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STUDIES ON BIOCHEMICAL, PHYSIOLOGICAL, AND MOLECULAR ABERRATIONS OF THE HEART INDUCED BY DIETARY COPPER RESTRICTION ALONE AND IN CONJUNCTION WITH HIGH DIETARY FAT IN RATS

DISSERTATION

Presented in Partial Fulfillment of the Requirements for the Degree Doctor of Philosophy in the Graduate School of The Ohio State University

By
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Chapter I
INTRODUCTION

Introduction

The impact of poor trace element nutrition upon biochemical and physiological function has been, and continues to be the subject of extensive research. The trace element, copper, has been under investigation in order to elucidate the dietary requirement for optimum health. Presently there is no Recommended Dietary Allowance (R.D.A.) for this micronutrient. However, in 1989 the National Research Council established a safe and adequate for dietary intake range of 1.5 - 3.0 mg Cu/day (N.R.C., 1989). At this time data from numerous studies on copper deficiency are actively being interpreted and integrated in order to establish a R.D.A. for copper. To facilitate this task there is a need for basic research on the ramifications of copper deficiency in mammalian systems. A better understanding of the biological aspects of copper deficiency will allow nutrition researchers to determine the recommended levels for copper intake.
Experiments performed on rats, mice and pigs have noted that copper deficient animals develop cardiac ultrastructural pathology, biochemical aberrations, and gross cardiac hypertrophy. Aneurysms, hemothorax and death from cardiac failure have been observed in copper deficient rats (Allen and Klevay, 1978). The specific type of hypertrophy associated with copper deficiency has been concentric cardiac hypertrophy (Medeiros et al., 1992), which is characterized by a thickened interventricular septum and left ventricular free wall, with a narrowing of the ventricular lumen in many instances. Examination of cardiac ultrastructure reveals an increased volume density occupied by mitochondria (Medeiros et al., 1991), fragmented mitochondria (Kopp et al., 1983), presence of excessive amounts of lipid droplets, myofibrillar disarray, and disruption and thickening of the basal lamina (Wildman et al., 1994). Other signs of copper deficiency include decreased activities of copper containing enzymes such as superoxide dismutase (DiSilvestro et al., 1992, Medeiros et al., 1993), cytochrome c oxidase (CCO) (Chao et al., 1993), ceruloplasmin (Prohaska and Heller, 1982) and lysyl oxidase (Romero-Chapman et al., 1991). Abnormal electrocardiogram activity has been demonstrated in copper deficiency as well. Bundle branch block, wandering pacemaker (Viestenz and Klevay,
1982), increased QT duration, and increased R wave voltage (Medeiros et al., 1992) have all been observed in copper deficient rodents. Further signs of copper deficiency in rats have also been noted at a molecular level. Expression of cardiac isomyosin has been observed to shift from the adult V1 form to the fetal V3 form (Liao et al., 1995a&b). Increased levels of cardiac fibronectin and decreased levels of cardiac laminin have also been demonstrated in copper deficient rats (Liao et al., 1995a&b). The latter two findings impact upon the integrity of the basal lamina. The nuclear encoded subunits of the electron transport chain enzyme cytochrome c oxidase appear to be depressed in copper deficient rat hearts. However, the mitochondrial subunits appear unaffected by copper status (Medeiros et al., 1993).

All of these factors collectively may have a role leading to the cardiomyopathy observed in copper deficiency. A potential larger problem presents itself when one considers the possibility of a long term diet poor in copper, yet not poor enough to lead to an overt deficiency. This type of chronic copper poor diet could possibly be another risk factor for the development of heart disease in related individuals and/or groups. Further research into the numerous aspects of copper deficiency upon the heart could produce information on
the events that lead to the development of cardiomyopathy. This information would also aid in understanding how much dietary copper is needed in order to achieve optimum cardiac health.

Objectives

The studies proposed here will investigate the impact a high fat diet has on copper restriction and sequelae of pathology observed during copper restriction. The first study is designed to determine if a diet relatively high in fat, which is typical of what westerners consume, will alter signs of copper deficiency including the relative severity of the cardiomyopathy.

The second study is designed to determine the temporal sequence of events during copper deficiency and how signs of copper status and cardiac aberrations vary as deficiency develops. The development of cardiac hypertrophy will be observed in relation to potential altered levels of nuclear encoded cardiac CCO peptides, fibronectin and laminin expressed in the heart. This study may give insights as to the potential mechanisms involved in cardiac hypertrophy.
Research Questions

The following questions will be addressed in two studies.

Study I
1) Will a copper adequate, high fat diet and a copper deficient high fat diet;
   a) Lower copper status of rats?
   b) Lead to more pronounced cardiac hypertrophy and greater dimensions of the heart?
   c) Further decrease the activity of CCO and expression of the nuclear encoded subunits of CCO?
   d) Lead to deleterious electrocardiographic activity pattern of the heart?
2) Will the high fat diet, independent of copper levels, result in alterations in any of the above parameters?

Study II
3) How long does it take for weanling rats fed copper restricted diets to develop cardiac hypertrophy?
4) When are the potential alterations in the levels of CCO, fibronectin, and laminin observed?
5) Will alterations in these proteins occur prior to, or concomitant with cardiac hypertrophy, thus possibly acting as one of the stimuli for hypertrophy?
Hypertrophic Cardiomyopathy (HCM)

The hypertrophy observed in HCM represents an unnatural growth response by some unknown molecular mechanisms that lead to heart dysfunction. Annual reported mortality rates for HCM are 3% and results from progressive cardiac failure and sudden death (Vassalli et al., 1994). Sudden death occurs typically in young patients who are asymptomatic or have mild symptoms of cardiomyopathy (Hecht et al., 1993). Cardiomyopathies are designated as primary or secondary. In primary cardiomyopathy the underlying mechanisms are due to a genetic defect. About 50% of these genetic defects are mapped to the beta-myosin heavy chain gene on chromosome 14q1 (Vassalli et al., 1994). Defects in genes coding for medium and long chain acyl-CoA dehydrogenases as well as mutations in mitochondrial DNA also lead to primary HCM (Kelly and
The mutations found in mitochondrial DNA are in genes that code for enzymes involved in oxidative phosphorylation. Other defects which can induce HCM are missense mutations in alpha tropomyosin genes linked to chromosome 15q2 (Thierfelder et al., 1994). Since the pathogenesis of this disease state remains a mystery (unless genetic analysis is done at necropsy) the term idiopathic cardiomyopathy is commonly used to describe primary HCM (Kelly and Strauss, 1994). Secondary cardiomyopathy results from factors such as hypertension or ischemic heart disease that lead to remodeling of the heart and myocardial disarray to compensate for changes in pressure, volume or death of functional myocytes (Kuribayashi and Roberts, 1992). The death of functional myocytes during HCM results in an even greater overload being added to surviving myocytes, thus leading to progressive deterioration and eventually cardiac failure (Katz, 1994).

One classic morphological abnormality of HCM is a nondilated hypertrophied left ventricle in the absence of any other cardiac or systemic disease capable of producing such hypertrophy (Maron, 1993). This is also known as a concentric pattern of hypertrophy since the wall of the heart chamber are thickened and the luminal space of the ventricle is reduced. This pattern can be brought about by aortic stenosis or hypertension which
results in pressure overload inside the left ventricle. The hypertrophy is an adaptive response to compensate for increase wall stress. When the load upon the heart is chronically elevated for a long period of time, as in hypertension, the compensation may progress to pump failure (Boluyt et al., 1994). The HCM observed during copper deficiency is of this concentric nature (Medeiros et al., 1993).

Ultrastructurally, the hypertrophy that occurs in the concentric pattern is brought about by parallel stacking of myofibrils which results in thickening of the individual myocytes (Anversa et al., 1986). The hypertrophy of the myocytes is demonstrated by increased cellular diameter (Sawada and Kawamura, 1991, Maron, 1993). Remodeling of the heart involves changes in the distribution of isomyosin as well which leads to a reduced sliding velocity of myosin (Yamashita et al., 1993).

Fibrosis is another common observation in the hypertrophied heart (Katz, 1994, Kuribayashi and Roberts, 1992). This fibrosis may be associated with ischemia in the heart. Abnormal coronary arteries are found in about 80% of patients who die of HCM (Maron, 1993). The blood vessels are thickened due to increased amounts of collagen, elastin, and smooth muscle cells being deposited in the tunica media of the blood vessels
which results in narrowing of the vessel's lumen (Maron, 1993). The resulting decreased luminal size may adversely affect blood flow in the heart itself and thus could be implicated in playing a role in the observed ischemia and necrosis associated with HCM (Maron, 1993).

Cardiac Hypertrophy and Copper deficiency

A concentric pattern of hypertrophy is observed in copper deficiency. This concentric hypertrophy is brought about by a parallel stacking of myofibrils (Medeiros et al., 1993) and proliferation of mitochondria in the diseased myocytes (Wildman et al., 1994). Heart to body weight ratios are found to be increased in copper deficient rats (Chao et al., 1993, Medeiros et al., 1992, Prohaska and Heller, 1982, Hoogeveen et al., 1994). Rats fed copper deficient diets from weaning develop a grossly thickened interventricular septum and left ventricular free wall (Medeiros et al., 1991, Fields et al., 1991, Medeiros et al., 1992). The mechanism as to why copper deficiency leads to cardiac hypertrophy remains unknown. The pattern of hypertrophy (concentric) observed at a gross level is similar to the hypertrophy induced by aortic stenosis (pressure overload). Since hypertension has
been an inconsistent result of copper deficiency (Medeiros et al., 1993, Wildman et al., 1994) it is likely that it can be ruled out as the trigger for cardiac hypertrophy in copper deficiency. Anemia has also been implicated in playing a role in cardiac hypertrophy. One study done by Fields et al. (1991) demonstrated that injection of red blood cells into copper deficient rats prevented the cardiac hypertrophy typically observed. The lack of cardiac hypertrophy in this study may have been due to some amounts of copper being unknowingly administered to the rats via the injected red blood cells. Anemia has been an inconsistent find in copper deficiency studies as some studies have found significantly lower hematocrit (Prohaska and Heller, 1982, Chao et al., 1994) and some studies have not (Medeiros et al., 1993, Chao et al., 1993). All of the previous studies, however, have observed cardiac hypertrophy, thus demonstrating that cardiac hypertrophy does occur even when anemia does not. Furthermore, Medeiros et al. (1991) demonstrated that anemia occurred after cardiac hypertrophy was observed. It should also be emphasized that anemia leads to volume overload in the heart that results in an eccentric pattern of hypertrophy, which is inconsistent with the concentric pattern of cardiac hypertrophy observed during copper deficiency.
Catacholamine imbalance has also been suggested to play a possible role in the onset of hypertrophy. Depletion of cardiac norepinephrine has been observed in copper deficient rats, and accompanied by an increase in dopamine, the precursor to norepinephrine (Prohaska and Heller, 1982, Seidel et al., 1991). The cuproenzyme, dopamine-beta-hydroxylase, catalyzes the formation of norepinephrine from dopamine (Mathews and van Holde, 1990). Depression of norepinephrine is typical in other models of HCM, but the depression observed in copper deficient hearts is particularly severe, suggesting that the impaired activity of the dopamine-beta-hydroxylase may be exacerbating norepinephrine depletion (Prohaska and Heller, 1982, Schoenemann et al., 1990). The fact that copper deficient hearts demonstrate significant depletion of norepinephrine before the onset of hypertrophy, lends strength to possible role norepinephrine may have in cardiac hypertrophy (Seidel et al., 1991)

Cardiovascular integrity and lysyl oxidase

The cardiovascular system in copper deficiency is compromised. Reports of ventricular aneurysms, hemothorax, cardiac rupture (Viestanz and Klevay, 1982) and even aortic rupture (in ostriches) (Vanhooser et al., 1994) have been published. These abnormalities may
be linked to the decreased activity of the copper-dependent enzyme lysyl oxidase. This enzyme is needed for cross linking of collagen fibers and elastin fibers (Alberts et al., 1994). Reduced number of cross links in collagen and elastin may impact upon the structural integrity of the heart and associated blood vessels as demonstrated by the cited defects in the ventricular and vascular integrity of copper deficient rodents.

Lysine, an amino acid found in collagen and elastin, is oxidized into alpha-aminoadipic-delta-semialdehyde by lysyl oxidase. This aldehyde is the precursor to the covalent crosslinks found in mature collagen (Kagan and Trackman, 1991). Two adjacent aldehydes on different collagen fibrils will spontaneously react to form an adol cross link, which is characteristic of mature collagen (Lowey et al., 1991). Copper deficiency in rats results in depressed lysyl oxidase activity in lung (Abdel-Mageed et al., 1994), skin (Romero-Chapman et al., 1991), and heart (Werman et al., 1995, Borg et al., 1985). This depressed lysyl oxidase activity leads to decreased collagen crosslinking (Borg et al., 1985) and results in the presence of a larger fraction of soluble collagen (Dawson et al., 1982).
Changes in cardiac ultrastructure

Ultrastructural cardiovascular abnormalities have revealed discrete areas of necrosis present which are associated with hemorrhaging and phagocyte invasion (Farquharson and Robins, 1991, Davidson et al., 1992). Foci of fibrosis have also been observed in the interstitial and pericapillary space of the myocytes (Farquharson and Robins, 1991, Davidson et al., 1993, Davidson et al., 1992). Phagocyte invasion has been noted in many of these fibrous areas as well (Medeiros et al., 1993). Interestingly, Davidson et al. (1993) reported that exercised copper deficient rats demonstrated a greater incidence of fibrosis as compared to copper deficient sedentary rats. Fibrosis has been observed at the endomysium as well. In one study, disruption of the endomysium due to proliferation of fibronectin was found to lead to dissociation of muscle fibers (Farquharson and Robins, 1991).

The basal lamina is thickened, sometimes distorted, or separated from its surroundings. Wildman et al. (1994) reported that copper deficiency led to the separation of basal laminae from the sarcolemma of the myocytes in the pericapillary space. Others have reported thickened and disrupted basal laminae (Chao et al., 1993, Davidson et al., 1992). This could impact upon normal myocyte function since the basal lamina is
involved in cell signaling, differentiation, and polarity (Alberts et al., 1994). Thickened basal laminae has also been hypothesized to possibly lead to local ischemia in the copper deficient heart (Davidson et al., 1992). This is in agreement with Maron (1993) who stated that fibrosis around capillary areas during HCM could lead to ischemia.

One of the more frequent ultrastructural observations in copper deficiency is the proliferation of mitochondria in the heart. Normally, there is a high number of mitochondria present in an orderly arrangement that is closely associated with myofibrils, resulting in ATP synthesis occurring in close proximity to an area which has high ATP consumption (Alberts et al., 1994). Increased mitochondrial:myofibrillar ratios have been commonly reported in the hearts of rats fed copper deficient diets (Medeiros et al., 1991, Chao et al., 1994, Davidson et al., 1992, Wildman et al., 1994). Mitochondria appear fragmented and vacuolized (Medeiros et al., 1991), the inner and outer mitochondrial membranes are fragmented and the cristae appear damaged as well (Kopp et al., 1983). Medeiros et al. (1991) proposed that mitochondrial proliferation, in addition to myofibrillar proliferation, may also contribute to the characteristic gross cardiac hypertrophy observed in copper deficiency. Models of pressure overload induced
hypertrophy also display increased mitochondrial volume density during the early stages of hypertrophic development (Meerson 1983). Mitochondrial proliferation may be a compensatory mechanism to attempt to overcome any functional impairments brought about by damage to existing mitochondria. Damage to the mitochondrial membranes would include free radical damage, lipid peroxidation, and decreased CCO activity (Medeiros et al., 1993). Additionally, a question has been raised as to whether mitochondrial swelling is a response mechanism in which increased surface area leads to maximized copper and oxygen uptake into these organelles, with the goal of increasing mitochondrial respiration (Medeiros et al., 1993).

Many studies have also reported myofibrillar disarray and decreased myofibril volume density within the myocytes (Davidson et al., 1992, Wildman et al., 1994, Medeiros et al., 1991). Other abnormalities include the excessive lipid droplets in myocytes and in lung cells (Richmond and Chi, 1993) and glycogen granules present (Chao et al., 1994, Wildman et al., 1994).
Cytochrome c oxidase (CCO)

Cytochrome c oxidase is a copper dependent enzyme that is located on the inner mitochondrial membrane (Alberts et al., 1994). This enzyme is the terminal enzyme in the electron transport chain. Molecular oxygen is reduced to water and the free energy derived from this reaction is used to pump protons across the inner mitochondrial membrane (Calhoun et al., 1994). Approximately 50% of this enzyme complex extends out into the space between the inner and outer mitochondrial membranes; 40% of the CCO complex is buried within the inner mitochondrial membrane; and the remaining 10% of the complex extends through the inner membrane and into the mitochondrial matrix (Zhang et al., 1991). CCO belongs to a superfamily of enzymes that are redox driven pumps which function as ion transporters (Calhoun et al., 1994). This superfamily is defined by two characteristics: 1) a bi-metallic active site consisting of a heme and closely associated copper atom which is the site where dioxygen is reduced to water; and 2) a very high degree of amino acid homology with subunit I of the enzyme (Calhoun et al., 1994). The branches of this superfamily of redox driven pumps that use cytochrome c as a substrate are known as cytochrome c oxidases.
Figure 1 Electron density map of bovine cardiac cytochrome c oxidase (CCO) showing location of metal sites (Tsukihara et al., 1995). The middle portion of the diagram represents the hydrophobic area imbedded in the inner mitochondrial membrane, the hydrophilic areas protrude out above and below the inner mitochondrial membrane.
Eukaryotic CCO is comprised of anywhere from 6 (yeast) to 13 (bovine) subunits. Mammalian subunits of CCO are designated as I, II, III, IV, Va, Vb, VIa, VIb, VIC, VIIa, VIIb, VIIc, and VIII (Zhang et al., 1991). Genes for subunits I, II, and III are all located within the mitochondrial genome, genes for all other subunits are located in the nucleus of the cell (Capitanio et al., 1994). Subunits I, II, and III comprise the catalytic core of this enzyme and are responsible for oxygen reduction and proton pumping (Alberts et al., 1994). Studies have observed that bacteria, which contain homologs of subunits I, II, and III, are just as efficient at catalyzing electron transfer and conducting proton pumping as the 13 subunit mammalian CCO, thus demonstrating location of core catalytic activity (Calhoun et al., 1994).

