CARNITINE PALMITOYLTRANSFERASE EXPRESSION
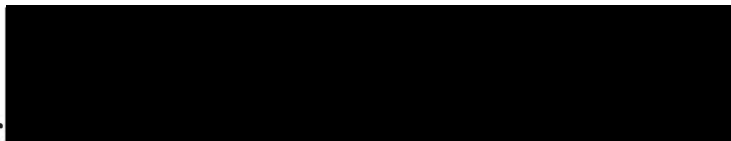IN RAT HEART

by

David E. Wu

A DISSERTATION

Presented to the Department of Physiology
and the Oregon Health Sciences University
School of Medicine
In partial fulfillment of
the requirements for the degree of

Doctor of Philosophy

May 1993

APPROVED:

....
   (Dr. Kent L. Thornburg, Professor in Charge of Thesis)

....
   (Dr. John A. Resko, Chairman, Graduate Council)

TABLE OF CONTENTS

LIST OF FIGURES

ACKNOWLEDGEMENT

I am deeply indebted to Dr. Kent Thornburg. Without his guidance and support, the timely completion of this thesis would not have been possible.

I would like to thank Dr. Mark Morton, Dr. Debra Anderson, Dr. Job Faber and Dr. John Resko for their generous help during my graduate study. I would also like to thank Dr. Barry Greenberg and Dr. McGarry for their generous gifts that greatly facilitated my work.

I would like to thank my parents who cultivated my curiosity in medicine and science.

I would like to thank my wife Miyoung and my children Anna and Alexander for their love, patience and support over the years. I also would like to thank Dr. and Mrs. William Sweetman for their support and friendship.

Lastly, I would like to thank the Medical Research Foundation of Oregon for the scholarship that made my medical education and this research project possible.

ABSTRACT

The heart has unique energy metabolic patterns at different times in life. Mammalian fetal myocardium is immature and has very limited ability to oxidize fatty acid. Fetal hearts rely heavily on carbohydrate oxidation for energy supply. Adult hearts, on the other hand, predominantly use fatty acid to supply energy. Therefore, a transition from fetal to adult type of energy metabolism is critical for species survival. It is well established that newborns take this critical adaptation soon after birth, however, the mechanisms that trigger this transition are still unknown.

Carnitine palmitoyltransferase (CPTase) is a rate limiting enzyme that regulates long chain fatty acid oxidation. CPTase activity is very low in fetal heart but increases dramatically after birth as part of the adaptation. The nature of the low CPTase activity is unclear.

Many disease states are associated with abnormal energy metabolism. Chronic heart failure is one example. It is known that failing hearts have impaired energy metabolism but the mechanism is unknown. Shortage of energy supply in the heart can lead to grave consequences.

There are two periods in life when significant changes in CPTase activity and fatty acid oxidation occur. During the fetal to newborn transition, CPTase becomes expressed. It is an ideal time to study the regulatory mechanisms on

viii

CPTase expression. During heart failure, the energy metabolism becomes abnormal and fatty acid oxidation appears to be suppressed, perhaps due to inhibition of CPTase activity. Therefore, this study is designed to monitor expression of CPTase at these two crucial times in the life of the rat.

In this study, a developmental pattern of newborn rat heart CPTase expression is established using ribonuclease protection assay and protein detection with antibody. CPTase mRNA expression in infarcted adult rat heart is also examined. I found that (1) CPTase mRNA and protein levels are low in the fetal rat hearts. CPT II expression in fetal hearts is 19±6% of the adult level (n=3). The difference is statistically significant ($p<0.05$); (2) mRNA levels are severely depressed in rat hearts with myocardial infarction (MI). CPT II/cyclophilin ratio for the MI group is 0.42 ± 0.163 (n=4) and for 2.17 ± 0.435 for normal hearts (n=3). The means of the two groups are significantly different ($p<0.01$).

These results suggest that CPTase is deficient in fetal hearts and is induced rapidly at birth. It provides evidence supporting the view that low CPTase activity in fetal rat hearts is due to CPTase deficiency, not masked enzyme activity. Depressed mRNA expression in infarcted rat hearts indicates that long term fatty acid oxidation may be regulated via gene expression.

INTRODUCTION

Successful transition from fetal to extrauterine life is critical for species survival. To meet the increased oxygen demand from muscle activity and thermogenesis at birth, newborn left ventricle output must increase 2-3 fold [91]. This, in turn, requires a steady source of energy to sustain increased cardiac work. Therefore, finding an abundant and readily available energy source becomes a critical step in newborn adaptation to the postnatal environment.

Mammalian fetal myocardium is immature and less able than adult myocardium to oxidize free fatty acids (FFA). Only 3% of the energy required for fetal cardiac work is derived from FFA; thus fetuses rely heavily on carbohydrate [35]. This inability of fetal heart to oxidize FFA is thought to be due primarily to a carnitine palmitoyltransferase (CPTase) deficiency [100].

CPTase activities are low throughout most of fetal life but a surge of cardiac CPTase activity occurs at birth [101]. This increase in CPTase activity coincides with a surge in serum FFA [95] and a switch from carbohydrate to FFA as a primary cardiac fuel source. It is well established that newborns take this critical step in their adaptation soon after birth [58,102]. However, not only are the mechanisms that trigger this change of energy source unknown, but also the

components of the birth process that invoke these mechanisms are not defined.

Adaptation is not only a problem for newborns. Adults have their share of problems with energy metabolism. Many disease states are associated with abnormal energy metabolism and the subsequent adaptations might be even more challenging than those encountered by newborns.

Adequate energy metabolism is particularly important in the adult heart which consumes a large quantity of energy uninterruptedly for decades. Any energy supply shortage can lead to grave consequences. In fact, myocardial ischemia, infarction and congestive heart failure are all associated with impaired energy supply and utilization.

The adult, newborn and fetal hearts are intricately related. When the adult heart becomes hypoxic, it switches back to a fetal type of metabolism. When the adult heart is pressure loaded and needs to grow rapidly, it manufactures fetal actin. Above all, the adult and fetal hearts share many secrets: How is energy metabolism regulated in the heart? Why are certain fuels preferred over the others at different times of life? Are various energy sources freely exchangeable?

The energy metabolism of the heart has been studied for years. A considerable amount of information has been collected from cultured cells, isolated mitochondria, tissue homogenates, perfused hearts and in vivo experiments, but

one big mystery remains unsolved: the regulation of CPTase expression and FFA oxidation.

There are two interesting periods in life when significant changes in CPTase activity and FFA oxidation occur. During the fetal to newborn transition, CPTase expression is increased. It is an ideal time to study the regulatory mechanisms of CPTase expression. During heart failure, the energy metabolism becomes abnormal and FFA oxidation appears to be suppressed. It is possible that the energy depletion is associated with CPTase activity because it is an essential enzyme in FFA oxidation.

Therefore, this study is designed to monitor expression of CPTase at two crucial times in the life of the rat.

Background

General Energy Metabolism

An uninterrupted energy supply is a prerequisite for life. To maintain an adequate energy supply, one must have reliable energy sources and a well coordinated and an efficient processing system to convert the energy into a biologically usable form. The primary energy sources for animals are carbohydrate, lipid and protein. Besides being used as fuel, these compounds also have other important biological functions such as providing building materials

for cells.   Therefore, a delicate balance between catabolism and anabolism must be carefully maintained.

Although each of the three energy sources is metabolized uniquely, the tricarboxylic acid (TCA) cycle is common to all. Key regulatory enzymes in each pathway are stimulated or inhibited by intermediate metabolites from the TCA cycle. Through intermediates of the TCA cycle, the three pathways communicate with each other and homeostasis of energy flux is maintained through complex networks of feedback control.

The degradation products of amino acid metabolism are acetyl-CoA and pyruvate. The end product of lipid breakdown and glycolysis is acetyl-CoA. Therefore, it is no surprise that all three pathways share the TCA cycle as their final step (Fig 1). Acetyl-CoA is fed into the TCA cycle in a reaction catalyzed by citrate synthase in which one acetyl-CoA and one oxaloacetate combine to form citrate. Intermediates in the TCA cycle are not consumed. The oxaloacetate is regenerated when each acetyl-CoA is oxidized to release energy and two carbon dioxide molecules.

The activity of the TCA cycle is tightly controlled according to the overall energy balance by regulating the level of TCA cycle intermediates. Several TCA cycle intermediates can be converted to amino acids by reversible transamination. Pyruvate can enter TCA cycle directly by a pyruvate carboxylase catalyzed reaction in which carbon
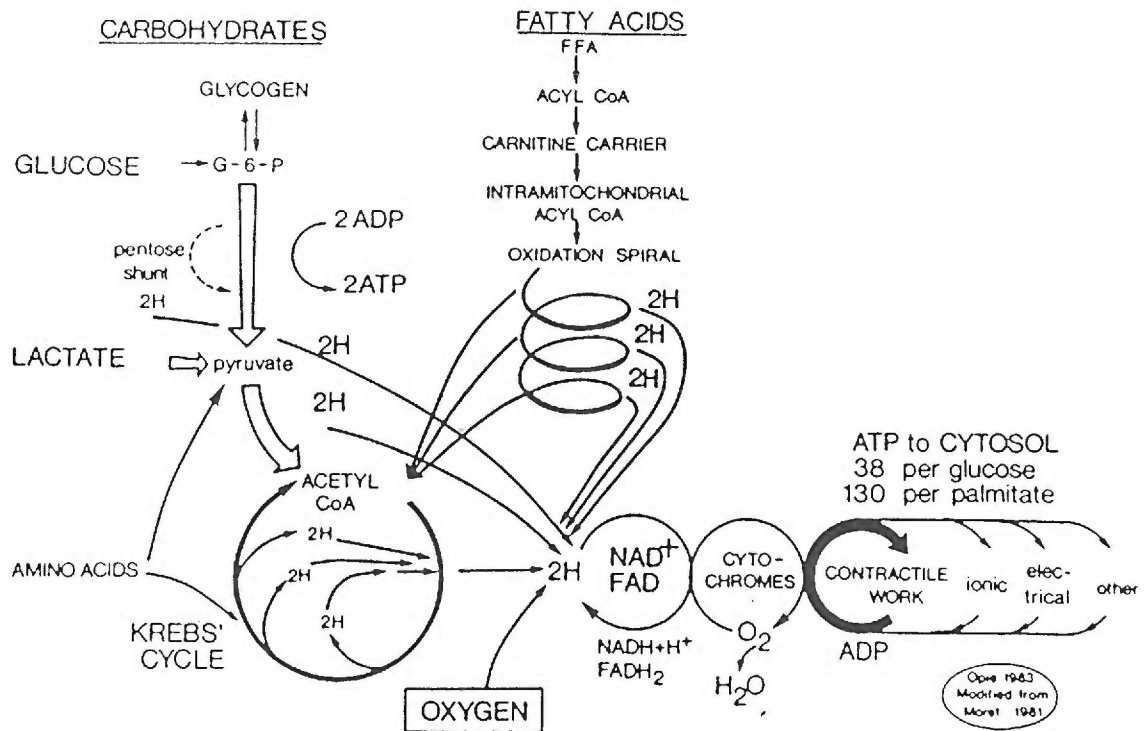
Fig. 1. Carbohydrate, fatty acid metabolism and the TCA cycle. Fatty acid and carbohydrate are the two primary energy sources for the heart under normal conditions. Acetyl-CoA is a common intermediate of both pathways. Acetyl-CoA enters the TCA cycle first and then is completely oxidized in the oxidative phosphorylation process. The end products of Acetyl-CoA oxidation are ATP, CO_2 and H_2O. This figure illustrates the intricate interaction between fatty acid and carbohydrate metabolism (Source: Opie LH. The Heart. 2nd Edition, 1991).

dioxide and pyruvate are converted into oxaloacetate. This particular reaction, however, is irreversible because it requires ATP. By adjusting the concentration of these intermediates, the rate of energy production is controlled and the flow of substrate can be directed towards reactions which represent the most pressing need.

Despite all of these intricate interconversions, acetyl-CoA cannot be directly converted to amino acids or glucose by mammals. Only the intermediates from the TCA cycle and pyruvate are precursors for gluconeogenesis. It should be noted, however, that gluconeogenesis requires sufficient amounts of acetyl-CoA. This is probably due to the fact that gluconeogenesis consumes significant amounts of energy and the abundance of acetyl-CoA indicates a state of energy surplus. In other words, although acetyl-CoA is not used in gluconeogenesis as a precursor, adequate energy derived from lipid metabolism is critical for the activities of other metabolic pathways.

Acetyl-CoA is the only substrate that is actually consumed in the TCA cycle. The primary products of TCA cycle are NADH and $FADH_2$; both are further oxidized through oxidative phosphorylation.

Oxidative phosphorylation, which occurs exclusively in mitochondria, is the process by which ATP is ultimately produced. Electrons pass through the electron transport system in the inner membrane and the energy released in this

process generates an electrochemical proton gradient across the inner mitochondrial membrane. This proton gradient is the driving force for ATP production. Protons move down the gradient and energize the membrane bound ATPase which uses this energy to produce ATP from ADP and inorganic phosphate. As is implied by its name, oxygen is an essential component of the oxidative phosphorylation system. The electrons, generated during substrate oxidation and passing through the electron transport system, are ultimately received by oxygen. Protons, after passing down the electrochemical gradient, combine with the reduced oxygen in the mitochondrial matrix and water is produced as a by-product of complete substrate oxidation.

Fatty Acid Metabolism

Lipids are the most abundant fuel storage form in animals. It has been calculated that an average size man has 141,000 kcal of energy reserve in fat storage in comparison with only 900 kcal of energy stored as glycogen. Phospholipids are also important building materials for cell membranes and intracellular organelles. Some lipid metabolites participate as messengers or mediators in neurohumoral regulation. Given its extensive involvement in a wide variety of biological functions, it may be helpful to examine some special aspects of lipid metabolism in more detail.

The net yield of complete oxidation of one molecule of glucose is 38 molecules of ATP. The energy conversion efficiency is 38%. Complete oxidation of one molecule of palmitic acid, a 16 carbon saturated fatty acid, produces 129 molecules of ATP, with a 40% conversion efficiency. Although both are nearly identical in efficiency of energy conversion, a comparison between the two shows that there are also some impressive differences.

The net energy yield from each glucose carbon is 6.3 ATPs but 8.1 ATPs for each carbon in palmitic acid. So, complete oxidation of one FFA carbon releases 28% more energy than a glucose carbon. On the other hand, complete oxidation of one glucose carbon consumes only one molecule of oxygen whereas complete oxidation of one FFA carbon requires 1.4 molecules of oxygen. Although FFA is the most energy rich substrate, there is a high cost in oxygen, approximately 10% extra per ATP, to extract the extra energy.

Another significant difference between carbohydrate and lipid metabolism is that glycolysis can produce 2 ATPs per glucose (6 carbons) when severe oxygen shortage occurs. FFA, on the other hand, is incapable of any anaerobic energy production. These two anaerobically produced ATPs may seem to be an insignificant amount when compared to the 38 ATPs produced from glucose and the 129 ATPs from FFA oxidation but they may allow survival of a particular tissue under

extreme hypoxic conditions.

Fatty Acid Synthesis

In animals, fatty acids are synthesized from acetyl-CoA in a two stage process (Fig 2). A commonly synthesized FFA is palmitic acid. In the first stage, malonyl-CoA is formed from acetyl-CoA by carboxylation. This reaction consumes ATP and is catalyzed by acetyl-CoA carboxylase which is regulated by citrate. Because citrate accumulates only when acetyl-CoA is abundant and the ATP/ADP ratio is high, it ensures that FFA synthesis will only occur when there is excess energy supply. Citrate also serves as a carrier to move acetyl-CoA from the mitochondrial matrix to the cytoplasm where fatty acid synthesis takes place. Once in the cytoplasm, citrate can be cleaved into oxaloacetate by citrate lyase and be used in gluconeogenesis. It can also be converted into malate and re-enter the TCA cycle.

In the second stage of FFA synthesis, malonyl-CoAs are added to acetyl-CoA or other existing short chain acyl-CoAs, one at a time. Each elongation cycle consumes 2 NADPH and elongates the chain by two carbons. In the process the third carbon is discarded as carbon dioxide.

Fatty Acid Oxidation

FFAs are degraded to acetyl-CoA by β-oxidation inside the mitochondria. First, a FFA molecule must be activated.

de novo fatty acid synthesis

$$\text{Acetyl - CoA} + CO_2 \quad \xrightarrow[\text{biotin}]{\text{Acetyl-CoA Carboxylase}} \quad \text{malonyl - CoA}$$

$$\text{Acetyl - CoA} + 7 \text{ malonyl-CoA} \quad \xrightarrow{\text{Fatty Acid Synthetase}} \quad \text{palmitoyl-CoA}$$

Mitochondrial Fatty Acid Elongation

$$\text{Acetyl-CoA} + \text{acyl-CoA} \quad \longrightarrow \quad \text{Acyl - CoA}_{(n+2)}$$

Microsomal Fatty Acid Elongation

$$\text{Malonyl-CoA} + \text{Acyl-CoA} \quad \longrightarrow \quad \text{Acyl}_{(n+2)}\text{-CoA} + CO_2$$
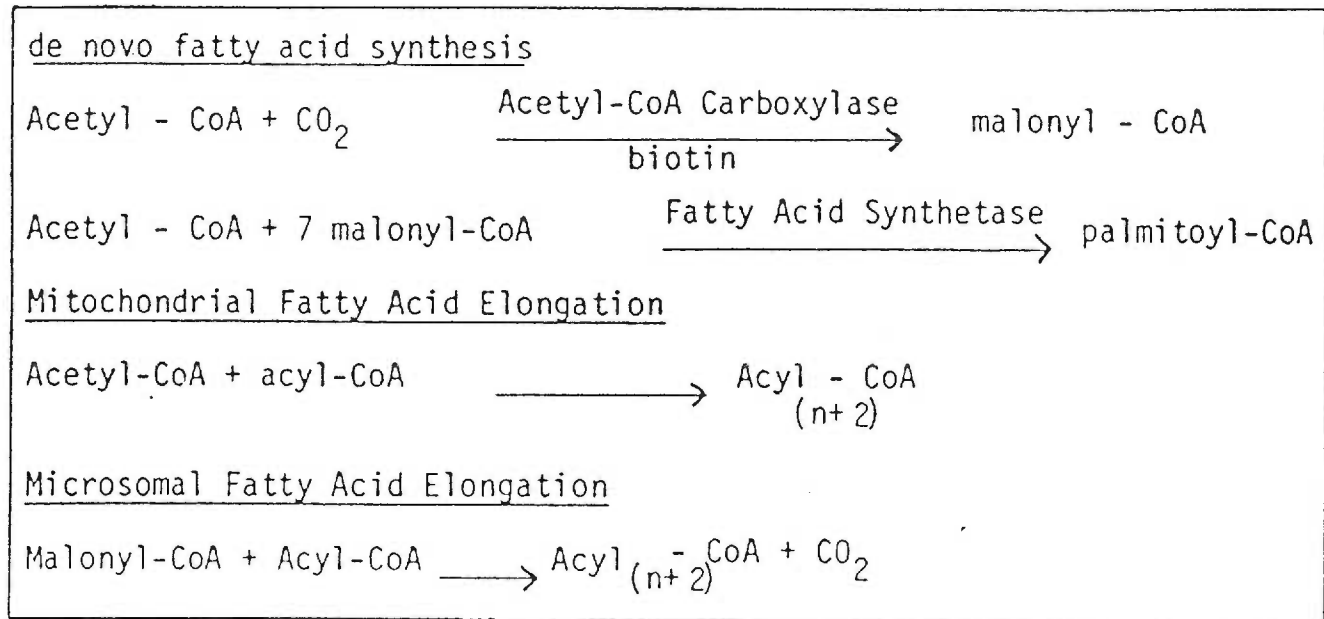
Fig. 2. Pathways of fatty acid synthesis. Fatty acid synthesis occurs in the cytosol and the mitochondria. Cytosolic fatty acid synthesis in the liver is the primary source of endogenous fatty acid. Heart has a limited ability to synthesis fatty acid via mitochondrial fatty elongation. Low lipogenic activity in the heart raises the issue of whether the regulatory roles of malonyl-CoA can be applied to the heart (Source: Warshaw JB. Seminars in Perinatology Vol. 3, p.132, 1979).

Activation is the formation of an acyl-CoA ester which "flags" the molecule for degradation. The activation process is irreversible and requires CoA and ATP. The acyl-CoA is then transported into the mitochondria where β-oxidation takes place. Each complete cycle of β-oxidation shortens the acyl-CoA by two carbons and releases one NADH, one $FADH_2$ and one acetyl-CoA (Fig 3). These products either enter the TCA cycle or the electron transport system for further oxidation.

The acetyl-CoA generated in the liver, however, cannot enter the TCA cycle efficiently because the intermediates are often drained for gluconeogenesis. Furthermore, accumulation of acetyl-CoA reduces the available CoA for other reactions. This problem is solved by a unique metabolic feature of the liver. Excess acetyl-CoAs are converted into ketone bodies and exported to other ketone consuming organs such as the heart and brain.

Ketone bodies are acetyl-CoA derivatives. Two acetyl-CoAs are condensed into acetoacetyl-CoA and in the process one of the CoAs is set free. The second CoA is released in the deacylation reaction catalyzed by hydroxymethylglutaryl-CoA synthase. The result of the overall reactions is the production of two free CoAs and one acetoacetate. Acetoacetate can be converted to acetone by decarboxylation, or be reduced to β-hydroxybutyrate. These three compounds are collectively called ketone bodies.

$$R-CH_2-CH_2-CH_2-COCoA \quad (C_{16})$$

FAD

FADH$_2$

$$R-CH_2-CH=CH-COCoA$$

H$_2$O

$$R \quad \overset{\cdot\cdot}{\underset{H \quad OH}{}} COCoA$$

NAD$^+$

NADH

$$R-CH_2-\overset{O}{\underset{\parallel}{C}}-CH_2-COCoA$$

CoA

$$R-CH_2-COCoA \quad +$$

⟶ acetyl-CoA

⟶ acetyl-CoA

⟶ acetyl-CoA

⟶ acetyl-CoA

⟶ acetyl-CoA

⟶ acetyl-CoA

acetyl-CoA
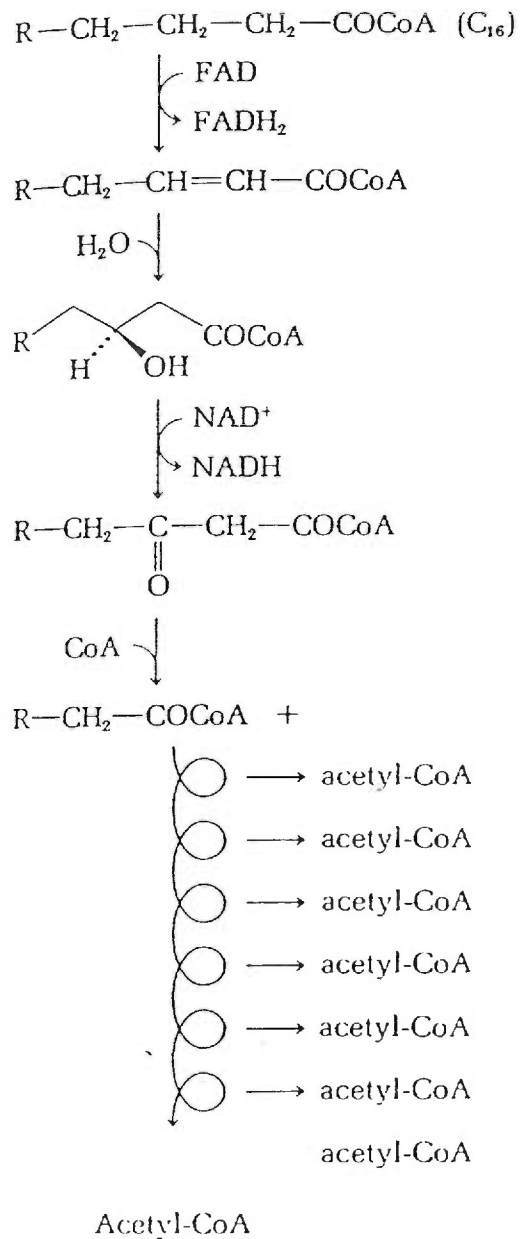
Acetyl-CoA

Fig. 3. β-oxidation of fatty acids. Fatty acids are degraded in the mitochondria through β-oxidation. The products are acetyl-CoA, NADH and FADH$_2$. Acetyl-CoA is further oxidized in the TCA cycle. Elevated acetyl-CoA levels indicate a state of energy surplus and favor synthetic activities. In CPTase deficiency, acetyl-CoA levels are depressed and the gluconeogenic activity is impaired (Source: Lehninger AL. Biochemistry 2nd Edition p.545, 1978).

Once ketone bodies have left the mitochondria, they diffuse out of the cell and travel in blood. In peripheral tissue, β-hydroxybutyrate is oxidized back to acetoacetate. The acetoacetate is first activated, which involves transferring a CoA from succinyl-CoA. The new acetoacetyl-CoA is then cleaved into two acetyl-CoAs which re-enter the TCA cycle for their final oxidation.

Because the inner mitochondrial membrane is impermeable to long chain acyl-CoA and because long chain FFAs must be activated in the cytoplasm before they can enter the mitochondrion, there is considerable interest in how FFAs are transported across the mitochondrial membranes.

As early as 1960, Bremer [18] and Fritz [37] independently discovered how long chain acyl-CoAs are transported into the mitochondrial matrix. A membrane bound CPTase converts acyl-CoA to acylcarnitine. The latter is transported across the inner mitochondrial membrane by an acylcarnitine translocase. Besides facilitating the exchange of various acylcarnitines, acylcarnitine translocase is also involved in a slower unidirectional transport that allows equilibration of carnitine concentration across the inner membrane. The palmitoyl-CoA is regenerated in the mitochondrial matrix for β-oxidation.

Early on, it was realized that CPTase was an essential component for long chain fatty acid oxidation. The regulatory role of CPTase in FFA oxidation, however, did not

become evident until much later. Borrebaek and his colleagues [13] noted that the capacity of the FFA activating enzyme exceeds the capacities of the acylating enzyme by seven fold. Soon after, McGarry [63] reported that CPTase was susceptible to malonyl-CoA inhibition. He hypothesized that because malonyl-CoA was the first committed intermediate in fatty acid synthesis, its inhibitory effect on CPTase must regulate the rate at which long chain FFA enter the mitochondrion. This in turn would determine the rate of long chain FFA oxidation.

Carnitine

The official chemical name of carnitine is $_L$-3-hydroxy-4-N-trimethylaminobutyric acid. This highly polar compound is widely distributed in nature and was first discovered around the turn of the century. Its biological usefulness was not realized until nearly a half century later when carnitine was recognized as an essential dietary factor for the growth of the mealworm.

