Sherry Davison Nichols

Education: Graduated from Arnett High School, Arnett, Oklahoma, in May 1991; received Bachelor of Science degree in Nutritional Sciences from Oklahoma State University in May 1995; completed the Requirements for the Master of Science degree at Oklahoma State University in July 1997

Professional Experience: Graduate Assistant, Nutritional Sciences Department, Oklahoma State University 1996-1997, Dietetic Intern, Oklahoma State University Dietetic Internship, 1996-1997

Professional Organizations: American Dietetic Association, Oklahoma Dietetic Association, Phi Kappa Phi, Kappa Omicron Nu, Phi Upsilon Omicron, Golden Key National Honor Society