In mammalian systems, as in all prokaryotic and eukaryotic systems, subunit I of CCO is the largest of all the subunits. Subunit I is comprised of 12 intermembrane helices and binds 2 heme groups, one between helix 2 and 10 (heme a) and another attached to helix 10 (heme a3) (Calhoun et al., 1994). Heme a3 is bound to a single histidine residue on helix 10 and is associated with a copper atom which is bound to two histidine residues on helix 7, one histidine residue on helix 6, and a tyrosine residue on helix 6 (Calhoun et
Figure 2 Diagram illustrating the flow of electrons through cytochrome c oxidase (CCO). The mobile electron carrier cytochrome c binds to subunit II of CCO. An electron is passed from cytochrome c to a CuA site composed of two copper atoms on subunit II. From here the electron is passed to a heme a site on subunit I and then to a bimetallic heme a3-copper site where molecular oxygen is tightly bound and will accept the electron. Molecular oxygen is converted to water using four electrons and hydrogen protons, the large concomitant energy release is used by CCO to fuel proton pumping into the mitochondrial inner membrane space (Gennis and Ferguson-Miller, 1995)
al., 1994). This copper atom is commonly referred to in the literature as CuB. Together heme a3 and CuB form the binuclear center of subunit I.

Subunit II of CCO is comprised of 2 transmembrane helices and contains the binding site for cytochrome c, which donates electrons to subunit II (Captiano et al., 1994). There are two copper atoms present that form a mixed valence center (Cu2+-Cu+), known as CuA which is bound to subunit II. There is also a magnesium atom located on subunit II (Tsukihara et al., 1995). The binuclear center of subunit II accepts electrons from cytochrome c and passes them along to subunit I where they will eventually be used to reduce molecular oxygen to water and thus facilitating proton pumping (Calhoun et al., 1994, Captiano et al., 1994).

To facilitate efficient proton pumping the inner mitochondrial membrane is quite impermeable to ions. The double phospholipid, cardiolipin, which is especially impermeable to ions, is a major component of the mitochondrial membranes (Alberts et al., 1994).

The precise role of the nuclear encoded subunits of CCO remains somewhat speculative. Possible roles of the nuclear encoded subunits may be to facilitate assembly of the functional CCO enzyme, stabilize the holoenzyme, and/or regulate activity of the functional oxidase (Captiano et al., 1994).
Activity of CCO has been observed to be depressed as a result of copper deficiency. Studies have shown the depression of CCO activity in spleen, thymus, liver, kidney, intestine, brain, (Prohaska, 1991) and heart (Medeiros et al., 1993, Chao et al., 1993). The decreases in CCO activity are differential and vary from organ to organ (Prohaska et al., 1991, Paynter et al., 1979). Decreases in CCO activity are presumably due to a lack of copper atoms for the CuA and CuB metallic centers of subunits I and II.

Several studies have observed that there appears to be a lack of nuclear encoded subunits present in the hearts of copper deficient rats (Medeiros et al., 1993b, Chao et al., 1994). Since the nuclear encoded subunits have been implicated in assembly, stabilization and regulating activity of CCO (Captiano et al., 1994), there exists a possibility that observed decreases in CCO activity during copper deficiency could also be due, in part, to the depleted amounts of nuclear encoded subunits as well as the lack of copper atoms present. Work by Keyhani et al. (1975) also demonstrated that copper is needed for CCO nuclear encoded peptides to assemble with the mitochondrial encoded peptides in the mitochondria of yeast cells. Subunit IV was the first nuclear encoded subunit identified as being decreased in copper deficient rat hearts (Medeiros et al., 1993b).
This subunit (IV) is predicted by Captiano et al. (1994) to consist of a single transmembrane alpha-helix with hydrophilic N terminus exposed at the matrix and a hydrophilic C termini exposed at the cytosolic phase. Subunit IV is the largest of the nuclear encoded and is proposed to regulate the access of protons from the inner mitochondrial space into the transmembrane proton translocating pathway of the oxidase (Captiano et al., 1994).

Due to the decreases in CCO activity during copper deficiency, studies were conducted to assess the impact upon ATP production. These studies here produced mixed results. Weisenberg et al. (1980) observed a progressive decrease in hepatic ATP of copper deficient rats. However, these rats had secondary iron deficiency which could have affected their ATP status. In another study by Kopp et al. (1983), decreases in hepatic, cardiac, and renal ATP levels were reported. Conversely, studies done by Rushinko and Prohaska (1985) and Chao et al. (1993) found no differences in cardiac ATP levels of copper deficient rats. Recently a novel CCO and ATP interaction has been observed, two binding sites for ATP have been found which regulate CCO activity (Taanman et al., 1994). One of these binding sites has been located on nuclear encoded subunit VIa, the location of the other remains unknown. In light of
the previous studies and this new information, it would seem that the relationship between CCO and ATP may be more complex than previously believed.

**Superoxide Dismutase (SOD)**

Superoxide free radicals are a natural by product in aerobic organisms. Metabolic reactions such as autoxidation of glyceraldehyde, autoxidation of reduced FMN, FAD, epinephrine, and tetrahydropteridines all generate superoxide radicals (Bordo et al., 1994). The superoxide anion is toxic due to its extreme reactivity with double bonds found in various molecules in the cell, such as polyunsaturated fatty acids found in cell membranes. The enzyme superoxide dismutase (SOD) catalyzes a dismutation reaction that produces hydrogen peroxide ($H_2O_2$) and oxygen ($O_2$) from 2 molecules of superoxide ($O_2^-$) and 2 protons ($H^+$) (Taniguchi, 1992).

There are three SOD isozymes found in mammalian cells, a mitochondrial SOD which contains a manganese atom in its structure (Mn-SOD), a cytosolic SOD containing copper and zinc in its structure (Cu,Zn-SOD), and an extracellular copper-zinc containing SOD (Taniguchi, 1992). In humans the cytosolic Cu,Zn-SOD is more prevalent than the extracellular isozyme (Crapo et al., 1992). The cytosolic Cu,Zn-SOD is also found in the nucleus and in small quantities in the mitochondrial
intermembrane space (Taniguchi, 1992). The cytosolic Cu,Zn-SOD, frequently used as a gauge of copper deficiency, exists as a dimer of two identical subunits with a total molecular weight of 32,000 kD (Bordo et al., 1994). The human Cu,Zn-SOD gene locus has been identified to be on chromosome 21q22. This 11 kb sequence consists of five exons and four introns (Taniguchi, 1992). Each subunit of Cu,Zn-SOD is composed of eight antiparallel beta-pleated sheets that form a flattened cylinder, and are joined by three external loops (Bordo et al., 1994). Copper$^{2+}$ is coordinated to four histidine residues (His-44, His-46, His-61, His-118) in a planar geometry. Zinc$^{2+}$ is coordinated to His-44, His-46, His-61, and Asp-81 which together form a tetrahedral geometry (Taniguchi, 1992). The common link between copper and zinc is histidine-61.

The chemical reaction of Cu,Zn-SOD is facilitated due to the relatively negative nature of the surface of the protein and the presence of a positively charged channel which electrostatically guides the negatively charged superoxide radical to the Cu$^{2+}$ active site (Fisher et al., 1991). This positively charged channel involves amino acid residues Lys-122, Glu-132, Glu-133, Lys-136, Thr-137, and Arg-143 (Fisher et al., 1991). The unpaired electron of superoxide (O$_2^-$) will reduce Cu$^{2+}$ to Cu$^+$, thus converting the O$_2^-$ into molecular
oxygen (O$_2$). Another O$_2^−$ radical will enter the positively charged channel and will receive an electron from Cu$^+$ along with two protons resulting in the formation of H$_2$O$_2$ and the regeneration of Cu$^{2+}$ (Roberts et al., 1991).

Copper restricted diets lead to low Cu,Zn-SOD activity in rats (Davidson et al., 1992, Rossi et al., 1994, Medeiros et al., 1993) and mice (Prohaska, 1991, Lynch and Klevay, 1994). Organ specific changes in Cu,Zn-SOD activities have been reported in copper deficient mice and rats. Liver Cu,Zn-SOD appears to be the most sensitive to copper deficiency (Prohaska, 1991, Rossi et al., 1994, Paynter et al., 1979). Further, Rossi et al. (1994) observed that Cu,Zn-SOD can be reactivated by adding copper to tissue extracts, thus indicating that Cu,Zn-SOD is, at least in part, post-translationally modulated. In the liver and heart, the decrease in Cu,Zn-SOD activity is accompanied by a concomitant increase in mitochondrial Mn-SOD (Kostantinova and Russanov, 1994, Lai et al., 1994). In a copper deficiency study done by Lai et al. (1994) Cu,Zn-SOD and Mn-SOD was examined for changes in mRNA, immunoreactive protein content, and activity in liver, heart and brain of rats. Results of this study indicated that in liver and heart the amount of immunoreactive protein levels, mRNA, and activity Cu,Zn-
SOD are down, while conversely all these parameters are increased for Mn-SOD. Brain Cu,Zn-SOD and Mn-SOD was unaffected by the treatment in this study. Copper marginal diets have also been reported to result in a depression of Cu,Zn-SOD activity in rat aorta (Nelson et al., 1992).

One of the consequences of copper deficiency is increased lipid peroxidation. This is presumably due to increased free radical (O$_2^-$) activity due to inadequate function of Cu,Zn-SOD. A study done by Nelson et al. (1992) found that lipid peroxidation was increased by 85% in copper deficient rats with a concomitant decrease in prostacyclin production and Cu,Zn-SOD activity. Lawrence and Jenkinson (1987) also reported increased lipid peroxidation as induced by tetrachloride in copper deficient rats. Free radical damage, however, is most likely not responsible for cardiac hypertrophy during copper deficiency as work done by Chao et al. (1994) observed cardiac hypertrophy and alterations in CCO in copper deficient rats in spite of antioxidant therapy (Dimethy Sulfoxide).

Ceruloplasmin

Ceruloplasmin is a blue-copper oxidase found in the serum of vertebrae species. Ceruloplasmin accounts for 90-95% of all circulating plasma copper (Takahashi et
Ceruloplasmin is an alpha-2 glycoprotein composed of a single polypeptide chain 1046 amino acids in length. This protein is synthesized in hepatocytes and secreted into the plasma after being bound with 6 atoms of copper (Takahashi et al., 1984). Using somatic cell hybrids and polymerase chain reaction (PCR), the rat ceruloplasmin gene has been assigned to chromosome 2 (Miura et al., 1994). Functions of ceruloplasmin include copper and iron transport and metabolism, antioxidant defense, immunological activation, and coagulation (Tereda et al., 1995). Copper is incorporated into ceruloplasmin as a post-translational modification. The sites of copper incorporation have been suggested to be the rough endoplasmic reticulum (Sato and Gitlin, 1991) or the golgi apparatus (Tereda et al., 1995). Copper levels do not appear to influence ceruloplasmin gene expression as no change has been noted in mRNA levels of copper deficient rats (Gitlin et al., 1992). In the absence of copper ceruloplasmin continues to be released into the plasma as apoceruloplasmin. This protein does not have oxidase activity and appears to have a different conformational structure as demonstrated by SDS-PAGE resolution (Sato and Gitlin, 1991). Many studies conducted with copper deficient rats have reported the oxidase activity of plasma ceruloplasmin to be low compared with copper adequate rats (Wapnir and
Devas, 1995, Prohaska and Heller, 1982, Werman et al., 1995, DiSilvestro et al., 1992). Two active species of rat holoceruloplasmin having molecular weights of 79 and 84 kD have been identified by Sato and Gitlin (1991) as well as a 132 kD apoceruloplasmin species. Pulse chase studies have also indicated that during copper deficiency secretion kinetics of holo and apoceruloplasmin are identical. However, there is a 60% reduction of serum ceruloplasmin concentration occurring during this state which is due to increased ceruloplasmin turnover (Gitlin et al., 1992).

Fat and Copper deficiency

Thus far very little work has been done to examine the effects of high levels of dietary fat in conjunction with copper deficiency. Much of the work in the literature examining effects of other dietary factors have focused on manipulating carbohydrate source during copper restriction. Fields et al. (1984) reported that fructose exacerbated signs of copper deficiency when compared with starch or glucose as the primary carbohydrate source during copper restriction. Lower liver SOD activity, lower hematocrit, higher plasma cholesterol, and lower body weights were demonstrated by Fields et al. (1984). Redman et al. (1988) observed that copper restricted rats fed fructose or sucrose
appear to have greater ventricular hypertrophy, myocardial inflammation, and myocardial degeneration as compared to rats fed copper restricted, starch diets.

Since the typical American diet has been repeatedly characterized as being high in fat and saturated fat, (Braitman et al., 1985, Stephen and Wald, 1984) the question of the impact of fat level and type upon the copper deficient state could be relevant to the human nutrition requirement for copper.

A study reported by Lynch and Strain (1989) was the first to include a high fat component to a copper deficient diet. Lynch and Strain fed copper deficient diets high in saturated fat or unsaturated fat (200 g per kg feed) to male Wistar rats and reported increases in both xanthine oxidase activity and liver copper levels from rats fed a high saturated fat diet as compared to rats fed a high polyunsaturated fat diet. Increased lipid peroxidation was also noted in both copper deficient high fat groups as compared with controls.

Wapnir and Devas (1995) observed that a high fat (hydrogenated vegetable oil) copper deficient diet did not affect liver copper levels compared to rats fed copper deficient, normal fat diets. Decreases in liver glucose-6-phosphate dehydrogenase activity and malic enzyme were observed in the copper deficient high fat
treatment as compared to the copper deficient low fat treatment (Wapnir and Devas, 1995).

Only one study exists that examined hypertrophic effects of fat upon the heart. Jenkins and Medeiros (1993) observed that copper deficient diets high in corn oil led to concentric hypertrophy whereas a copper deficient diet high in coconut oil and cholesterol led to eccentric cardiac hypertrophy. Activity of SOD was reported to be increased in the corn oil treatment as compared to the coconut oil + cholesterol treatment (Jenkins and Medeiros, 1993).

Even though the accumulation of lipid droplets has been a common observation in copper deficient rat hearts (Wildman et al., 1994, Davidson et al., 1993), whole body respiratory quotient (RQ) has demonstrated that fat oxidation is increased in copper deficient rats (Hoogeveen et al., 1994). Hoogeveen et al. also noted that fat mass was reduced in the rats fed copper deficient diets.

All of these studies examined different aspects regarding the role of fat in copper deficiency. Though the results are few and inconsistent, it becomes clear that the type and amount of dietary fat can influence the biochemical and pathological alterations observed in copper deficient animals.
The basal lamina, also known as the basement membrane, is a cell associated extracellular matrix that serves several purposes. The extracellular matrix will contribute to tissue architecture, provide fixed ligands for cellular receptors, aids in tissue regeneration after injury, and form selectively permeable barriers between the tissue compartments (Alberts et al., 1994, Yurchenco and O'Rear, 1994). The basal lamina is also known to influence cell polarity, cell metabolism, cell differentiation, cell migration, and to help organize proteins in adjacent plasma membranes (Alberts et al., 1994). The components of the basal lamina include type IV collagen, laminin, entactin, perlecan, fibulin-1, SPARC (secreted protein acidic and rich in cysteine) (Yurchenco and O'Rear, 1994). In nerve tissue additional components are found, agrin and netrin. Laminin and entactin are glycoproteins, and perlecan, fibulin-1, and SPARC are heparin sulfate proteoglycans.

The components of the basal lamina are secreted by the surrounding cells, these components will self-assemble into a basal lamina that is like a flexible, thin mat (Alberts et al., 1994). Basal lamina is found under epithelial cell sheets, around individual muscle cells, fat cells, and Schwann cells. The basal lamina itself appears to be organized into a three dimensional
network of anastomosing strands (Inoue 1994). There are three layers which are identified by electron microscopy; the lamina lucida, lamina densa, fibroreticularis (Merker, 1994). Recently, it has been reported that the lamina lucida of the basal lamina may be an artifact resulting from fixation and dehydration procedures currently employed in electron microscopy sample preparation (Chan and Inoue, 1994). If this is the case, then the basal lamina is composed only of lamina densa and fibroreticularis.

It has been reported that during chronic heart failure the basal lamina of human skeletal muscle is marginally increased with localized thickening present (Lindsay et al., 1994). This same phenomenon has been reported in the cardiac muscle of copper deficient rats as well (Wildman et al., 1994, Chao et al., 1993, Davidson et al., 1992). Lindsay et al. (1994) also reported abnormal morphology and localized thickening of skeletal muscle basal lamina in three patients with ischemic cardiomyopathy.

Laminin

Laminin is a basal lamina specific protein which is composed of three subunits; a large A chain (MW 400,000 kD), a B1 chain (MW 210,000 kD), and a B2 chain (MW 200,000 kD) (Kleinman et al., 1993). Laminin is also a
glycoprotein containing 12-15% carbohydrate, 4-6% of this carbohydrate is in the form of sialic acid (Lowey et al., 1991). The three subunit chains of laminin come together to form a cross shaped structure of approximately 850,000 kD (Alberts et al., 1994, Lowey et al., 1991). The triple stranded laminin structure is assembled via an alpha helical coiled-coiled domain which is toward the c-termini of the three chains which comprise the molecule. Furthermore, the c-terminus of the A chain initiates the assembly of the triple-stranded molecule (Utani et al., 1994). It is the alpha helical coiled-coiled portion of the molecule which is the characteristic "long arm" in the crosslike laminin structure. Nomizu et al. (1994) reported that isoleucine residues in the A2 and B1 chains are responsible for the stabilization of the triple stranded coiled-coiled structure. The three classes of laminin chains, A, B1, and B2, are further divided into subclasses. There are three variants of class A chains, four variants of class B1 chains, and two variants of class B2 chains (Tryggvason, 1993). Currently there are seven different assembly forms of laminin present (Timpl and Brown, 1994).

In the basal lamina, laminin is bound onto a highly crosslinked network type IV collagen. The laminin-type IV collagen link occurs through a bridge formed between
laminin and type IV collagen by a protein known as entactin (Alberts et al., 1994). Entactin binds to a cysteine-rich repeat located on the B2 chain of laminin and onto type IV collagen, the nature of the entactin-collagen binding remains unknown (Tryggvason, 1993). Laminin is known to undergo self-assembly in a concentration and temperature dependent manner. By virtue of this self assembly, it is further thought that laminin is able to form an independent, free standing network in newly forming basal lamina, as observed during morphogenesis (Tryggvason, 1993).