In mammals, carnitine is synthesized primarily in the liver. Two essential amino acids, methionine and lysine, are required for carnitine synthesis. Extrahepatic tissues have to obtain their carnitine from the circulation. Mammals have little, if any, ability to metabolize carnitine. Therefore, carnitine is mainly excreted by kidney, either directly or as acyl conjugates.

Normally carnitine is transported into muscle cells against a large concentration gradient [45]. This ability to concentrate carnitine is specially impressive in the heart. The myocardial carnitine concentration is reportedly 60 fold higher than in plasma [66].

Cantrell and Borum [24] identified a cardiac carnitine binding protein in 1982. This protein appears to be associated with the plasma membrane and is distinct from other binding proteins involved in carnitine transport in the mitochondrial membrane. Carnitine carriers in muscle are probably different from those in liver. This might explain why there is a wide difference in carnitine affinity amongst tissues.

Apparently only L-carnitine is the biologically active form in mammals. Yet D-carnitine competes with the L-isomer for binding sites, therefore delaying the transport of L-carnitine. Ingestion of both carnitine isomers can produce myasthenia like symptoms. Injection of D-carnitine was shown to cause carnitine deficiency in rat heart but liver function was not impaired [8]. Carnitine transport is also inhibited by acylcarnitines [66].

Carnitine is found to be slowly released from muscle cells. This release is stimulated by carnitine or its analogues. The actual mechanism of this release is not known, but it is probably not a simple reversal of the

uptake process.

In 1955, Fritz first demonstrated that carnitine was essential to the maintenance of energy metabolism. In the early 1960's, Bremer [18] and Fritz [37] independently demonstrated the critical role of carnitine in the β-oxidation of fatty acids. Since the 1970's, abnormalities in carnitine metabolism have been linked to a number of congenital metabolic diseases and other clinical illnesses.

Carnitine deficiency is subclassified into primary and secondary deficiencies. Primary carnitine deficiency is rare and is defined as defects in the biosynthesis of carnitine. Primary carnitine deficiency is probably inherited in an autosomal dominant pattern. Most reported carnitine deficiencies, however, are "secondary" and are caused by a wide variety of conditions. The etiologies range from a low carnitine diet, poor gastro-intestinal function to severe renal disease. Common manifestations of this disease are muscle weakness, failure to thrive, and in severe cases, encephalopathy and cardiomyopathy. Many patients with carnitine deficiency benefit from carnitine supplement.

In some cases, the state of carnitine metabolism also seems to correlate with the functional state of the heart [81]. Patients with more severe heart failure have lower total carnitine level in the heart. The decrease in total cardiac carnitine correlates with the severity of the heart

failure and has little relation to the etiology. The total plasma carnitine, on the other hand, moves in the opposite direction. Plasma carnitine is greatly elevated in heart failure patients.

Carnitine Palmitoyltransferease (CPTase)

The first description of CPTase appeared nearly 30 years ago and voluminous discussion of this enzyme has been published since. Despite intense research efforts and significant progress in our understanding of this enzyme system, many critical aspects of the structural and functional details remain illusive.

In 1963, Bremer [18] described a Coenzyme A dependent reaction in which palmitoylcarnitine was formed from carnitine and free palmitate. This reaction was catalyzed by liver mitochondrial enzymes. At about the same time, Fritz [37] observed the reverse reaction in which palmitoyl-CoA was formed from palmitoylcarnitine and CoA. It was determined that this reaction was the second step in the acyl activation process after acyl-CoA is formed. It was reasoned that this reaction must be freely reversible because there was no requirement for hydrolysis of high energy bonds. A few years later, CPTase was reportedly purified to homogeneity [22]. Probably no one would foresee that twenty years later we would be still trying to determine what CPTase really is.

Several groups reported that there were two CPTase activities in mitochondria. It was suggested that an overt CPTase activity was loosely bound to the outer surface of the inner mitochondrial membrane and catalyzed the forward reaction described by Bremer [18]. There was another latent CPTase activity which was firmly bound to the inner membrane and catalyzed the reverse reaction described by Frtiz [22,37,43]. In 1977, McGarry [63] discovered that CPTase was susceptible to strong malonyl-CoA inhibition and was in a position to play an important role in regulating fatty acid oxidation. This potentially important role of CPTase inspired many investigators to unveil the secret of CPTase. By then, it was known that there was more than one acyltransferase, and each seemed to catalyze acylcarnitine formation in only a small group of fatty acids.

As time passes by and we learn more about CPTase, there is less agreement among researchers in the field. Their disagreement centers on three aspects of this enzyme system.

1. Are there two CPTases?

2. If so, where are they located?

3. How do they interact with malonyl-CoA?

Rat liver CPTase was first characterized by Miyazawa and his colleagues [65]. The purified CPTase had a molecular weight of 280-300 kDa in its native form and was thought to be a polymer of several 69 kDa polypeptides. They characterized the CPTase as quite stable during the

purification and immunologically distinct from the other two known acyltransferases. A comprehensive study was undertaken by a group headed by McGarry [29,106,107]. They characterized CPTase using inhibitors, detergents and antibodies.

CPTase was first treated with a reversible inhibitor (malonyl-CoA) and several irreversible inhibitors (glycidyl-CoA derivatives including tetradecylglycidyl-CoA). In addition, 2-bromopalmitoyl-CoA, a non-glycidyl-CoA derivative, was also used as an irreversible inhibitor. The goal was to determine whether inhibitors bind to the catalytic unit or a separate regulatory site. They found that in the presence of malonyl-CoA, binding of various other labeled ligands was reduced. On the other hand, the binding of labeled malonyl-CoA was abolished in the presence of any of the other irreversible ligands. Furthermore, only a single protein, with a molecular weight of 90 kDa, was tagged by radiolabeled tetradecylglycidyl-CoA. This protein was considerably larger than the 68 kDa size of CPTase previously reported.

The conclusion was that malonyl-CoA and the glycidyl-CoA ligand appeared to be competing for the same binding site. In addition, it was believed that 2-bromo-palmitoyl-CoA must occupy the same site as palmitoyl-CoA, yet it also competed with malonyl-CoA. Therefore, it was concluded the inhibitors must interact with CPT I either at the catalytic

site or at a site very close to it.

Based on these observations, McGarry [29] proposed a model in which CPT I regulator and catalytic units consisted of three sites, each site specific for palmitoyl CoA, malonyl-CoA and carnitine, respectively. These sites were close together and once malonyl-CoA occupied its binding site it interfered with normal catalytic activities. He further reasoned that if the three sites were so close and the labeled tetradecylglycidyl-CoA only identified a single protein, then the malonyl-CoA binding site must be on CPT I itself.

When detergents were used, two populations of CPTase were found. One CPTase was only loosely associated with the mitochondrial membrane. It could be easily solubilized with mild detergent treatment, such as Tween-20, while leaving the other CPTase intact on the membrane. The solubilized CPTase was relatively stable but was insensitive to malonyl-CoA inhibition. The other CPTase was tightly bound to the mitochondrial membrane and its activity was inhibited by malonyl-CoA. When solubilization was attempted with strong detergents, all membrane bound CPTase activities were lost.

Rabbit anti-rat polyclonal antibodies were raised against the purified protein representing the loosely associated CPTase. When these antibodies were used to probe Western blots containing all mitochondrial membrane proteins, only a 68 kDa molecular weight protein was

detected. If mitochondrial membrane was extracted first with Tween-20, leaving one CPTase tightly bound to the membrane and removing all loosely bound CPTases, the antibodies no longer detected anything.

Together, their results strongly indicated that there were two CPTases with strikingly different properties. The tightly bound CPTase was designated "CPT I" whereas the loosely associated CPTase became "CPT II".

Murthy and Pande [67] believed that the CPT I was a transmembrane protein across the outer mitochondrial membrane. If so, they reasoned that the malonyl-CoA binding site and CPT I catalytic site would be affected differently when treated with mild protease. They treated purified outer mitochondrial membrane vesicles with Nagarse, a protease, and found that CPT I activity was resistant to Nagarse digestion whereas the malonyl-CoA sensitivity was greatly affected by the protease. Their conclusion was that the malonyl-CoA binding site was on the outside surface of the outer membrane and the catalytic site was on the inside of that same membrane.

Following the same line of reasoning, Zammit et al. focused on the regulating units of CPT I [109]. A purified outer membrane preparation was used in radiation inactivation analysis. The prediction was that if the malonyl-CoA binding unit and CPT I were indeed two proteins of different sizes, then they would be destroyed by

radiation at different rates. The larger the protein, the faster the destruction. A differential decay should be reflected on CPT I sensitivity to malonyl-CoA inhibition. Indeed, after irradiation the remaining CPT I activity became more susceptible to malonyl-CoA inhibition. The conclusion was that CPT I catalytic and malonyl-CoA binding units were proteins of different sizes within the outer membrane. The molecular weights were 83 kDa for the CPT I catalytic unit and 60 kDa for malonyl-CoA binding site.

Armed with antibodies against CPT II, McGarry and his colleagues [108] probed CPTase from a variety of tissues in several species. They found that although the protein size of CPT II varied somewhat among different tissues and species, there was considerable conservation of amino acid sequence. They maintained that their antibodies detected CPT II only and did not interact with CPT I.

However, some of the immunological findings by McGarry et al. [106,107,108] were disputed by others. Other investigators reported antibody cross-reactivity with both CPT I and CPT II. Several authors [15,52] reported the solubilization of intact CPT I, which was also in disagreement with McGarry and his colleagues.

Brady and Brady [15] purified a protein which was extracted with Tween-20 and had a molecular weight of 68 kDa. Using the polyclonal antibodies against this protein, they precipitated all measurable CPTase activity in

different mitochondrial fractions. They concluded that all CPTase must be immunologically identical.

Ghadiminejad et al. [39] treated the outer mitochondrial membrane with sodium cholate and did not see an appreciable change in CPT I activity but lost all the sensitivity to malonyl-CoA inhibition. After ultra centrifugation, 40% of the CPT I was solubilized. The solubilized CPT I exhibited no sensitivity to malonyl-CoA but malonyl-CoA was still able to bind to some solubilized proteins. The addition of polyethyleneglycol 6000 to the supernatant restored the malonyl-CoA sensitivity to the solubilized CPT I. They reasoned that this restoration was probably due to reorientation of the CPT catalytic and regulatory units in micelles forming a kinetically productive complex.

If CPT I and CPT II are immunologically identical, then the malonyl-CoA binding site extracted from the outer membranes must be able to confer malonyl-CoA sensitivity on CPT II solubilized from the inner membrane. When CPT II was extracted from the inner mitochondrial membrane and added to polyethyleneglycol 6000, no sensitivity to malonyl-CoA was observed. When outer mitochondrial membrane protein was solubilized and added to the mixture, the sensitivity to malonyl-CoA was restored [39].

These results imply that CPT I catalytic and malonyl-CoA binding sites are separate proteins, and that CPT II is sensitive to malonyl-CoA if reconstituted with the malonyl-

CoA binding units extracted in a functional form from the outer mitochondrial membrane.

Recently, two other groups reported that CPT I could be solubilized. Furthermore, they confirmed that the malonyl-CoA binding site could be separated from the catalytic site. But they could not agree on whether CPT I and CPT II were identical proteins. Kerner and Bieber [52] extracted malonyl-CoA sensitive CPT I with octyl glucoside from heart mitochondria. They noted that exposing the solubilized CPT I to KCl resulted in rapid loss of malonyl-CoA sensitivity but not CPT activity. Their polyclonal antibodies indiscriminately precipitated all solubilized CPT activities. After CPT was bound to a immunoglobulin column, malonyl-CoA binding unit was dissociated from CPT activity during salt elution. The purified CPT has a molecular weight of 68 kDa. The malonyl-CoA binding units had a molecular weight of 39 kDa and were associated with β-oxidation enzymes. They concluded that CPT I and CPT II were different catalytic expressions of the same protein and were located on the inner mitochondrial membrane (because they were associated with β-oxidation enzymes).

Murthy and Pande [68] extracted CPTase from outer mitochondrial membrane vesicles with octyl glucoside in the presence of glycerol. CPT I and CPT II were separated on an hydroxyapatite HPLC column. The two CPTases retained their different properties even after passed through the column.

They found a phospholipid environment was critical to sustain CPT I activity but was not needed for CPT II activity. Reconstitution of CPT I into asolectin liposomes enhanced its activity and sensitivity to malonyl-CoA but no changes were seen with CPT II. Binding of a radiolabeled CPTase inhibitor occurred only with CPT I and the molecular weight of CPT I was determined to be 90 kDa.

In the 1990's, CPTase research entered the era of molecular biology like all other biomedical research. Knowledge of CPTase expanded to the genetic level. cDNAs for CPT II have been recently cloned and sequenced.

Woeltje et al [108] published the sequence of rat liver CPT II. The cDNA clone is approximately 2.2 kb in length and encodes 658 amino acids in the reading frame. The predicted molecular weight of the protein product is 74 kDa. Human liver cDNA was cloned and sequenced by Finocchiaro [34]. The human clone is also approximately 2.2 kb long and encodes the same number of amino acids. The predicted amino acid composition has 82.2% homogeneity with rat liver CPT II. The human CPT II gene is located on chromosome 1 [34].

At the time I started this project, the following views were generally held (Fig 4):

1. CPT I and CPT II catalyze the same reaction but in the opposite directions.
2. CPT I is located on the inner surface of the outer mitochondrial membrane and CPT II is
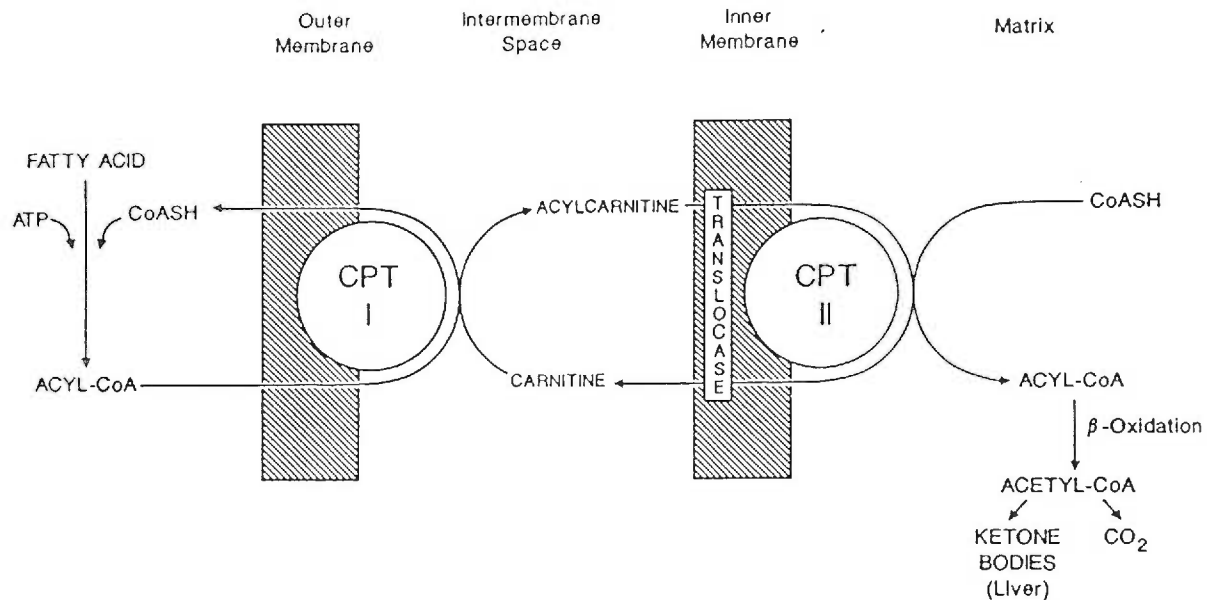
Fig. 4. Mitochondrial transport of fatty acids. Fatty acids must be activated before being metabolized by β-oxidation. The inner mitochondrial membrane is impermeable to acyl-CoA. Thus, a transmembrane transport system is required to move fatty acids into the mitochondrion. Acyl-CoA is converted to acyl-carnitine prior to its transport across the inner mitochondrial membrane. This figure illustrates the current model of this transport system. CPT I and CPT II are now believed to be two different proteins although both are capable of catalyzing the same reaction (Source: McGarry JD et al. Biochemie Vol 73, p. 78, 1991).

loosely associated with the inner surface of the inner membrane.

3. CPT I is inhibited by malonyl-CoA and is therefore the one that is under regulatory control.

There is still no consensus on whether CPT I and CPT II are immunologically distinct; nor is there agreement on the nature of the regulatory unit or the malonyl-CoA binding site. Even the location of the two enzymes is not agreed upon by all. Only recently (March 1993), McGarry et al. [32] provided evidence which may finally put this matter to rest. This group isolated peptides from a truncated CPT protein. A cDNA sequence encoding the protein was expressed to produce malonyl-CoA sensitive CPT I. Although these experiments yield only indirect evidence, they lend strong support to the two distinct CPTase protein hypothesis.

An inability to discriminate between CPT I and CPT II has not only frustrated biochemists and molecular biologists, it has also made it very difficult for physiologists to study the regulation of long chain fatty acid oxidation.

Birth and Adaptation

Fetal energy metabolism

Fetuses receive glucose and amino acids from the

maternal circulation and large quantities of lactate synthesized by the placenta [7]. Fetal gluconeogenesis activity is very low due to the low activity level of cytosolic phosphenolpyruvate carboxykinase (PEPCK) in fetal liver [40]. Normally, fetal glucose homeostasis is maintained through continuous uptake from the maternal circulation and is a function of maternal nutritional state. Glucose oxidation, however, contributes only 50% of the energy derived from oxidative phosphorylation in "fed sheep"; the ratio drops to 17% during fasting [94]. The remainder of the energy demand is supplied by oxidation of lactate 25%, amino acids 20% and acetate 5% [7].

There is no significant passage of FFA from ewe to fetus despite the fact that fetal serum FFA concentration is only 1/10 of the maternal value [49]. There is, of course, a large variation of placental transport of FFA among different species. Human, rabbit and guinea pig placentas are quite permeable to FFA. The interspecies difference in placental transport, however, does not seem to be a factor in the rate of FFA oxidation. Neither sheep nor rabbit newborns are able to oxidize long chain FFA to any significant extent [58,100].

Most mammalian fetuses have the capacity to synthesize fatty acid in their liver and adipose tissue [7], but these tissues are also resistant to lipolysis [23]. These findings indicate that the fetus is probably programmed not to use

FFA, regardless of its availability.

What is the fate of the fetal FFA if they are not consumed to supply energy? To answer this question, Christie et al.[26] infused radiolabeled palmitic acid directly into fetal sheep femoral arteries. They found that after rapid uptake, FFA are partially oxidized and used in resynthesis in the liver. It has been reported that long chain fatty acid esters are stored in the heart as well. Zierler found that there is a delay of 30 minutes from fatty acid uptake to oxidation and suggested that all fatty acids first go through esterification before being oxidized [110]. If this is true, then fetal storage of fatty acids probably is given a higher priority over oxidation.

Contrary to the common believe that fetuses generate their energy anaerobically because they are hypoxemic, fetal metabolism is actually fully aerobic under physiological conditions. The mode of energy metabolism, however, does vary with the availability of oxygen. While increasing oxygen delivery to the fetus does not increase fetal oxygen consumption [6], oxygen depletion without a corresponding decrease in consumption does lead to net lactate production [102]. Fetal metabolism is normally aerobic but fetuses have tremendous anaerobic metabolic reserve to survive low oxygen condition. Although all animals (including adults) can extract some energy anaerobically, fetuses have the unique ability of producing enough ATP anaerobically during oxygen

deprivation to make up 40% of the aerobic production [85].

Birth

In mammals, birth begins the adaptation to extrauterine life and this process modifies physiological functions in many organ systems over a relatively short period of time.

Birth is a stressful process for the fetus. Uterine contractions can reduce placental blood flow considerably and cause episodes of hypoxia. This could be detrimental to the fetus, especially in prolonged labor, because most organ systems require a constant oxygen supply to maintain their proper function. Once fetuses leave the protective environment of the uterus, they have to rely on thermogenesis to maintain their body temperature. The onset of successful thermogenesis depends on the rapid onset of fatty acid oxidation and the expression of an uncoupling protein which is the rate-limiting factor in brown fat thermogenesis. The induction of uncoupling protein at birth is regulated by thyroid hormone [78].

The first breath marks the end to dependency on the placental circulation for oxygen supply. Pulmonary vascular resistance decreases with the expansion of the lungs and decreases further with oxygenation [47,83]. Increased oxygenation is also known to facilitate the closure of the ductus arteriosus and to reduce right to left shunts [4]. After birth, the pulmonary and systemic circulations are

connected in series, which is a dramatic change from the parallel arrangement of the fetus [91]. This arrangement allows oxygenation of venous blood before it enters the left ventricle. With better oxygenation and the elimination of the right to left shunt, the over all oxygen delivery is markedly improved in newborns.

It is well documented that complex neurohormonal changes occur at birth as well. Catecholamine levels increase dramatically. A surge in serum glucagon and a sharp decrease in insulin level are observed within minutes to hours after birth. Growth hormone level is also elevated in newborns [87]. These changes are not just coincidence. It was demonstrated that suppression of specific hormone surges prior to birth can selectively abolished many adaptive physiologic changes [1,88]. After birth, the rich supply of lactate and glucose from the placenta is replaced by dietary fat and carbohydrate from milk. However, both fetal heart and liver have only limited ability to oxidize FFA. With only limited glycogen storage, the need for a rapid adaptation to the abrupt change in fuel supply is urgent, especially when energy demand has increased so dramatically by thermogenesis and increased muscle and organ activity.

Cardiac Energy Metabolism

Most mammalian fetuses rely primarily on carbohydrates to provide energy for cardiac work throughout their fetal

life. On the other hand, adult mammalian hearts prefer long chain FFA as an energy source except under hypoxic conditions. The reason for this preference is still unknown. Newborns consume more oxygen per kilogram of body weight than fetuses. The higher oxygen consumption demands an increased oxygen delivery and subsequently more cardiac work.

Warshaw reported that both fetal and neonatal calf heart mitochondria lack the capacity to oxidize long chain FFA. These mitochondria, however, were able to efficiently oxidize medium chain FFA and the carnitine derivatives of long chain FFA [100] (Fig.5). This indicates that the oxidative apparatus in mitochondrial matrix is mature in these animals but the transport system is not.

This inability to utilize long chain FFA by the heart in the very young, with some interspecies variation in the degree of the deficiency, appears to be quite common among mammals [36]. Unable to oxidize FFA, fetal hearts rely heavily on lactate (60% -80%) and glucose (30%) for their energy needs [35]. Comparing this to the total body lactate consumption (lactate contributes to only 25% of total energy supply) [94] fetal hearts derive a disproportionately large share of their energy from lactate oxidation.

Fetal and adult hearts both obtain carnitine from the circulation because hearts lack the ability to synthesize carnitine [9]. The carnitine concentration in the fetal

Ia

M + BSA
Palmityl CoA
ADP
Carnitine
(25)
(80)
Palmitylcarnitine
(90)

6 Week Calf

Ib

Palmityl-CoA
M BSA ADP
Carnitine
Palmitylcarnitine
(13)
(79)
ADP
(13)

⊢2 MIN⊣

7 Month Fetus
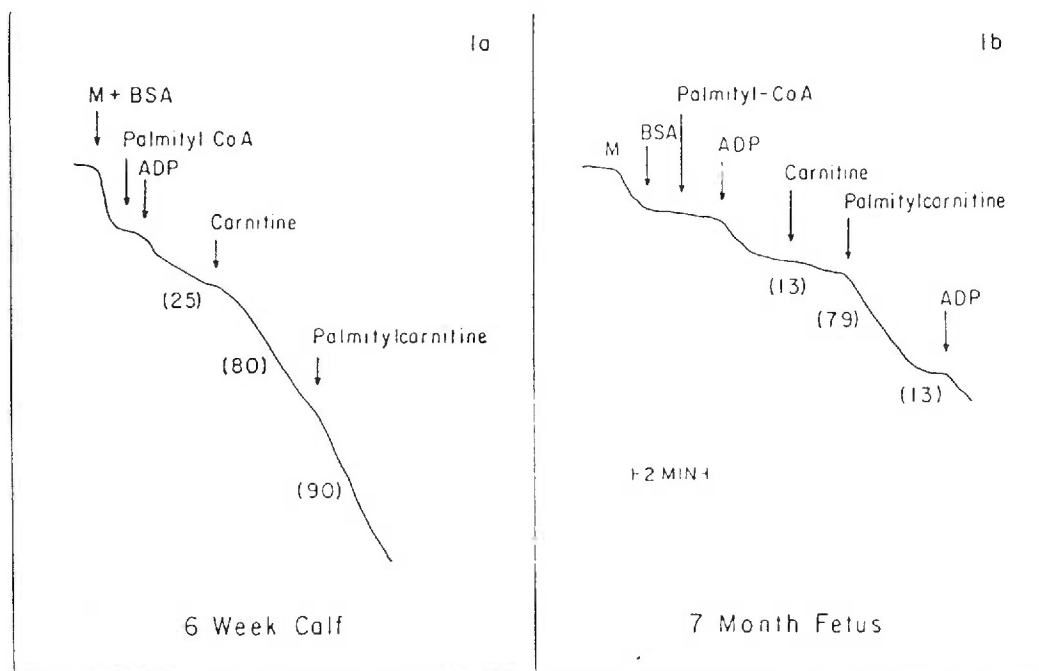
Fig. 5. Fatty acid oxidation rate in fetal and 6 week old calf hearts. Heart homogenates were used in these experiments. Oxygen consumption was monitored using polarography. M=mitochondrial homogenate, BSA=bovine serum albumin, ADP=adenosine diphosphate. The numbers in parenthesis indicated the rate of oxygen consumption in η atoms oxygen/mg protein/minute. These data showed that the fetal calf heart's ability to oxidize fatty acyl-CoA was much lower than for the newborn calf but the rate of acyl-carnitine oxidation was similar to those of the newborn calf. The author concluded that the low fetal heart fatty acid oxidation rate was a reflection of the deficiency of CPTase in the fetal heart (Source: Warshaw JB. J. Cell Biology Vol.44, p.356, 1970).

circulation is reported to be very low [96,100]. The combination of low concentrations of carnitine and low CPTase activities are believed to be the major contributing factors to the low FFA oxidation rate in fetuses.

Fetal and adult hearts are both incapable of gluconeogenesis and have limited abilities to synthesize fatty acids via reversal of β-oxidation in mitochondria [28]. Therefore, all fuels must be extracted from the circulation.

After being mobilized from storage by intracellular lipase, FFA is carried by serum albumin. At any given time, there is only a small amount of FFA (about 5% of total plasma lipid) in adult plasma [110]. The turnover rate, however, is extremely high. With a half-life of only three minutes, up to 300 gram of fat can be transferred daily [55].

In the adult mammalian heart, at least 60% of the energy requirement is derived from FFA [35]. It has been recently reported that FFA uptake in the heart is not by passive diffusion as previously thought, but is transported by a 40-kDa membrane binding protein [97]. The single pass extraction of FFA in the myocardium is 40-60%. Under normal conditions the rate limiting step for FFA oxidation is the FFA uptake at low plasma FFA concentration. Under conditions of a high muscle workload, elevated catecholamine levels stimulate FFA release and transmitochondrial transport via CPTase becomes rate limiting.

Induction of CPTase

How do fetuses select their primary fuels?

It is thought that fetuses oxidize only limited FFA because 1) their lipolysis in liver and adipose tissue is inhibited, 2) tissue carnitine concentration are low, 3) activity of CPTase is low, 4) mitochondria are "immature", 5) respiratory chain enzyme activities are low [93]. All of theses are attributed to the immaturity of the fetus. These may explain low fetal FFA oxidation but they do not explain why these functions are immature in fetal life. The observation that the fetus lives with immature biochemical systems might be better explained teleologically on the basis of fetal function under the environmental conditions in which they live.

The primary function of fetal metabolism is to ensure continuous growth. Fetuses grow rapidly especially in late gestation. Large quantities of fatty acid are required for development of the nervous system, cell membranes and lipid containing structures. In addition, increasing amounts of fat must be stored as fuel in preparation for postnatal survival. Given the importance of fatty acid and its limited supply, fatty acid is much too precious a commodity to be used as fuel, especially when abundant lactate and glucose are readily available. Therefore, fetal FFA metabolism is storage oriented.

Fetuses extract large amounts of lactate from the

placenta. When tissue lactate increases, oxidation of long chain FFA is reduced [10], presumably through inhibition of liver CPTase. Lactate also stimulates acetyl-Co A carboxylase in rat liver which elevates malonyl-Co A concentration. The end result is the further inhibition of the already low CPTase activity. All of these biochemical changes preserve fatty acid for more pressing needs.

The total oxidative capacity of the fetal heart is a direct function of tissue oxygenation [93] and maturation [31]. For example, full term fetuses are more responsive to an epinephrine surge than the preterm lambs [73]. However, the induction of some key metabolic enzymes in newborns is relatively independent of maturation. It has been suggested that the birth process, not the developmental stages or nutritional factors, is responsible for the induction of liver PEPCK [40] and perhaps CPTase induction, also.

The suddenly increased mechanical demand on the newborn heart has been thought to be the inducer of energy metabolism changes at birth. Tietel believes that the transition from fetal to immediate postnatal circulation pattern can be achieved solely by ventilation, oxygenation and umbilical cord occlusion without any of the other components of birth [91]. Yet many attempts have been made to induce postnatal levels of FFA oxidation by ventilation, oxygenation and cord occlusion. All have failed.

Dietary fat intake was originally thought to regulate

the development of the enzyme pathway required for FFA oxidation [101]. This appears not to be the case. When newborn rabbits were divided into two groups and fed high or low fat diets after birth, no evidence was found to support the suggestion that the development of enzymes in the FFA oxidation pathway was influenced by the amount of lipid in the diet [3].

Two mechanisms have been proposed to be responsible for the immediate increase in FFA oxidation capacity in the liver at birth. They are 1) decreased lipogenesis and 2) decreased sensitivity of CPTase to malonyl-Co A inhibition [41]. These are now thought to occur due to changes in catecholamine and glucagon levels in the perinatal period [87,88].

Malonyl-Co A, being an intermediate in lipid synthesis, is in perfect "position" to regulate the activity between synthesis and oxidation of FFA in maintaining a balance in energy flux. Since the liver is the metabolic center for the body, it would be an ideal place for malonyl-CoA to exert its regulatory effect. That is probably one of the reasons why most investigators have choosen to study liver CPTase.

The regulatory effects of malonyl-Co A on liver CPTase has been extensively studied. It is well established that malonyl-Co A strongly inhibits CPTase in the liver and suppresses FFA oxidation in anabolic states. Dramatic

changes in fatty acid storage and oxidation can occur within hours in starved rat liver. Changes in liver carnitine levels also correlate positively with ketogenic potential. In the newborn rat, the acquired ability to oxidize FFA seems to be associated with falls in the malonyl-CoA concentration. However, Prip-Buss [80] found that the decrease in malonyl-CoA was insufficient to induce FFA oxidation.

Besides tissue malonyl-CoA level, certain properties of CPTase are readily changed as well. When an animal is in a state of enhanced fatty acid oxidation (low malonyl-CoA), liver CPTase exhibits diminished sensitivity to malonyl-CoA inhibition. After birth, CPTase sensitivity to malonyl-CoA inhibition is also decreased. When CPTase sensitivity to malonyl-CoA was decreased by adding glucagon or cAMP to cultured fetal rabbit hepatocytes, oxidation of FFA increased [80]. This indicates that altered CPTase sensitivity to malonyl-CoA is an important regulating mechanism in FFA oxidation. Prip-Buss [80] believes that the fetal to newborn transition is tied to changes in malonyl-CoA sensitivity.

Malonyl-CoA inhibition is the center piece of CPT regulation in liver. Malonyl-CoA serves to balance the energy flux through the two branches of fat metabolism (Fig 6). This regulatory mechanism appears to be involved in lactate inhibition of CPT and FFA oxidation. It has been
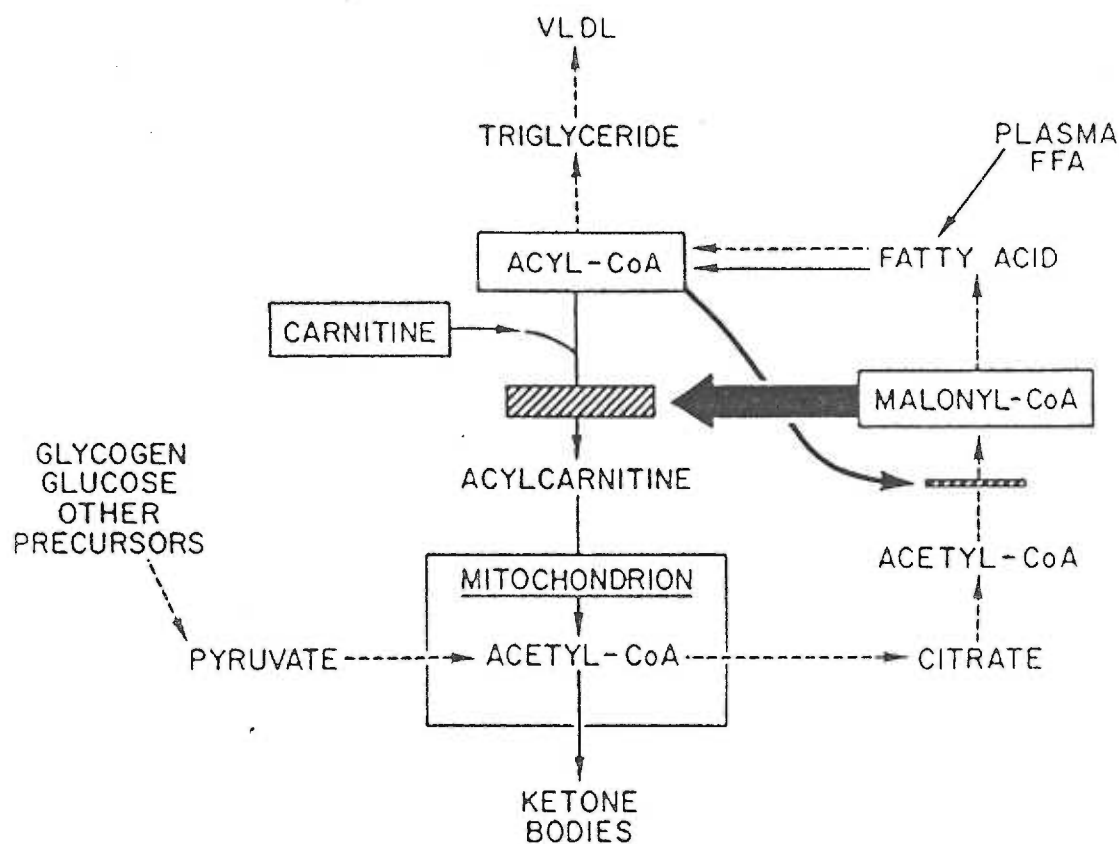
Fig. 6. Interaction between fatty acid synthesis and fatty acid oxidation. Malonyl-CoA is believed to be the regulator between fatty acid synthesis and its oxidation in liver. In addition, malonyl-CoA is believed to coordinate the energy flow between fatty acid and carbohydrate metabolic pathways. The regulatory role of malonyl-CoA is illustrated in this figure. Dashed lines indicate fatty acid synthesis; solid lines represent oxidation of fatty acid. When fatty acid is actively synthesized, malonyl-CoA concentration increases and fatty acid oxidation is inhibited via inhibition of CPTase activity. As indicated in the figure, fatty acid oxidation rate is also influenced by the activity in the carbohydrate pathway (Source: McGarry JD. Diabetes/Metabolism Reviews. Vol.5, p.274, 1989).

shown that lactate inhibits CPT in isolated rat hypothecates, presumably through stimulating acetyl-CoA carboxylase activity. This in turn increases malonyl-CoA concentration and inhibits CPT and FFA oxidation [10].

Unfortunately, we know little about how cardiac CPTase is regulated, either in fetal hearts or in adult hearts. Whether the observations on liver CPTase can be applied to explain how cardiac CPTase is regulated remains to be seen.

There are some established facts supporting the regulatory role of malonyl-CoA in the heart. Malonyl-Co A is found in the heart and it is reported that cardiac malonyl-CoA concentrations do vary in the same direction as their concentrations in liver. Malonyl-Co A also inhibits cardiac CPTase in isolated mitochondria. However, the source of cardiac malonyl-Co A and its intracellular distribution is not known.

To make matters even more confusing, there is little fatty acid synthesis activity in the heart. Lipogenesis in the heart occurs only inside the mitochondria and relies on elongation of existing FFA. This pathway does not generate malonyl-Co A. CPT inhibition by malonyl-CoA may be an essential regulatory function in the liver to suppress FFA oxidation when energy is abundant, but what function does it serve in the heart? Myocardium normally prefers FFA as a fuel so why inhibit FFA oxidation when energy supply is plentiful?

It is known that heart and liver react differently to changes in nutritional state. The heart is more resistant to changes induced by starvation and hormone administration. This difference might in part be explained by the fact that catabolic ability is limited in the heart.

Even if malonyl-CoA can be established as the sole regulator of cardiac CPTase, it probably would act through short-term feedback mechanisms. Little is known about the long term regulation of CPTase. The abrupt change in CPTase activity at birth provides a golden opportunity to study the long term regulation of this enzyme.

Dramatic changes in expression of several specific enzyme systems at birth are well documented. Liver PEPCK, the rate limiting enzyme in the gluconeogenesis pathway, has an induction pattern similar to CPTase. PEPCK depends on β-oxidation products to function. Fetal liver has only low levels of PEPCK activity during fetal life but reaches adult level in 24 hours after birth [41].

The induction of PEPCK follows the birth related surge of catecholamines and glucagon. Insulin levels are suppressed. The markedly increased rate of PEPCK synthesis after birth correlates with changes in its RNA level. Fetal rat liver has no detectable PEPCK mRNA, but within hours of birth PEPCK mRNA rapidly accumulates in nuclei and cytosol [38].

Based on this information, I hypothesize that CPTase

expression is probably activated in a similar manner as
PEPCK. Indeed, the hormonal changes which occur at birth
also coincide with the surge of CPTase activity. It is
likely that the same hormones are playing important roles in
events related to FFA oxidation.

The importance of catecholamines in postpartum
adaptation has been clearly demonstrated. In sheep, the
catecholamine surge is abolished with fetal adrenalectomy;
so are the postpartum surges of serum glucose and FFA [88].

Surges of serum glucagon are seen at birth in the
human, sheep, rabbit and rat. Serum insulin levels are
usually maintained at a basal level after falling initially
and are less sensitive to physiological stimuli.

Some of these hormonal changes at birth are thought to
be secondary to the catecholamine surge [88]. The combined
effect of hormonal changes at birth, high epinephrine, high
glucagon, high growth hormone and low insulin, would
mobilize glucose through glycogenolysis and possibly
gluconeogenesis and would activate lipolysis and promote
ketogenesis. In addition, adrenocorticotropic hormone
(ACTH), thyroid hormone and cortisol levels also change
dramatically in the perinatal period. Since all of these
hormones have strong regulatory effects on FFA oxidation,
they, too, could be the potential triggers for CPTase
induction at birth.

However, birth related factors other than hormones

must be involved because prolonged infusion of glucagon and epinephrine in fetal lambs failed to elicit evidence of gluconeogenesis [99]. The onset of gluconeogenesis in newborn lamb liver follows oxygenation. It has been hypothesized that higher levels of oxygenation after birth might be the triggering factor for PEPCK expression [99]. But Warnes et al. [99] and Gleason [42] were not able to induce the expression of liver PEPCK in utero with either epinephrine infusion or oxygenation.

It is clear that PEPCK induction is regulated at the mRNA level but is it possible that long term regulation of PEPCK is also controlled at gene level? There is evidence to indicate so. Refeeding rats starved for 24 hours induces PEPCK mRNA expression within 2 hours and the mRNA half-life is about 40 minutes [27].

There are very little data on the half-life of CPTase and present data are controversial. Brady reported that the rat liver CPTase half-life is 2 days [17]. The half life reported by Miyazawa was 6.6 days for CPTase [65]. Nonetheless, these data indicate that CPTase needs to be replenished regularly rather rapidly.

Clinical Significance

Given the central role of CPTase in FFA oxidation and the importance of FFA oxidation in sustaining the energy supply, it would be reasonable to expect that changes in

CPTase activity could result in diseases associated with abnormal FFA oxidation.

CPTase activities fluctuate greatly according to the health and nutritional state of the animal. In diabetes and starvation, rat liver CPTase activity is elevated 2-3 fold [17]. In severe diabetes, patients rely very much on FFA oxidation because of their low glucose oxidation. When rats were fed a riboflavin deficient diet, their liver CPTase activity increased [16]. Several hypoglycemic drugs are known inhibitors of CPTase. CPTase is deficient in several hereditary diseases. The symptoms range from mild exercise intolerance to lethal metabolic disorders. CPTase deficiency was once considered to be a rare disease but increasing numbers of CPTase deficiency cases have been reported. In fact, CPTase deficiency is now considered a more common cause of muscle pain and myoglobinuria [74].

Although CPTase deficiency was known to cause clinical problems as early as 1973 [30], the different clinical manifestations of the two types of CPTase deficiency were not realized until 1981 [14]. It was thought then that CPT I deficiencies were rare and occurred mostly during infancy. Severe hypoglycemia and low levels of ketogenesis are common in CPT I deficiency and these symptoms often are triggered by fasting. CPT II deficiencies, on the other hand, were perceived as occurring a little later in life and were often thought to be muscle diseases.

However, more recent evidence suggests that CPTase deficiencies are far more complex and impair many organs. Falik-Borenstein and co-workers [33] reported a new case of CPT I deficiency with severe hypoglycemia and distal renal tubular acidosis. This infant responded well to medium chain fatty acid treatment as would be expected of a CPT I deficiency.

It is now known that CPT II deficiency affects more infants than previously thought and is often lethal. A new case report by Hug and his colleagues confirmed several earlier reports on the severity of infant CPT II deficiency. The newborn in their case had markedly decreased CPT II activity throughout major organs, but the heart and skeletal muscles were most severely affected. The CPT II activities were only 2% in the heart and 1% in the muscles when compared to normal levels. The child's clinical symptoms began with hypothermia and hypoglycemia, but quickly developed hypotonia, hyperreflexia, seizure and cardiac arrhythmia. The child died of unexplained diseases of the heart and brain when she was only 5 days old. At autopsy, lipid droplets were deposited in hepatocytes, ventricular muscle cells and renal tubular epithelium [45].

One of their findings was especially interesting. Cardiac CPT I activity was actually higher than normal and CPT I activity was not increased in other organs. This might indicate that the heart isoform of CPT I is regulated

independently from those of other organs.

In coronary artery disease FFA metabolism is also altered. It is known that FFA oxidation is suppressed and long chain acyl-Co A accumulates in myocardium after acute ischemia. Excess FFA is thought to have deleterious effects on cardiac function. Thomassen found that in patients with stable angina, the resting myocardial uptake of free fatty acids is only 50% of control whereas their cardiac glucose and lactate uptake is almost doubled [92]. Altered sensitivity of CPTase to malonyl-Co A inhibition in ischemic hearts has also been reported [76].

In chronic heart failure, the myocardium undergoes hypertrophy but the number of capillaries do not change. Without an accompanying increase in the capillary bed, oxygen and nutrient supply is reduced. A correlation between decreased myocardial ATP content and impaired cardiac function has been reported [5]. It is proposed that cardiac energy deficit could potentially be one of the causes of chronic heart failure [50].

Cardiac carnitine concentration is markedly decreased in end stage heart failure [61]. This implies that there is a distinct possibility that oxidation of long chain FFA is impaired. It has been reported that chronic inhibition of CPT by administration of the CPTase inhibitor tetradecyl-glycidic acid resulted in diastolic dysfunction [57]. The specific mechanisms of energy depletion in heart failure are

still poorly understood. Heart failure is a complex process and is not the primary topic of this thesis. However, in the following sections I would like to briefly review some aspects of the failing heart that will establish the relationship between energy metabolism and heart failure.

General Concept of Heart Failure

Pumping adequate amounts of blood to meet the oxygen demand of the organism is the only function of the heart. Therefore heart failure can be generally defined as pump failure. Like everything else in medicine and science, our knowledge of heart failure accumulates over time. The next section will give an outline on how our understanding of the complex pathophysiology of heart failure evolved over the last century.

Nearly a century ago, Osler [70] divided the clinical state of congestive heart failure into three stages. There was a period of "development", followed by a period of "full compensation" and at last came the final stage of "broken compensation". It was recognized then that the heart is not normal even in the first two stages of the illness when symptoms are few. At the turn of the century, Sir MacKenzie [60] was convinced that "heart failure is due to the exhaustion of the reserve force of the heart muscle".

Half a century later, we began to appreciate the nature of MacKenzie's "reserve force of the heart muscle" in

biochemical and physiological terms. The primary problem in heart failure was attributed to the loss of myocardial contractility. Not surprisingly, the traditional treatments mainly consisted of measures to increase myocardial contractility. Now, it is recognized that diastolic dysfunction in heart failure is as important as myocardial contractility. Furthermore, we are now studying abnormalities at cellular and subcellar organelle levels. With our better understanding of heart failure, recommended treatment is changing, too. Current treatments for heart failure focus on "unloading" the heart and reducing cardiac energy expenditure and oxygen consumption.

Numerous events occur during the course of heart failure. We now know that β-receptor density in the failing heart is reduced about 50% [21]. Remaining β-receptors are desensitized as well. The receptor down regulation is also highly selective; only the β_1 receptors in the heart are affected [71].

Calcium homeostasis is also abnormal in heart failure. This abnormality must contribute to the deteriorating cardiac function in heart failure because of calcium's pivotal role in both contraction and relaxation [51].

Cardiac Adaptation

Coronary artery disease is now considered the most common cause of chronic heart failure. Hypertension is a

close second. Coronary artery disease impairs myocardial contractility whereas the hallmark of hypertension is pressure overload. These altered loads represent the two basic conditions the heart must adapt to. Numerous studies over the year provided voluminous information on how heart failure evolves.

Because heart failure is a pump failure, the primary deficit is reduced perfusion pressure. To maintain adequate systemic blood pressure, all neurohormonal systems involved in pressor regulation will be activated.

Normally, vasoconstriction is a life saving mechanism in acute situations such as hypovolemia. Vasoconstrictors are released immediately whenever the blood pressure becomes too low to maintain adequate perfusion, especially of the brain. However, the only purpose of the elevated pressor response is to maintain blood pressure. Once the crisis is over, vasoconstrictor release is quickly inhibited through the feedback loop involving baroreceptors. These acute compensating mechanisms are thought to be vital functions preserved through evolution. It was reasoned that in the early days, man hunted for survival and blood loss from injury was common. These traits among the most fit were selected and passed on to modern human being. They seem to have served us well. These same compensating mechanisms are no doubt working in acute and chronic heart diseases. Why are the outcomes so different?

The pathophysiology differs significantly between acute and chronic heart failures. The severity of the underlying diseases plays a significant role in determining whether the heart failure is acute or chronic. It would be reasonable to say that the chronic and acute heart failure really differ in the time they have to adapt.

When severe cardiac function impairment occurs acutely, the heart is unable to compensate to any significant extent. Most of the compensating mechanisms such as myocardial hypertrophy, blood volume expansion and chamber dilatation do not have enough time to work because of the rapid deterioration. In acute failure, cardiac output decreases sharply and ventricular filling pressure increases. If the loss of myocardial contractility cannot be quickly restored, the elevated filling pressure will back-up into the left atrium and the pulmonary vasculature. This ultimately leads to pulmonary hypertension and pulmonary edema which in turn impairs oxygenation of the blood. Without any means to boost cardiac output, blood pressure is maintained at the expense of local perfusion through the actions of pressor agents. Cardiac work and oxygen expenditure is greatly increased while pumping against a higher resistance. At the same time, oxygen delivery to the tissues remains impaired. Tissues begin to rely more and more on anaerobic metabolism which leads to metabolic acidosis. The vicious cycle perpetuates itself. Cardiac function deteriorates quickly

and the final result is acute heart failure.

Since hearts in chronic failure have time to adapt, do they follow a different course? For many years, it was believed that the same mechanisms are at work and these self-reinforcing events lead to progressive disfunction of the heart and eventually failure. In essence, it was thought chronic heart failure is really an extension of the acute failure at a slower pace.

It is now clear that the adaptation to overload is far more complex than originally imagined. Chronic heart failure is not simply a longer version of acute failure and the response to overload is not simply a mirror image of the much simpler reactions seeing in hypovolemia.

When cardiac output is chronically inadequate, increasing peripheral resistance becomes a frequently used tactic to maintain blood pressure. The catecholamine level is persistently elevated in these patients. Apparently, the feedback control no longer works in chronic heart failure patients. Through unknown mechanisms, atrial and arterial baroreceptors lose some of their ability to suppress the release of vasopressin from the pituitary gland. The consequence of this baroreflex dysfunction is a persistent state of excessive activation of pressor release. Thus the transient beneficial pressor response becomes a potentially detrimental problem.

Instead of deteriorating in a downward death spiral as

in acute failure, the chronic failing heart adapts to the adverse environment and survives. It has been found that heart rate and peripheral vascular tone tend not to increase significantly in heart failure patients who have persistently elevated circulating catecholamine levels. Therefore, there must be protective mechanisms that are activated to counteract the detrimental effects of chronically elevated plasma catecholamines.

This adaptation is believed to be accomplished by modifying the response to catecholamines in end organs. Since β-receptors control the adrenergic pathway, it is possible that down regulation eventually "shuts down" the cAMP conversion and reduces the inotropic and chronotropic effects of catecholamines. It is also known that despite elevated circulating levels of catecholamines, the failing myocardium is depleted of catecholamines. The depletion of catecholamines in the myocardium itself may attenuate the effects of elevated circulating catecholamine levels. Locally released vasodilators may also counter the vasoconstrictive effect of the high catecholamine state without altering β-receptor function.

The usual sequence of events in pressure overload is that the ventricular muscle undergoes hypertrophy, which allows improved contractility because hypertrophy increases the contractile mass. In the cases of volume overload, the chambers will dilate to take advantage of the Frank-Starling

mechanism and some degree of hypertrophy may also compensate for the enlarged radius of curvature. A thicker wall will help to lower wall stress as well.

Katz [51] believes that myocytes in the heart become overloaded regardless of etiology. Furthermore, he believes that hypertrophy and the neurohormonal discharges that help to maintain blood pressure are beneficial, at least in the early stages and that these responses become detrimental only when the overloading is sustained.

Insufficient peripheral perfusion decreases renal blood flow and stimulates the renin-angiotensin-aldosterone system. The subsequent salt and water retention increases venous return to the heart as expected. Again, as an early response, electrolyte and fluid retention augment preload. They became a problem only when the volume retention becomes chronic as more burden is added to the already overloaded heart, causing pulmonary congestion.

An inadequate oxygen supply forces the capillary beds to increase regional blood flow. Since the heart is already compensating, vasodilation will only lower the pressure further without improving the perfusion. The decrease in blood pressure activates the sympathetic system and vasoconstriction occurs. The higher peripheral resistance increases the blood pressure and afterload for the already burdened heart. Ultimately, the increased cardiac energy expenditure accelerate the rate of myocardium damage in over

loaded heart [51].

Hypertrophy

Hypertrophy has been studied in much detail
morphologically. Grossly, hypertrophied hearts have
markedly increased muscle mass without accompanying
increases in capillary numbers. This increases the volume
of tissue supplied by each capillary and efficiency of
nutrient delivery decreases as the diffusion distances
become longer. Eventually, the mismatch becomes so large
that the muscle mass outgrows its own blood supply. To make
the matter worse, the coronary reserve is markedly reduced
in hypertrophied hearts. This was observed in a series of
198 patients with hypertensive hypertrophy but normal
coronary arteriogram [89]. Many of these patients were
younger than 30 years old. One possible explanation is that
the coronary bed is compressed by hypertrophied heart
muscle.

Cellular abnormalities appear to make their
contribution to the energy starvation in the chronically
over loaded failing heart as well. In later stages of
myocardial hypertrophy, the increased myofibril mass
occupies a larger portion of cell volume. The space
available for mitochondria decreases and mitochondrial
numbers decrease. This change increases the
myofibril/mitochondria ratio, compounding the problem

associated with reduced substrate supply due to fewer
capillaries.

If chronic hypertension develops slowly over time,
hypertensive heart failure begins with a pressure loaded
normal heart. A similar condition is also seen in endurance
training in which the heart is normal but and the pressure
is mildly elevated. One might ask what makes the athelte's
heart respond differently from the hypertensive heart?