Laminin appears early during the course of vertebræ development and is thus thought to play important roles in cell attachment, migration, differentiation, and basal lamina assembly (Kleinman et al., 1993). Laminins are known to have binding sites for at least six different integrins and are involved in many cell-extracellular matrix interactions (Timpl and Brown, 1994). Processes such as embryonic development, tissue homeostasis, and tissue remodeling are all affected by such interactions (Timple and Brown, 1994). In vitro cell migration is also observed to be mediated by laminin, again indicating interaction of cells with various sites on laminin (Kleinman et al., 1993). Involvement of laminin and integrins in tissue remodeling is of interest since cardiac remodeling is a
consistent and documented observation in copper deficient rat (Medeiros et al., 1993, Wildman et al., 1994, Medeiros et al., 1992, Jenkins and Medeiros, 1993). Using SDS-PAGE and Western blotting, Liao et al. (1995) reported that rats fed copper deficient diets for three weeks displayed decreased levels of cardiac laminin as compared to control rats. In light of the structural role played by laminin in the basal lamina, Liao's observations correlate well with previous reports of basal lamina distortion and fragmentation (Wildman et al., 1994, Davidson et al., 1992). Besides the issue of compromised basal lamina integrity, other questions remain as to the effect of decreases in levels of laminin on cell signaling, cardiac remodeling, and cell attachment, all of which could be factors during hypertrophic cardiomyopathy.

**Fibronectin**

Fibronectin is an adhesive glycoprotein which serves to organize the extracellular matrix and allow surrounding cells to attach themselves to the basal lamina (Alberts et al., 1994). Fibronectin is found as multiple isoforms and is expressed in a temporal and cell dependent manner (Farhadian et al., 1994). The fibronectin molecule itself is a dimer with each subunit having a molecular weight of 220-250 kD (Potts and
Campbell, 1994). The two monomers are joined together by cysteine via a pair of disulfide bonds located near the carboxyl terminus of each subunit (Sottile and Wiley, 1994). Fibronectin is made up of units of modular protein, each of these modules correspond to exons of the fibronectin gene (Patthy, 1991). There are three types of protein modules which are found in fibronectin: Type I modules, 45 amino acids long (also called Fn1), Type II, about 60 amino acids long (Fn2), and Type III, about 90 amino acids long (Fn3) (Potts and Campbell, 1994). These modules make up domains within the fibronectin molecule that serve as binding sites for collagen, integrins, heparin, thrombospondins, and other molecules (Potts and Campbell, 1994). The Type III is the most common module found on fibronectin, it is found in 15-17 places on the molecule, depending on the isomform (Alberts et al., 1994). Within the Type III module there is a specific tripeptide sequence known as the RGD sequence (Arg-Gly-Asp) which facilitates cell binding to fibronectin (Alberts et al., 1994). RGD sequences are also found on other extracellular matrix proteins and are recognized by several types of integrins. Besides the RGD sequence, the first five Type I modules in the amino terminal end of fibronectin are also critical for cell surface binding (Sottile and Wiley, 1994). These Type I modules are located on a 70
kD region on the amino terminal end of the fibronectin monomer (Sottile and Wiley, 1994).

An adhesive protein such as fibronectin plays important roles due to the interaction between cells and the extracellular matrix. Common processes such as development and wound healing are dependent upon proper interaction between the extracellular matrix and the surrounding cells (Potts and Campbell, 1994). Fibronectin is implicated in some disease states such as cancer and cardiovascular disease where abnormal adhesion plays a role in the etiology of the disease. Left ventricular failure observed during heart failure may be mediated in part by alterations in the basal lamina components (Zellner et al., 1991). The left ventricular force production of the heart is dependent in part on the attachment of myocytes to the extracellular matrix, which is mediated through the basal lamina (Zellner et al., 1991). In a study done by Zellner et al. (1991) it was observed that there was a significant reduction in myocyte attachment to fibronectin and laminin during chronic supraventricular tachycardia (SVT). SVT results in left ventricular dilation and dysfunction (eccentric hypertrophy). Upon ultrastuctural examination Zellner et al. (1991) also found focal disruptions in the basal lamina-sarcolemmal interface. These results are similar to cardiac
ultrastructural pathology of copper deficient rats with cardiomyopathy reported by Wildman et al. (1994) and Davidson et al. (1993).

Another study done by Farhadian et al. (1994) reported that pressure overload induced cardiac hypertrophy (via aortic stenosis) caused no change in total amount of fibronectin mRNA in rats. Farhadian et al. (1994) concluded that pressure induced cardiac hypertrophy does not require increased amounts of fibronectin mRNA to occur. However, it may be that signs of cardiac hypertrophy alone would not necessarily be concomitant with altered fibronectin expression. Boluyt et al. (1994) observed that there is no change in fibronectin mRNA during stable cardiac hypertrophy in rats, but there is a four to five fold increase in fibronectin mRNA in hypertrophic rats with actively failing hearts. It has also been reported that there is a rapid and significant increase in fibronectin mRNA and protein expression after myocardial infarction as well, thus suggesting a role in cardiac repair (Knowlton et al., 1992). Boluyt et al. (1994) also noted that there was an increase in mRNA expression of the EIIIA fibronectin isoform, a variant which is expressed during conditions of wound healing and pressure overload hypertrophy (concentric hypertrophy), again suggesting a
function in repair mechanisms. A study done by Mamuya and Brecher (1992) observed that in drug induced cardiac hypertrophy, fibronectin mRNA increased transiently then returned to normal levels, but fibronectin protein levels increased progressively over the experimental period. Similar to Boluyt et al. (1994), Mamuya and Brecher (1992) also observed variants of fibronectin expressed during the drug induced cardiac hypertrophy.

Humans with essential hypertension are subjected to focal increases in myocardial fibronectin as well (Pardo-Mindan and Panizo, 1993). In addition to fibronectin increases, type I and III collagen and laminin protein levels were also observed to be elevated by Pardo-Mindan and Panizo (1993), they concluded that this may be due to the remodelling of the heart during hypertension.

Copper deficient rats may also have increased levels of cardiac fibronectin. Liao et al. (1995) reported increased levels of cardiac fibronectin present in rats on a copper deficient diet for three weeks.

To conclude, it appears that fibronectin levels are altered as cardiac hypertrophy progresses. The more severe cases of hypertrophy exhibit the most obvious changes in fibronectin levels. Fibronectin may play roles in remodelling and repair of the injured and progressively failing heart.
Chapter III

METHODS AND MATERIALS

Study I: Copper restricted & adequate low and high fat diets.

Study II: Temporal aspects of copper restricted & adequate diets

Animals and Diets for Study I

Forty male weanling Long-Evans rats were purchased from Harlan Sprague Dawley (Indianapolis IN) and randomly placed into one of four dietary treatment groups in a 2x2 factorial design. Treatments were as follows: adequate-copper, low fat (Cu+, low fat, n=10); adequate-copper, high fat (Cu+, high fat, n=10); deficient-copper, low fat (Cu-, low fat, n=10); deficient-copper, high fat (Cu-, high fat, n=10). Due to some rats failing to thrive or premature death the final sample sizes were as follows: Cu+, low fat, n=7; Cu+, high fat, n=8; Cu-, low fat, n=8; Cu-, high fat, n=9. Diets were prepared to contain copper concentrations of 6.0 mg Cu/kg (94.5 μmol/kg) in the
adequate diets and <1 mg Cu/kg (<15.8 μmol/kg) in the deficient diets and were verified by flame atomic absorption spectrophotometry (Varian Spectr AA-5, Varian, Victoria, Australia). The fat source was formulated so as to contain a 2:1 of saturated (coconut oil, US Biochemical, Cleveland, OH) to polyunsaturated fatty acids (Mazola Corn oil, Krogers, Cincinnati, OH).

Diets were prepared based on the recommendations of the American Institute of Nutrition, AIN-76A (AIN 1977) with modifications regarding the amount and type of fat (see Appendix A, B, C). Diets were made by mixing the following ingredients (g/kg diet) in a Hobart A-200 FT mixer (Troy, OH): 500 g sucrose, 250 g casein, 3 g D-L-methionine, 50 g fiber, 35 g AIN-76 mineral mix, 10 g AIN vitamin mix, and 2 g choline bitartrate. For the high fat diet 150 g of fat mixture and 50 g of corn starch were added, for the low fat diet 50 g of fat mixture and 150 g of cornstarch were added. Mineral mix omitting copper was used for the copper deficient diets whereas the standard mineral mix was used for the copper adequate diets. Dietary copper was in the form of cupric carbonate. Corn and coconut oils were analyzed for fatty acids using a Hewlett-Packard gas chromatograph equipped with a flame ionization detector, this was done to determine the saturated and polyunsaturated fatty acids present in order to
formulate the desired 2 to 1 ratio. Previous analysis of the fatty acids composing corn oil revealed that 12.5% was saturated fat and 54.7% was polyunsaturated fat, with the balance, 32.8%, being mono-unsaturated fat. Coconut oil is found to contain 86.8% saturated fat, 4.0% polyunsaturated fat, and 9.3% mono-unsaturated fat. Using this information, 1000 g of corn oil was mixed with 1240 g of coconut oil to yield 2240 g of a fat mixture containing 1201 g saturated fat and 596 g polyunsaturated fat or the desired 2:1 saturated to polyunsaturated formulation, which was confirmed by further analysis using gas chromatography.

Upon arrival rats were housed singly in stainless steel cages in a room with a 12 hour light:dark cycle and controlled temperature. Animals were visually examined and fed once daily and weighed weekly during the 40 day experimental period. Rats were given free access to deionized-distilled water and their appropriate diet. This protocol was reviewed and approved by The Ohio State University Institutional Laboratory Animal Care Committee.

**Animals and diets for Study II**

Thirty three male weanling Long-Evans rats were purchased from Harlan Spraque Dawley (Indianapolis, IN) and randomly assigned to a copper adequate dietary
treatment, n=15, 6.0 mg Cu/kg (94.5 μmol/kg) feed; or a copper deficient dietary treatment, n=15, < 1.0 mg Cu/kg (<15.8 μmol/kg) feed; or sacrificed immediately for baseline parameters, n=3, no dietary treatment. Copper concentrations in the diet were determined by flame atomic absorption spectrophotometry (Varian Spectr AA-5, Varian, Victoria, Australia). Three animals from each treatment were sacrificed on a weekly basis for a time period of one week up to 5 weeks.

The diets were prepared based on the recommendations of the American Institute of Nutrition, AIN-76A (AIN 1977). Diets were prepared by mixing the following ingredients in a Hobart A-200 FT mixer (Troy, OH): 500 g sucrose, 250 g casein, 150 g cornstarch, 3 g D-L-methionine, 50 g fiber, 50 g corn oil, 35 g AIN-76 mineral mix, 10 g AIN vitamin mix, and 2 g choline bitartrate. The copper source used was cupric carbonate and the copper deficient diet was made using mineral mix omitting copper.

Upon arrival rats were housed singly in stainless steel cages in a room with a 12 hour light:dark cycle and controlled temperature. The rats had free access to appropriate diet and deionized-distilled water and were fed and visually examined once daily. Rats were also weighed once weekly. This protocol was be reviewed by
the Ohio State University Institutional Laboratory Animal Care Committee.

Electrocardiogram and heart morphometric analysis (Study I)

After 40 days of treatment animals were anesthetized with a mixture of ketamine and xylazine (85 mg/kg:15 mg/kg), which was injected intraperitoneally. Electrocardiogram (ECG) leads I, aVF, and V3 were recorded at 100 mm/sec paper speed on a photographic oscillograph with frequency response flat to over 1000 Hz. Clamp type electrodes were used with the animals in right lateral recumbancy. Using leads I, aVF, and V3, two axes projection of cardiac vectors were obtained with lead I being the X axis, aVF being the Y axis, and V3 allowing for detection of ventricular depolarization and repolarization as described by Medeiros et al. (1992). QT interval, QRS, PQ, S wave height, R wave height, and heart rate were statistically analyzed. Visual assessment of notching, repeated deflections (such as presence of R R' peaks) and other abnormal tracings were also recorded.

After the ECGs were recorded the thoracic cavity was opened and, using cardiac puncture, a blood sample was taken from each rat with an aliquot placed in heparinized tubes for hematocrit determination and
another aliquot placed in separate heparinized tubes for plasma cholesterol, and plasma lactate dehydrogenase (LDH) determinations. Rats were then sacrificed by severing the aorta. Heart and liver were excised from each animal for further analysis. Liver was blotted and frozen for superoxide dismutase (SOD) activity and copper level determinations as described below. The heart was blotted and rinsed of blood, weighed, and cut in a median coronal plane. The ventral (posterior) half of the heart was immediately frozen for later use in various biochemical assays and the dorsal (anterior) section of the heart was placed in 10% buffered formalin solution for morphometrical analysis.

The following cardiac morphometrical dimensions were measured using precise calipers: left ventricular lesser dimension, traversing the ventricle just below the site of the papillary muscle; the left ventricular major dimension, which spans the ventricle lengthwise to the apex; and thickness of the left ventricular free wall; the right ventricular free wall, and the interventricular septum, and the left ventricular apical dimension. Left ventricular free wall, right ventricular free wall, and interventricular septum were measured at a point 2/3 down the length of the heart toward the apex. The left ventricular lesser dimension and the left ventricular major dimension were measured
to assess potential decreases in the left ventricular luminal size.

Hematocrit (Study I&II), plasma cholesterol, and plasma lactate dehydrogenase (Study I)

A small portion of heparinized blood from each animal was transferred to microhematocrit tubes and centrifuged in a microcapillary centrifuge to measure packed cell volume to the nearest 0.005. Plasma was separated from the heparinized blood to determine cholesterol levels and LDH activity. Total cholesterol was determined spectrophotometrically (Beckman Du-68 Spectrophotometer) using an enzymatic cholesterol kit (Stanbio Laboratory Inc., San Antonio, TX). LDH activity was also be assessed spectrophotometrically utilizing a kit that measures the NAD+ produced during the lactate dehydrogenase reaction (LDH optimized Lactate dehydrogenase kit, Sigma Diagnostics, St. Louis, MO).

Liver copper status (Study I&II)

A 0.5 g sample of liver was weighed and digested in concentrated nitric acid in order to measure copper content using a flame atomic absorption spectrophotometer (Varian Spectr AA-5, Varian, Victoria, Australia). Copper content was expressed as µg Cu per
gram of liver tissue. Bovine liver standard, purchased from the National Institute of Standards and Technology, (Gaithersburg, MD) was used to verify accuracy of these analytical techniques.

**Plasma ceruloplasmin (Study II)**

Plasma ceruloplasmin oxidase activity was assessed using the method of Schosinsky et al. (1974). Briefly, 0.75 ml of acetate buffer (1.36% sodium acetate•3H₂O, and 0.26% glacial acetic acid, pH=5), 0.2 ml o-dianisidine dihydrochloride reagent (pre-incubated in 30°C water bath), and 0.5 ml plasma were incubated for 5 min and 15 min in a 30°C water bath. Following the 5 min or 15 min incubation time, 2.0 ml of 9 M sulfuric acid was added to the tubes and mixed immediately. The absorbencies of these solutions were measured at 540 nm in a 1 cm wide cuvet using a Beckman Du-68 spectrophotometer. The blank used was distilled water. Ceruloplasmin oxidase activity was calculated as the difference in absorbance between 15 min and 5 min multiplied by 62.5 U/L.

**Liver superoxide dismutase (Study I)**

Liver SOD activity was assessed by homogenizing 1.0 g sample of liver with 5 ml of phosphate-buffered saline (Sigma Diagnostics, St. Louis, MO). SOD activity was
based on the ability of the homogenate to inhibit the autooxidation of pyrogallol as described by Marklund and Marklund (1974) and modified by Prohaska (1983). Protein content of the extract from the SOD assay was determined by using the method described by Lowry et al. (1951).

Heart cytochrome c oxidase (Study I&II) and lactate dehydrogenase activity (Study I)

Heart samples (0.1 g) were homogenized as detailed by McCormick et al., (1989). Working cytochrome c was prepared by dissolving 25-35 mg cytochrome c (Bovine Heart, Sigma Diagnostics, St. Louis, MO) in 1.0 ml of degassed phosphate buffer (pH=7.0). Excess sodium hydrosulfate was added to change the cytochrome c from an oxidized to a reduced state. A 1.5cm X 27cm sephadex G-25 column was used to separate out the sodium hydrosulfate from the reduced cytochrome c. Cardiac cytochrome c oxidase activity was assayed by measuring the decrease in optical density at 550nm (Beckman Du-68 spectrophotometer) of reduced cytochrome c as it is oxidized by the enzyme as described by Prohaska and Wells (1974). The molar extinction coefficient of 19,600 was used for the cytochrome c. Units of CCO activity were based on the amount (µmol) of reduced working cytochrome c oxidized per minute by the CCO in
the sample. The protein content of the heart was obtained using the method described by Lowry et al. (1951) on the nonmyofibrillar protein fraction isolated for the CCO assay and data were expressed as units/ug cardiac nonmyofibrillar protein.

Cardiac LDH activity was determined using the nonmyofibrillar protein fraction described above. The assay was done spectrophotometrically (Beckman Du-68 spectrophotometer) using a kit obtained from Sigma Diagnostics (St. Louis, MO). Cardiac LDH activity was expressed as units activity/ug nonmyofibrillar protein, where one unit was defined as the amount of enzyme which catalyzes the formation of 1 umol/L of NAD+/minute under assay conditions.

**Urea-SDS-PAGE for cytochrome c oxidase analysis and Western Blots (Study I&II)**

The electrophoresis procedure used followed the method described by Chao et al. (1994) and Estey et al. (1990). A Bio-Rad mini gel apparatus was used to cast slab gels 80 x 0.75 mm containing 16% acrylamide (2.67% crosslinked with bis) and 6 mol/liter urea. The stacking gels (15 x 80 x 0.75 mm) contained 0.1% SDS and 5% acrylamide-6 mol/L urea. Nonmyofibrillar proteins were denatured in the same volume of dissociation buffer (0.1 mol/L Tris-HCL, pH=6.2)
containing 5% SDS, 8 mol/L urea, 1% B-mercaptoethanol and 1% glycerol. A methyl green dye solution (1/10 volume of dissociation buffer, 0.5% methyl green and 30% sucrose in water) was then used as an indicator for protein migration. Samples were incubated with the dissociation buffer and methyl green dye for 45 min before electrophoresis at 37°C. Diluted (1:15) bovine heart cytochrome c oxidase standard (74 g/L) was denatured and incubated in the same manner as the samples. After incubation, 25 μl of sample (74 ± 2 μg protein) and 15 μl of standard (23 μg protein) were loaded to the wells in the stacking gel. Electrophoresis was performed at a constant 10 mA/gel for 1.25-1.50 hours. The running buffer used consisted of 0.1% SDS-192 mmol/L glycine-25 mmol/L Tris at pH=8.3.