To answer this question, we must first look for the
distinction between physiologic and pathologic hypertrophy.
Wikman-Coffelt [104] defined physiological hypertrophy as
"accompanied by a normal or augmented contractile state in
which the maximum rate at which myosin hydrolyzes ATP and
the maximum velocity of muscle shortening are either normal
or elevated. Pathological hypertrophy, on the other hand,
is associated with depressed contractility without
necessarily concordant heart failure, in which case the rate
of myosin ATPase activity and the rate of myosin shortening
are decreased".

Most hypertrophies induced by pressure overload alone
have a gradual onset and are preceded by a prolonged period
of functional adaptation. It has been shown that
mitochondrial mass increases before there is an increase in
myofibril mass [62]. This suggests that an increase in
oxidative phosphorylation capacity is already under way
while cardiac function is still optimal. Workload has long

been considered an important determinant of ventricular mass. Myocardium responds to an increased load by adding more sarcomeres. This appears to occur rapidly following load increases. It has been observed that the activities of RNA transcription, mRNA transport and protein synthesis are increased within hours of pressure loading [104].

The characteristic structural and functional adaptations in pressure loaded left ventricle are being linked to changes in enzyme activities and substrate utilization. It is now believed that these biochemical changes are possible signals that induce hypertrophy.

During pressure loading, the relative growth of myofibril mass eventually surpasses that of the mitochondria. There is also an increase in the surface to volume ratio in hypertrophied muscle cells. This development is particularly unfavorable for these cells with their increasing demand for energy.

Different myosin isoforms may be synthesized in response to overload, at least in rodents. Pressure load induces increased expression of the V_3 myosin isoform to replace the V_1 isoform. V_1 myosin heavy chain has higher ATPase activity and faster shortening velocity whereas V_3 myosin heavy chain is a slower contracting type with lower ATPase activity. The results are a reduction in contractility but the tension generated during systole is increased. These changes have an overall negative inotropic

effect and actually might reduce energy demand in early stage of overload.

It is believed that in rats V_1 myosin synthesis is increased in physiological hypertrophy whereas the V_3 myosin is dominant in pathological hypertrophy. This finding may not apply directly to human hypertrophy but it illustrates that changes do occur in gene expression and that these changes may determine the myosin ATPase activity and muscle shortening velocity.

Besides preferentially expressing genes of certain adult isoforms during hypertrophy, genes of fetal myocardial proteins are also expressed in selective sites in rat hearts, presumably through alternative splicing. The newly synthesized myosin heavy chains first appear in subendocardial regions of the left ventricle. The fetal isoform of actin, on the other hand, is produced and distributed uniformly throughout the heart.

Why do adult rat hearts produce fetal proteins under pressure load? Adult myocardium is terminally differentiated and has lost its mitotic ability. The normal rate of protein synthesis in adult myocardium is relatively slow. It has been speculated that the capability of rapid protein synthesis in the heart is only present during development. In response to pressure load, cardiac myocytes need to accelerate protein synthesis and revert to the earlier pattern of production. The result is preferential

synthesis of several proteins in their fetal isoforms [51].

There are other differences between physiological and pathological hypertrophy. The duration of loading also plays an important role. Taegtmeyer and Overturf [90] observed that specific metabolic changes occur 8 weeks after consistent, moderate pressure load. This indicates that intermittent loading during endurance training may not induce the same metabolic changes seen in chronic hypertension. Myocardial hypertrophy might be reversible under certain circumstances. The determining factors are the health of the heart and the degree and duration of the load. The load in pathological hypertrophy usually is of much longer duration and is rarely removed. The fibrosis associated with hypertrophy further diminishes the chance of reversal in pathologically hypertrophied hearts even if the load is removed.

Relaxation Abnormality

The relaxation properties of the myocardium may have been affected long before the heart starts to loose its contractile capability in hypertrophy. To describe the events in diastole, one may begin with what happens in the immediately preceding systole.

When the sarcolemma is depolarized, Ca^{+2} enters the myoplasm through voltage sensitive calcium channels on the sarcolemma. This small surge in Ca^{+2} current triggers a

much larger activator Ca^{+2} release from sarcoplasma reticulum (SR) into myoplasm. Ca^{+2} binds to troponin-C on the myofilaments and initiates the contraction. The magnitude of the contractile response is determined by the amount of Ca^{+2} in the myoplasm. A single action potential releases enough Ca^{+2} to fully activate the contractile apparatus. The Ca^{+2} in the myoplasm, however, is pumped back into SR even before the muscle reached its maximum contraction. Therefore the rate of Ca^{+2} reuptake determines the force of the contraction.

The rate of relaxation of ventricular muscle depends on the rate of calcium removal from the myocyte cytosol. The first step in relaxation is the dissociation of Ca^{+2} from troponin. Ca^{+2} is rapidly removed from myoplasm by SR sequestration which is an energy dependent process. In addition to SR uptake, Ca^{+2} is extruded into the extracellular space by a gradient driven Na^+/Ca^{+2} and ATP dependent Ca^{+2} pumps (Fig 7).

Maintaining normal calcium metabolism is vital to ventricular function. The total myocyte calcium content seems to remain constant in heart failure but the intracellular distribution changes dramatically. Calcium uptake by SR is reduced during relaxation due to depressed activity of Ca^{+2} activated ATPase [46]. Less activator Ca^{+2} is available for the subsequent contraction [77]. This alteration in calcium distribution occurs quite early in the
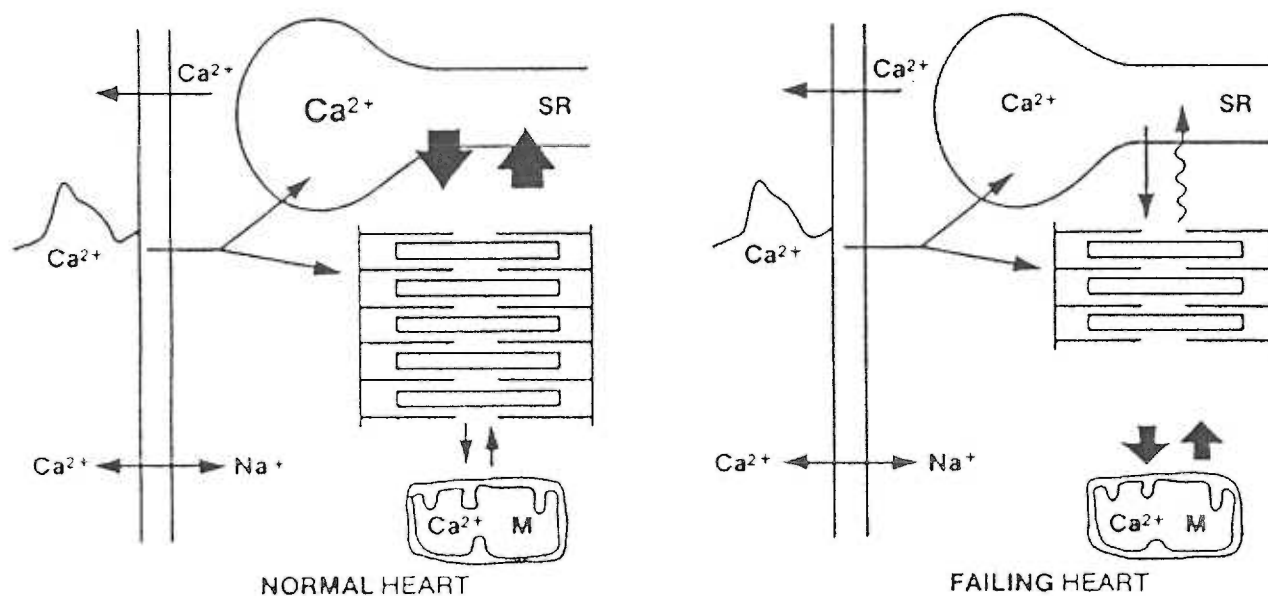
Fig. 7. Calcium transport in normal and failing heart. Normally calcium enters the myocyte following an action potential. Calcium ions are then released from the SR. The concentration of intracellular calcium during systole determines the contractility of the muscle. During relaxation, most of the calcium is actively pumped into SR or expelled into the extracellular space. A small amount is picked up by the mitochondria. The calcium homeostasis is maintained through energy dependent ion pumps. If energy metabolism becomes abnormal, the calcium homeostasis becomes disturbed. Cytosolic calcium levels elevate, leading to impaired relaxation. Intracellular calcium redistribution also occurs which causes contractile and relaxation abnormalities. It is believed that changes in diastolic function may actually precede changes in contractile functions (Source: Remme WJ. Cardiovascular Pharmacology Vol 8,(Suppl. 1), p.S40, 1986).

disease and could be a contributing factor to the impaired contractility. Besides affecting contractility, the delayed clearance of cytoplasmic calcium may exacerbate the relaxation abnormality in the failing heart. Arrhythmias may be another consequence of calcium overload. Because relaxation is energy dependent, any imbalance between energy production and consumption may contribute to abnormalities in relaxation. Relaxation has a major pathophysiologic role in heart failure.

Mitochondrial calcium uptake is increased in chronic heart failure. This contributes to the shift in the intracellular calcium distribution, too. Furthermore, this portion of the calcium pool is practically not available to excitation-contraction coupling because the rate of calcium release from mitochondria is very slow [82]. It was suggested that the elevated mitochondrial calcium concentration might also exert deleterious effect on oxidative phosphorylation.

Summary

As shown in this brief review, the scientific community has learned much regarding the heart but many unanswered questions remain. It is safe to conclude that cardiac CPTase induction is probably controlled by a multitude of stimuli associated with birth. This, however, is about all that we can say.

Ventilation, oxygenation and cord occlusion are thought

to be the primary candidates that might contribute to the induction of cardiac CPTase because all of them have the ability to stimulate a catecholamine surge [42,47,73,99]. The catecholamine surge induced by these stimuli is far in excess of the level required to induce PEPCK expression in cultured cells [72], yet all in vivo experiment, designed to elicit induction of gene expression have failed without exception.

Much effort has been expanded to elucidate the nature of CPTase and the pathophysiology of energy depletion in cardiac failure. Despite the intense effort, both of these fields are still full of unknowns. Despite all the promising hypotheses on energy deficiency and heart failure, no conclusive evidence has been found to support them. There is, however, a bright spot in CPTase research. The CPT I sequence, recently cloned by McGarry [32], may finally provide an adequate tool to advance physiology research.

With the limited means at hand, two aspect of CPTase regulation were studied. The perinatal period is chosen because of the flurry of inductive activities. It is the most ideal period in life to unlock the secret of cardiac CPTase regulation. The failing heart is selected to investigate the relationship between CPTase expression and abnormalities in cardiac energy metabolism.

<u>MATERIAL AND METHODS</u>

Time Bred Sprague-Dawley rats were purchased from Bantin & Kingman. The pregnant rats were housed in separate cages in the Department of Animal Care and allowed access to water and feed pellets ad lib. Rats were anesthetized with halothane vapor and rat hearts were collected from the following age groups: fetuses of 20 days gestation, newborns of 1, 5, 10, 15 days of age, and adults. The tissues were immediately frozen in liquid nitrogen and stored at -80°C.

Western Blot Protein Quantification

<u>Isolation of heart mitochondria.</u> Heart mitochondria were isolated using a modified differential centrifugation methods of Clarke and Bieber [9,28]. Rat hearts were weighed and processed in 8 ml ice-cold Buffer A per gram of tissue. The tissue were homogenized on ice for 30 seconds using a polytron grinder. All subsequent procedures were performed at 4°C. The homogenate was then centrifuged at 500 x g for 15 minutes, the supernatant was transferred to another tube and centrifuged at 15,000 x g for 15 minutes. The pellet was resuspended in the original volume of Buffer B and centrifuged again at 500 x g for 15 minutes to remove large debris. The supernatant was transferred to a clean tube and centrifuged at 11,000 x g for 15 minutes. The pellet was resuspended in the same volume of Buffer B and centrifuged again at 7,000 x g for 15 minutes. The final pellet

containing purified mitochondria was resuspended in 0.5 ml
ice-cold Buffer B per gram of tissue and stored at -80°C.
Buffer A: 0.25 M sucrose, 5 mM HEPES, 0.25 mM EDTA, pH 7.7.
Buffer B: 130 mM KCl, 20 mM Tris-HCl, 1 mM EDTA pH 7.5.
Protein determination. The protein concentration was
determined using a DC Protein Assay from Bio-rad. It is a
modified Lowry method based on colorimetric reactions
between protein, alkaline copper tartrate and Folin reagent
[59]. Bovine serum albumin (BSA) was used as a standard. A
1.39 mg/ml BSA solution was prepared and a serial dilution
of this was made to produce a standard curve.

Fifty μl mitochondrial suspension and 50 μl distilled
water were pipetted into a clean tube. Then 500 μl of the
alkaline copper tartrate solution was added to the
mitochondrial sample and briefly mixed. Four ml of the
Folin reagent was added to the tube and vortexed
immediately. The final mixture was allowed to react at room
temperature for 20 minutes and the absorbance read at 750
nm. A standard BSA sample was always included for each
mitochondrial protein determination. The color change was
less than 5% per hour after 15 minutes of development. The
protein concentration of each sample was calculated from its
absorbance value, based on the standard curve.
Protein denaturation. A small aliquot of "quench" solution
was added to the mitochondrial suspension. The "quench"
solution contained 6% SDS, 0.24 M dithiothreitol, 20% (w/v)

sucrose and a trace amount of a marking dye bromophenol
blue. To ensure complete reduction of the disulfide bonds
and 100% SDS binding, the final SDS/protein ratio was always
greater than 3:1 and the final dithiothreitol concentration
greater than 40 mM. The mixture was then heated in boiling
water for 10 minutes to achieve complete protein
denaturation. The protein concentration of the denatured
sample was recalculated taking into account the dilution due
to the addition of the "quench" solution.

SDS-Page gel. A 7.5% gel SDS denaturing gel was poured
according to the method described by Laemmli [11]. The 38%
acrylamide: bisacrylamide (37.5 : 0.5) stock was mixed with
an adequate amount of Tris-Glycine buffer and SDS to yield
the desired 7.5% concentration [11]. After degassing the
mixture, ammonium persulfate and TEMED was added to initiate
polymerization. The gel mixture was quickly poured into the
1 mm thick gel mold and a small volume of 0.1% SDS overlay
was added to ensure a smooth, flat meniscus. The gel was
allowed to set at room temperature for 30 minutes. After
polymerization of the separating gel is complete, a
stacking gel was poured over the separating gel and a 10-
well teflon comb inserted to form sample wells.

After assembling the gel apparatus the gel itself was
placed between the upper and lower buffer chambers
containing "running buffer", so that the gel became the
only conductor between the two chambers. The running buffer

consisted of 50 mM Tris-Base, 0.4 M glycine and 0.1% SDS, pH 7-9. A protein sample of 5-20 μg was loaded into each well. A current of 25 mA per plate was applied, the gel run at room temperature for 2 hours (with water cooling the inner core to prevent overheating).

Blotting. When the gel electrophoresis was complete, the gel was carefully peeled from the glass plates and washed for 15 minutes in blotting buffer to remove the salt and detergent. Blotting buffer consisted of 25 mM Tris-Base, 192 mM glycine, 20 % (v/v) methanol, pH 8.3, prechilled to 4°C.

The gel was transferred onto a piece of thick filter paper saturated with blotting buffer. A sheet of presoaked nitrocellulose membrane was placed on top of the gel and trapped air bubbles were removed. Another thick filter paper was placed on top of the membrane and the whole sandwich placed between two fiber supporting pads in the gel holder. The gel holder was placed in the buffer tank so that the gel faced the cathode and the nitrocellulose membrane faced the anode. The tank was filled with cold blotting buffer and an ice pack. Constant voltage was applied to transfer the protein from the gel to the nitrocellulose membrane (100V, 250-300 mA). Transfer was complete in 1 hour at room temperature without overheating.

Protein Detection. Total protein was determined by staining with the Coomassie Brilliant Blue method [11]. The gel was

removed from the glass plates and immersed in a staining solution consisting of 50% (v/v) methanol, 10% (v/v) acetic acid, 40% (v/v) water and 0.1% Coomassie Brilliant Blue for 1 hour at room temperature. The gel was then rinsed and destained overnight in 10% acetic acid.

CPT protein was detected using the Bio-rad alkaline phosphatase immun-blot assay (with or without amplification). The first antibody (RK40 rabbit anti-rat polyclonal antibodies) was a generous gift from Dr. McGarry, University of Texas Southwestern Medical Center [108]. All procedures were performed at room temperature. The first antibody solution was consisted of RK40 diluted 1/5,000 in Tween-20, Tris-buffered solution (TTBS). The second antibody solution was 1/3,000 diluted alkaline phosphatase/goat anti-rabbit antibody conjugate (Bio-rad) in TTBs.

After the protein was transblotted onto the nitrocellulose membrane, the membrane was removed from the apparatus and placed in a clean Petri dish. The blot was blocked with fat-free dry milk protein reconstituted in TTBS for 1-2 hours at room temperature on a shaking platform. After blocking, the blot was washed twice for 5 minutes each in TTBS. Then, the blot was incubated in the first antibody solution overnight with gentle agitation, then the second antibody solution was added to the blot after washing the blot for 5 minutes twice in TTBS. After 1-2 hours of

shaking incubation, the blot was again washed 5 minutes each for three times in TTBS. If the amplified method was used, the membrane was incubated with streptavidin - biotinylated alkaline phosphatase for 1 hour and washed again; otherwise, I proceed directly to the color development.

The color developing reagent, which contains 5-bromo-4-chloro-3-indolyl phosphate and nitroblue tetrazolium, was mixed immediately before use and added to the washed blot. The purple color appears immediately and the blot was allowed to develop up to 30 minutes undisturbed for optimum results. The blot was then washed in deionized water to rinse off the reagents. The color on the blot is stable at this point and can be photographed or used in densitometry measurements.

Ribonuclease Protection Assay (RPA)

RPA offers several benefits in mRNA detection compared to Northern hybridization methods. It is much more sensitive and eliminates the need to extract mRNA, especially for tissues with low copy numbers of mRNA. It has a high resolution so that it is possible to separate mRNA species differing by only a few nucleotides in size. The RPA is based on the theory that double stranded RNA will not be digested in the presence of specific ribonucleases. Therefore the portion of the mRNA that is complementary to the probe will be protected by a radiolabeled strand of antisense RNA generated from a cDNA template. The RNA probe

is called a riboprobe. Riboprobes are short, usually around 200-300 nucleotides, and therefore do not require a full length mRNA. The disadvantage of RPA is that it is more difficult to do and is more labor intensive. It also requires a perfect match between the probe and mRNA, and therefore may not be suitable for hybridizations between different species.

RNA extraction. Total RNA is extracted using the acid phenol method developed by Chomczynski and Sacchi [25].

Solution D: 4 M guanidinium thiocyanate, 25 mM sodium citrate, 0.5% sarcosyl, pH 7.0. 0.1 M 2-mercaptoethanol is added immediately before use.

The standard protocol is for 100 mg of tissue. It can be proportionally expanded to extract RNA from larger quantities of tissue, if needed.

Frozen tissue was weighed and immediately homogenized in 1 ml of solution D using a polytron homogenizer. 100 μl of 2 M sodium acetate pH 4.0, 1 ml of water saturated phenol and 200 μl of chloroform-isoamyl alcohol (49:1) were added with thorough mixing between each addition. The above steps were all performed at room temperature. The mixture was then vortexed vigorously for 10 seconds and cooled on ice for 15 minutes.

The following steps were all performed at 4°C. The samples were centrifuged at 10,000 x g for 20 minutes. The aqueous phase containing RNA was carefully transferred to a

clean tube with pipets and mixed with 1 ml of isopropanol.
The RNA/isopropanol mixture was placed in a -20°C freezer
for a minimum of 1 hour to facilitate crystal formation.
The sample was centrifuged at 10,000 x g to precipitate RNA.
The pellet was dissolved in 300 μl solution D and
precipitated with equal volume of isopropanol at -20°C for 1
hour. The sample was centrifuged again at 10,000 x g for 10
minutes to collect the RNA and the pellet washed in 75%
ethanol. The final pellet was resuspended in 50 μl of 0.5%
SDS and incubated at 65°C for 10 minutes. At this point the
RNA is ready to be used for Northern hybridization or
ribonuclease protection assay.

cDNA Reconstruction. A CPT II.4 cDNA, cloned into a
pBluescript KS+, was a generous gift from Dr. McGarry [108].
The 2.5 kb cDNA, however, was too long to produce a useful
probe for RPA. In addition, the first 300 nucleotides
downstream from the T7 promoter is a non-transcribed portion
that does not have matching sequences in the mRNA. These
problems were discovered after much effort in getting the
RPA to work. To eliminate these problems, the CPT II.4 cDNA
was reconstructed to obtain a shorter sequence from the
middle of the original cDNA.

The fragment with the highest homology between human
and rat is from 450-900 bp so it was selected for the probe
[34,108]. CPT II.4 was cut with Sac I which cleaves the cDNA
into several pieces but leaves the 0-900 bp fragment intact.

The pBluscript containing the 0-900 bp fragment was extracted from the gel and ligated using T4 ligase. The newly ligated plasmids were used to transform E. Coli strain JM101. The successfully transformed bacteria were selected on LB agar with 50 μg/ml ampicillin and verified to have a linearized cDNA of the correct length. This plasmid was named CPT II.6.

To remove the fragment from 0-450 bp, CPT II.6 was cut with Xho I and the above procedure repeated. The final plasmid yielded the desired cDNA which is short and has the best homology with the human DNA sequence. This plasmid was named CPT II.7.

RNA Probe Synthesis. Anti-sense CPT II RNA probe was synthesized from the CPT II.7 cDNA template using T7 RNA polymerase. The plasmid was linearized by restriction enzyme Xho I. Anti-sense cyclophilin RNA probe was synthesized from a cyclophilin cDNA template containing 140 nucleotides. This cDNA was a PCR fragment subcloned into the plasmid pGem5zf. The plasmid is linearized by the restriction enzyme Apa I. The 3' overhangs generated from Apa I cut were removed by a Klenow enzyme. SP6 RNA polymerase is used to generate the RNA probe.

The MAXIscript kit from Ambion Inc. was used for all RNA probe synthesis. After linearization, the templates were extracted with phenol/chloroform and precipitated in ammonium acetate/ethanol.

The reaction mixture contains:

    1 μg of linearized template DNA

    1 μl 200 mM DTT

    1 μl 10 mM ATP

    1 μl 10 mM CTP

    1 μl 10 mM GTP

    2 μl 10x transcription buffer

    1 μl RNase inhibitor

    5 μl ^{32}P-labeled UTP

    6 μl 100 uM UTP

    1 μl RNA polymerase

The mixture was incubated at room temperature for 2-3 hours and cDNA template was removed with DNase. Unincorporated ribonucleotides are removed by first extracting with phenol/chloroform, then passed through a Bio-rad spin column. The cut-off size of the column was 30 bp.

The probes were further purified on a 8 M urea 5% polyacrylamide gel. After an one and a half hour run at 200 V, the gel is used to expose an X-ray film for 1 minute. The X-ray film was later used as the template to excise full length probes. The probes that are less than full length usually are only a small fraction of the total probe.

The gel purified probe was eluded from the gel overnight in probe elution buffer (0.5 M ammonium acetate/1 mM EDTA/0.2% SDS). The final RNA probes were stored at -80°C.

RPA protocol. Hybridization was carried out in solution of rat heart total RNA and labeled probe, 5-20 μg total RNA were used for solution hybridization. 200-600 pg of labeled probes (50,000-100,000 cpm) was used for each RNA sample. The probes were added to the RNA sample and the mixture precipitated in 0.5 M ammonium acetate/ethanol. The pellet was collected by centrifugation and resuspended in 30 μl hybridization buffer (80% formamide, 40 mM PIPES pH 6.4, 0.4 M NaCl, 1 mM EDTA). After heating to 90°C, the hybridization mixture was incubated at 42°C overnight.

On the next morning, a stock solution of RNase is diluted 1/100. The RNase activity in the stock solution is RNase A 250 U/ml and RNase T1 10,000 U/ml. This combination gives the most complete single strand digestion. The dilution is made with the ribonuclease digestion buffer (10 mM Tris-HCl, 300 mM NaCl, 5 mM EDTA). Three hundred fifty μl RNase digestion mixture was added to each sample and the digestion was complete in one hour at 37°C.

Two and one-half μl Proteinase K (20 mg/ml) and 10 μl of 20% SDS were added to each tube and incubated at 37°C for 15 minutes. The digested mixture was then extracted with phenol/chloroform and precipitated with 0.5 M ammonium acetate.

The final pellet was resuspended in 5 μl of 80% formamide loading buffer and run on 8 M urea 5% polyacrylamide gel at 200 V constant voltage. The

separation usually took about two hours. The gel was carefully transferred onto a filter paper and dried under vacuum at 80°C for two hours. Autoradiography was performed to yield a permanent record.

To test the specificity of the probe, yeast total RNA was used as control in a separate solution hybridization. No protected bands were seen on autoradiography. This indicates that the riboprobe is highly specific and does not form a double strand with yeast RNA.

An RNA sense strand was synthesized to be used as a positive control and to determine the optimum linear density range for densitometer analysis of autoradiograph.

Probes unprotected by mRNA were used to determine the efficiency of RNase and to determine the optimum digestion conditions. To avoid probe loss during the process, tRNA was always used as a carrier.

Northern Hybridization

DNA Probe Synthesis. DNA probes were synthesized using CPT II.4 as a template. The cDNA insert was cleaved from the plasmid using restriction enzymes Xba I and Bam HI. The insert is separated from the rest of the plasmid on 1% agarose gel. The band with the correct length was excised from the gel and extracted using the Qiaex extraction kit from Qiagen. The probes are extracted with phenol/ chloroform and precipitated in sodium acetate/ethanol. The

final pellet was resuspended in TE buffer and stored at -80°C for future use.

On the day of northern hybridization, DNA probes were synthesized using the random primer method which builds a homologous strand using radiolabeled nucleotides. The reagents were purchased from Boehringer Mannheim Biochemicals.

Reaction mixture:

1 μg CPT II.4 cDNA (denatured at 95°C, then cooled on ice)

1 μl 0.5 mM dATP

1 μl 0.5 mM dGTP

1 μl 0.5 mM dTTP

2 μl 10x reaction buffer

5 μl ^{32}P labeled dCTP

1 μl Klenow enzyme

Water was added to bring the final volume to 20 μl; the mixture was incubated at 37°C for 1-2 hours. Unincorporated nucleotides were removed by passing the reaction mixture through a Bio-rad spin column. Cleaned probes were used directly for Northern hybridization.

Northern Protocol. cDNA probes were tested first on Northern blots to ensure that the excised cDNA insert was indeed CPT II.4. Rat liver total RNA was used for this hybridization.

50-100 µg of rat liver total RNA are loaded onto a formaldehyde agarose gel after heat denaturation at 95°C for 10 minutes in 80% formamide loading buffer. The 1.2% gel was run at 20 V constant current overnight in 1x MOPS buffer at room temperature.

On the next morning, the gel was removed from the gel tray and photographed. A paper towel blotting method was used overnight to transfer the RNA bands to Genescreen nylon membrane. When the blotting was complete, the nylon membrane was exposed to UV light for 3 minutes to cross-link the RNA with the membrane.

The cross-linked blot was prehybridized for at least 4 hours at the desired temperature (usually at 42°C). Radiolabeled probes were added to the blot in a hybridization solution. In my experience, the best results are obtained when the blots were hybridized overnight. The hybridized blot was then washed vigorously in 1x SSC with 0.1% SDS to remove excess probe. Autoradiographs were made from the finished blot.

Infarcted Rat Hearts

Tissue from failing rat heart were generously provided by Barry Greenberg, M.D. Cardiology, Oregon Health Sciences University. These rats underwent left anterior descending artery ligation approximately 3 months prior to sacrifice. Four animals were given various medications for 16-32 days

in Dr. Greenberg's drug treatment experiment. Two control animals without drug treatment were available. One animal had a sham operation but did not have infarction. The other one had infartction but did not receive drug treatment.

Five animals (four experimental, one control) had an infarction of the left ventricle and the infarct size was approximately 35% of the left ventricle free wall. The hearts were frozen in liquid nitrogen within 3-5 minutes after sacrifice.

Pieces of myocardium from right and left ventricles were carefully removed from the non-infarcted area outside of the infarct zone. Total RNA and mitochondria are extracted for analysis.

Data analysis

Prior to using RPA for routine detection of CPT II mRNA, a series of experiments were carried out to determine the optimum condition of the assay. First, a fixed amount of probe was used with increasing RNA samples. The minimum amount of total RNA required to generate a measurable signal and the linear range of the assay were determined. Then, the total RNA was held constant and the amount of probe was varied over a wide range of concentrations. This ensured that there was a considerable excess of probes relative to the CPT II mRNA species.

Cyclophilin, a housekeeping gene with relatively

constant expression, was used as an internal standard. CPT II mRNA was expressed as a ratio of the density of CPT II/cyclophilin bands. This methods minimized the potential sample variation associated with RNA degradation.

CPT II mRNA expression in the developmental series was first measured as the CPT II/cyclophilin ratio then expressed as a percentage of the adult level because the absolute values from densitometry vary considerably due to interday variations in probe activity and exposure time. Three different sets of rat hearts were used to ensure that the changes that I observed were not due to a single sample bias. The mean and the standard deviation were calculated for each age group. The analysis of variance method was used to determine the statistical significance between the means of each group.

CPT II protein concentration in the developmental series was measured as the density of the protein band and expressed as a percentage of the adult level. These samples were already pooled because a single heart did not have enough protein for Western Blot analysis. Thus, these protein measurements already represented a "mean" value.

CPT II mRNA expression in the MI study was determined as a CPT II/cyclophilin ratio. The ratio was directly used in statistical analysis to calculated the mean and the standard deviation. An unpaired t-test was used to determine statistical significance.

RESULTS

The first outcome of my project was the reconstruction of a cDNA probe. This probe has not been used previously in this laboratory. Several steps have been taken to ensure that the new cDNA probe is correctly reconstructed.

Verification of CPT II.4 probe. CPT II.4 cDNA was a generous gift from Dr. McGarry [108]. For various reasons, cDNA acquired from other laboratories were not always the correct ones (I have received useless probes that cost me considerable amounts of time). It is important to verify the identity of the probe prior to laboratory application. According to the original work by McGarry, his full length probe was 2.3 kb long and could be excised from the plasmid by using restriction enzymes Xba I and Bam HI. The cDNA template was excised from the plasmid and used to direct DNA probe synthesis as described in the methods section. The radiolabeled probe was used in a Northern hybridization to verify that the probe was complementary to only one 2.3 kb mRNA species in the total liver RNA. The result was shown in Fig 8. The probe detected a single band on the autoradiograph and the mRNA species had the expected size. The faint band also indicated that the copy numbers of CPT II mRNA were very low as was shown in McGarry's original work on rat liver [108].

CPT II cDNA reconstruction. Due to reasons described in the methods section, a shorter cDNA is needed as the

<u>RESULTS</u>

The first outcome of my project was the reconstruction of a cDNA probe. This probe has not been used previously in this laboratory. Several steps have been taken to ensure that the new cDNA probe was correctly reconstructed.

<u>Verification of CPT II.4 probe</u>. CPT II.4 cDNA was a generous gift from Dr. McGarry [108]. For various reasons, cDNA acquired from other laboratories were not always the correct ones (I have received useless probes that cost me considerable amounts of time). It is important to verify the identity of the probe prior to laboratory application. According to the original work by McGarry, his full length probe was 2.3 kb long and could be excised from the plasmid by using restriction enzymes Xba I and Bam HI. The cDNA template was excised from the plasmid and used to direct DNA probe synthesis as described in the methods section. The radiolabeled probe was used in a Northern hybridization to verify that the probe was complementary to only one 2.3 kb mRNA species in the total liver RNA. The result was shown in Fig 8. The probe detected a single band on the autoradiograph and the mRNA species had the expected size. The faint band also indicated that the copy numbers of CPT II mRNA were very low as was shown in McGarry's original work on rat liver [108].

<u>CPT II cDNA reconstruction</u>. Due to reasons described in the methods section, a shorter cDNA is needed as the
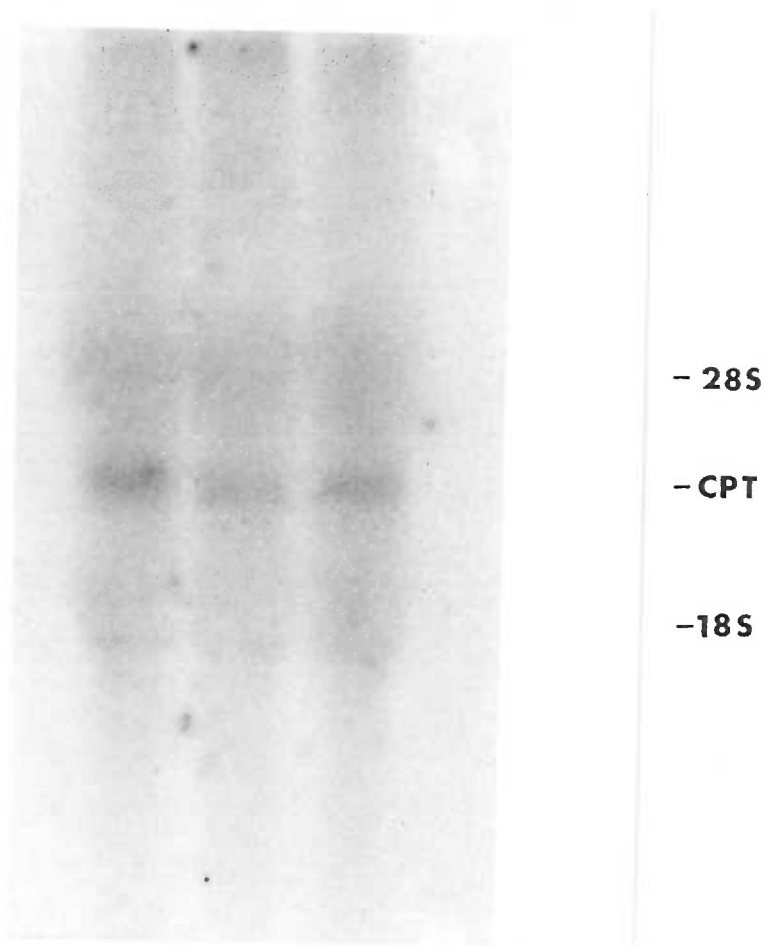
− 28S

− CPT

−18S

Fig. 8. Probe verification on Northern blot. All three lanes contain 100 µg of rat liver total RNA. The Northern blot was probed with ^{32}P-labeled DNA probes synthesized from CPT II.4 cDNA template. The cDNA template was excised from pBluescript KS+ using restriction enzyme Xba I and Bam HI as describe in the methods section. The faint band is 2,300 bp long and is the correct size for CPT II mRNA. This indicates that the copy numbers of CPT II are very low in the rat. Therefore, I used RPA to detect CPT II mRNA because it is 10-100 times more sensitive.

template for riboprobe synthesis. CPT II.4 was first cut with Sac I and the result is shown in Fig 9. The large bands shown in lanes 2-4, contain the plasmid and the truncated cDNA (0-900 bp), were extracted from the agarose gel and ligated by T4 ligase to generate CPT II.6. CPT II.6 plasmids obtained from bacterial culture on ampicillin-LB agar, were again cut with Xho I to verify the composition of the probe. The DNA fragments were separated on the agarose gel as shown in Fig 10. The large DNA band in lane 1 was approximately 3,400 bp and the small band was about 500 bp. Both DNA fragments had the predicted lengths. The large band was again extracted and ligated to generate CPT II.7. This plasmid contained the central region (450-900 bp) of the original cDNA and was used in riboprobe synthesis.

Developmental CPTase mRNA expression. A CPT II deficiency was not expected in the fetal or newborn hearts according to historical data [100,101]. My experimental results differed from those of the past. The autoradiograph of an RPA in Fig. 11 shows a developmental sequence of rat heart CPT II mRNA expression. The density of the bands were roughly proportional to the amount of mRNA hybridized by the radiolabeled probes. In order to ensure that changes in density were not merely due to uneven loading, a cyclophilin probe was included in each sample. Cyclophilin is a "housekeeping" gene with relatively constant expression. Therefore, it is suitable as an internal standard. The CPT
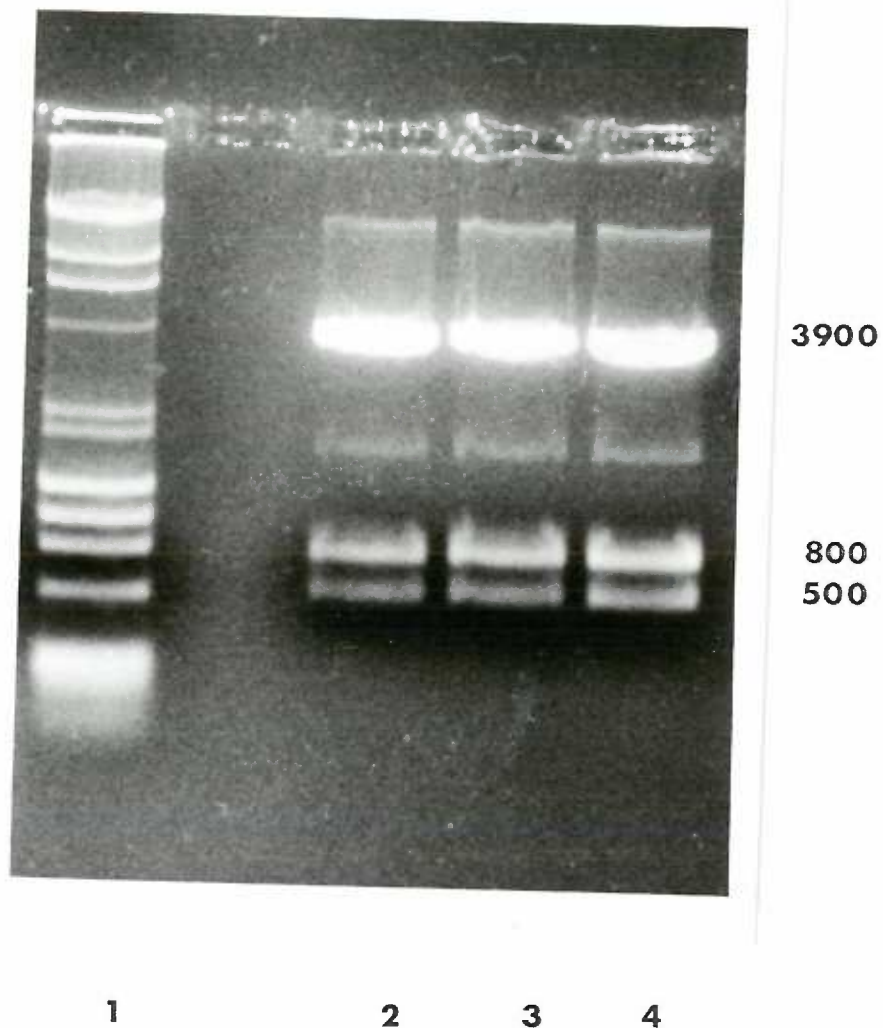
3900

800
500

1   2   3   4

Fig. 9. cDNA reconstruction. CPT II.4 was cut with Sac I which removes 2 fragments from the cDNA (900-1,700 and 1,700-2200 bp). The fragments are shown as the two lower bands in lanes 2-4. Lane 1 is a DNA standard. The plasmid and the remaining cDNA is represented by the 3,900 bp fragment which was extracted and ligated by T4 ligase to yield CPT II.6.
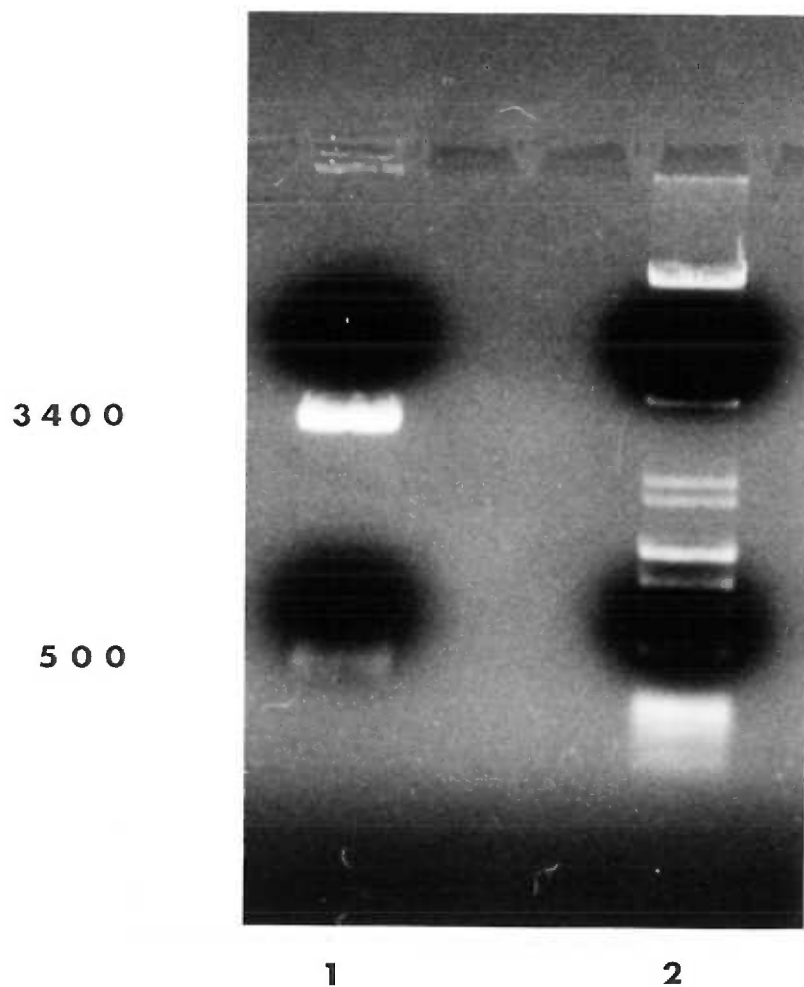
Fig. 10. cDNA reconstruction. CPT II.6 was cut with Xho I which removes a fragment from the cDNA (0-480 bp). This fragment is shown as the lower band in lane 1. Lane 2 is a DNA standard. The plasmid and the remaining cDNA is represented by the 3,400 bp fragment which was extracted and ligated by T4 ligase to yield CPT II.7.

II densities were determined by densitometry and expressed as CPT II/cyclophilin ratio relative to the adult level (see Fig. 12). These data clearly demonstrate a developmental increase in CPT II and a postnatal surge in CPT II mRNA levels. Although there is an increase in mRNA expression in less than 12 hours after birth, newborn cardiac CPTase expression is still far below adult levels. After the first week of life, the mRNA expression resumes and continues to increase until it reaches adult levels. This developmental pattern is seen in different sets of rat hearts (n=3).

Developmental CPTase protein expression. Fig 13 and 14 are Western blots probed with RK40 antibody. An enhanced method using biotinylated alkaline phosphatase is shown in Fig. 13. This method detected more than one protein band. Non-immunized rabbit serum was used as a control to identify non-specific antibody binding (data not shown). A CPT II protein band was identified by comparing the RK40 blot with control blots. Although this method is 20 times more sensitive than the standard alkaline phosphatase method and was thought to be necessary to detect the low CPTase protein, the extra bands were indeed a nuisance. Experimenting with different components used in the enhanced detection method, I found that a biotinylated alkaline phosphatase conjugate had a tendency to adhere to certain proteins. These bands represent non-specific binding. They are not caused by endogenous alkaline phosphatase either.
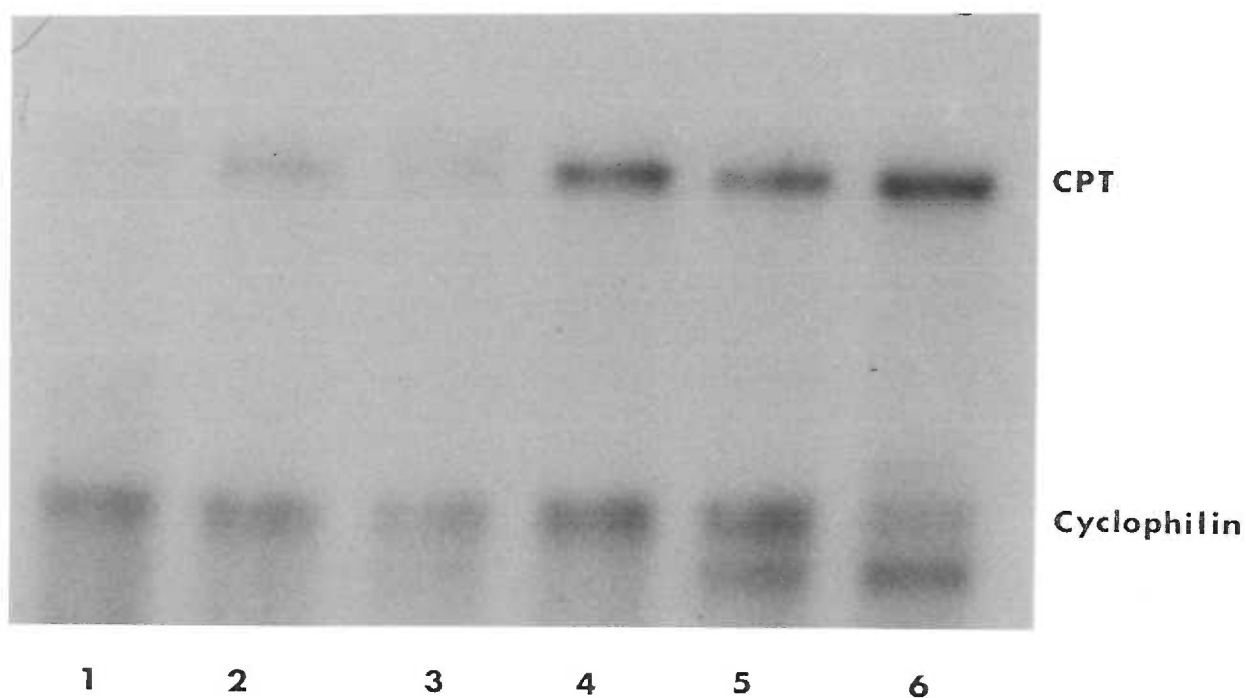
CPT

Cyclophilin

1   2   3   4   5   6

Fig. 11. An RPA gel used to show developmental CPT II mRNA expression. Each lane contains 20 μg of total rat heart RNA. Lane (1) fetal, (2) newborn (<12 hours), (3) 5 day, (4) 10 day, (5) 15 day and (6) adult rats. Each sample was probed with CPT II and cyclophilin RNA probes. The complementary binding between CPT II mRNA and the probe forms a segment of double stranded RNA which is protected from ribonuclease digestion. The protected CPT II mRNA is 250 bP in length. The cyclophilin probe is 140 bp long.
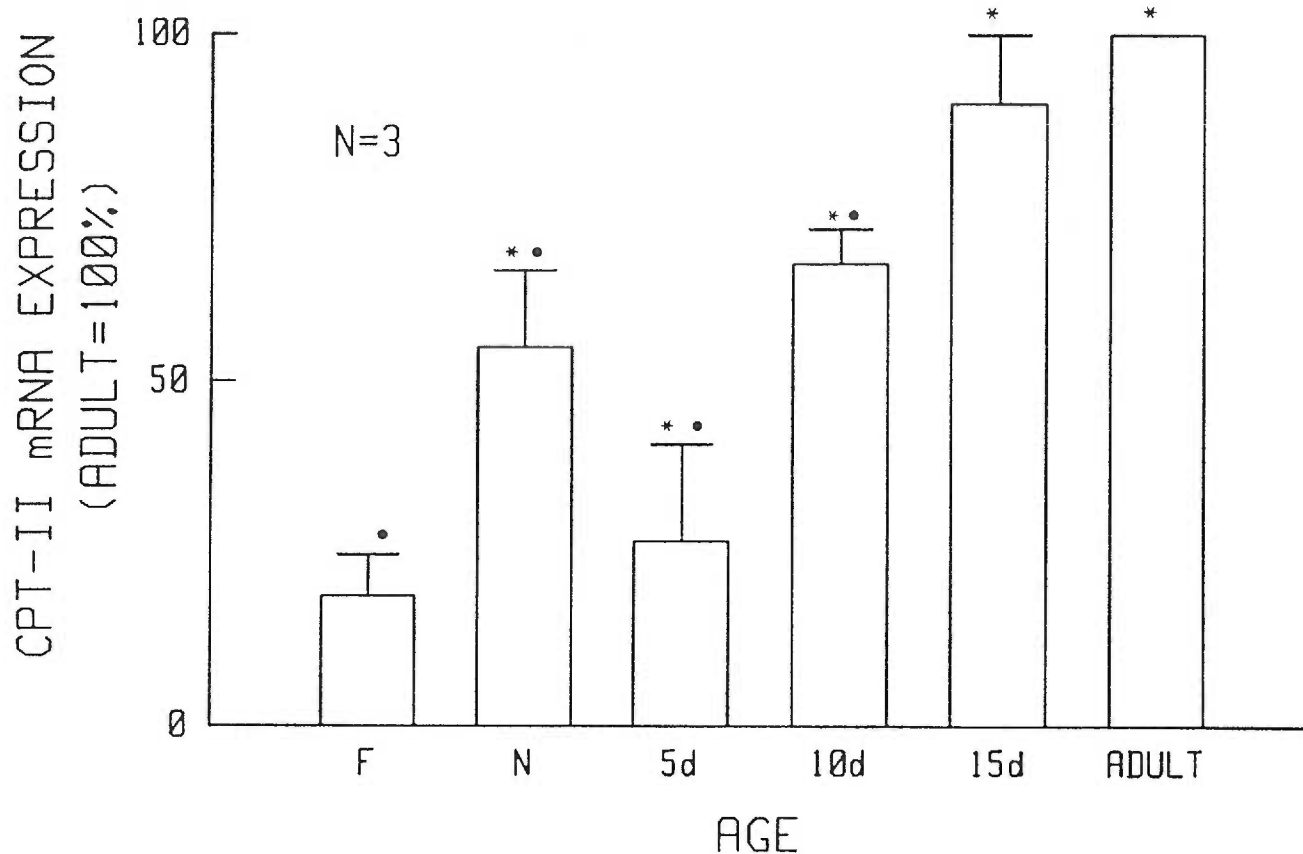
Fig. 12. Developmental time course of CPT II mRNA expression. The CPT II/cyclophilin ratio was determined by densitometry (CPT II mRNA levels are standardized using cyclophilin). The result is expressed relative to the adult value which is defined as 100%. These data were obtained from three different sets of rats. The values plotted were fetal 19 ± 6%, newborns 55 ± 11%, 5d 27 ± 14%, 10d 67 ± 5%, 15d 90 ± 10%. * significantly different from fetal value (p<0.05). • significantly different from adult value (p<0.05).

Since the standard alkaline phosphatase detection method does not use biotinylated compounds, I reasoned that it should produce cleaner blots if there were sufficient amounts of CPT II protein present.