Following electrophoresis the proteins on the gel were transferred to nitrocellulose membranes using the method of Towbin et al. (1979) as described by Medeiros et al. (1993b). Western blots were prepared as detailed by Chao et al. (1994). Briefly, membranes were incubated with the primary polyclonal antibody (using a 1:1000 dilution) for cytochrome c oxidase overnight at room temperature in a 5% milk-TBS solution. Blots were then washed several times with a 5% milk-TBS solution for 30-45 min. Next a secondary goat
antirabbit antibody conjugated to alkaline phosphatase (Sigma Diagnostics, St. Louis, MO) was incubated with the membrane for 1 hour in a 5% milk-TBS solution. Excess antibody was then removed by washing the membrane with 5% milk-TBS and gently rinsing the membranes with dionized distilled water to remove remnants of the 5% milk-TBS solution. Development of the blots was done by placing them in a solution containing NBT (nitro blue tetrazolium) in 70% N,N-dimethylformamide (50:1) and BCIP (5-bromo-4-chloro-3-indolyl phosphate) in a developing buffer (0.1 mol/L Tris, 0.1 mol/L NaCl and 0.01 mol/L MgCl₂) for approximately 15 min. Development of the membranes was quenched by using a 20 mmol/L Tris and 5 mmol/L EDTA stop buffer solution for 5 min. After blots air dried they were photographed and analyzed using a Hoefer GS 300 Transmittance/Reflectance densitometer in the reflective mode (Hoefer Scientific Instruments, San Francisco, CA).

Fibronectin Isolation (Study II)

A 0.04 g sample of cardiac tissue in 0.5 ml cold phosphate buffered saline (PBS) was homogenized in a cold Polytron apparatus. The PBS contained the following protease inhibitors aprotinin (5 trypsin inhibitory units/ml), 10 mM leupeptin, 1 mM pepstatin A, and 1 mM phenylmethylsulfonylfluoride (PMSF), all at a
12:1 ratio of buffer volume to wet tissue weight (Mamuya and Brecher, 1992). The homogenate was centrifuged at 11,000 x g for 2 min at a temperature of 4° C. The supernatant was removed and saved. The pellet was removed and resuspended in 4% SDS (4 g lauryl sulfate in 100 ml dd water) and heated to 100° C for 4 min, then centrifuged at 11,000 x g at room temperature for 2 min. The supernatant contained the denatured fibronectin and was stored at -80° C. Fibronectin extraction following this method has been verified by Western blots (Liao et al., 1995a, 1995b). The protein content of the sample was determined using the method described by Lowry et al. (1951).

**Laminin Isolation (Study II)**

Using a cold Polytron homogenizer, a 0.06 g sample of cardiac tissue was homogenized in 0.5 ml cold PBS buffer containing 0.5 M NaCl, 0.05 M Tris HCl, 10 mM leupeptin, aprotinin (5 trypsin inhibitory units/ml), 1 mM PMSF, 1 mM pepstatin A as described by Nishi (1988). The homogenized sample was centrifuged at 11,000 x g for 2 min at 4° C. Following centrifugation the supernatant, which contained laminin, was decanted and stored at -80° C. Laminin extraction following this method has been verified by Western blots (Liao et al.,
The protein content of the sample was determined as described by Lowry et al. (1951).

Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE) for Study II

Preparation of samples and electrophoresis of both fibronectin and laminin followed the same protocol. Samples in a 1:1 dilution of equilibration buffer (pH=6.8) consisting of 0.0625 M Tris-HCl, 2.3% SDS, 5% beta-mercaptoethanol, 10% glycerol, and a small amount of bromophenol were boiled at 100°C for 6 min. Following the boiling, the samples were centrifuged at 11,000 x g for 8 min. Fibronectin samples were loaded at 10 µg of protein per well and laminin samples were loaded at 100 µg of protein per well in a 7.5% SDS-polyacrylamide gel (Laemmli, 1970). Gels were electrophoresed for 2 hours at a constant current of 10 mA per gel at room temperature using a Bio Rad Mini-Protean II Dual Slab Cell apparatus (Bio Rad Laboratories, Richmond, CA). The wattage limit was set past 100 W and current limit set for 100 mA in a ISCO Electrophoresis Power Supply (Model 494, Lincoln, NE). Gels were stained with coomasie blue and destained with a 10% acetic acid solution overnight. Following the period of destaining, gels were analyzed using a Hoefer GS 300 Transmittance/Reflectance densitometer in the
transmittive mode (Hoefer Scientific Instruments, San Francisco, CA).

Statistical Analysis for Study I

Differences in the 2 X 2 experimental design with two levels of copper and fat were analyzed by the general linear model analysis of variance using the Statistical Analysis System (SAS Institute, Cary, NC). Significant interactions were analyzed using the Least Square Differences (LSD) procedure. The alpha level was set a priori at 0.05.

Statistical Analysis for Study II

The differences by week and level of dietary copper were analyzed by a one way analysis of variance, with diet as the main effect. (Systat, Inc., Evanston, IL). The alpha level was set a priori at 0.05.
Chapter IV
RESULTS

Study I

At the end of the 40 day experimental period the animals in the Cu-, low fat group showed visible signs of copper deficiency. Some grayish hairs were observed and rats appeared physically weaker than their copper adequate counterparts. Rats from the Cu-, high fat group appeared healthier than the Cu-, low fat group, but still displayed physical weakness when handled. Body weights were lower in the Cu- groups (Table 1) with the Cu-low fat animals exhibiting lower weights than the Cu-, high fat group (P < 0.01).

Cardiac hypertrophy was observed in the Cu- groups (Table 1) as demonstrated by greater heart:body weight (P < 0.01). Despite the differences in heart:body weight, a copper effect was not detected with heart weights alone. Fat and Cu x fat effects, however, were detected (P < 0.01) in heart weights with the Cu-, high fat rats having greater heart weights than the others (P<0.01) and the Cu+, high fat being greater than the
## TABLE 1

**Study I**: Body and heart weights, blood variables, and lactate dehydrogenase activities of rats fed copper restricted and adequate low and high fat diets

<table>
<thead>
<tr>
<th>Treatment groups</th>
<th>ANOVA P Values $^2$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Cu Effect</td>
</tr>
<tr>
<td>Cu-</td>
<td></td>
</tr>
<tr>
<td>low fat</td>
<td>n=8</td>
</tr>
<tr>
<td>Cu+</td>
<td>n=9</td>
</tr>
</tbody>
</table>

<p>| | | | | |
|                  |               |               |               |     |
| Body wt (g)      | 184$^c$      | 269$^a$      | 248$^b$      | 276$^a$ |
|                  | 0.0001       | 0.0001       | 0.0002       | 18.3 |
| Heart wt (mg)    | 0.86$^c$    | 1.32$^a$    | 1.00$^{bc}$  | 1.10$^b$ |
|                  | NS          | 0.0001       | 0.0015       | 0.139 |
| Heart:Body wt (mg: g) | 4.70 | 4.89 | 4.02 | 4.04 |
|                  | 0.0001       | NS           | NS           | 0.463 |
| Hematocrit       | 38          | 40          | 38           | 38    |
|                  | NS          | NS          | NS           | 5.8   |
| Plasma Cholesterol (mmol/L) | 2.02 | 2.72 | 1.94 | 2.40 |
|                  | NS          | 0.0487      | NS           | 0.552 |
| Plasma LDH$^3$   | 950         | 803         | 922          | 891   |
|                  | NS          | NS          | NS           | 240   |</p>
<table>
<thead>
<tr>
<th>Cardiac</th>
<th>127</th>
<th>190</th>
<th>166</th>
<th>157</th>
<th>NS</th>
<th>NS</th>
<th>NS</th>
<th>42</th>
</tr>
</thead>
</table>

1. Values are means; means with different superscripts are significantly different, $P < 0.05$.
2. NS, $P > 0.05$.
3. LDH = Lactate dehydrogenase.
Cu-, low fat rats (P < 0.01). Hematocrit levels were not significantly different between treatments. Rats in the high fat groups had higher serum cholesterol than their low fat counterparts (P < 0.05), regardless of copper levels. No differences in LDH activities of serum and nonmyofibrillar cardiac proteins were observed in this study.

Liver copper levels were lower in the Cu- groups (P < 0.01) on both a per weight and a per mg protein basis (Table 2). The Cu-, high fat rats exhibited significantly lower liver copper stores on a per weight basis than the Cu-, low fat group (P < 0.01) with copper levels being half the amount found in the Cu-, low fat. High dietary fat also served to lower liver copper stores (P < 0.01). The Cu- groups had decreased SOD activity compared with the Cu+ groups (P < 0.01, Table 2). Decreased activity of cardiac CCO was noted in the Cu- groups (P<0.01). Additionally, the Cu-, high fat group had lower CCO activity (P < 0.01) as compared to the Cu-, low fat group (Table 2).

Western blots and subsequent reflectance densitometry measures demonstrated that the nuclear encoded subunits of CCO (IV, Va,b, VIa,b,c) were visibly depressed in the Cu-, low fat rats (Figure 3 and 4). The Cu-, high fat group displayed an even further
<table>
<thead>
<tr>
<th>Treatment groups</th>
<th>ANOVA P Values$^2$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Cu Effect</td>
</tr>
<tr>
<td>Liver Cu 60.5c (nmol/g liver)</td>
<td>0.0001</td>
</tr>
<tr>
<td>n=8</td>
<td></td>
</tr>
<tr>
<td>Liver Cu 228 (nmol/mg protein)</td>
<td>0.0001</td>
</tr>
<tr>
<td>n=9</td>
<td></td>
</tr>
<tr>
<td>Liver SOD 8101 (U/g liver)$^3$</td>
<td>0.0001</td>
</tr>
<tr>
<td>n=7</td>
<td></td>
</tr>
<tr>
<td>Liver SOD 303 (U/mg protein)$^3$</td>
<td>0.0001</td>
</tr>
<tr>
<td>n=8</td>
<td></td>
</tr>
<tr>
<td>Cardiac CCO 2.2b (U/µg non-myofibrillar protein)$^4$</td>
<td>0.0001</td>
</tr>
</tbody>
</table>

$^1$ Liver copper, liver superoxide dismutase (SOD) activity, cardiac cytochrome c oxidase (CCO) activity as indicators of copper status in rats fed copper restricted and adequate low and high fat diets.

$^2$ ANOVA P Values for comparison of copper status between different groups.

$^3$ Results expressed as units per gram of liver or protein.

$^4$ Results expressed as units per microgram of non-myofibrillar protein.
TABLE 2 (continued)

Values are means; means with superscripts are significantly different, P < 0.05.

2NS, P > 0.05.

3U = units of activity for SOD: the amount of sample needed to reduce the oxidation of pyrogallol by 50%.

4U = units of activity for CCO: μmol cytochrome c that is oxidized/min/μg cardiac non-myofibrillar protein.
Figure 4 (Study I) Western blot of cardiac cytochrome c oxidase (CCO) from rats fed diets consisting of Cu+, low fat; Cu+, high fat; Cu-, low fat; Cu-, high fat. Rats fed copper adequate diets showed depressed amounts of nuclear encoded subunits of CCO. Subunits IV, Va, Vb, VIA, VIb, and VIC are all decreased in comparison to the copper adequate animals (Cu+, low fat; Cu+, high fat). Even more severe depression of the nuclear encoded subunits is observed in the Cu-, high fat rats, indicating that fat played a role in exacerbating this sign of copper deficiency. Std= bovine CCO standard.
Figure 5  Densitometer pherograms taken from Western blots of cytochrome c oxidase (CCO) from rats fed diets consisting Cu+, low fat; Cu+, high fat; Cu-, low fat; Cu-, high fat. CCO nuclear encoded subunits IV, Va, Vb, VIa, VIb, and VIc are depressed in the Cu-, low fat; Cu-, high fat treatments. In the Cu-, high fat group an almost complete absence of IV, Va, and Vb is seen. Subunit II, coded for in the mitochondrial genome, is unaffected by the copper deficient treatments.
lowering of the CCO nuclear encoded subunits, almost to a point of complete absence.

Left ventricular free wall thickness differed between the Cu- and Cu+ rats (P < 0.05) and between the low fat and the high fat groups (P < 0.05, Table 3) with greater thickness in the Cu- groups and in the high fat groups. Other areas of the heart exhibiting hypertrophy were the right ventricular free wall and interventricular septum (P < 0.01) as thickening was noted in the high fat groups. Left ventricular lesser dimension was lower in the Cu-, low fat; Cu-, high fat; Cu+, high fat treatments (P < 0.05) as compared with the Cu+, low fat group (Table 3). Left ventricular major dimension was lower in the Cu-, low fat group (P < 0.05) compared to the Cu-, high fat and Cu+, low fat groups. Gross visual examination of the coronal heart sections revealed decreased ventricular luminal space in both Cu- groups as well as thickened musculature of the hearts (Figure 5).

Electrocardiogram lead V3 was analyzed for any alterations in cardiac electrical activity and heart rate. Heart rate did not appear to vary statistically among the treatments, though the Cu+, low fat group appeared to have the lowest heart rate (Table 4). The QT interval, a measure of the time involved in ventricular depolarization and repolarization, was
TABLE 3

Study I: Heart morphometric data: thickness (in millimeters) of ventricular walls, interventricular septum, apex and dimensions of ventricular lumens in hearts of rats fed copper restricted and adequate low and high fat diets

<table>
<thead>
<tr>
<th>Treatment groups</th>
<th>Cu- low fat</th>
<th>Cu- high fat</th>
<th>Cu+ low fat</th>
<th>Cu+ high fat</th>
</tr>
</thead>
<tbody>
<tr>
<td>n=8</td>
<td>n=9</td>
<td>n=7</td>
<td>n=8</td>
<td></td>
</tr>
<tr>
<td>Left ventricular free wall</td>
<td>3.51</td>
<td>3.94</td>
<td>3.13</td>
<td>3.65</td>
</tr>
<tr>
<td>Right ventricular free wall</td>
<td>1.37</td>
<td>1.63</td>
<td>1.16</td>
<td>1.49</td>
</tr>
<tr>
<td>Apical dimension</td>
<td>2.60</td>
<td>2.92</td>
<td>2.10</td>
<td>2.47</td>
</tr>
<tr>
<td>Interventricular septum</td>
<td>2.85</td>
<td>3.62</td>
<td>2.79</td>
<td>3.11</td>
</tr>
<tr>
<td>Left ventricular lesser dimension</td>
<td>2.61b</td>
<td>2.76b</td>
<td>3.46a</td>
<td>2.70b</td>
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</table>

ANOVA P Values

<table>
<thead>
<tr>
<th>Cu Effect</th>
<th>Fat Effect</th>
<th>Cu x Fat Effect</th>
<th>SE</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.0343</td>
<td>0.0042</td>
<td>NS</td>
<td>0.394</td>
</tr>
<tr>
<td>NS</td>
<td>0.0114</td>
<td>NS</td>
<td>0.272</td>
</tr>
<tr>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>0.625</td>
</tr>
<tr>
<td>NS</td>
<td>0.0015</td>
<td>NS</td>
<td>0.360</td>
</tr>
<tr>
<td>NS</td>
<td>NS</td>
<td>0.0511</td>
<td>0.560</td>
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</table>
TABLE 3 (continued)

<table>
<thead>
<tr>
<th>Left ventricular</th>
<th>major</th>
<th>7.27\textsuperscript{b}</th>
<th>8.68\textsuperscript{b}</th>
<th>8.70\textsuperscript{a}</th>
<th>7.58\textsuperscript{ab}</th>
<th>NS</th>
<th>NS</th>
<th>0.0155</th>
<th>1.255</th>
</tr>
</thead>
</table>

\(^1\)Values are means; means with different superscripts are significantly different, \( P < 0.05 \).

\(^2\)NS, \( P > 0.05 \).
**Figure 5** (Study I) Photograph of representative hearts from rats fed diets consisting of Cu+, low fat; Cu+, high fat; Cu-, low fat; Cu-, high fat. Rats on copper deficient diets (Cu-, low fat; Cu-, high fat) exhibited higher heart:body weight ratio (P< 0.01) and thickening of the left ventricular free wall (P< 0.01), both indicative of cardiac hypertrophy.
<table>
<thead>
<tr>
<th>Cu$^{-}$</th>
<th>Cu$^{+}$</th>
<th>Cu$^{+}$</th>
<th>Cu$^{-}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>HF</td>
<td>HF</td>
<td>LF</td>
<td>LF</td>
</tr>
</tbody>
</table>

Figure 5
lower in the high fat groups \( (P < 0.01) \). Additionally, the Cu-, high fat treatment demonstrated decreased QT \( (P < 0.05) \) as compared with all other groups. A trend was observed for Cu-, low fat group to have a higher R-wave voltage than the other groups, but this was not statistically significant \( (P < 0.06) \). S-wave voltage, however, was significantly greater in the Cu- treatments \( (P < 0.01) \).
### TABLE 4

**Study I: Electrocardiogram data using lead V3 from rats fed copper restricted and adequate low and high fat diets**

<table>
<thead>
<tr>
<th>Treatment groups</th>
<th>ANOVA P Values</th>
<th>Cu</th>
<th>Fat</th>
<th>Cu x Fat</th>
<th>SE</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Cu- low fat</td>
<td>Cu- high fat</td>
<td>Cu+ low fat</td>
<td>Cu+ high fat</td>
<td>Cu Effect</td>
</tr>
<tr>
<td>n=8</td>
<td>n=9</td>
<td>n=7</td>
<td>n=8</td>
<td></td>
<td></td>
</tr>
<tr>
<td>QRS (ms)</td>
<td>19</td>
<td>20</td>
<td>19</td>
<td>20</td>
<td>NS</td>
</tr>
<tr>
<td>PQ (ms)</td>
<td>49</td>
<td>49</td>
<td>44</td>
<td>47</td>
<td>NS</td>
</tr>
<tr>
<td>QT (ms)</td>
<td>97&lt;sup&gt;a&lt;/sup&gt;</td>
<td>71&lt;sup&gt;b&lt;/sup&gt;</td>
<td>90&lt;sup&gt;a&lt;/sup&gt;</td>
<td>86&lt;sup&gt;a&lt;/sup&gt;</td>
<td>NS</td>
</tr>
<tr>
<td>R wave (mV)</td>
<td>0.84</td>
<td>0.46</td>
<td>0.53</td>
<td>0.62</td>
<td>NS</td>
</tr>
<tr>
<td>S wave (mV)</td>
<td>0.26</td>
<td>0.22</td>
<td>0.12</td>
<td>0.15</td>
<td>0.01</td>
</tr>
<tr>
<td>Heart rate (beats/min)</td>
<td>294</td>
<td>300</td>
<td>275</td>
<td>310</td>
<td>NS</td>
</tr>
</tbody>
</table>

<sup>1</sup>Values are means; means with different superscripts are significantly different, P < 0.05.

<sup>2</sup>NS, P > 0.05
Study II

During the course of the five week experimental period body weights differed only at the third week of dietary treatment. Rats fed copper restricted diets showed a decrease in body weight at three weeks, but not at any other time point during the study (P < 0.05, Tables 5 and 6). The presence of cardiac hypertrophy was assessed by measuring heart weight and calculating a heart to body weight ratio, which normalized for differences in body weights. Differences in absolute heart weights were found by the fourth week of copper restriction (P < 0.048, Table 6). Significantly greater heart:body weight was observed after only three weeks of copper restriction (P < 0.007, Table 6) and persisted for the remainder of the experimental period.

Copper status was assessed by measuring liver copper levels and plasma ceruloplasmin activity. Liver copper was found to be significantly lower by the third week of copper restriction (P < 0.018, Table 6). Rats fed the copper restricted diets demonstrated lower plasma ceruloplasmin activity after two weeks of dietary treatment (P < 0.014, Table 6). Liver iron levels were measured in the same fashion as liver copper in order to examine any changes in iron stores. There was no significant difference in liver iron stores at any time
Table 5

Study II: Initial body weight, heart weight, heart:body weight, hematocrit, indicators of copper status, and liver iron of rats taken at weaning (baseline)^1

Baseline (n=3)

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body Weight (g)</td>
<td>57 ± 2</td>
</tr>
<tr>
<td>Heart Weight (g)</td>
<td>0.43 ± 0.09</td>
</tr>
<tr>
<td>Heart:body Weight</td>
<td>7.5 ± 1.6</td>
</tr>
<tr>
<td>Hematocrit^2</td>
<td>48 ± 3</td>
</tr>
<tr>
<td>Ceruloplasmin^3</td>
<td>45 ± 8</td>
</tr>
<tr>
<td>Liver Copper (μg/g liver)</td>
<td>5.9 ± 3.6</td>
</tr>
<tr>
<td>Liver Iron (μg/g liver)</td>
<td>656 ± 189</td>
</tr>
</tbody>
</table>

^1Values are expressed as means ± standard deviation
^2Hematocrit is expressed as a percent of the blood that is packed cell volume.
^3Ceruloplasmin activity is expressed as the absorbance difference between 15 min and 5 min multiplied by 62.5 U/L.
TABLE 6

Study II: Weekly body and heart weight, heart:body weight, hematocrit, and indicators of copper status of rats fed copper restricted and adequate diets

<table>
<thead>
<tr>
<th></th>
<th>Adequate copper (n=3)</th>
<th>Restricted copper (n=3)</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Week One</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Body Weight (g)</td>
<td>57 ± 25</td>
<td>48 ± 5</td>
<td>NS</td>
</tr>
<tr>
<td>Heart Weight (g)</td>
<td>0.48 ± 0.20</td>
<td>0.39 ± 0.01</td>
<td>NS</td>
</tr>
<tr>
<td>Heart:body weight</td>
<td>8.5 ± 0.3</td>
<td>8.3 ± 0.9</td>
<td>NS</td>
</tr>
<tr>
<td>Hematocrit&lt;sup&gt;2&lt;/sup&gt;</td>
<td>49 ± 1</td>
<td>44 ± 5</td>
<td>NS</td>
</tr>
<tr>
<td>Ceruloplasmin&lt;sup&gt;3&lt;/sup&gt;</td>
<td>50 ± 14</td>
<td>33 ± 6</td>
<td>NS†</td>
</tr>
<tr>
<td>Liver Copper (µg/g liver)</td>
<td>5.3 ± 1.2</td>
<td>4.2 ± 0.9</td>
<td>NS</td>
</tr>
<tr>
<td>Liver Iron (µg/g liver)</td>
<td>661 ± 623</td>
<td>460 ± 18</td>
<td>NS</td>
</tr>
<tr>
<td><strong>Week Two</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Body Weight (g)</td>
<td>66 ± 24</td>
<td>77 ± 12</td>
<td>NS</td>
</tr>
<tr>
<td>Heart Weight (g)</td>
<td>0.40 ± 0.12</td>
<td>0.57 ± 0.20</td>
<td>NS</td>
</tr>
<tr>
<td>Heart:body weight</td>
<td>6.2 ± 1.0</td>
<td>7.3 ± 1.4</td>
<td>NS</td>
</tr>
<tr>
<td>Hematocrit&lt;sup&gt;2&lt;/sup&gt;</td>
<td>47 ± 2</td>
<td>42 ± 3</td>
<td>NS†</td>
</tr>
</tbody>
</table>
TABLE 6 (continued)

<table>
<thead>
<tr>
<th></th>
<th>Week Three</th>
<th></th>
<th>Week Four</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Ceruloplasmin&lt;sup&gt;3&lt;/sup&gt;</td>
<td>48 ± 12</td>
<td>14 ± 8</td>
<td>199 ± 12</td>
<td>NS</td>
</tr>
<tr>
<td>Liver Copper (μg/g liver)</td>
<td>4.8 ± 1.4</td>
<td>2.5 ± 1.3</td>
<td>1.29 ± 0.19</td>
<td>≤0.048</td>
</tr>
<tr>
<td>Liver Iron (μg/g liver)</td>
<td>308 ± 171</td>
<td>450 ± 181</td>
<td>6.43 ± 0.6</td>
<td>≤0.027</td>
</tr>
<tr>
<td>Body Weight (g)</td>
<td>195 ± 9</td>
<td>142 ± 33</td>
<td>198 ± 96</td>
<td>NS</td>
</tr>
<tr>
<td>Heart Weight (g)</td>
<td>0.96 ± 0.08</td>
<td>1.10 ± 0.40</td>
<td>29 ± 2</td>
<td>≤0.005</td>
</tr>
<tr>
<td>Heart:Body Weight</td>
<td>4.9 ± 0.3</td>
<td>7.8 ± 0.9</td>
<td>4.7 ± 0.6</td>
<td>≤0.018</td>
</tr>
<tr>
<td>Hematocrit&lt;sup&gt;2&lt;/sup&gt;</td>
<td>47 ± 2</td>
<td>30 ± 2</td>
<td>2.1 ± 0.1</td>
<td>≤0.001</td>
</tr>
<tr>
<td>Ceruloplasmin&lt;sup&gt;3&lt;/sup&gt;</td>
<td>53 ± 13</td>
<td>13 ± 7</td>
<td>1.0 ± 0.5</td>
<td>≤0.01</td>
</tr>
<tr>
<td>Liver Copper (μg/g liver)</td>
<td>2.1 ± 0.1</td>
<td>1.0 ± 0.5</td>
<td>1.1 ± 0.6</td>
<td>≤0.018</td>
</tr>
<tr>
<td>Liver Iron (μg/g liver)</td>
<td>240 ± 152</td>
<td>198 ± 96</td>
<td>30 ± 2</td>
<td>≤0.005</td>
</tr>
</tbody>
</table>

*NS* indicates not significant.
TABLE 6 (continued)

<table>
<thead>
<tr>
<th></th>
<th>Ceruloplasmin</th>
<th>Liver Copper (µg/g liver)</th>
<th>Liver Iron (µg/g liver)</th>
<th>Body Weight (g)</th>
<th>Heart Weight (g)</th>
<th>Heart:Body Weight</th>
<th>Hematocrit</th>
<th>Ceruloplasmin</th>
<th>Liver Copper (µg/g liver)</th>
<th>Liver Iron (µg/g liver)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>44 ± 12</td>
<td>2.4 ± 0.3</td>
<td>299 ± 206</td>
<td>193 ± 21</td>
<td>1.02 ± 0.26</td>
<td>5.3 ± 1.2</td>
<td>45 ± 5</td>
<td>45 ± 5</td>
<td>2.8 ± 0.5</td>
<td>202 ± 27</td>
</tr>
<tr>
<td>Week Five</td>
<td>10 ± 2</td>
<td>1.0 ± 0.2</td>
<td>227 ± 7</td>
<td>180 ± 10</td>
<td>1.26 ± 0.04</td>
<td>7.02 ± 0.32</td>
<td>33 ± 6</td>
<td>12 ± 3</td>
<td>0.9 ± 0.4</td>
<td>260 ± 113</td>
</tr>
</tbody>
</table>

1Values are expressed as means ± standard deviation
2Hematocrit is expressed as a percent of the blood that is packed cell volume.
3Ceruloplasmin activity is expressed as the absorbance difference between 15 min and 5 min multiplied by 62.5 U/L.

Trend present at P < 0.14.
Trend present at P < 0.057.
Trend present at P < 0.005.
Trend present at P < 0.004.
Trend present at P < 0.05.
Trend present at P < 0.001.
Trend present at P < 0.006.
during the five week period (Table 6). Through visual inspection of the data, it appears that both liver iron and liver copper stores are greatest when the animal was very young and became lower with time (Tables 5 and 6).

Hematocrit levels declined in rats fed copper restricted diets. After two weeks a strong trend toward declines in hematocrit levels were observed ($P < 0.057$, Table 6) with significant differences occurring at week three of copper restriction ($P < 0.001$, Table 6).

Cardiac fibronectin was isolated and subjected to SDS-PAGE (sodium dodecyl sulfate polyacrylamide gel electrophoresis) and analysis with transmittance densitometry. The amounts of the protein fibronectin did not differ between the copper adequate and copper restricted animals at any time point in this study (Table 7, Figure 6). Cardiac laminin levels were assessed in the same manner. Visual examination indicated lower levels of laminin at week three and four of copper restriction (Figures 6 and 7). Statistical analysis, however, revealed no significant differences in laminin protein levels between the copper adequate and restricted treatments at any time period (Table 7, Figures 6 and 7).
**TABLE 7**

Study II: Weekly cardiac laminin and fibronectin protein levels of rats fed copper restricted and adequate diets

<table>
<thead>
<tr>
<th></th>
<th>Adequate copper n=3</th>
<th>Restricted copper n=3</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Baseline laminin (µg), n=3 (week 0)</td>
<td>42.4 ± 5.1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>week 1, laminin (µg)</td>
<td>60.5 ± 14.8</td>
<td>67.2 ± 6.6</td>
<td>NS</td>
</tr>
<tr>
<td>week 2, laminin (µg)</td>
<td>65.5 ± 17.8</td>
<td>70.6 ± 9.5</td>
<td>NS</td>
</tr>
<tr>
<td>week 3, laminin (µg)</td>
<td>79.4 ± 28.2</td>
<td>53.2 ± 12.8</td>
<td>NS</td>
</tr>
<tr>
<td>week 4, laminin (µg)</td>
<td>76.8 ± 15.5</td>
<td>65.4 ± 14.5</td>
<td>NS</td>
</tr>
<tr>
<td>week 5, laminin (µg)</td>
<td>79.7 ± 12.9</td>
<td>89.5 ± 16.0</td>
<td>NS</td>
</tr>
<tr>
<td>Baseline fibronectin (µg), n=3 (week 0)</td>
<td>28.1 ± 11.6</td>
<td></td>
<td></td>
</tr>
<tr>
<td>week 1, fibronectin (µg)</td>
<td>39.5 ± 16.5</td>
<td>69.8 ± 41.0</td>
<td>NS</td>
</tr>
<tr>
<td>week 2, fibronectin (µg)</td>
<td>48.0 ± 38.7</td>
<td>35.6 ± 22.4</td>
<td>NS</td>
</tr>
<tr>
<td>week 3, fibronectin (µg)</td>
<td>40.6 ± 8.8</td>
<td>38.9 ± 28.9</td>
<td>NS</td>
</tr>
<tr>
<td>week 4, fibronectin (µg)</td>
<td>68.0 ± 31.2</td>
<td>44.9 ± 15.8</td>
<td>NS</td>
</tr>
<tr>
<td>week 5, fibronectin (µg)</td>
<td>33.9 ± 17.2</td>
<td>31.5 ± 11.9</td>
<td>NS</td>
</tr>
</tbody>
</table>

Values are expressed as mean µg protein ± standard deviation
Figure 6 (Study II) SDS-PAGE of cardiac fibronectin and laminin from rats fed copper restricted (-) and copper adequate (+) diets from time of weaning up to five weeks. After analysis using transmittance densitometry, no significant differences were found at any time period between the copper restricted group and copper adequate group. Fib = fibronectin, Lam = laminin, Wk = week.
Figure 7 (Study II) SDS-PAGE of cardiac laminin from rats fed copper restricted (-) and copper adequate (+) diets from time of weaning up to five weeks. After analysis using transmittance densitometry no significant differences were found at any time point between the copper adequate and copper restricted groups. Lam = laminin, Wk = week.
Cardiac cytochrome c oxidase was isolated, electrophoresed on a 16% urea gel and Western blots were obtained. The density of the bands on the Western blots were analyzed by reflectance densitometry and reported in Table 8 as percent nuclear encoded subunits:percent mitochondrial encoded subunit II. After one week of copper restriction there was a strong trend toward the copper restricted group having lower amounts of nuclear encoded peptides as the copper adequate group (Figure 8, Table 8, P < 0.075). A similar trend also was also present at week two, but not as strong (Figure 8, Table 8, P < 0.20). Statistical differences between the copper adequate and copper restricted groups occurred at week three of the study with the copper restricted group having the lowest levels of CCO nuclear encoded peptides (Figure 8, Table 8, P < 0.0001). These differences were present during week four and week five as well (Figure 8, Table 8, P < 0.0001).

Plotting the percent nuclear encoded subunits:mitochondrial encoded subunit II and heart:body weight on the same graph was done in Figure 9. A trend is present such that differences in percent nuclear encoded subunits:mitochondrial encoded subunit II appear to be preceding the development of cardiac hypertrophy.
Study II: Weekly cytochrome c oxidase (CCO) data from rats fed copper restricted and adequate diets\(^1\)

<table>
<thead>
<tr>
<th>Week</th>
<th>Adequate copper n=3</th>
<th>Restricted copper n=3</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Baseline, n=3 (week 0)</td>
<td>5.73 ± 0.42</td>
<td></td>
<td></td>
</tr>
<tr>
<td>week 1</td>
<td>7.00 ± 2.85</td>
<td>2.97 ± 0.55</td>
<td>NS (0.075)</td>
</tr>
<tr>
<td>week 2</td>
<td>5.97 ± 1.00</td>
<td>4.53 ± 2.00</td>
<td>NS (0.20)</td>
</tr>
<tr>
<td>week 3</td>
<td>11.90 ± 0.66</td>
<td>3.27 ± 0.29</td>
<td>0.0001</td>
</tr>
<tr>
<td>week 4</td>
<td>3.10 ± 0.46</td>
<td>1.00 ± 0.97</td>
<td>0.0001</td>
</tr>
<tr>
<td>week 5</td>
<td>3.93 ± 0.68</td>
<td>1.87 ± 0.21</td>
<td>0.0001</td>
</tr>
</tbody>
</table>

\(^1\)Values are means ± standard deviations of the percent nuclear encoded subunits:percent mitochondrial encoded subunit II as determined by reflectance densitometry analysis of Western blots.
Figure 8  (Study II) Composite photo of Western blots for cardiac cytochrome c oxidase (CCO) from rats fed copper restricted (Cu-) and copper adequate (Cu+) diets from time of weaning up to five weeks. Nuclear encoded subunits of CCO begin to show signs of depression by the first week of copper restriction. Statistical differences in the amounts of nuclear encoded subunits occur at week 3 ($P < 0.0001$). St = bovine cardiac CCO standard, Wk = week.
Figure 8
Figure 9 (Study II) Comparison of changes in percent nuclear encoded peptides: percent mitochondrial encoded peptide of cardiac cytochrome c oxidase (CCO) vs changes in heart:body weight. Data taken from rats fed copper deficient and copper adequate diets from baseline up to five weeks. This figure uses data from baseline to week 3. Changes, although non-significant, in CCO are observed before the development of cardiac hypertrophy as determined by heart:body weight.
Chapter V
DISCUSSION

Study I

Consistent with other studies using 5% corn oil in the diet as a fat source, (Al-Othman et al. 1992, Chao et al. 1994, Hoogeveen et al. 1994, Prohaska and Heller 1982), this study revealed that rats fed Cu- diets, regardless of fat level showed similar signs of copper deficiency. The Cu-, low fat rats failed to thrive and exhibited lower final body weights compared to the Cu+ rats. The Cu-, high fat rats, however, had body weights similar to the Cu+, high fat and Cu+, low fat rats. It would seem that the excess energy provided by the high fat diet offsets the effect of copper restriction to slow weight gain. This fat effect was also paralleled in the Cu+, high fat rats as they exhibited greater final body weights than the Cu+, low fat rats.

The copper restricted treatments resulted in lower copper stores as indicated by liver copper levels. The Cu-, high fat group was noted to have lower copper
stores than the Cu-, low fat group on a wet weight basis, but not when using liver protein levels as a comparison. In contrast to our observations, previous work done by Wapnir and Devas (1995) demonstrated that a high saturated:polyunsaturated fatty acid (45% diet by weight) Cu- diet resulted in higher, but not statistically different, liver copper content as compared to low fat (5% diet by weight) Cu- controls. In our study high dietary fat seemed to play a role by exacerbating copper depletion, perhaps through lower copper uptake in the gastrointestinal tract in presence of high fat, or greater copper losses through bile excretion. In such a condition bile production may be increased to accommodate the high levels of dietary fat thereby leading to greater copper loss, as a portion of the bile is destined to follow normal excretory routes. Stool analysis should be done to determine if the Cu-, high fat rats are excreting more copper through the stool as compared to the Cu-, low fat rats.