The blot in Fig 14 was probed with RK40 and developed with standard alkaline phosphatase method. A single species of protein was detected. This blot, however, lacks a suitable molecular size marker that could be detected by the color developing reagent. To verify that this band is indeed the same protein detected by the enhanced method, a set of duplicate samples were loaded on a single SDS-Page gel. After electrophoresis and transblotting, the nitrocellulose blot was cut in half and incubated with RK40, CPT II protein on one of the blot was detected by standard alkaline phosphatase method and the other blot was detected using the enhanced method. The two halves were then rematched to located the size of the detected band (results not shown). In this way I avoided the uncertainty associated with the lack of size marker on the blot shown in Fig. 14. Although the fetal CPT II protein can be detected by either alkaline phosphatase methods, its concentration is very low. Quantities of total protein reaching the maximum loading capacity of the gel were needed to obtain the faint band in the fetal sample (lane 6) when the standard method was used. It is clear that CPT II protein levels increase with age except during the first week of life, consistent
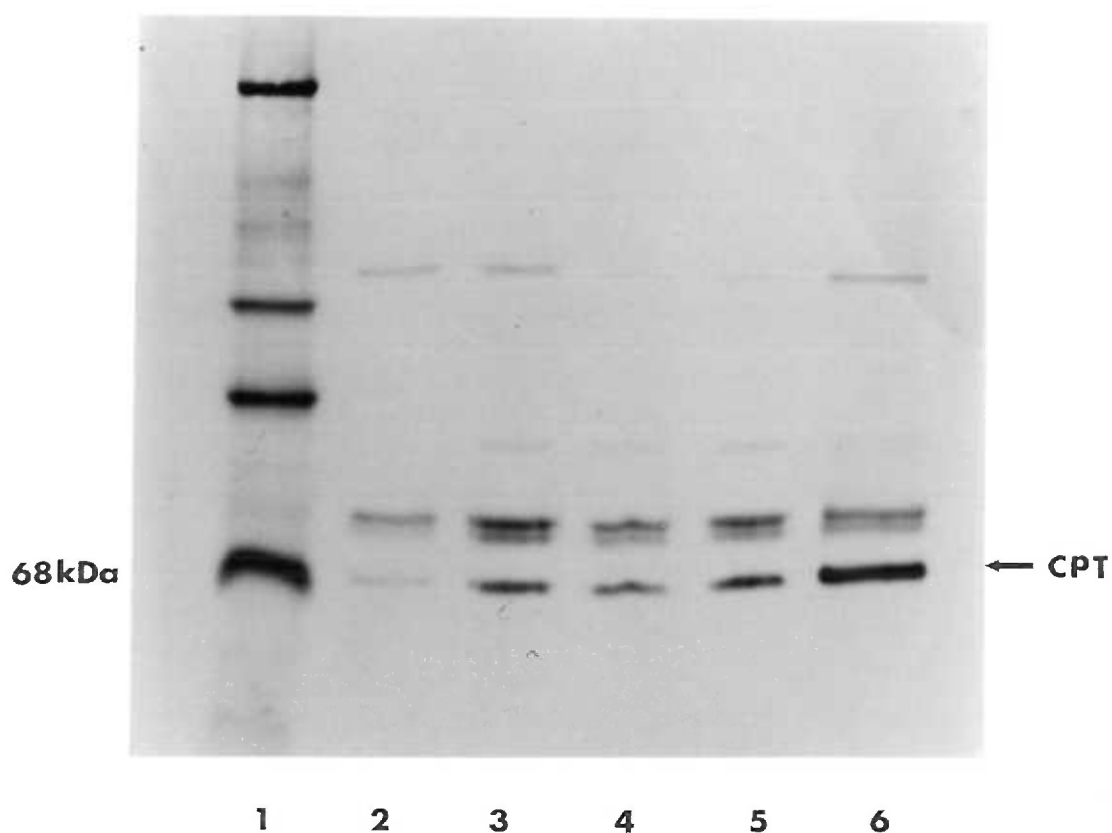
Fig. 13. Developmental expression of CPT II protein. Total rat heart mitochondrial protein was denatured in a "quench" solution containing SDS and dithiothreitol. Each lane contains 5 μg of total rat heart mitochondrial protein which is separated on 7.5% SDS-Page gel. The Western blot was probed with RK40 antibody as described in methods section. The 2nd antibody was a biotinylated goat anti-rabbit IgG. The CPT II protein band is indicated by the arrow and has a molecular weight of approximately 68 kDa. Other bands are from non-specific binding of biotinylated alkaline phosphatase and are not antibody related. lanes: (1) biotinylated protein standard, (2) fetal, (3) newborn (<12 hours), (4) 5 day, (5) 10 day and (6) adult rat hearts.
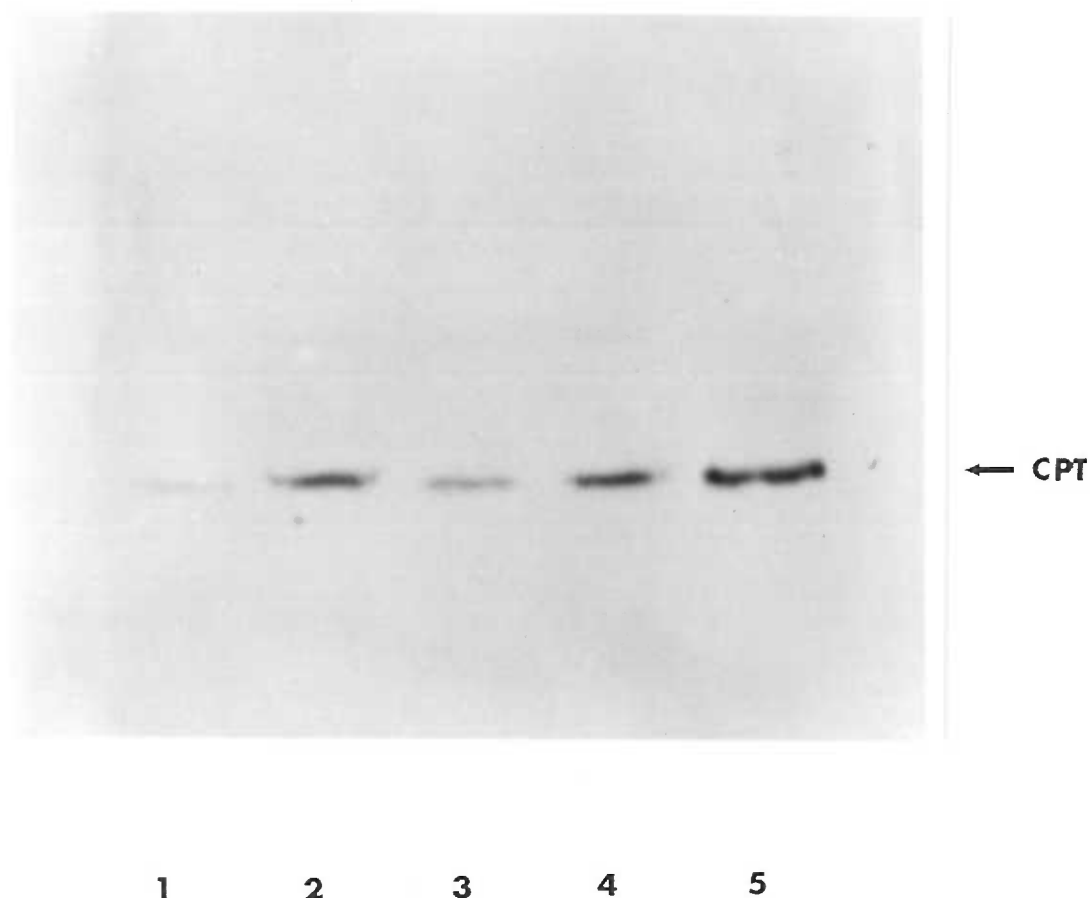
← CPT

1 2 3 4 5

Fig. 14. Developmental expression of CPT II protein. Denatured total rat heart mitochondrial protein is subjected to Western blot protein detection. Each lane contains 20 μg of total rat heart mitochondrial protein which is separated on 7.5% SDS-Page gel. The Western blot is probed with RK40 antibody as described in the methods section. The 2nd antibody was an alkaline phosphatase conjugated to goat anti-rabbit IgG. This detection method is less sensitive but it eliminates the non-specific binding seeing in Fig 13 in the biotinylated method. The CPT II protein band is indicated by the arrow and has a molecular weight of approximately 68 kDa. lanes: (1) fetal, (2)newborn (<12 hours), (3) 5 day, (4) 10 day and (5) adult rat hearts.

with the findings on mRNA expression. The relative
concentrations of CPT II protein was determined by
densitometry and plotted relative to adult levels which are
defined as 100 % in Fig 15. A total protein stain was
included in Fig 16 to show the specificity of RK40 antibody.

CPTase expression in MI rat hearts. I believe that
energy depletion is an important component in chronic heart
failure and hypothesize that CPTase is inhibited in failing
hearts because FFA oxidation in these hearts is known to be
impaired. The results of the myocardial infarction (MI)
studies are presented in Fig. 17 and 18. An autoradiograph
of a RPA gel is shown in Fig. 17. Rat heart mRNA was
extracted from 4 different rats (lanes 1-4), all of which
were determined to be in failure based on Dr. Greenberg's
data. Sham operated animals (n=14) have a mean left
ventricular end diastolic pressure (LVEDP) of 5.7 ± 1.2
mmHg; mean dP/dt 9,764 ± 281 mmHg/minute. For the MI group,
mean LVEDP is 15.1 ± 3.8 mmHg; mean dP/dt 3,000 ± 283 mmHg.
Three of the four animals were treated with ramipril, an
angiotensin converting enzyme (ACE) inhibitor. One was
treated with ramipril and HOE 140. HOE 140 is an experiment
al medication that antagonizes the bradykinin effect of the
angiotensin activation system and its chemical name is $_D$-
Arginyl-$_L$-arginyl-$_L$-prolyl-$_L$[(4R)-4-hydroxyprolyl-glycyl-$_L$-
[3-(2-thienyl)alanyl]-$_L$-seryl-$_D$-(1,2,3,4-tetrahydroiso-
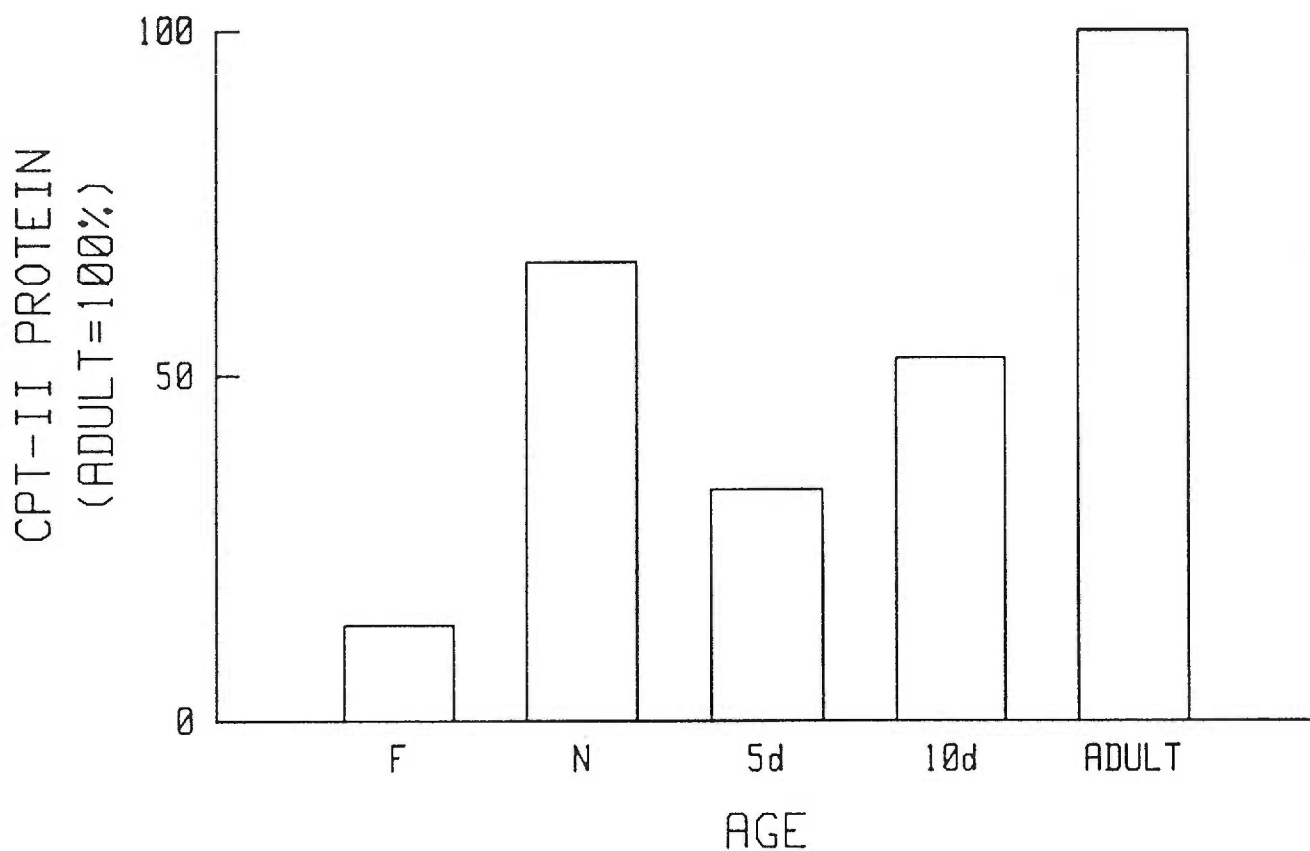quinolin-3-ylcarbonyl)-$_L$-[(3aS,7aS)-octahydroindol-2-

Fig. 15. Developmental time course of CPT II protein based on Western Blot densities. CPT II protein levels were determined by densitometry. The result is expressed relative to the adult value which is defined as 100% (Day 15 newborn was not measure due to the lack of protein).

−70k
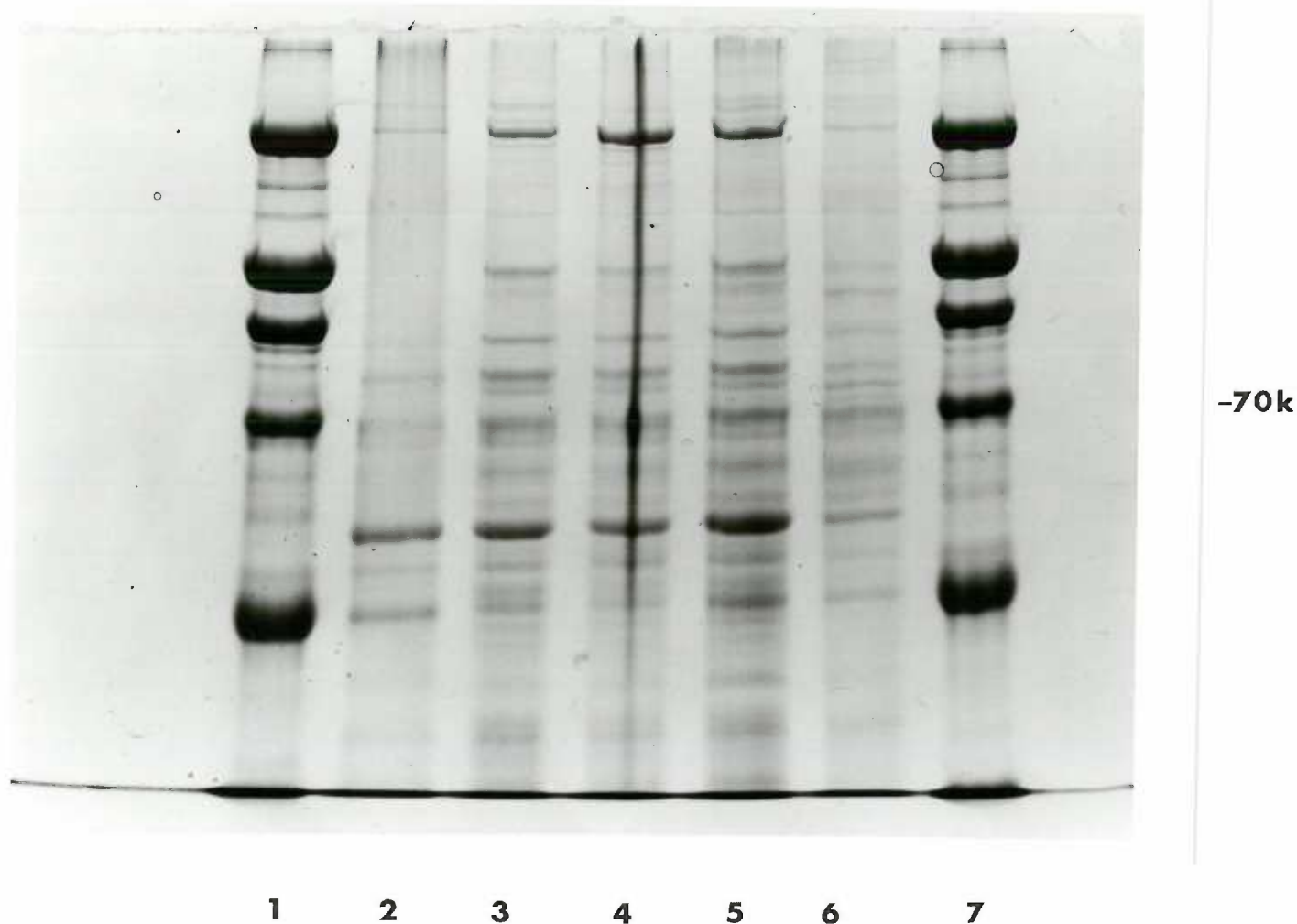
Fig. 16. Protein separation on 7.5% SDS-Page gel. Denatured total rat heart mitochondrial protein was separated on SDS-Page gel and stained by Coomassie Blue. Lanes 1 and 7 are the protein standard. Lanes: (2) adult, (3) 10 day, (4) 5 day, (6) fetus. Each lane contains 20 μg of total rat heart mitochondrial protein. This stained blot confirms that the 68 kDa protein has a very low concentration because it is not readily identifiable. This figure demonstrates the myriad of proteins among which CPT II had to be identified.

ylcarbonyl]-$_L$-arginine acetate. These drugs were administrated in Dr. Greenberg's original experimental protocol. Unfortunately, the effect of the drug on cardiac CPTase is not known and may complicate our data interpretation. One sham operated animal (without drug treatment) showed that mRNA level was 90% of the normal value; LVEDP was normal at 5.1 mmHg and dP/dt was moderately decreased to 5,300 mmHg/minute. One control animal (without drug treatment) was also available. The CPT II mRNA level was only 30% of normal value as were other MI hearts, LVEDP was 15.7 mmHg; dP/dt was 3,000 mmHg/ munite. Although more control data are needed, the present data lend support to my believe that the ACE inhibitors do not suppress CPTase expression. The results clearly demonstrated that CPT II mRNA expression in the failing rat hearts was severely depressed compared to the normal adult rat hearts (lane 5-7). The severity of depression in mRNA expression was nearly identical in all four MI hearts as shown in Fig 17. The CPT II mRNA density was standardized using cyclophilin as a standard and the CPT II/cyclophilin ratio was determined by densitometry. This ratio was used for statistical analysis. The ratio for the MI group was 0.42 ± 0.163, for the normal hearts 2.17 ± 0.435 (p<0.01). These result are plotted in Fig 18.
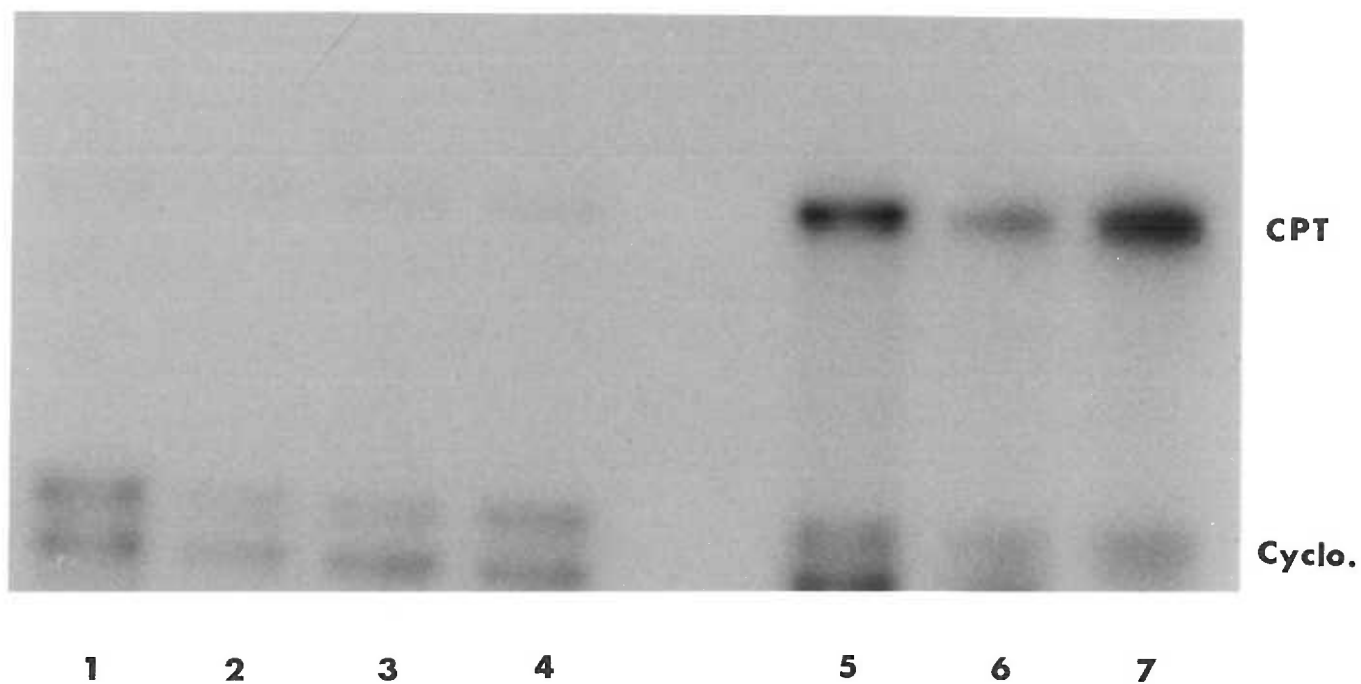
Fig. 17. CPT II mRNA expression in rat hearts with myocardial infarction. Each lane contains 20 μg of total rat heart RNA. Lanes 1-4 are RNA from 4 rats hearts all of which have an approximately 35% infarcted left ventricular free wall. Lanes 5-7: normal adult rat hearts. Each sample is probed with CPT II and cyclophilin RNA probes. The complementary binding between CPT II mRNA and the probe forms a segment of double strained RNA which is protected from ribonuclease digestion.

Fig. 18. CPT II mRNA expression in normal vs. post-myocardial infarction (MI) hearts. CPT II densities are determined by densitometry and CPT II mRNA is standardized using cyclophilin as denominator. The ratio for normal is 2.17 ± 0.435 (n=3); for MI is 0.42 ± 0.163 (n=4). * ($p < 0.01$).

DISCUSSION

Part I

Developmental Series

The importance of CPTase in controlling overall energy metabolism first became evident in the early 70's. It was found that CPTase helps transfer long chain FFA into 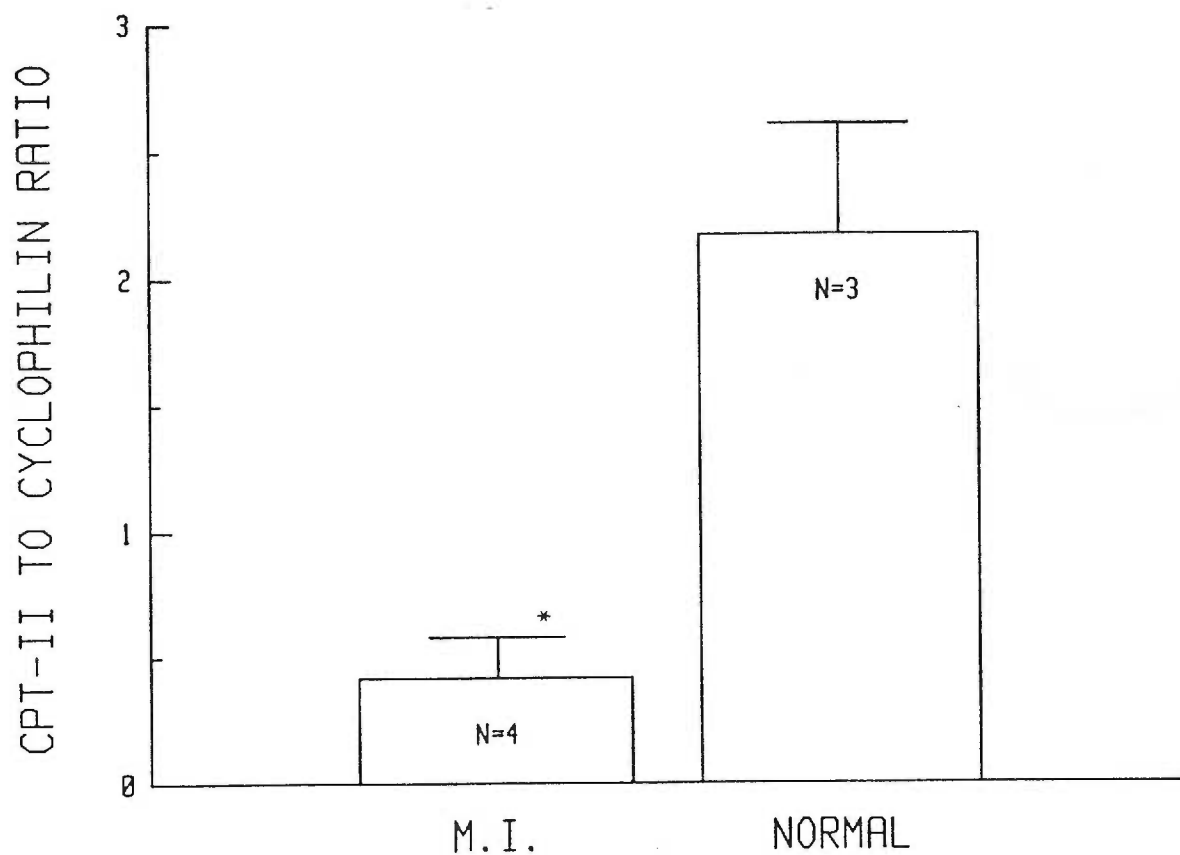mitochondria which are otherwise impermeable to such compounds [100]. CPTase was also linked to metabolic defects in human [30].

A detailed study on the development of rat energy metabolism was first published by Warshaw in 1972 [101]. As noted in his paper, CPTase activity is very low in fetal liver and heart. There is virtually no ability to oxidize palmitoyl-CoA in fetal rat heart. CPTase activity rises markedly during the early days of life. This change in CPTase activity correlates with a markedly increased ability to oxidize palmitoyl-CoA in the presence of carnitine.

The earliest fetal rat heart CPTase activity (17 day gestation) was measured by Warshaw [101]. CPTase activity was very low and remained so until birth. In the first day of life CPTase activity nearly doubled. The activity of CPTase continued to rise until 30 days of age when it reached the adult level (Fig 19).