Liver SOD activity was also depressed by the Cu- treatments. The Cu-, high fat rats exhibited a trend toward lower SOD activity than the Cu-, low fat rats on both wet weight and per mg protein basis, but this was not statistically significant. The trend toward lower SOD activity in the Cu-, high fat rats may further support the thought that the Cu-, high fat rats could be
in a more depleted copper state than the Cu-, low fat rats. A fat main effect was also observed since the rats fed high fat diets had lower SOD activity compared to those fed low fat diets.

Cardiac CCO, a cuproenzyme involved in the electron transport chain, was observed to have significantly lower activity in both Cu- treatments. Additionally, the Cu-, high fat rats demonstrated lower CCO activity compared to the Cu-, low fat rats. This data corresponds with the indicators of copper status discussed above (liver copper, liver SOD activity) which shows Cu-, high fat rats to be further depleted than Cu-, low fat rats.

Previous studies done by our laboratory demonstrated through Western blots that the nuclear encoded subunit peptides of CCO are markedly reduced in hearts of copper deficient rats (Chao et al. 1994, Medeiros et al. 1993b). This study observed similar results with the Cu-, low fat rats exhibiting lower amounts of cardiac CCO nuclear encoded subunit IV, and the Cu-, high fat rats demonstrating even further depression of this subunit. Other nuclear encoded subunits (Va,b and VIa,b,c) were also lower. Therefore the low CCO activity observed during copper restriction may be attributed to, at least in part, a lack of a functional protein complex present in the mitochondria.
These data lead to a few possible scenarios: 1) there may be increased degradation of the subunits' mRNA (perhaps through decreased stability of the mRNA structure); 2) translation of the mRNA into peptides may be lower; 3) degradation of the nuclear encoded CCO peptides could be greater; 4) transport of these nuclear encoded peptides into the mitochondria could be lower during copper deficiency. The possibility of copper regulating genes in the nucleus encoding the peptides does not appear likely at this time as Northern hybridizations done in our lab indicate no appreciable change in the levels of mRNA from subunits II (mitochondrial encoded) and IV (nuclear encoded).

Previous studies have reported high plasma cholesterol levels during copper deficiency (Al-Othman et al. 1992, Medeiros et al. 1993b). In this study there was no observed copper effect upon plasma cholesterol levels. A fat effect was observed, however, such that the Cu-, high fat and Cu+, high fat rats had greater plasma cholesterol levels compared to the low fat groups.

Studies have reported the accumulation of lipid droplets and glycogen granules in cardiac myocytes during copper deficiency (Davidson et al. 1992, Medeiros et al. 1991). These observations could be due to a
possible metabolic shift from fat oxidation to increased carbohydrate oxidation in order to meet energy needs (Wildman et al. 1994). If such a scenario were occurring lactate dehydrogenase activity would be expected to increase. The data obtained on cardiac and serum LDH do not support this hypothesis, nor does recent work done by Hoogeveen et al. (1994) who demonstrated increased fat utilization in Cu- rats by performing whole body respiratory quotients (RQ). Organ oxidation of substrate, however, could be considerably more variable than a whole body RQ, which can be considered to be an average of substrate oxidation of all the organs and tissues in an organism. Additionally, Wapnir and Devas (1995) reported that a high fat Cu- diet resulted in lower activity of glucose-6-phosphate dehydrogenase, which would further indicate a low glycolytic activity.

Cardiac hypertrophy is an adaptive mechanism to conditions of pressure or volume overload. The cardiac remodeling which occurs is a temporary fix and will eventually result in cardiac failure. Hypertrophic cardiomyopathy (HCM), a non-dilated cardiomyopathy with a concentric pattern of hypertrophy, is not common, with diagnosis occurring in 0.1% - 0.5% of the American population (Maron 1993).
Hypertrophic cardiomyopathy generally leads to premature death due to cardiac failure from infancy to middle age depending on the severity of the case. There is likely, though, a larger population with HCM that goes undetected due to mild symptoms which, although unnoticed, could still lead to premature death (Maron 1993). Hearts of Cu- rats were hypertrophied as demonstrated by greater heart:body weights. Heart weights by themselves displayed a significant fat effect, but not a copper effect. It appeared odd that the Cu-, low fat group had the lowest heart weight, however, this data should not be interpreted by itself. The Cu-, low fat rats were by far the lightest animals in the study so it appears reasonable that they would also have the smallest hearts as compared with other groups, even with concomitant cardiac hypertrophy relative to their own body weight. A more accurate measure of the hypertrophic effect of the treatments is illustrated in heart:body weight which standardizes the hypertrophic effect in relation to body weight.

Morphometrical measurements of left ventricular free wall, interventricular septum, left ventricular lesser dimension, left ventricular major dimension all indicated a concentric pattern of hypertrophy present.
Concentric cardiac hypertrophy is characterized by a thickened left ventricular free wall, occasionally thickened interventricular septum, and decreased left ventricular luminal space (Maron 1993). The Cu- hearts displayed thickened left ventricular free walls, a smaller left ventricular major dimension, and smaller left ventricular lesser dimension, which is in agreement with the definition of concentric hypertrophic cardiomyopathy. The interventricular septum was thickened by the high fat treatments, regardless of the copper treatments. However, such an effect could be due to the large animals in the high fat groups simply having larger hearts than the small animals in the low fat groups.

Electrocardiograms allowed for an examination of how cardiac electrical activity was affected by the treatments. Curiously, the Cu-, high fat rats displayed a short QT interval, indicative of a more rapid ventricular depolarization-repolarization. One would expect a more rapid heart rate to accompany such a consequence. This study did not find a concomitant rapid heart rate in the Cu-, high fat rats. Possibly the short QT interval in the Cu-, high fat rats could be a result of the cardiac hypertrophy which was the greatest in this group. In previous studies Cu- treatments have resulted in longer QT intervals
(Al-Othman et al. 1992, Davidson et al. 1992 and 1993, Medeiros et al. 1993). In this study the Cu-, low fat rats did exhibit a non-significant trend for having the longest QT interval (97 mS) which is in direct opposition to the short QT intervals observed in the Cu-, high fat group. It would seem that the high dietary fat is in some way responsible for the shorter QT intervals observed.

S-wave voltages was observed to increase as a result of the Cu- treatments. This was accompanied by a strong trend (P<0.06) for increased R-wave voltages in the Cu-, low fat rats. Other studies have reported greater R-wave voltages, but not S-wave voltages (Al-Othman et al. 1992, Medeiros et al. 1992). The higher voltages could be an adaptation by the heart to insure conductance through a hypertrophied myocardium. Further analysis of EKGs did not reveal any overt notching or other aberrations isolated to any one treatment group.

The role of dietary fat and copper in the etiology of hypertrophic cardiomyopathy remains somewhat speculative. Data in this study leads to the conclusions that amount of dietary fat will exacerbate some of the indices associated with copper deficiency induced cardiomyopathy and also further decrease copper status. Studies on the influence of fat level upon marginal copper intake may provide useful information on
the likelihood of such diets affecting human heart disease incidence. Furthermore, fatty acid saturation and chain length influences may provide insights as to other factors in addition to fat levels that could be a factor in determining copper requirements.

Study II

In contrast to other studies with weanling rats fed copper restricted diets, this study did not observe any consistent differences in body weight (Davidson et al., 1992, Wildman et al., 1994). The rats fed copper restricted diets demonstrated decreased body weight at the third week of treatment, but not at any time after.

During the second week of the study, rats fed copper restricted diet demonstrated a statistically non-significant trend ($P < 0.10$) toward lower liver copper levels. By the third week of the study the rats fed copper restricted diets were observed to have significantly lower liver copper levels than the copper adequate rats ($P < 0.018$). Another study (Medeiros et al. 1992) observed rats to have low liver copper levels after only two weeks of consuming copper restricted diets. Liver copper levels appear to decline as a function of age as well. The levels of liver copper in
the rats fed copper adequate diets for five weeks were half that of the baseline values (Tables 5 and 6).

Consistent with other studies, plasma ceruloplasmin activity was found to be significantly depressed in the copper restricted group (DiSilvestro et al., 1992, Prohaska 1991, Prohaska and Heller, 1982). After only one week of consuming a copper restricted diet, a statistically non-significant trend for lower ceruloplasmin activity was observed (P < 0.14, Table 6). Statistical differences occurred at two weeks of copper restriction (P < 0.014, Table 6) with the copper restricted rats demonstrating lower ceruloplasmin activity. This is a noteworthy observation as changes in the biological activity of a cuproenzyme are observed before there are any significant differences in liver copper stores. It appears that copper stores do not need to be depleted to result in a biochemical aberration; only moderate decreases in liver copper stores result in significant drops in plasma ceruloplasmin activity. Perhaps plasma ceruloplasmin should be given more consideration as a sensitive method for copper assessment in test animals as well as humans in questionable copper status.
Presumably due to the rapid and severe decline in ceruloplasmin activity, hematocrit levels were also negatively impacted. Ceruloplasmin has ferroxidase activity and is needed for normal iron metabolism (Krause and Mahan, 1984). Decreased hematocrit levels indicative of anemia were observed after three weeks of copper restriction as compared to the copper adequate animals (Table 6). There was a strong trend (P < 0.057) toward anemia at week two of copper restriction as well. Examination of the data reveals that there is approximately one week of lag time between the significant decline of ceruloplasmin activity (week two) and the development of anemia (week three).

Cardiac hypertrophy consistently occurs in weanling rats and mice raised on copper deficient diets (Chao et al., 1994, Medeiros et al., 1992, Prohaska and Heller, 1982). This study also observed cardiac hypertrophy in the rats fed copper restricted diets as demonstrated by increased heart weight at week four and increased heart:body weight at week three (Table 6). Medeiros et al. (1992) also observed increases in rat heart:body weight after three weeks of copper restriction. In this study cardiac hypertrophy, as demonstrated by increased heart:body weight, occurred concomitantly with significant declines in liver copper stores. In a study done by Medeiros et al. (1992) decreases in
liver copper preceded observed cardiac hypertrophy by one week. These data from both studies indicate that liver copper stores are depleted before cardiac hypertrophy occurs.

Electron microscopy has demonstrated fragmentation and separation of cardiac basal lamina in rats fed copper restricted diets (Chao et al., 1994, Wildman et al., 1994). The basal lamina plays roles in aspects of normal cell function such as cell polarity, signal transduction, and adhesion (Alberts et al., 1994, Yurchenco and O'Rear, 1994) and the components of basal lamina have been found to be increased during cardiac hypertrophy (Boluyt et al., 1994). Two important components of the basal lamina are fibronectin and laminin (Alberts et al., 1994). Studies done by Liao et al. (1995a,b) observed increased amounts of fibronectin and decreased amounts of laminin present in the hearts of copper deficient rats. These biochemical changes in laminin and fibronectin could be in part responsible for the alterations observed in the cardiac basal lamina.

One of the aims of this study was to determine the time point at which the changes in basal lamina proteins fibronectin and laminin were occurring. We did this in order to gain insight as to whether or not these protein alterations were acting as some sort of stimulus for the
development of cardiac hypertrophy during copper deficiency. Analysis by SDS-PAGE and transmittance densitometry revealed no changes in fibronectin or laminin between the copper restricted rats and copper adequate rats during the course of this study (figure 6, 7, table 7). These observations are in contrast to those made by Liao et al. (1995a,b). With regards to laminin, the conflicting results to Liao's work can be in part explained by the small sample sizes used in each diet treatment group (n=3), along with the large amount of variability present in the data.

From a visual perspective it appeared that at week three and four there were slight declines in laminin levels, but not at week five (figure 7). It should be emphasized, however, these results were not statistically significant and even if they had been significantly different, they are occurring after the development of cardiac hypertrophy as evidence by heart:body weight. Additionally, cardiac hypertrophy occurred despite no observable change in fibronectin levels. Based on these data alterations in fibronectin and laminin can be ruled out as a stimulus for cardiac hypertrophy.

Cytochrome c oxidase (CCO) is a copper dependent enzyme that is the terminal electron acceptor in the electron transport chain. Not only does it contain 3
atoms of copper, but also 2 heme groups, one magnesium atom, and one zinc atom (Tsukihara et al., 1995). Mammalian cytochrome c oxidase is composed of 13 peptide subunits, genes for subunits I, II, III are found in the mitochondria, and genes for subunits IV, Va, Vb, VIA, VIb, VIC, VIIa, VIIb, VIIc, and VIII are found in the nucleus of the cell (Zhang et al., 1991). Through Western blots, several studies have observed that there appears to be a lower amounts of nuclear encoded subunits present in the hearts of copper deficient rats as compared with copper adequate controls (Medeiros et al., 1993, Chao et al., 1994). These studies also report that the levels of mitochondrial encoded subunits appear unaffected. This study has also made similar observations. Western blots of cardiac nonmyofibrillar proteins demonstrated that the nuclear encoded peptides IV, Va, Vb, VIA, VIb, and VIC were all consistently lower in rats fed copper restricted diets and the levels of mitochondrial encoded subunits appear unaffected (Figure 8). Using reflective densitometry, the Western blot membranes were analyzed for the relative density of the nuclear encoded subunits and each band was assigned a percent value corresponding to its relative density. A ratio was taken between the percent amounts of nuclear encoded peptides to percent mitochondrial encoded peptides. This ratio was analyzed using ANOVA to detect
differences between the copper restricted dietary group and the copper adequate dietary group (Table 8). After only one week of consuming a copper restricted diet, rats demonstrated an inconsistent decline in the amounts of nuclear encoded peptides observed on the Western blots (Figure 8). Statistically this was observed to be a strong trend ($P < 0.075$, Table 8). Similar observation were made after two weeks of dietary copper restriction as well. Statistically significant differences occurred at three weeks of copper restriction ($P < 0.001$, Table 8, Figure 8). Similar to ceruloplasmin activity, CCO appeared to show some signs of decrease before there were statistically significant differences in liver copper. These data suggest that these copper dependent enzymes are adversely affected while liver copper stores are decreasing. However, it appears that liver copper stores need not be significantly depleted before changes occur.

One aim of this study was to gain insight to the initial stimulus that may lead to cardiac hypertrophy. During the course of this study changes in peptide subunits of CCO, as demonstrated by Western blots, were assessed in order to determine if CCO could be providing the initial stimulus for cardiac hypertrophy. In brief, we suspect that the alterations of CCO stimulate an increase in volume density of mitochondria which may
play a major role in increasing the size of the myocyte and subsequently of the heart itself. Previous studies examining cardiac ultrastructure of rats raised on copper restricted diets have observed significantly increased mitochondrial volume density, and increased ratio of mitochondria:myofibrils (Chao et al., 1994, Wildman et al., 1994). It has been suggested that increased mitochondrial volume density may be making a significant contribution to cardiac hypertrophy (Medeiros et al., 1991).

Chao et al. (1994) observed that copper restricted rats treated with the antioxidant dimethyl sulfoxide (DMSO) appeared to be protected against the cardiac morphological damage presumably induced by oxidative stress. Chao et al. (1994) further reported that cardiac CCO nuclear encoded peptides were low in the copper restricted, DMSO groups and that mitochondrial swelling, increased mitochondrial volume density, and cardiac hypertrophy were present. These data indicate that oxidative damage may only play an accessory role in development of cardiac hypertrophy and that the primary changes observed during copper deficiency are what may be causing mitochondrial swelling, damage and cardiac hypertrophy. Swelling of mitochondria and increases in mitochondrial volume density may be a compensatory
mechanism responding to a relative drop in CCO activity within the mitochondria.

During this study we found that statistical differences in CCO occurred at the same time that cardiac hypertrophy was observed, at week three. However, visual inspection of the Western blots revealed differences in the levels of nuclear encoded subunits before week three (Figure 8). The relationship of changes in CCO compared to changes in heart:body weight is illustrated in Figure 9. These data are consistent with a theory implicating alterations in CCO acting as a stimulus for cardiac hypertrophy. Further work needs to be done using electron microscopy to examine changes in mitochondrial architecture over the same time period and Western blots or immunogold staining of the CCO nuclear encoded peptides in order to better evaluate the role CCO may be playing in cardiac hypertrophy.
Chapter VI

SUMMARY & CONCLUSIONS

The impact of copper restriction over a period of time and in conjunction with high dietary fat was examined in the two studies presented here. Study I found that high dietary fat, in tandem with copper restriction resulted in exacerbating some of the known signs of copper deficiency. Liver copper levels, on a per weight basis, were significantly lower in rats fed Cu-, high fat diets compared to rats fed Cu-, low fat diets. Other indicators of copper status, liver copper on a per gram protein basis and liver SOD activity also displayed trends toward a similar reduction in the Cu-, high fat group compared to the Cu-, low fat group. These data indicate that the Cu-, high fat animals may have had more compromised copper status than the Cu-, low fat animals. The poor copper status of the both Cu-groups resulted in cardiac hypertrophy as demonstrated by thickening of left ventricular free wall, thickening interventricular septum, greater heart weight, and greater heart:body weight. There were no differences in
these parameters between the Cu-, high fat and the Cu-, low fat animals. High fat also exerted a hypertrophic effect upon the heart as it independently led to thickening of the left ventricular free wall and interventricular septum of the heart.

Cytochrome c oxidase activity was found to be low in both Cu- groups compared to the Cu+ groups. The Cu-, high fat rats displayed significantly lower CCO activity compared to the Cu-, low fat rats, presumably due to exacerbated poor copper status. A similar result was also observed when CCO Western blots were analyzed. Both Cu- groups displayed low nuclear encoded subunits compared to Cu+ groups, and the lowest levels of nuclear encoded subunits were observed in the Cu-, high fat group.

The results of this study indicate that a high level of dietary fat may play a significant and detrimental role during the course of copper restriction. In hindsight, two questions are left unanswered; how is cardiac ultrastructure affected by high fat and would the high dietary fat lead to more rapid development of cardiomyopathy? These questions are of concern due to the chronic high fat intake that characterizes the typical American diet. The heart itself may be more susceptible to ultrastructural damage
and disease under the influence of high dietary fat and poor copper intake.

The temporal sequence of events occurring during copper restriction was examined in study II. Data from this study demonstrated that there does not need to be overt copper deficiency for biological systems to be altered from normal. A trend was present toward low liver copper levels after two weeks of copper restriction with significantly lower liver copper occurring after three weeks. After only one and two weeks of copper restriction ceruloplasmin activity and hematocrit levels, respectively, displayed strong tendencies to be low compared to copper adequate rats. One additional week of copper restriction produced significantly lower ceruloplasmin activity and hematocrit levels compared to the copper adequate animals. Cardiac hypertrophy, as demonstrated by greater heart:body weight, was observed after three weeks of copper restriction. Cardiac fibronectin and laminin levels were also assessed by SDS-PAGE and were not found to be different at any time during the study.