Fetal, newborn and adult rat heart CPTase activities have been compared in rat as have those of many other species [36]. Low fetal cardiac CPTase activity is common.
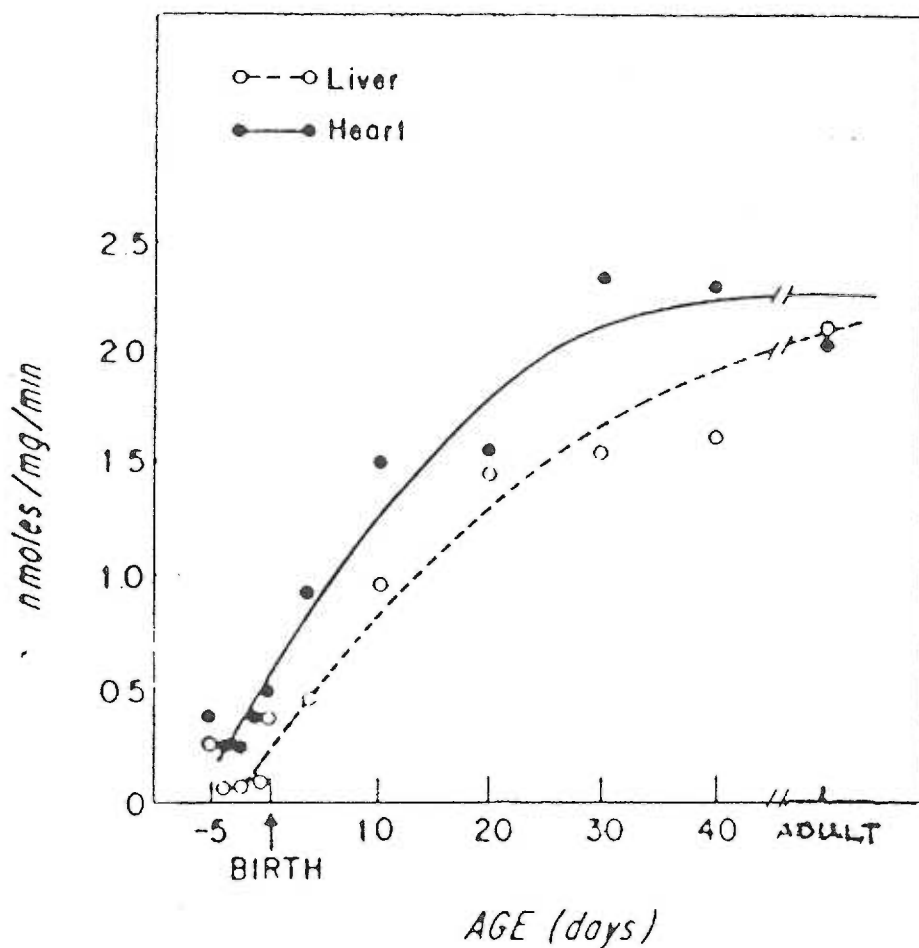
Fig. 19. Developmental changes in CPTase activity in rat. Heart and liver homogenates were used in these experiments. The reaction acyl-CoA + carnitine --> acyl-carnitine + CoA was measured using ^{14}C labeled palmitoyl-CoA. CPTase activities were low in both rat liver and heart. Low CPTase activity was associated with low levels of fatty acid oxidation. At birth, the CPTase activity increased rapidly and reached adult levels in a month. The dramatic change in CPTase activity was of considerable interest in research studying CPTase expression (Source: Warshaw JB. Developmental Biology Vol 28, p.540, 1972).

Whenever the enzyme activity appears to be low, it is either due to a depressed enzyme level or a normal level of enzyme which has, for various reasons, low activity. It is generally assumed that low fetal cardiac CPTase activity is due to enzyme deficiency, not the inhibition of enzyme activity.

This assumption, however, was not accepted by all. McMillin-Wood [64] treated neonatal rat heart mitochondria with phospholipase C and found that treated neonatal hearts had comparable CPTase activity as the adult heart though phospholipase C treatment had no effect on adult rat heart CPT. The neonatal heart mitochondria had 3-4 times higher palmitoyl-Co A hydrolase activity than adult mitochondria. This raised the possibility that the low neonatal heart CPTase activity might be due to a masking effect of palmitoyl-Co A hydrolase or other outer membrane protein. Phospholipase C can selectively destroy the outer mitochondrial membrane. So it was thought that treatment with phospholipase C might have removed the these interfering reactions.

Brosnan and Fritz [22] showed evidence that bovine fetal heart mitochondria oxidized palmitoyl-Co A in the presence of carnitine as well as calf and adult rat heart. At nearly the same time Warshaw [100] reported that fetal calf heart CPTase was considerably less active than in the young calf; furthermore, the addition of carnitine did not help. It had

been argued that if CPTase activity was latent in the heart and could be easily activated, then the low FFA oxidation might have to be explained by reasons other than CPTase deficiency [64].

These conflicting data were the source of considerable confusion. Part of the discrepancy might have been the result of the heterogeneous methods that were employed. Some experiments were conducted using tissue homogenates, and others used isolated mitochondria. We now know that CPTase activities are known to be markedly influenced by detergent, ionic strength, pH and mitochondrial integrity. Despite of all these controversies regarding CPTase activity, no direct quantification of enzyme levels and gene expression in fetal transition was ever reported.

Since the ability to oxidize FFA is nearly absent in fetal hearts but is present in newborn hearts, something must trigger this transition. Birth related events are the prime candidates because of their timing. One can further argue that it should be possible to identify the key stimulus if we could isolate and control the onset of each individual cpmponent.

With the advance in research technology, it is now feasible to test these ideas in fetal and newborn animals. Stress, lung inflation, oxygenation, alterations in temperature, energy source and thermogenesis are the primary candidates as "triggers". Over the last few years, many

attempts have been made to induce FFA oxidation in the fetus but none have succeeded. In a concentrated effort by Iwamoto and her colleagues [47], acutely instrumented near term fetal sheep underwent ventilation, oxygenation, and umbilical cord compression to simulate birth. Their typical experiment lasted about two hours and all three experimental manipulations were implemented. Oxygen consumption and substrate utilization were monitored regularly during the experiment. They too, failed to induce FFA oxidation despite an elaborate experimental design.

After many of these well thought-out but failed attempts to elicit FFA oxidation in fetus, one can not help but wonder if it is possible to induce fetal FFA oxidation. Although I am not studying the regulator of CPTase induction as part of the thesis, I do plan to investigate such "triggers" in the future. I still believe that this is the best opportunity to study induction of CPTAse in the life of an animal. Furthermore, the problem is of great physiological importance. Why did all these investigators fail to "turn on" gene expression? A closer examination of those acute experiments raises some concern as to how effective the investigators were able to monitor CPTase induction. In Iwamoto's protocol for example [47], the experiment lasted about two hours. This left only about 20-30 minutes or so to observe fetal responses to each experimental manipulation. The short time frame may

severely limit their ability to see a response after initiation of the stimulus. Even if all stimuli have a cumulative effect, the total experimental time may not have been sufficient to elicit the expression of CPTase.

A second problem may compound the difficulty encountered from the short experimental time. The common "assay" method, chosen by many authors, is to monitor changes in metabolic products. Unfortunately, these changes are the last steps in an induction process. First, the gene has to be expressed and transcribed into mRNA. The mRNA is then translated into protein which may have to be further processed before it possesses catalytic activity. The enzyme activity is also influenced by other environmental conditions. And lastly, the product of metabolism itself depends on the availability and uptake of specific substrate which may or may not be present.

A third problem is related to the time course of CPTase induction. According to Warshaw's data, CPTase activity doubles in the first 24 hours after birth but reaches adult levels 3-4 weeks later. While it is always impressive to have something doubled, the baseline level of CPTase activities in fetal heart is extremely low. In fact, even at the end of the first day, newborn rat heart CPTase activity is only approximately 1/3 of the adult level. No doubt the actual level achieved in the first few hours will be considerably lower. Therefore, even if CPTase can be

induced, the chance to detect the induction by measuring actual FFA oxidation is very low.

An alternative approach to this problem would be to measure something upstream in the induction process. The best candidates would be the level of mRNA expression or the level of CPTase protein itself. Direct measurement of CPTase enzyme activity is less ideal because of the problems mentioned earlier. It is extremely important to determine the natural time course of CPTase expression for a very simple reason. If it takes longer than a few hours to induce CPTase under the optimum condition, i.e. natural birth, no in utero experiment will ever be successful in a shorter time. Since there are no data of any kind on heart CPTase gene expression, I decided to collect data for a natural time course of CPTase expression in newborn rats hearts.

My working hypothesis is that the apparent low cardiac CPTase activity in the very young animal is secondary to low CPTase level and not due to a normal enzyme level which may be immature or inhibited. If this hypothesis is correct, I could expect to see an increase in CPTase gene expression and protein synthesis after birth.

The best way to test this hypothesis is to determine the enzyme protein and CPTase mRNA levels in various age groups. In addition to newborn rats of various age, fetal and adult mRNA and protein levels were also measured for

extreme values. I decide against measuring CPTase activity because it is not only less reliable, it has also been done before [101].

My original plan was to use Northern hybridization to quantify mRNA concentration changes. This plan was abandoned due to the extremely low copy numbers of this mRNA and the extremely small quantity of tissue available from each animal. I turned instead to the ribonuclease protection assay (RPA). RPA is a much more sensitive method and has less stringent requirements for mRNA integrity. The disadvantage of RPA is that it requires a high degree of homogeneity between the probe and the messanger species thus making interspecies mRNA detection virtually impossible.

Besides providing a time course for CPTase induction, a direct determination of CPTase protein level may have added benefits, at least when the experiments were designed. I had hoped that the relationship between mRNA expression and protein level would provide additional information on the controversy over CPT I and CPT II.

The antibody (RK40) was raised against rat liver CPT II as was the cDNA probe. I was well aware of the disputed nature of the CPTase and I anticipated two possible outcomes based on the two most prominent models of CPTase at the time. In the first case, CPT I and CPT II are considered as distinct proteins. If the antibody and cDNA probes react exclusively with CPT II, only CPT II expression would be

followed. Since CPT II activity is thought to be adequate at all ages, I would have expected relatively constant mRNA expression and CPT II protein levels in all age groups. In this case mRNA and CPTase protein level would not reflect the newly acquired ability to oxidize FFA in newborn hearts which is attributed primarily to the induction of CPT I.

In the second case, if CPT I and CPT II are similar proteins with regulatory units associated with CPT I then the antibody and cDNA probe should react to both types of CPTase. Therefore the mRNA expression and total CPTase protein level will reflect CPTase induction and I could expect to see an increase in both mRNA expression and CPT protein levels.

The results showed that a surge of mRNA level occurs immediately after birth. This new level of mRNA expression is not maintained during the first week of life, but a further steady increase in CPTase mRNA resumes about 10 days after birth and reaches a level close to the adult a few days later. Changes in CPTase protein levels follow a similar pattern. The protein is hardly detectable in fetal hearts. Once the animal is born, CPTase protein becomes readily detectable. The protein level drops off a little at 5 days and rebounds in 10 day old newborns. Adult rat heart has the highest level of CPTase protein.

These experimental results are expected if the second scenario is correct. There is an overall increase in mRNA

level during development with an accompanying increase in CPTase protein level. The association between mRNA expression and protein synthesis, however, is not tightly coupled. Protein synthesis seems to lag the mRNA expression in the older newborns. Based on my experiments and all available information, I was ready to conclude that the developmental pattern seemed to support the hypothesis that CPT I and CPT II were immunologically similar.

A very recent publication by McGarry (March 1993) [32] provided strong, if not definitive, evidence that CPT I and CPT II are distinct proteins. McGarry showed that CPT I does not react with RK40. Furthermore, he published the CPT I cDNA sequence and raised a new antibody against a truncated form of CPT I. His evidence is convincing. McGarry now believes that CPT I isoforms from various tissues are also immunologically distinct. The sequence of the truncated CPT I that he published is 4.7 kb. The predicted peptide contains 773 amino acids with a molecular weight of 90 kDa.

In light of McGarry's new finding, we have to conclude that CPT II mRNA expression increases immediately at birth and gradually reaches the adult level. This corresponds to an increase in CPT II protein synthesis. By the time the newborns are 5 days old, there is a considerable delay between mRNA expression and the protein synthesis. The mRNA levels approach their adult value at day 10-15 but protein

synthesis levels off until reaching the adult level at a later time.

This conclusion certainly does not agree with the prediction based on historical data. If what we showed is indeed the developmental pattern of CPT II, then there must be a CPT II deficiency in the fetus. This means that the historical data are incorrect and fetal cardiac CPT II function is not at the newborn level.

How reliable is the generally agreed upon notion that CPT II is not deficient? To answer this question, one must take into account the historical background. Given what we know now and what was done in the early 1970's, it is entirely possible that Warshaw and other investigators could have been measuring the combined activities of both CPTases. If that is indeed the case, the reported pattern of changes in activity during development may be reflecting complex changes in both enzymes. It is possible that the combined CPTase activities increase rapidly after birth.

Warshaw's [101] original measurement on the calf heart was done in tissue homogenate, a crude method by today's standards. Based on his experiments, it was deduced that the dissociation of the palmitoylcarnitine is not deficient in fetal calf heart because palmitoylcarnitine could be oxidized but palmitoyl-CoA could not. This reasoning was taken one step further to conclude that the depressed FFA oxidation in fetal heart was really the consequence of low

CPT I. The truth is that fetal heart CPT II activity was never directly assessed and as a matter of fact, it was not even mentioned in Warshaw's papers.

Does a model with CPT II deficiency make sense? Perhaps so. If CPT I is deficient in the fetal heart, there is little reason to have large quantities of CPT II. Normally palmitoylcarnitine is available only in very limited quantities in the fetal heart. Without the products from the first reaction catalyzed by CPT I, the activity of CPT II would be useless. Secondly, if there is sufficient CPT II in the fetal heart to function at the newborn level, there is little reason for the dramatic induction at birth. Thirdly, We found fetal heart CPT II protein is almost undetectable. It would be difficult for the fetal heart to maintain normal function without sufficient enzyme concentration unless the specific activity of CPT II was so high that very few enzyme molecules are needed for normal activities. But again, if CPT II activity is so enormous, why does the newborn rat synthesize relatively large amounts of CPT II at birth? All of these are, of course, only deductions but it does make one wonder why the fetus would maintain an enzyme that has no practical function. If CPT II is deficient in fetal rat hearts then rapid CPT II synthesis is necessary to increase FFA oxidation after birth. The rapid protein synthesis is indeed confirmed in our observation.

Why is there a transient decrease in both CPT II mRNA and protein levels in the first 5 days of life? One possible explanation is that the original surge is to activate the expression of CPTase but the actual maturation of the system is a far slower process. Clark and Clark [28] believe that there are actually two phases in postnatal adaptation. The first phase is an emergency measure with the emphasis on utilizing endogenous fuel supply immediately after birth. The second phase is the true adaptation to the continuous supply of exogenous fuel. The hormonal response to diet is established gradually after birth. This process involves maturation of the feedback control system and receptor coupling to cAMP etc [28]. These are all interesting areas for future research.

The last unanswered question is probably the least important one physiologically because it exists only under experimental conditions, nonetheless it is challenging. How do fetal hearts dissociate palmitoylcarnitine if there is a CPT II deficiency? I don't know the answer. If I had to speculate, it would be interesting to look into the chemical properties of palmitoyl-CoA and palmitoylcarnitine. The inner mitochondrial membrane is impermeable to palmitoyl-CoA whereas palmitoylcarnitine can be readily transported with the help of palmitoylcarnitine translocase. Production of palmitoylcarnitine is impaired in a pure CPT I deficiency. Therefore it is impossible to transport long chain acyl-CoA

into the mitochondria. Since β-oxidation occurs in the mitochondrial matrix only, FFA will could be oxidized.

The situation is different in a pure CPT II deficiency. Now the problem is the dissociation of palmitoylcarnitine not its transport into the mitochondron. When palmitoylcarnitine is added to the reaction mixture, as was done in Warshaw's experiment, it would enter the mitochondria and in turn elevate intramitochondrial concentration of palmitoylcarnitine. It is possible that if intramitochondrial palmitoylcarnitine concentration is increased dramatically, mass action would drive the equilibrium in favor of dissociation.

It is clear that there is a lag in protein synthesis. This indicates that CPTase translation could be regulated by other mechanisms in addition to changes in CPTase mRNA expression.

Part II

Chronic Heart Failure and CPT II Expression

The ultimate goal of CPTase research is to understand the role of FFA metabolism in various disease states. Actively manipulating CPTase activity may have far reaching effects on metabolic abnormalities in many illnesses. In fact, our limited knowledge of CPTase is already being applied to clinical treatments.

Some hereditary CPTase deficiencies can be successfully

treated with medium chain FFA. The treatment principle is based on the fact that the mitochondrial membranes are permeable to medium chain FFAs thus bypassing the CPTase deficiency which blocks the long chain FFA transport into the mitochondria. Hypoglycemia is common in these patients and is thought to be a direct consequence of impaired FFA oxidation. After administration of medium chain FFA, sufficient ketogenesis resumes, gluconeogenic activity returns to normal and the hypoglycemia is corrected.

Knowledge of CPTase function is also used to treat other illnesses such as diabetes. Some hypoglycemic medications are CPTase inhibitors by nature. These medications suppress CPT I activity which results in decreased β-oxidation of FFA. The energy supply is then shifted to glucose oxidation. When glucose becomes the primary source of energy, gluconeogenesis is also inhibited because it limits the TCA cycle intermediates being "siphoned" out of the system. The net result is markedly lower blood glucose levels. There are also proposals to treat acute myocardial infarction with CPT I inhibitors.

My interest, of course, is in CPTase's role in a number of cardiac ailments, especially in chronic heart failures. I believe that the function of CPTase is altered and could be, at least partially, responsible for the deterioration of cardiac energy metabolism in heart failure.

Over the years, more and more evidence emerges to

support the idea that energy metabolism is impaired in chronic heart failure. Now it is clear that most forms of heart failure are accompanied by a state of energy depletion. Advancements in biochemistry and molecular biology made it possible for us to monitor events occurring at subcellular levels.

Because adequate energy supply is central to all cardiac function and its deficiency is seen in all types of heart failure, it has been hypothesized that chronic deficiency in energy supply contributes directly to heart failure [51].

The foundation of this hypothesis is that both contraction and relaxation are energy dependent functions. Therefore chronic energy deficiency may cause dysfunction in both phases of the cardiac cycle. More specifically, calcium homeostasis may be affected by energy depletion because it is maintained through energy dependent ion pumps.

It has been further speculated that relaxation may be affected earlier than contractile function because it is more sensitive to energy fluctuation. The rationale is that the excitation-contraction process is a rapid phenomenon. Most of the calcium released during the process flows passively down the concentration gradient and requires no energy expenditure. On the other hand, intracellular calcium concentration is actively maintained by inherently slower processes. All of the ion pumps involved in calcium

removal require direct energy input from hydrolysis of ATP. Even the Na^+/Ca^{+2} exchanger, operating on a concentration gradient ultimately requires energy to maintain its function. The accumulating intracellular sodium, entered in exchange for calcium, must be pumped out by ATP consuming Na^+/K^+ pumps. Therefore, relaxation will be more susceptible to energy depletion.

These, of course, are only eloquent deductions based on normal physiology. Finding evidence to support this hypothesis is entirely a different matter and so far no direct evidence has been reported to support thse ideas. Despite the lack of direct evidence, these hypotheses remain attractive to many and more research is now directed at diastolic dysfunction in failing hearts.

Energy depletion in the failing heart is usually considered a secondary problem, based on the pathophysiology of chronic failure. The contributing factors are: (1) increasing work load; (2) decreasing ability to deliver nutrients; (3) a lower ratio of mitochondrial to myofibril mass; (4) carnitine depletion; (5) impaired coronary reserve. No doubt these all contribute to the energy depletion in the failing heart. Each of these problems is expected to worsen with each increment of hypertrophy. More oxygen will be consumed for the higher pump activity. A larger muscle mass means that the limited capillaries must supply more myofibrils. At the same time, coronary flow is

reduced due to higher compression pressure, especially in the endocardial regions. Myofibril mass displaces the mitochondria and worsens its own energy supply. Less carnitine depresses energy metabolism even further. The overall result is gross energy depletion and deteriorating contraction and relaxation. Eventually the heart may outgrow its energy supply and become "decompensated".

Heart failure can be due to a variety of causes that follow different courses in their development. The above sequence of events may be adequate to explain the pattern of end stage hypertensive failure but it cannot account for the energy depletion in non-hypertrophic failures or events occuring much earlier in the course of developing heart failure. I prefer an alternative explanation in which energy metabolism is considered a primary defect.

Normally, energy production is not a limiting factor for cardiac function because the contractility and relaxation can be markedly increased as needed. Therefore, it is fair to say that energy production in the normal heart is regulated by demand. This of course is no longer true in failing hearts but there must be a transition from having significant reserve in normal hearts to having energy depletion in failing hearts. After all, chronic heart failure is usually a gradually progressing process.

What happens during the transition period toward failure? It has been reported that FFA oxidation is

suppressed quite early when the rat heart is subjected to a pressure load, maybe even before the onset of hypertrophy [90]. If this is true, it is unlikely that lipid metabolism will improve as heart failure becomes more advanced. In fact, abnormal lipid metabolism in failing hearts has been recognized as early as 1968 [105]. Lipid metabolism involves many enzymes and cofactors. After decades of research, are we now in a position to say which step(s) of the lipid metabolism are affected in heart failure? Although we still do not have the complete picture parts of the puzzle are coming together.

Regardless of etiology, the final picture of all end stage heart failures have striking similarities in their energy metabolism. The first clue for the puzzle came from patients with systemic carnitine deficiency. The characteristic feature of this disease is low circulating carnitine. Their myocardial FFA oxidation has been shown to be inhibited and many of these patients develop cardiomyopathy. In these patients, carnitine treatment seems to improve the mechanical function of the heart [8]. These data suggest that suppressed carnitine levels might cause some forms of cardiomyopathies.

It is now clear that heart carnitine content is actually decreased in all end stage heart failure. This seems to be true regardless of the etiology. Masumura et al. [61] reported decreased free myocardial carnitine in end

stage heart failure patients with valvular disease. Regits et al. [81] reached the same conclusion in patients with end stage heart failure caused by dilated cardiomyopathy, coronary artery disease and other causes.

Cardiac fuel metabolism in chronic heart failure is known to be altered in favor of glucose utilization over FFA. Failing hearts are also starving hearts. It has been established that the cardiac ATP content is lower in failing hearts, an indication that their energy metabolism could be significantly impaired.

Because 90% of the carnitine in the heart is in the cytoplasm, the carnitine dependent function in the cytoplasm might be more susceptible to the loss of carnitine. The most important role of carnitine, of course, is to form acyl-carnitine in FFA transport. This particular step takes place in the cytoplasm. Based on this information it has been proposed that loss of carnitine may lead to abnormal FFA oxidation in the heart.

How do hearts loose their carnitine in the diseased state? To answer this question, we need to look for conditions that are associated with decreased cardiac carnitine levels. It seems that cell membrane integrity would be an important factor in determining the intracellular carnitine level, especially if one takes into account the 60 fold concentration gradient across the cell membrane. The non-specific damage of cell membranes

occurring after myocardial ischemia was identified as a potential cause of carnitine leaking from the heart. It was shown that 60 minutes of acute myocardial ischemia results in a depletion of total tissue carnitine in the ischemic area and depletion was not restored with reflow [56].

Another possible cause of low cardiac carnitine is its uptake. Because carnitine has to be transported into the cell, any inhibition of the transport system will impair carnitine uptake. It is known that diphtheria toxin impairs the carnitine carrier system and leads to a decrease in cardiac carnitine level [19]. These findings demonstrate that carnitine homeostasis could indeed be disrupted under specific conditions and it could lead to heart failure as in the case of systemic carnitine deficiency. This, however, is not enough to explain all the other carnitine deficiencies observed in failing hearts.

Many types of heart failure do not start with intrinsic myocardial impairment. They are secondary to diseases such as hypertension, valvular disease etc. The myocardium in these patients often starts out normal. It is not characteristic for these hearts to show evidence of early cell membrane damage. If cell membrane integrity is preserved, there is little reason for carnitine to leak out and heart failure should be averted. Yet, all failing hearts lose their carnitine.

I believe that the carnitine loss in failing hearts is

more likely a secondary phenomenon. As mentioned earlier, myocytes have a high carnitine concentration. Given the fact that the carnitine uptake is against a steep gradient, it must be by an active transport system. All active transport systems require exogenous energy inputs that are usually coupled to metabolism. Since energy metabolism is impaired in failing hearts, the failure in carnitine transport will ultimately lead to cardiac carnitine deficiency. In fact, this might be the reason that the degree of carnitine loss reflects the severity of heart failure.

If this is the case, then what causes the impairment of energy metabolism? One important fact is that very early in the evolution of heart failure, lipid metabolism is already suppressed in favor of glucose consumption. Under hypoxic conditions, cardiac FFA oxidation is immediately suppressed and glucose becomes the primary fuel source. Acute myocardial ischemia is one good example. Although the actual mechanism for this switch is not known, one often cited reason is that the suppression of FFA oxidation is due to inadequate oxygen supply, as it was often assumed in fetal metabolism.

The loss of ability to oxidize FFA under acute conditions is usually a temporary phenomenon, as it has been shown many times that FFA oxidation is usually fully recovered immediately after reflow. Perfused rat hearts

normally obtain over 90% of the ATP from FFA and only 6% from glucose and this proportion remains the same during reflow after 25 minutes of global ischemia [58]. This type of response is probably regulated through the malonyl-CoA inhibition of CPT I. It has been reported that modification of CPT I sensitivity to malonyl-CoA inhibition occurs in acute myocardial ischemia [76].

In hypertensive hearts, oxygen supply should still be adequate for a time. Hypoxia, therefore, is not expected to be a problem in these patients in the early stages. This is indirectly supported by the fact that complete oxidation of glucose must pass through the TCA cycle just like FFA. As a matter of fact, it takes only 10% more oxygen to obtain a given amount of ATP by oxidizing FFA than by oxidizing glucose. Unless the hypoxia is severe, i.e. the available oxygen is reduced to within 10% above the anaerobic threshold, it should make no difference which fuel is used as far as ATP generation is concerned. Therefore, hypoxia is not likely to cause an early inhibition of FFA oxidation in heart failure. I believe the long term inhibition of FFA oxidation in chronic failure is induced through a completely different mechanism, a mechanism that alters gene expression.

In addition, the energy demands in acute myocardial infarction versus chronic failure are quite different. Anaerobic energy metabolism is insufficient to provide ATP

for long term cardiac function. Although, theoretically the maximal anaerobic rate under optimal conditions is expected to produce a significant portion of the energy requirement of the heart, in reality, it is questionable whether this optimal rate could ever be achieved. Furthermore, it has been shown that the rate of lactate production in human patients with angina pectoris is too low to account for the energy needs of a normal contracting heart [69].

To test the hypothesis that CPTase is depressed in failing hearts, we have determined CPTase gene expression in failing adult rat hearts. All of the four hearts had approximately 35% of their left ventricle free wall infarcted and demonstrated highly abnormal hemodynamic parameters. Although these data only reflect the changes late in the disease, they are sufficient to show that there is indeed a significant alteration in CPTase expression.

The mRNA level of cardiac CPT II is severely depressed in these hearts. The overall effect of gene regulation is still hard to grasp because of our inability to monitor the rate limiting enzyme, CPT I. However, the change in CPT II expression alone is sufficient to suggest that the FFA oxidation is impaired by a mechanism that has not been previously described.

Unfortunately, all four rats were treated with angiotensin converting enzyme (ACE) inhibitors for other purposes. The potential effect of the medication must be

eliminated as a cause of the suppressed mRNA expression. This will require further studies on "control" animals with infarction.

The likelihood of a drug effect, however, is relatively small. Only one paper relating ACE inhibitor to energy metabolism has been published. In it, ACE inhibitors were thought to improved energy balance through reducing workload and oxygen demand on the failing heart [86]. No information is available on ACE inhibitor interaction with CPT system and FFA oxidation.