Western blots done for CCO displayed strong tendencies toward low nuclear encoded peptides after only one week of copper restriction with significant differences occurring after three weeks. There is a possibility that observed alterations in CCO nuclear
encoded peptides may be acting as a stimulus for increasing mitochondrial volume density which in turn contributes to development of cardiac hypertrophy. The data from this study supports this hypothesis as trends for low CCO nuclear encoded peptides were observed before development of cardiac hypertrophy.

These data suggest that the rat need not be depleted of liver copper stores to display some signs of copper deficiency including low ceruloplasmin activity and low levels of CCO nuclear encoded peptides. Additionally, it appears that typical signs of copper deficiency are observed after only three weeks of copper restriction from weaning. The conclusions drawn from this study are that biological alterations occur in a relatively short amount of time, pathology of copper deficiency occurs in the rat after only three weeks of consuming a copper restricted diet, and alterations in CCO nuclear encoded peptides begin to occur before any sign of cardiac hypertrophy. The question of alterations in cardiac ultrastructure over time is one which deserves attention, especially if the link between alteration in CCO nuclear encoded peptides, increase in mitochondrial volume density, and cardiac hypertrophy is to made.
Over years the copper deficient rat heart has been the subject of investigation in an attempt to fully characterize cardiac status. Now that there is a firm foundation of work in this area, future directions of research should concentrate on the impact of copper marginal diets alone and combined with high dietary fat. This may more closely resemble the typical American diet and would shed light on the dietary requirement for copper. Such work is needed in order to assess any possible risk that may be associated with such a diet. Additionally, other dietary and environmental factors should be examined combined with a marginal or restricted copper intake. It may be that marginal or restricted dietary copper may lead to greater disease vulnerability to factors such as cigarette smoking, drug use, possible carcinogenic agents in foods, air pollutants and general environmental pollutants.
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APPENDIX A

Composition of diets
TABLE 9
Composition of low fat and high fat diets for study I

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Low fat</th>
<th>High fat</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sucrose</td>
<td>500 g</td>
<td>500 g</td>
</tr>
<tr>
<td>Casein</td>
<td>200 g</td>
<td>200 g</td>
</tr>
<tr>
<td>Cornstarch</td>
<td>150 g</td>
<td>50 g</td>
</tr>
<tr>
<td>Fat mixture[^2]</td>
<td>50 g</td>
<td>150 g</td>
</tr>
<tr>
<td>Celufil fiber</td>
<td>50 g</td>
<td>50 g</td>
</tr>
<tr>
<td>Mineral mix[^3]</td>
<td>35 g</td>
<td>35 g</td>
</tr>
<tr>
<td>Vitamin mix[^4]</td>
<td>10 g</td>
<td>10 g</td>
</tr>
<tr>
<td>D-L-methionine</td>
<td>3 g</td>
<td>3 g</td>
</tr>
<tr>
<td>Choline bitartrate</td>
<td>2 g</td>
<td>2 g</td>
</tr>
<tr>
<td><strong>Total Energy</strong></td>
<td>15.5 kJ/g</td>
<td>17.1 kJ/g</td>
</tr>
</tbody>
</table>

[^1]: Ingredients expressed as amount needed to make 1 kg of diet.
[^2]: Fat mixture is a mixture of coconut and corn oils designed to yield a 2:1 saturated to unsaturated fatty acid ratio.
[^3]: Copper was omitted from mineral mixture used for Cu-diet, Cu+ diets contained 94.5 mmol/kg copper.
[^4]: Vitamin mix is based on the AIN-76A recommendations.
TABLE 10
Composition of diet for study II

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>g/kg diet</th>
<th>% (by weight)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sucrose</td>
<td>500</td>
<td>50.0</td>
</tr>
<tr>
<td>Cornstarch</td>
<td>150</td>
<td>15.0</td>
</tr>
<tr>
<td>Casein</td>
<td>200</td>
<td>20.0</td>
</tr>
<tr>
<td>Celufil fiber</td>
<td>50</td>
<td>5.0</td>
</tr>
<tr>
<td>Corn oil</td>
<td>50</td>
<td>5.0</td>
</tr>
<tr>
<td>Mineral mix(^1)</td>
<td>35</td>
<td>3.5</td>
</tr>
<tr>
<td>Vitamin mix</td>
<td>10</td>
<td>1.0</td>
</tr>
<tr>
<td>D-L-methionine</td>
<td>3</td>
<td>0.3</td>
</tr>
<tr>
<td>Choline bitartrate</td>
<td>2</td>
<td>0.2</td>
</tr>
</tbody>
</table>

Total Energy 15.5 kJ/g

\(^1\) Copper was omitted in the mineral mix used for Cu-diets.
APPENDIX B

Composition of AIN Vitamin and AIN Mineral mix used in formulating diet for rats.
### TABLE 11

AIN mineral mix for rats

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>g/kg of diet</th>
</tr>
</thead>
<tbody>
<tr>
<td>Calcium monophosphate (CaHPO$_4$)</td>
<td>500.000</td>
</tr>
<tr>
<td>Potassium citrate (K$_3$C$_6$H$_5$O$_7$)</td>
<td>220.000</td>
</tr>
<tr>
<td>Sodium chloride (NaCl)</td>
<td>74.000</td>
</tr>
<tr>
<td>Potassium sulfate (K$_2$SO$_4$)</td>
<td>54.000</td>
</tr>
<tr>
<td>Magnesium oxide (MgO)</td>
<td>24.000</td>
</tr>
<tr>
<td>Manganese carbonate (MnCO$_3$)</td>
<td>3.500</td>
</tr>
<tr>
<td>Ferric citrate (FeC$_6$H$_5$O$_7$)</td>
<td>1.600</td>
</tr>
<tr>
<td>Zinc Carbonate (ZnCO$_3$)</td>
<td>1.600</td>
</tr>
<tr>
<td>Chromium potassium sulfate (CrKSO$_4$)</td>
<td>0.550</td>
</tr>
<tr>
<td>Potassium Iodate (KIO$_3$)</td>
<td>0.010</td>
</tr>
<tr>
<td>Sodium selenite</td>
<td>0.010</td>
</tr>
<tr>
<td>Copper carbonate (CuCO$_3$)</td>
<td>(Cu$^+$)$^1$ 0.398</td>
</tr>
<tr>
<td></td>
<td>(Cu$^-$)$^2$ 0.000</td>
</tr>
</tbody>
</table>

$^1$Cu$^+$ represents copper adequate diet.

$^2$Cu$^-$ represents copper deficient diet.
## TABLE 12
AIN 76A vitamin mix for rats

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>mg/kg diet</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nicotinic acid</td>
<td>3000.0 mg</td>
</tr>
<tr>
<td>D-Calcium pantothenate</td>
<td>1600.0 mg</td>
</tr>
<tr>
<td>Retinyl palmitate</td>
<td>800.0 mg</td>
</tr>
<tr>
<td>Pyridoxine HCl</td>
<td>700.0 mg</td>
</tr>
<tr>
<td>Thiamin</td>
<td>600.0 mg</td>
</tr>
<tr>
<td>Riboflavin</td>
<td>600.0 mg</td>
</tr>
<tr>
<td>Folic acid</td>
<td>200.0 mg</td>
</tr>
<tr>
<td>D-L-alpha-tocopherol acetate</td>
<td>20.0 mg</td>
</tr>
<tr>
<td>D-biotin</td>
<td>20.0 mg</td>
</tr>
<tr>
<td>Menaquinine</td>
<td>5.0 mg</td>
</tr>
<tr>
<td>Cholicalciferol</td>
<td>2.5 mg</td>
</tr>
<tr>
<td>Cyanocobalamine</td>
<td>1.0 mg</td>
</tr>
</tbody>
</table>
APPENDIX C

Fatty acid profiles of coconut and corn oil used to formulate a 2:1 saturated to unsaturated fat mixture.
TABLE 13
Fatty acid profile of coconut oil and corn oil used to formulate the fat mixture used in the high and low fat diets for Study I.1

<table>
<thead>
<tr>
<th>Fatty acid</th>
<th>Corn oil (g/100 g oil)</th>
<th>Coconut oil (g/100 g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>6:0</td>
<td>-</td>
<td>9.1 g</td>
</tr>
<tr>
<td>8:0</td>
<td>-</td>
<td>9.4 g</td>
</tr>
<tr>
<td>10:0</td>
<td>-</td>
<td>5.6 g</td>
</tr>
<tr>
<td>12:0</td>
<td>-</td>
<td>35.5 g</td>
</tr>
<tr>
<td>14:0</td>
<td>0.05 g</td>
<td>16.9 g</td>
</tr>
<tr>
<td>16:0</td>
<td>10.9 g</td>
<td>8.3 g</td>
</tr>
<tr>
<td>16:1</td>
<td>0.06 g</td>
<td>0.21 g</td>
</tr>
<tr>
<td>18:0</td>
<td>1.6 g</td>
<td>2.0 g</td>
</tr>
<tr>
<td>18:1</td>
<td>21.3 g</td>
<td>4.9 g</td>
</tr>
<tr>
<td>18:2 (n-6)</td>
<td>51.9 g</td>
<td>1.4 g</td>
</tr>
<tr>
<td>18:3 (n-3)</td>
<td>0.62 g</td>
<td>-</td>
</tr>
<tr>
<td>Total saturated fatty acids$^2$</td>
<td>12.5 g</td>
<td>86.8 g</td>
</tr>
<tr>
<td>Total polyunsaturated fatty acids$^3$</td>
<td>54.7 g</td>
<td>4.0 g</td>
</tr>
</tbody>
</table>
Using this information it was calculated that 1000 g of corn oil mixed with 1240 g coconut oil would yield a 2:1 saturated to unsaturated fatty acid mixture.  

Saturated fatty acid total for corn oil is the sum of 16:0 and 18:0 fatty acids. For coconut oil it is the sum of 6:0, 8:0, 10:0, 12:0, 16:0, and 18:0.

Polyunsaturated fatty acid total for corn oil is the sum of 18:2 (n-6), 18:3 (n-3) and longer chain PUFA. For coconut oil it is the sum of 18:2 (n-6) and longer chain PUFA.
APPENDIX D

Superoxide Dismutase (SOD) assay

1. Solutions needed

a) **3 N NaOH**
   - 120 g NaOH pellets
   - bring to 1 L with dd H2O in a volumetric flask

b) **TAPS buffer, pH=8.2**
   - 2.43 g TAPS
   - 78 mg Detapac (diethylenetriaminepentaacetic acid)
   - bring to 200 mL with dd H2O in volumetric flask, bring to pH 8.2 with 3 N NaOH, stir well before use
   - store at room temperature, solution is good for approximately one month

c) **Phosphate Buffered Saline (PBS)**
   - add 1 pack of PBS powder into 1 L dd H2O

d) **Ethanol-Chloroform solution (25:15)**
   - 25 mL 100% ethanol
   - 15 mL chloroform
   - store in -20 °C

e) **0.01 N HCl, 1 L**
   - 86 mL stock HCl
   - bring to 1000 mL with dd H2O in volumetric flask

f) **Pyrogallol solution, 40 mL**
   - 20 mg pyrogallol
   - 40 mL 0.01 N HCl
   - mix well, store in sealed dark flask, discard after 3 days or if solution turns yellow
Preparation of tissue for SOD assay

1. Place 1 g of tissue sample in 5 mL of cold PBS. Homogenize with polytron until tissue is liquified. Rinse polytron rotor with dd H2O and dry between samples. Do all samples from one treatment group together.

2. Transfer 1 mL of homogenate to a microfuge tube, add 0.4 mL ethanol-chloroform to each tube and mix by inversion. Spin samples at 11,000 x g for 5 min at 4°C.

*For serum or plasma samples, add 1 mL of sample to 0.4 mL ethanol-chloroform solution, mix, spin for 5 min as described above.

3. Remove precipitate and discard. Respin supernatant and ethanol chloroform for 3 min at 11,000 x g, 4°C. Transfer supernatant to another microfuge tube and store -20°C. Use samples for assay within 1 month.

Setting up Beckman Du 68 Spectrophotometer

1. Turn on machine and allow it to warm up for 30 min.

2. Use UV light source for assay. Using the step function go to program 6 (enzyme kinetics). Enter the following program parameters:

- edit = 0 will appear, enter wavelength 320 with the lambda key
- number of cells = 6
- blanks = 0
- tabulate: yes
- initial time: 30 (sec)
- T time: 2 (min)
- 1 cycle
- span: 1
- 0 = cal
- results: yes
Determination of Pyrogallol quantity to add to TAPS

The goal is to determine the amount of pyrogallol to be used which will give a 0.016 change in absorbance per 30 seconds (dA/30 s).

1. Place 1 mL of TAPS buffer in each cuvette. Add varying amounts of pyrogallol to each cuvette. The amount of pyrogallol to yield a dA/30 of 0.016 is usually within a range of 15-50 µL. Mix TAPS and pyrogallol well in cuvette and read immediately.

Example:

<table>
<thead>
<tr>
<th>cuvette</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
</tr>
</thead>
<tbody>
<tr>
<td>pyrogallol</td>
<td>16</td>
<td>18</td>
<td>20</td>
<td>22</td>
<td>24</td>
<td>26</td>
</tr>
<tr>
<td>dA/30 s</td>
<td>.0170</td>
<td>.012</td>
<td>.0133</td>
<td>.0145</td>
<td>.0151</td>
<td>.0175</td>
</tr>
</tbody>
</table>

The correct volume of pyrogallol should lie between 24 and 26 µL.

<table>
<thead>
<tr>
<th>cuvette</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
</tr>
</thead>
<tbody>
<tr>
<td>pyrogallol</td>
<td>24</td>
<td>24.5</td>
<td>24.5</td>
<td>25</td>
<td>25</td>
<td>25.5</td>
</tr>
<tr>
<td>dA/30 s</td>
<td>.015</td>
<td>.0155</td>
<td>.0158</td>
<td>.016</td>
<td>.0163</td>
<td>.0171</td>
</tr>
</tbody>
</table>

2. In this example the correct amount of pyrogallol to be used in the assay would be 25 µL. The quantity of pyrogallol to be used should be re-calculated each day the assay is done, even if the pyrogallol supply used is from the day before.

Preparation of sample

1. Dilute sample 1:25 (960 µL PBS + 40 µL sample) with PBS in a microfuge tube, store unused homogenate in -20°C.

2. Using the same approach as above, determine sample volume that will reduce the dA/30 s by 50% (reduce it to 0.008).

3. To each cuvette add:

- 1 mL TAPS
- calculated volume of pyrogallol from above
- varying amounts of sample
Calculation of SOD activity

\[(V_C + V_S) \times D_S \times 1.4[(W_S + 5) + W_S] = U\]

\(V_C = \) total volume in cuvette
\(V_S = \) volume of sample yielding a 50% reduction of dA/30
\(D_S = \) dilution factor (25)
\(W_S = \) weight of liver sample homogenized
APPENDIX E

Plasma Ceruloplasmin oxidase activity assay

Solutions needed

1. Acetate buffer solution, pH=5
   -2.6 mL glacial acetic acid
   -13.608 g sodium acetate·3H₂O
   -bring up to 1000 mL in a volumetric flask
   -store at 4° C

2. Sulfuric acid, 9 mol/L
   -100 mL 18 mol/L sulfuric acid added slowly to 100 mL dd H₂O

3. o-dianisidine dihydrochloride, 7.88 mM/L
   -250 mg o-dianisidine dihydrochloride
   -bring to 100 mL with dd H₂O in a volumetric flask
   -store at 4° C, material is light sensitive so use a brown glass jar or a clear jar wrapped with foil

Procedure

1. Prepare 2 cuvets (1 cm diameter) with 0.375 mL of acetate buffer in each, add 25 µL of plasma to each, allow them to sit 5 min at room temperature.

2. Add 100 µL of o-dianisidine dihydrochloride solution to both cuvets, allow one cuvet to sit for 5 min and add 1 mL of 9 M H₂SO₄, read absorbance at 540 nm. The other cuvet should be allowed to sit for 15 min before 1 mL of 9 M H₂SO₄ is added, read absorbance at 540 nm.

Calculation of Ceruloplasmin Oxidase activity

oxidase activity = (Ab₁₅ - Ab₅) x 0.3125 U/mL

*Ab₁₅ = absorbance at 540 nm after 15 min incubation with the o-dianisidine solution

*Ab₅ = absorbance at 540 nm after 5 min incubation with the o-dianisidine solution
APPENDIX F

Isolation of Cytochrome C Oxidase
Stock solutions needed

1. 1.5% Triton X solution
   - 98.5 mL of 0.1 M KCl
   - 1.5 mL pure Triton X

Sample preparation

1. Add 2 mL of Triton X solution to test tubes and place on ice. Remove heart samples from -80°C freezer and cut a 0.2 g slice of left ventricle with a razor blade and place in a test tube. Place the heart sample back in the -80°C freezer as soon as possible. The ratio of sample:Triton X solution should be 10:1 by weight. Homogenize the sample with the polytron. Centrifuge the homogenate at 3000 rpm (1086 x g) for 20 min. Remove the supernatant, which contains the non-myofibrillar proteins, and aliquot as desired in microfuge tubes (50 or 100 μL portions), store in -80°C. The pellet can also be saved in the -80°C freezer if desired as it contains the myofibrillar protein fraction.
APPENDIX G

Cytochrome C Oxidase activity assay
PRINCIPLE: The activity of Cytochrome C Oxidase is measured by the decrease in optical density of the reduced Cytochrome C as it is oxidized by the enzyme at 550 nm.


Stock solutions and materials needed

1. 0.25 M Potassium phosphate dibasic buffer
   -43.60 g potassium phosphate dibasic (M.W.=174.2)
   -bring up to 250 mL with dd H2O

2. 0.25 M Potassium phosphate monobasic buffer
   -34.025 g potassium phosphate monobasic (M.W.=136.1)
   -bring up to 250 mL with dd H2O

3. 0.1 M Working phosphate buffer
   -titrate dibasic phosphate buffer with monobasic to pH 7 using the pH meter. Start by adding 100 mL of monobasic. Store at 4°C.
   -dilute 200 mL of this buffer with dd H2O up to 2 L (produces the 0.1 M solution).
   -degas 1 L of the working phosphate buffer to be used for the assay.

4. Cytochrome C standard- purchase from Sigma Chemical Co., horse heart type IV

5. Sodium Hydrosulfite (Sodium dithionite)- purchase from Sigma Chemical Co.

6. Sephadex G25-80

Preparing Column

10 g of Sephadex G-25-80 is swelled with 100 mL of 0.1 M degassed phosphate buffer (pH=7) overnight. Add the swelled gel in equal volumes of buffer to a 1.5 cm x 27 cm column and allow it to settle in. Wash the column with 2 volumes of buffer (each volume equal to the volume of settled gel in the column), adjust the flow rate to 0.5 mL/min.
Preparing working Cytochrome C

Dissolve 25-35 mg of Cytochrome C standard in 1.0 mL of 0.1 M degassed phosphate buffer. Add excess sodium hydrosulfite (20-40 mg) and vortex. The Cytochrome C solution will change from an oxidized (red color) to a reduced state (orange color). Load prepared column with Cytochrome C and adjust flow rate to 0.5 mL/min. Collect pink fraction as it comes off the column (watch carefully as the pink fraction is quick to come off the column). Continue running column with at least 200 mL of 0.1 M phosphate buffer to wash out sodium hydrosulfite.

Set up for working Cytochrome C

1. Run absorbance reading on the Cytochrome C collected.
   -place 1.9 mL of 0.1 M phosphate buffer with 0.1 mL of Cytochrome C collected in a cuvette
   -zero spectrophotometer with 2.0 mL phosphate buffer
   -run absorbance at 550nm, then at 565 nm

2. Take ratio of Ab 550/Ab 565, ratio must be greater than 6 in order to use the cytochrome c collected. Usual range is between 12-18.

3. To assay the activity of sample CCO isolated from tissue, 50 µmol of working cytochrome c will be needed for each cuvette. To calculate the amount of working cytochrome c to use for each cuvette in the assay perform the following calculations:

   \[
   \frac{(Ab\ 550 \times D)}{19,600\ \text{mol/L/cm}} \times 10^5 = W\ \text{µmol}
   \]

   \[(50\ \text{µmol} \times 2000\ \text{µl}) + W = Y\ \text{µL needed}\]

*definition of variables

-Ab 550 = absorbance of working cytochrome c at 550 nm
-D = dilution factor, equals 20
-19,600 mol/L/cm = molar extinction coefficient of cytochrome c
-50 µmol = amount of working cytochrome c needed per cuvette
-2000 µl = total volume in each cuvette
$-Y =$ volume of working cytochrome c needed per cuvette for CCO activity assay.

*each cuvette will need $X \mu L$ working cytochrome c (calculated above) and $X \mu L$ of phosphate buffer to equal a total volume of 1900 $\mu L$.

**Set up for Beckman Du-68 spectrophotometer**

- select visible as light source
- select program 6 with step function
- edit = 0 will appear, enter 550 nm as wavelength
- # cells = 6
- blanks = 0
- tabulate = yes
- initial time = 30 (seconds)
- total time = 2 min
- 1 cycle
- span = 1
- results = yes

1. Add 1.9 mL working cytochrome c solution into a cuvette. Quickly add 100 $\mu L$ of sample, mix thoroughly and run through Beckman Du-68 spectrophotometer. Begining with time 0, record absorbances at 30 second intervals.

**Calculation for Cytochrome C Oxidase activity**

1. Calculate protein concentration of the sample

   - run Lowry protein assay to determine protein concentration of samples
   - keep track of dilution factor used for sample
   - mg/mL x dilution factor = concentration of sample
   - determine total protein content of sample added to reaction tube. Convert this to $\mu g$ of protein.

2. Run a simple regression on the absorbance points on a sample in order to determine the slope of the linear portion of the curve. This is the change in optical density/min of the sample. $Y = bX + A$, slope = $b$

   - divide change OD/min by the extinction coefficient for CCO, which is 19,600 mole/L/cm
-since 2 mL of reaction media was used to measure absorbance and we need to convert from moles to micromoles, take the value obtained in previous step and multiply it by 0.002 L/10^-6, or 2000. This conversion yields the total μmol of CCO oxidized in the tube during the assay.

-divide the μmol CCO oxidized from previous step by the total μg protein added to the cuvette via the sample from step 1. This will yield the final value of μmol CCO oxidized/min/μg protein.
APPENDIX H

Urea gel electrophoresis and Western blot for Cytochrome C Oxidase

Stock solutions

1. 30% acrylamide stock solution (30:0.8)

- 30 g acrylamide monomer
- 0.8 g methylene-bis-acrylamide
- add some dd H₂O to dissolve solids
- transfer to 100 mL volumetric flask and add dd H₂O up to 100 mL
- degas the solution
- filter and store at 4°C

2. 1.5 M Tris-HCl, pH=8.8 (lower gel separating buffer)

- 18.17 g Tris base
- add about 85 mL dd H₂O in a beaker to dissolve solids
- adjust pH to 8.8 with concentrated HCl
- transfer to volumetric flask and bring to 100 mL volume with dd H₂O
- store at 4°C

3. 0.5 M Tris-HCl, pH=6.8 (stacking gel buffer)

- 6.06 g Tris base
- add about 85 mL dd H₂O to dissolve solid
- adjust pH to 6.8 with concentrated HCl
- transfer to volumetric flask and bring to 100 mL volume with dd H₂O
- store at 4°C

4. 10% Sodium dodecyl sulfate (SDS)

- 10 g SDS (also called lauryl sulfate)
- bring to 100 mL with dd H₂O

5. Indicator solution (0.5% methyl green/30% sucrose)

- 0.05 g methyl green
- 3 g sucrose
- bring to 10 mL with dd H₂O
6. Dissociation buffer (10 mL)- this solution consists of 5% SDS, 8 M urea, 0.1 M Tris-HCl, 1% beta-mercaptoethanol, and 1% glycerol.

-2.5 mL 20% SDS
-2.67 mL 30 M urea
-2.0 mL 0.5 M Tris-HCl
-100 µL beta-mercaptoethanol
-100 µL glycerol
-2.63 mL dd H2O

Preparation of dual slab mini-gels with 16% urea gels

1. Assemble slab gels following instructions on plastic Bio-Rad spacer card. Make sure to seal the bottom with parafilm.

2. 16% urea gel- to make 10 mL, enough for 2 mini-gels.

-3.6 g urea
-5.33 mL acrylamide stock
-2.5 mL 1.5 M Tris-HCl, pH=8.8
-2.065 mL dd H2O
-degas
-100 µL 10% SDS
-50 µL 10% ammonium fresh persulfate (0.100 g ammonium persulfate and 1 mL dd H2O)
-5 µL TEMED
-mix gently, pour gel using a Pasteur pipette, carefully float a layer of dd H2O on top of the gel to remove air bubbles and to smooth it out
-allow 30-60 min or overnight to harden

3. 5% urea stacking gels- enough for 2 mini-gel stacks

-1.8 g urea
-0.835 mL acrylamide stock
-1.25 mL 0.5 M Tris-HCl, pH=6.8
-2.83 mL dd H2O
-degas
-50 µL 10% SDS
-25 µL 10% fresh ammonium persulfate from previous step
-4 µL TEMED
-pour gel on top of hardened running gel, place combs into the stacking gel
-allow 30-60 min to harden
-prepare samples while stacking gel is hardening
4. Running buffer

-0.5 g SDS
-1.5 g Tris base
-7.2 g glycine
-bring to 500 mL volume with dd H₂O in volumetric flask

Sample preparation

1. To make enough sample prep for a Western and a sister gel follow this formula

-30 μL sample
-30 μL dissociation buffer
-3 μL indicator solution
-heat at 37 °C for 45 min

2. For CCO standard, enough for 2 gels, use 10 μL/gel

-10 μL CCO standard
-30 μL dissociation buffer
-5 μL indicator solution
-heat at 37 °C for 45 min (with samples)

*if standard is too concentrated and unreadable, dilute with 30 μL dd H₂O while preparing next time

3. For molecular weight marker, enough for two gels

-use 20 μL/gel
-heat at 37 °C for 45 min

4. Loading samples

-use at 30-35 μL/well of sample for gels which Westerns will be performed on, this will maximize the antibody reaction
-use 15 μL/well of sample for sister gels
Running conditions

1. Run gels using ISCO power supply set at following:
   - wattage limit set past 100
   - voltage limit set past 100
   - milliamps set at 100 mA
   - current set at 10 mA/gel (20 mA total for 2 gels)
   - allow gels to run for approximately 1.5 hours or until dye runs off the gel

Staining and destaining gels and membranes

1. Solutions needed
   a) coomassie brilliant blue R-250
      - mix 2.0 g coomassie blue
      - 500 mL 50 % methyl alcohol
      - 120 mL 12 % acetic acid
      - bring to 1 L with dd H2O
      - filter solution
   b) 5 % acetic acid
      - 50 mL glacial acetic acid
      - 450 mL dd H2O

2. Staining gel
   - cut stacking gel off and stain gel with coomassie brilliant blue R-250 for about 30 sec
   - destain gel with 5 % acetic acid overnight
   - store gel in a baggie

3. Staining membrane
   - stain membrane in 0.1 % Ponceau S for 1 min
   - destain with 5 % acetic acid for 5 min
   - rinse gently with small amount of dd H2O and place on kimwipes and filter paper to dry
   - after drying, store membranes in airtight baggie if Western is not to be performed immediately
Western Blot for Cytochrome C Oxidase

1. Solutions needed- Refrigerate all solutions but allow to come to room temperature before using.

   a) 5% milk-TBS (Tris buffered saline) at pH=7.5

      - 6.06 g Tris (50 mM)
      - 9.93 g NaCl (170 mM)
      - 50 g nonfat powdered milk
      - Bring to 1000 mL volume with dd H$_2$O in volumetric flask
      - Adjust pH to 7.5 with concentrated HCl

   b) BCIP (5-Bromo, 4-Chloro, 3-Indoyl Phosphate)

      - 10 mg BCIP (stored at 4° C)
      - 200 µL dd H$_2$O (if BCIP is not disodium salt then use 70% Dimethyl formamide instead)
      - Mix in microfuge tube for final concentration of 50 mg/mL

   c) NBT (Nitro Blue Tetrazolium)

      - 15 mg NBT (stored at 4° C)
      - 300 µL 70% Dimethyl formamide (made with 210 µL Dimethyl formamide + 90 µL dd H$_2$O)
      - Mix in microfuge tube for final concentration of 50 mg/mL

   d) 10X Alkaline Phosphate buffer

      - 60.5 g Tris (1 mM)
      - 29.2 NaCl (1 mM)
      - 5.1 g MgCl$_2$ (50 mM)
      - Bring to 500 mL with dd H$_2$O in volumetric flask

   e) 10X stop buffer pH=8.0

      - 12.1 g Tris (0.2 mM)
      - 50 mL of 0.5 EDTA stock (50 mM) or 9.306 g EDTA per 50 mL of dd H$_2$O
      - Bring to 500 mL with dd H$_2$O in a volumetric flask
      - Adjust pH if needed
2. Using a gentle shaking apparatus at room temperature, wash the nitocellulose membrane in 50 mL of 5% milk-TBS for 1 hour changing the solution every 15 min.

3. Add 50 mL fresh 5% milk-TBS to membrane along with 50 μL of CCO antibody (stored at -80° C). Cover dish with plastic wrap, place on shaker and incubate for at least 3 hours or overnight.

4. Wash membrane with 3 (50 mL) changes of fresh 5% milk-TBS for 30 min.

5. Add 50 mL of 5% milk-TBS and 30 μL of goat-antirabbit antibody conjugated with alkaline phosphatase. Allow to incubate for 2 hours at room temperature with gentle shaking.

6. Wash membrane with 3 (50 mL) changes of 5% milk-TBS for 30 min.

7. To develop blots
   - dilute 10X alkaline phosphate buffer to 1X (10 mL 10X AP + 90 mL dd H2O) to make 100 mL 1X per membrane
   - rinse membrane briefly with dd H2O
   - put 100 mL of 1X AP buffer in a dish, add 200 μL of BCIP solution and 300 μL of NBT solution
   - add membrane to the dish with the above solutions and allow to develop undisturbed for 30-60 min.

8. After developing blots
   - rinse blots briefly with dd H2O and allow them to air dry on a kimwipe.
   - store membranes wrapped in foil in a dark place (they are light sensitive) and photograph ASAP as the blots will fade with time.
APPENDIX I

SDS-PAGE for Fibronectin and Laminin
Materials Needed for SDS-PAGE

1. Stock Solutions

a) Buffer A: Acrylamide stock solution.
   - acrylamide-bis-acrylamide 30 g : 0.8 g
   - bring up to 100 mL with dd H₂O in volumetric flask

b) Buffer B: Lower gel buffer, 3.0 M Tris-HCl
   - pH=8.8.
   - 36.3 g Tris
   - 48 mL of 1 M HCl
   - bring up to 100 mL with dd H₂O in volumetric flask

c) Buffer C: Upper gel buffer, pH=6.8.
   - 6.0 g Tris
   - 48 mL of 1 M HCl
   - bring up to 100 mL with dd H₂O in volumetric flask

2. 7.5 % acrylamide solution, 47 mL total.

   - 12.5 mL Buffer A
   - 6.25 mL Buffer B
   - 28.25 mL dd H₂O

3. Running Buffer for SDS-PAGE, need to make fresh for each run.

   - 1.52 g Tris
   - 0.5 g SDS (Lauryl Sulfate)
   - 7.2 g glycine
   - bring to 500 mL with dd H₂O in volumetric flask
Extraction of Laminin from cardiac tissue

(Mamuya and Brecher, J. Clin Invest 89:392-401, 1992)

1. Extraction Buffer: Used to extract a relatively high amount of laminin.

-2.922 g 0.5 M NaCl
-0.6055 g 0.005 M Tris-HCl
-bring to 100 mL in a volumetric flask

*add the following protease inhibitors (to prevent degradation over long term storage) for the final concentrations listed

-10 mM Leupeptin
-1 mM PMSF
-1 mM Pepstatin A
-5 trypsin IU/mL Aprotinin

2. Cut 0.06 g of cardiac muscle from the area of the left ventricle, rinse with dd H2O to wash away blood. Place in 0.5 mL of laminin extraction buffer and keep on ice until homogenization. Homogenize cardiac tissue in cold polytron apparatus. Centrifuge at 11,000 x g (Microfuge) for 2 min at 4° C (place microfuge in cold room for 1 hour to reach this temperature). Remove supernatant which contains laminin, aliquot into desired portions and store at -80° C.

3. Measure total protein content by method of Lowry et al., 1951.

Extraction of Fibronectin from cardiac tissue

(Mamuya and Brecher, J Clin Invest 89:392-401, 1992)

1. Solutions needed

a) Fibronectin Extraction Buffer: Phosphate buffered saline (PBS), make this with pre-mixed packets mixed with 1 L of dd H2O, store at 4° C. Add the following protease inhibitors (to prevent degradation over long term storage) for a final concentration of:

-10 mM leupeptin
-1 mM pepstatin A
-1 mM PMSF
-5 trypsin IU/mL aprotinin
b) 4% SDS solution

- 4 g SDS
- bring to 100 mL in volumetric flask

2. Cut 0.04 g cardiac tissue from left ventricle, rinse free of blood with dd H$_2$O, place in tube with 0.5 mL fibronectin extraction buffer and put on ice until homogenization. Using cold polytron apparatus, homogenize tissue, centrifuge homogenate at 11,000 x g (using Microfuge) for 2 min at 4° C. Remove supernatant and resuspend pellet in 200 µL 4% SDS. Heat suspension at 100° C for 4 min and centrifuge at 11,000 x g for 2 min at room temperature. The supernatant contains denatured fibronectin, aliquot as desired and store at -80° C.

3. Measure total protein content by using method of Lowry et al., 1951.

Preparation of dual slab mini-gels for SDS-PAGE

1. Assemble two slab gels by following instructions on plastic Bio-Rad spacer card. Seal bottom of slabs with parafilm and place slabs on gel drier.

2. Two running gels for dual slab apparatus.

- 7.05 mL 7.5% acrylamide solution
- 75 µL 10% SDS
- 375 µL 1.5% fresh ammonium persulfate (0.015 g ammonium persulfate with 1.0 mL dd H$_2$O).
- 6 µL TEMED (hardens the gel)
- using a Pasteur pipette, pour solution into slab, leave 2 cm space at top for the stacking gel
- float a 1 cm layer of dd H$_2$O on top of gel
- allow gel to harden for 1 hour or overnight

3. Two stacking gels for dual slab apparatus.

- 0.625 mL Buffer A
- 1.25 mL Buffer C
- 2.825 mL dd H$_2$O
- 50 µL 10% SDS
- 250 µL fresh 1.5% ammonium persulfate
- 4 µL TEMED (hardens the gel)
- place comb in stacking gel, watch for air bubbles
- allow gels to harden for 30-60 min, while gel is hardening prepare samples for SDS-PAGE
4. Running conditions for SDS-PAGE.

- Wattage and voltage limit set past 100
- Milliamps set at 100 mA
- Current limit set at 10 mA/gel
- Allow gel to run 1.5-2.0 hrs or until dye runs off the bottom of the gel

Preparation of fibronectin and laminin for SDS-PAGE

1. Equilibration buffer: needed to buffer proteins in sample.

- 2.3 g SDS
- 5 mL beta-mercaptoethanol
- 10 mL glycerol
- 0.755 g Tris
- Dissolve in dd H_2O, adjust pH to 6.8 with HCl and bring to final volume of 100 mL.
- Add a pinch of bromophenol blue dye

2. Prepare a 1:1 dilution of sample extract to equilibration buffer, boil for 6 min. Centrifuge at 11,000 x g at room temperature for 8 min. Load supernatant into wells of slab gels. Load 15 μL laminin/well (10 μg protein) and 10 μL fibronectin/well (100 μg protein)

* Prepare high molecular weight marker in same manner as fibronectin and laminin samples.

Staining and de-staining of gels

1. Carefully cut off stacking gel and place running gel in coomassie blue dye for approximately 30 sec. Remove gels from dye and place in 10% acetic acid solution overnight to de-stain background.

2. Remove gels from acetic acid solution and place in plastic to store, if gel is of acceptable quality, perform densitometry and/or photograph as soon as possible.