In addition, results from the two control animals (without drug treatment) also demonstrated that the depression of mRNA expression was probably not drug induced. Nonetheless, the limited data from control animals are insufficient to rule out an ACE inhibitor effect. However, even if our observation would prove to be a purely drug related phenomenon, it still is a worthy finding.

Our results have not yet demonstrated CPTase inhibition as a primary event in failing hearts. Such evidence can only be obtained with a time course study which is planed as a follow up project.

There are data suggesting that the effect of CPTase inhibition may go beyond its role in overall energy metabolism. With confidence, we can say that the energy supply of the myocardium is probably not impaired in the early hypertensive heart. The only early metabolic change

so far known is the replacement of FFA with glucose as a fuel. Overall energy metabolism is still adequate. One may argue that as long as the total energy flow is adequate, the source of fuel should matter little. This statement, however, may not be true. It has been shown over the years that energy supply derived from glucose and FFA may not be completely exchangeable and that suppression of FFA oxidation may affect specific cardiac function.

Feeding rats a fat free diet is known to result in cardiac hypertrophy without first developing overt malnutrition [75]. New hypoglycemic medications used in diabetic treatments are known to induce cardiac hypertrophy and diastolic disfunction while inhibiting FFA oxidation [57]. Furthermore, 2-tetradecylglycidic acid induced hypertrophy can be prevented by supplementing medium chain FFA [54]. Lopaschuck [58] found that FFA can be lowered significantly in rat heart perfusate, but once complete removed, cardiac function cannot be sustained by glucose alone. Although we cannot separate the effects of enhanced glucose oxidation from those of suppressed FFA oxidation, the alteration of the two fuel sources seems to be associated with changes in cardiac morphology and function.

Taegtmeyer and his colleagues [90] found that altered metabolism was induced as early as 8 weeks after pressure loading the rabbits. The rate of ketone body consumption was decreased and the rate of glucose utilization was

increased in the hypertensive rabbit hearts. The activities of glycolytic enzymes were increased while two of the three enzymes involved in ketone body metabolism had lower activities. The metabolic changes were very specific and were not accompanied by higher activities in other mitochondrial enzymes, such as key enzymes of TCA cycle. This indicates that the altered metabolism is truly a preferential use of glucose not an overall elevation of mitochondrial activity. Interestingly, these metabolic changes occurred only in the left ventricle and not in the right ventricle which was shielded from the pressure load.

Rupp et al. [84] studied subcellular organelle changes in rat heart hypertrophy induced by pressure load, CPT I inhibition or a combination of both. They found that inhibition of CPT I caused biventricular hypertrophy whereas pressure load only induced left ventricular hypertrophy.

"Compartmentilization" is another concept in dispute concerning the notion that energy generated in different pathways are equally exchangeable. It was postulated that ATP from glycolysis might preferentially supply membrane ion pumps [20]. Studies on acute myocardial infarction also provided evidence that preferential use of energy sources might have significant impact on recovery from myocardial infarction. It was shown that enhancing glucose oxidation protects post-ischemic myocardium, presumably by inhibiting CPT I activity [58]. Weiss [103] demonstrated that energy from

oxidative phosphorylation is preferentially used for contractile function whereas glycolytic energy is primarily for sarcolemmal function.

Normally, glucose oxidation is suppressed by FFA oxidation. High concentration of acetyl-CoA suppresses pyruvate dehydrogenase activity and the overall glucose flux into the TCA cycle. Inhibition of CPTase will lead to a disproportionate energy supply from glucose oxidation. A chronically suppressed long chain FFA oxidation combined with the reciprocal increase in glucose utilization adversely affects cardiac structure and function. Cardiac hypertrophy and diastolic function are the early manifestations of the altered energy metabolism with or without accompanying pressure overload [57,84]. If the altered energy metabolism is not corrected early, it may eventually lead to heart failure.

Altered CPTase activity and FFA oxidation may also serve as a signal for cardiac growth. Rupp [84] showed that hypertrophy may be induced by either inhibition of CPT I or by hypertension. Do they act through a common mechanism ? The answer is probably not because etomoxir is not known to change hemodynamic parameters. Therefore it must be functioning through a different mechanism. In this case it may be through altered fuel metabolism.

Although these are still fragmented pieces of information, they make a strong case for continuing research

in the cardiac energy metabolism. With an interdisciplinary approach, the chance of determining the interrelationships between lipid metabolism, heart failure and growth will be greatly increased.

SUMMARY AND CONCLUSION

My results demonstrate that CPT II is indeed deficient in fetal hearts and is induced rapidly at birth. The fetal rat hearts (n=3) CPT II expression is $19 \pm 6\%$ of the adult level but the newborn hearts CPT II increases to $55 \pm 11\%$ of the adult level. These dat indicates that a significant surge in mRNA expression and CPTase protein synthesis occurs immediately after birth, at least in the rat. These type of changes in cardiac CPTase expression have not been previously reported. These results also raise interesting questions about the status of fetal heart CPT II and its induction during adaptation at birth.

The results from the infarcted rat hearts indicate that CPT II mRNA expression is significantly depressed in failing hearts (some were treated with ACE inhibitors). CPT II/cyclophilin ratio is 0.42 ± 0.16 (n=4) for the MI group and 2.17 ± 0.43 (n=3) for normal animals.

Both findings will be the stepping stone leading to a much better understanding of CPTase induction mechanism and its role in chronic heart failure.

Clearly, more study is need to clarify the unanswered questions, especially after we develop the capability to evaluate the developmental pattern of CPT I directly.

REFERENCES

1.   Agata Y, Padbury J, Ludlow JK, Polk DH, Humme JA. The effect of chemical sympathectomy on catecholamine release at birth. Pedia. Res. 20:1338-1344, 1986.

2.   Anderson DF, Bissonnette JM, Faber JJ, Thornburg KL. Central shunt flows and pressures in the mature fetal lamb. Am. J. Physiol. 241:H60-H66, 1981.

3.   Aprille JR. Dietary lipid and postnatal development. II. palmityl coenzyme A oxidation in heart and liver. Pediat. Res. 10:982-985, 1976.

4.   Assali NS, Kirschbaum TH, Dilts Jr. W. Effects of hyperbaric oxygen on uteroplacental and fetal circulation. Circ. Res.22:573-588, 1963.

5.   Bashore TM, Magorien DJ, Letterio J, Shaffer P, Unverferth DV. Histologic and biochemical correlates of left ventricular chamber dynamics in man, J.A.C.C. 9:734-742, 1987.

6.   Battaglia FC, Meschia G, Makowski EL, Bowes W. The effect of maternal oxygen inhalation upon fetal oxygenation. J. Clin. Invest. 47:548-555, 1968.

7.   Battaglia FC, Meschia G. Principle substrates of fetal meta-bolism. Physiological Review. 58:499-527, 1978.

8.   Bazzato G, Coli U, Landini S, Mezzina C, Ciman M. Myesthenia-like syndrome after D,L- but not L-carnitine (letter). Lancet 1:1209, 1981.

9.   Bieber LL. Carnitine. Ann. Rev. Biochem. 57:261-283, 1988.

10.   Bielefeld DR, Vary TC, Neely JR. Inhibition of carnitine palmitoyl-Co A transferase activity and fatty acid oxidation by lactate and oxfenicine in cardiac muscle. J. Mol. Cell Cardiol. 17:619-625, 1985.

11.   Blackshear PJ. Systems for polyacrylamide gel electrophoresis. Methods Enzm. 104:237-255, 1984.

12.   Blanco CE, Martin CB, Rankin J, Landauer M, Phernetton T. Changes in fetal organ flow during intrauterine mechanical ventilation with or without oxygen. J. Dev. Physiol. 10:53-62, 1988.

13.   Borrebaek B, Christiansen R, Christophersen BO, Bremer J. The role of acyltransferases in fatty acid utilization. Supp. 1, Circ. Res. 38:I16-I 21, 1976.

14.   Bougneres PF, Saudubray JM, Marsac C, Bernard O, Odievre M, Girard J. Fasting hypoglycemia resulting from hepatic carnitine palmitoyl-transferase deficiency. J. Pediatr. 98:742-746, 1981.

15.   Brady PS, Brady LJ. Hepatic carnitine palmitoyltransferase turnover and translation rates in fed, starved, streptozotocin-diabetic and diethylhexyl phthalate-treared rats. Biochem. J. 246:641-649, 1987.

16.   Brady PS, Feng Y, Brady LJ. Transcriptional regulation of carnitine palmitoyltransferase synthesis in riboflavin deficiency in rats. J. Nutr. V118:1128-1136, 1988.

17.   Brady LJ, Brady PS. Regulation of carnitine palmitoyltrans-ferase synthesis in spontaneously diabetic BB Wistar rats. Diabetes. 38:65-69, 1989.

18.   Bremer J. Carnitine in intermediate metabolism. J.Biol. Chem. 238:2774-2779, 1963.

19.   Bressler R, Wittels B. The effect of diphtheria toxin on carnitine metabolism in the heart. BBA. 104:39-45, 1965.

20.   Bricknell OL, Daries PS, Opie LH. A relationship between adenosine triphosphate, glycolysis and ischemic contracture in the isolated rat heart. J. Mol. Cell Cardiol. 13:941-945, 1981.

21.   Bristow MR, Ginsburg R, Minobe W, Cubicciotti R, Sageman WS, Lurie K, Billingham M, Harrison D, Stinson E. Decreased catecholamone sensitivity and B-adrenergic receptor density in failing human hearts. New Eng. J. Med. 307:205-211, 1982.

22.   Brosnan JT, Kopec B, Fritz IB. The isolation of carnitine palmitoyltransferase on the inner membrane of bovine liver mitochondria. J. Biol. Chem. 248:4075-4083, 1972.

23.   Cannon B, Nedergaard J. The function and properties of brown adipose tissue in the new born. The biochemical development of the fetus and neonate. C.T. Jones Editor. Elsevier, Amsterdam, 697-730. 1982.

24.   Cantrell CR, Borum PR. Identification of a cardiac carnitine binding protein. J. Biol. Chem. 257:10599-10604, 1982.

25. Chomcznski P, Sacchi N. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. Analy. Biochem. 162:156-159, 1987.

26. Christie WW, Calvert DT, Ahand JH, Noble RC. The metabolism of palmitic acid in the fetal lamb. Comp. Biochem. Physiol. 80B:617-621, 1985.

27. Cimbala MA, Lambers WH, Nelson K, Monahan JE, Yoo-Warren H, Hanson RW. Rapid changes in the concentration of phosphoenol-pyruvate carboxykinase mRNA in rat liver and kidney. J. Biol. Chem. 257:7629-7636, 1982.

28. Clark JB, Clark Jr. CM. The growth and metabolism of the developing heart. Biochemical development of the fetus and neonate. Jones, editor. Elsvier Biochemical Press, 185-212, 1982.

29. Declercq PE, Falck JR, Kuwajima M, Tyminski H, Foster DW, McGarry JD. Characterization of the mitochondrial carnitine palmitoyl-transferase enzyme system. I. Use of inhibitor. J. Biol. Chem. 262:9812-9821, 1987.

30. Dimauro S, Dimauro PM. Muscle carnitine palmitoyltransferase deficiency and myoglobinuria. Science 182:929-930, 1973.

31. Edwards EM, Dhand UK, Jeacock M, Shepherd DAL. Activities of enzymes concerned with pyruvate and oxaloacetate metabolism in the heart and liver of develping sheep. Biochim. Biophy. Acta. 399:217-227, 1975.

32. Esser V, Britton CH, Weis BC, Foster DW, McGarry JD. Cloning, sequencing, and expression of a cDNA encoding rat liver carnitine palmitoyltransferase I. J. Biol. Chem. 268:5817-5822, 1993.

33. Falik-Borenstein ZA, Jordan SC, Saudubray JM, Brivet M, Demaugre F, Edmond J, Cederbaum SD. Brief report: renal tubular acidosis in carnitine palmitoyltransferase type I deficiency. New Eng. J. Med. 327:24-27, 1992.

34. Finocchiaro G, Taroni F, Rocchi M, Liaris Martin A, Colombo I, Torri Tarelli G, DiDonato S. cDNA cloning, sequence analysis, and chromosomal localization of the gene for human carnitine palmitoyltransferase. Proc. Natl. Acad. Sci. U.S.A. 88:661-665, 1991.

35. Fisher DJ, Heymann MA, Rudolph AM. Myocardial oxygen and carbohydrate consumption in fetal lambs in utero and in adult sheep. Am. J. Physiol. 238:H399-H405, 1980.

36. Fisher DJ. Oxygenation and metabolism in the developing heart. Seminars in Perinatology. V8(3):217-224, 1984.

37. Fritz IB, Kaplan E, Yue KTN. Specificity of carnitine action on fatty acid oxidation by heart muscle. Am. J. Physiol. 202:117-121, 1962.

38. Garcia Ruiz JP, Ingram R, Hanson RW. Changes in hepatic messenger RMNA for phosphoenlpyruvate carboxykinase (GTP) during development. Proc. Natl. Acad. Sci. U.S.A. 75:4189-4193, 1978.

39. Ghadiminejad I, Saggersn ED. Carnitine palmitoyltransferase (CPT II) from liver mitochondrial inner membrane becomes inhibitable by malonul-CoA if reconstituted with outer membrane malonyl-CoA binding protein. FEBS 269:406-408,1990.

40. Girard J. Gluconeogenesis in late fetal and early neonatal life. Biol. Neonate. 50:237-258, 1986.

41. Girard J. Metabolic adaptations to change of nutrition at birth. Metabolic problems of the newborn. Biol.Neonate 58(suppl 1):3-15, 1990.

42. Gleason CA, Ruldoph AM. Oxygenation does not stimulate hepatic gluconeogenesis in fetal lamb. Pediat. Res. 20:532-535, 1986.

43. Hoppel CL, Tomec RJ. Carnitine palmitoyltransferase: localization of two enzymatic activities in rat liver mitochondria. J. Biol. Chem. 247:832-841, 1972.

44. Hoppel CL. Carnitine and carnitine palmitoyltransferase in fatty acid oxidation and ketosis. Fed. Proc. 41:2853-2857, 1982.

45. Hug G, Bove KE, Soukup S. Lethal neonatal multiorgan deficiency of carnitine palmitoyltransferase II. New Eng. J. Med. 325:1862-1864, 1991.

46. Ito Y, Suko J, Chidsey CA. Intracellular calcium and myocardial contractility. V. calcium uptake of sacoplasmic reticulum fractions in hypertrophied and failing rabbit hearts. J. Mol. Cell Cardol. 6:237-247, 1974.

47. Iwamoto HS, Teitle DF, Ruldolph AM. Effect of birth-related events on blood flow distribution. Pedia. Res. 22:634-640, 1987.

48. Iwamoto HS, Teitle DF, Ruldolph AM. Effect of birth-related events on metabolism in fetal sheep. Pedia. Res.

30:158-164, 1991.

49. James E, Meschia G, Battaglia FC. A-V differences of free fatty acids and glycerol in the ovine umbilical circulation. P.S.E.B.M. 138:823-826, 1971.

50. Katz AM. Cellular mechanisms in congestive heart failure. Am. J. Cardio. 62:3A-8A, 1988.

51. Katz AM. Cardiomyopathy of overload. New Engl. J. Med.322:100-110, 1990.

52. Kerner J, Bieber L. Isolation of a Malonyl-CoA sensitive CPT/B-oxidation enzyme complex from heart mitochondria. Biochem. 29:4326-4334, 1990.

53. Langer GA. Ionic movements and the control of contraction. In: Langer GA, Brady AJ. eds. The mammalian myocardium. New York: Wiley, 193-218, 1974.

54. Lee SM, Bahl JJ, Bressler R. Prevention of the metabolic effects of 2-tetradecylglycidate by octanoic acid in the genetically diabetic mouse (db/db). Biochem. Med. 33:104-109, 1985.

55. Lehninger AL. Biochemistry. 2nd edition. Part 3 Biosynthesis and the utilization of phosphate-bond energy. Page 825 & 841, 1975.

56. Liedtke AJ, Demaison L, Eggleston AM, Cohen LM, Nellis SH. Changes in substrate metabolism and effects of excess fatty acids in reperfused myocardium. Circ. Res. 62:535-542, 1988.

57. Litwin SE, Raya TE, Gay RG, Bedotto JB, Bahl JJ, Anderson PG, Goldman S, Bressler R. Chronic inhibition of fatty acid oxidation: new model of diastolic dysfunction. Am. J. Physiol. 258:H51-H56, 1990.

58. Lopaschuk GD, Spafford MA. Energy substrate utilization by isolated working hearts from newborn rabbits. Am. J. Physiol. 258:H1274-H1280, 1990.

59. Lowry OH, Rosebrough NJ, Farr AL, Randall RJ. Protein measurement with the Folin phenol reagent. J. Biol. Chem. 195:265-275, 1951.

60. Mackenzie J. Disease of the heart. London: Oxford medical publications, 1908.

61. Masumura Y, Kobayashi A, Yamazaki N. Myocardial free carnitine and fatty acylcarnitine levels in patients with

chronic heart failure. Japn. Circ. J. 54:1471-1476, 1990.

62.   Matlib MA, Rembert JC, Millard RW.   Mitochondrial function in canine experimental cardiac hypertrophy.   J. Mol. cell Cardiol. 15:221-232, 1983.

63.   McGarry JD, Mannaerts GP, Foster DW. A possible role of malonyl-CoA in the regulation of hepatic fatty acid oxidation and ketogenesis.   J. Clin. Inves. 60:265-270, 1977.

64.   McMillin-Wood J.   Carnitine palmitoyltransferase in neonatal and adult heart and liver mitochondria. J. Biol. Chem. 250:3062-3066, 1974.

65.   Miyazawa S, Ozasa H, Osumi T.   Purification and properties of carnitine octanoyltransferase and carnitine palmitoyltransferase from rat liver. J. Biochem. V94:529-542, 1983.

66.   Molstad P, Bohmer T, Eiklid K. Specificity and characteristics of the carnitine transport in human heart cells (CCL 27) in culture. BBA. 471:296-304, 1977.

67.   Murthy MSR, Pande SV. Malonyl-CoA binding site and the overt carnitine palmitoyltransferase activity reside on the opposite sides of the outer mitochondrial membrane. Proc. Natl. Acad. Sci. U.S.A. 84:378-382, 1987.

68.   Murthy MSR, Pande SV. Characterization of a solublilized malonyl-CoA sensitive carnitine palmitoyltransferase from mitochondrial outer membrane as a protein distinct from the malonyl-CoA insensitive carnitine palmitoyltransferase of the inner membrane. Biochem. J. 268:599-604, 1990.

69.   Olson RE.   In discussion on: Evaluation of myocardial metabolism in ischemic heart disease, by Gorlin R. Circulation 40:(Suppl 4) 155-167, 1969.

70.   Osler W. The principles and practice of medicine.   New York: D. Appleton, pp 634, 1892.

71.   Packer M. Neurohormonal interactions and adaptations in congestive heart failure. Circulation. 77:721-730, 1988.

72.   Padbury JF, Ludlow JK, Ervin MG, Jacobs HC, Humme JA. Thres-holds for physiological effects of plama catecholamines in fetal sheep. Am. J. Physiol. 252:E530-E537, 1987.

73.   Padbury JF, Agata Y, Ludlow JK, Ikegami M, Baylen B,

Humme J. Effect of fetal adrenalectomy on catecholamine release and physiologic adaptation at birth in sheep. J. Clin. Invest. 80:1096-1103, 1987.

74. Pande SV, Murthy MSR. Carnitine: Vitamin for an insect, vital for man. Biochem. Cell Biol. 76:671-673, 1989.

75. Panos TC, Finerty JC. Effect of a fat-free diet on growing female rats, with special reference to the endocrine system. J. Nult. 49:397-418, 1953.

76. Paulty DF, Kirk KA, McMillin JB. Carnitine palmitoyltrans-ferase in cardiac ischemia. Circ. Res. 68:1085-1094, 1991.

77. Perreault CL, Meuse AJ, Bentivegna LA, Morgan JP. Abnormal intracellular calcium handling in acute and chronic heart failure: role in systolic and diastolic dysfunction. Europ. Heart J. 11:C:8-21, 1990.

78. Polk DH. Thyroid hormones effects on neonatal thermogenesis. Seminars in Perinatology. 12:151-156, 1988.

79. Pouleur H, Marechal G, Balasim H. Effects of dobutamine and sulmazol (AR-L115 BS) on myocardial metabolism, coronary, femoral and renal blood flow: a comparative study in normal dogs and in dogs with chronic volume overload. J Cardiovas. Pharmacol. 5:861-867, 1983.

80. Prip-Buss C, Pegorier JP, Duee PH, Kohl C, Girard J. Evidence that the sensitivity of carnitine palmitoyltransferase I to inhibition by malonyl-CoA is an important site of regulation of hepatic fatty acid oxidation in the fetal and newborn rabbit. Biochem. J. 269:409-415, 1990.

81. Regitz V, Shug A.L., Fleck E. Defective myocardial carnitine metabolism in congestive heart failure secondary to dilated cardiomyopathy and to coronary, hypertensive and valvular diseases. Am. J. Cardiol. 65:755-760, 1990.

82. Remme WJ. Congestive heart failure-pathophysiology and medical treatment. J. Cardiovas. Pharmac. 8:S36-S52, 1986.

83. Reid DL, Thornburg KL. Pulmonary pressure-flow relationships in fetal lamb during in utero ventilation. J. Appl. Physiol. 69:1630-1636, 1990.

84. Rupp H, Elimban V, Dhalla N. Modification of subcellular organells in pressure-overloaded hear by etomoxir, a carnitine palmitoyltransferase I inhibitor. FASEB J. 6:2349-2353,1992.

85.   Scheurer J.   The effect of hypoxia on glycolytic ATP production. J. Mol. Cell. Cardiol. 4:689-692, 1972.

86.   Schultheiss HP. Effect on the myocardial energy metabolism of angiotensin-converting enzyme inhibition in chronic heart failure. Am. J. Cadiol. 65:74G-81G, 1990.

87.   Sperling MA.   Integration of fuel homeostasis by insulin and glucagon in the newborn. Momogr. Pediatr. 16:39-58, 1982.

88.   Sperling MA, Ganguli S, Leslie N, Landt K.   Fetal-perinatal catecholamine secretion:role in perinatal glucose homeostasis. Am. J. Physiol. 247:E69-E74, 1984.

89.   Strauer BE. Significance of coronary circulation in hupertensive heart disease for development and prevention of heart failure. Am. J. Cardiol. 65:34G-41G, 1990.

90.   Taegtmeyer H, Overturf KL.   Effects of mederate hypertension on cardiac function and metabolism in the rabbit. Hypertension 11:416-426, 1988.

91.   Teitel DF.   Circulation adjustment to postnatal life. Seminars in perinatalogy. 12:96-103, 1988.

92.   Thomassen A, Bagger JP, Nielson TT, Henningsen P. Altered global myocardial substrate preference at rest and during pacing in coronary artery disease with stable angina pectoris. Am. J. Cardiol. 62:686-693, 1988.

93.   Tripp ME. Developmental cardiac metabolism in health and disease. Pediatr. Cardiol. 10:150-158,1989.

94.   Tsoulos NG, Colwell JR, Battaglia FC, Makowski EL, Meschia G. Comparison of glucose, fructose and oxygen uptakes by fetuses of fed and starved ewes. Am. J. Physiol. 221:234-237, 1971.

95.   Van Duyne CM, Parker HR, Havel RJ, Holm LW.   Free fatty acid metabolism in fetal and newborn sheep. Am.J.Physiol. V199(6):987-990, 1960.

96.   Vary TC, Reibel DK, Neely JR.   Control of energy metabolism of heart muscle.   Ann. Rev. Physiol. 43:419-430, 1981.

97.   Vyska K, Meyer W, Stremmel W, Notohamiprodjo G, Minami K, Machulla H-J, Gleichmann U, Meyer H, Korfer R.   Fatty acid uptake in normal human myocardium.   Circ. Res. 69:857-870, 1991.

98.   Wang L, Brady PS, Brady LJ.   Hormonal regulation of carnitine palmitoyltransferase synthesis in H4IIE cells. In Fatty Acid Oxidation: Clinical, biochemical and molecular aspects. page 209-216, Alan R. Liss Inc. 1990.

99.   Warnes DM, Seamark RF, Ballard FJ.   The appearance of gluconeogenesisat birth in sheep (activation of the pathway associated with blood oxygenation). Bioch.J. 162:627-634,1977.

100.   Warshaw JB, Terry ML.   Cellular energy metabolism during fetal development. II. Fatty acid oxidation by the developing heart. J. Cell Biology. V44:354-360, 1970.

101.   Warshaw JB.   Cellular energy metabolism during fetal development. IV. Fatty acid activation, acyl transfer and fatty acid oxidation during development of chick and rat. Developmental Biology. 28:537-544, 1972.

102.   Warshaw JB.   Fatty acid metabolism during development. Seminar in Perinatology. 3:131-139, 1979.

103.   Weiss J, Hiltbrand B. Functional compartmentation of glycolytic versus oxidative metabolism in isolated rabbit heart. J. Clin. Invest. 75:436-447, 1985.

104.   Wikman-Coffelt J, Parmley WW, Mason DT. The cardiac hypertrophy process: Analyses of factors determining pathological vs. physiological development. Circ. Res. 45:697-707, 1979.

105.   Wittels B, Spann JF Jr. Defective lipid metabolism in the failing heart.   J. Clin. Invest. 47:1781-1794, 1968.

106.   Woeltje KF, Kuwajima M, Foster DW, McGarry JD. Characterization of the mitochondrial carnitine palmitoyltransferase enzyme system. II. Use of detergents and antibodies. J. Biol. Chem. 262:9822-9827, 1987.

107.   Woeltje KF, Esser V, Weis BC, Cox WF, Schroeder JG, Liao ST, Foster DW, McGarry JD.   Inter-tissue and inter-species characteristics of the mitochondrial carnitine palmitoyltransferase enzyme system.   J. Biol. Chem. 265:10714-10719, 1990.

108.   Woeltje KF, Esser V, Weis BC, Sen A, Cox WF, McPhaul MJ, Slaughter CA, Foster GW, McGarry JD. Cloning, sequencing, and expression of a cDNA encoding rat liver mitochondrial carnitine palmitoyltransferase II. J. Biol. Chem. V265(18):10720-10725, 1990.

109.   Zammit VA, Corstorphine CG, Kolodziej MP. Target size

analysis by radiation inactivation of carnitine palmitoyltransferase activity and malonyl-CoA binding in outer membrane from rat liver mitochondria. Biochem. J. 263:89-95, 1989.

110. Zierler KL. Fatty acid as substrate for heart and skeletal muscle. Circ. Res. 38:459-463, 1976.