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Autocrine/paracrine regulation of ATP citrate lyase

Smith, Katherine Sue, Ph.D.

The Ohio State University, 1990
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AUTOCRINE/PARACRINE REGULATION OF ATP CITRATE LYASE

DISSERTATION

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

by

Katherine Sue Smith, B.S.

The Ohio State University

1990

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To Mom and Don
ACKNOWLEDGMENTS

My deepest gratitude to my parents, all the teachers, professors and friends who have endured me over my many years of education. Throughout the course of my time at The Ohio State University I learned much about science and probably more about life thanks to my committee, Drs. Roehrig, McCune and Allred. They provided a great atmosphere for learning through their interactions with the students and their consistent encouragement. Some of the most valuable experiences came from simply talking freely with them. The Nutrition Science group consists of rare and special people who are not only good researchers, but are accomplished teachers. This genuine concern for students is almost extinct today. It would be a great loss to the students if the present funding crunch should get the best of them; please persevere.

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ABBREVIATIONS

ATP CL: ATP citrate lyase
CoA: coenzyme A
LDH: lactate dehydrogenase
OAA: oxaloacetate
HMG CoA: B-hydroxy-B-methylglutaryl-CoA
MAO: monoamine oxidase
Pi: phosphate
cAMP: cyclic AMP
KD: kilodalton
LDL: low density lipoprotein
TYR: tyrosine
SER: serine
THR: threonine
HEPES: N-Z-hydroxyethylpiperazine-N'-z-ethane sulfonic acid
SDS: sodium dodecyl sulfate
PAGE: polyacrylamide gel electrophoresis
ECM: extracellular matrix
CM: conditioned media
HD: high density (10⁷ cells/ml)
LD: low density (10⁵ cells/ml)
EGF: epidermal growth factor
PDGF: platelet derived growth factor
TGF-ß: transforming growth factor-beta
IGF: insulin-like growth factor

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CHAPTER I
INTRODUCTION

ATP citrate lyase (ATP CL) produces cytosolic acetyl CoA from citrate which is transported from the mitochondria to the cytosol. The provision of acetyl CoA is essential for synthesis of fatty acids and cholesterol. All of these processes are confined to the cytosol. There is an abundance of acetyl CoA in the mitochondria derived from catalysis of pyruvate to acetyl CoA by pyruvate dehydrogenase. However, acetyl CoA cannot permeate the mitochondrial membranes, so it is combined with oxaloacetate by the citrate synthase reaction, then citrate is delivered to the cytosol on the citrate transporter, integral to the mitochondrial membranes.

The ATP CL reaction is a requisite reaction in lipogenesis in non-ruminants, yet it is not usually considered regulatory. The acetyl CoA carboxylase reaction catalyzes the first committed step in fatty acid synthesis and has been touted as the site of regulation. However, ATP CL activity may be limiting under certain physiological or
nutritional circumstances where CoA levels are low (1) or the citrate transporter is inhibited (2).

The processes of cell growth and division require biogenesis of additional membranes. Cholesterol and phospholipids are the main lipid constituents of cell membranes. De novo lipid synthesis may be required for membrane biogenesis to occur. The ATP CL reaction provides acetyl CoA as the substrate for subsequent cholesterol and fatty acid synthesis. ATP CL would need to be activated in concert with or prior to proliferation in order for new membranes to be synthesized to accommodate cell division. It has been proposed that the de novo lipid synthesis is a prerequisite for cell proliferation. Therefore, the activation of lipogenesis would be an early event in proliferation. It would be reasonable for the mitogenic signal to stimulate ATP CL activity in addition to DNA replication.

The proliferative capacity of hepatocytes, and of most cell types in vitro, is only susceptible to mitogenic stimulation at low cell density. Hepatic ATP CL activity is stimulated by a putative autocrine/paracrine activator(s) secreted into the media by hepatocytes incubated at low cell densities, while incubation at high cell densities result in secretion of an inhibitor(s) of ATP CL activity (3).
Prolonged fasting results in a cessation of growth. The levels of insulin which facilitate normal growth are low. There is a deprivation of glucose required for production of the ribose sugar moiety of DNA and incorporation of glucose into membrane lipids, processes which are vital for cell growth.

The normal production of autocrine mediators may vary under different physiological conditions which elicit altered growth. Maintenance of normal growth is aberrant in the diabetic condition as is apparent with poor wound healing and development of retinopathy. Insulin deficiency may partially account for these effects, though other functions are also probably disrupted. Since wound healing and development of retinopathy rely on autocrine control mechanisms, it is likely that some anomaly in autocrine regulation exists in diabetes.

Growth factors are growth regulators often named for the tissue in which they exert their effect. Most growth factors act as mitogens, although some are inhibitors of proliferation. Insulin is the prototype growth factor, but its mechanism of action has not yet been elucidated. The cell membrane receptors for insulin and many growth factors possess inherent tyrosine (TYR) protein kinase activity which is activated upon ligand binding. However, the target
proteins to be phosphorylated on TYR residues by the integral protein kinases are as yet uncharacterized.

Growth factors regulate cell proliferation in vitro by exogenous addition or they can be produced in an autocrine manner by the cultured cells. Transformed cells which have escaped normal growth controls, are thought to be propagated uncontrollably through overproduction of autocrine stimulators or mitogens.

The regulation of ATP CL under a variety of various growth conditions was the focus of the research presented in this dissertation. Liver is one of the main organs involved in regulation of lipid synthesis. Liver is also one of the few tissues which retains the capacity to regenerate. Establishment of hepatocyte cultures can only be attained by supplementation with epidermal growth factor (EGF) alone or in the presence of insulin. Hepatocytes are an ideal model to study the effect of growth regulators on a lipogenic enzyme activity since in vivo, liver is capable of proliferating and is also an important site for lipid regulation. Alteration of hepatic ATP CL activity by fasting, diabetes and exogenous addition of EGF was examined to shed some light on the putative role of growth regulators in the regulation of a lipogenic enzyme. A preliminary evaluation of a potential mechanism of ATP CL regulation was also conducted. The working hypothesis for this study was
that liver ATP CL is subject to autocrine/paracrine regulation which changes as a function of dietary and hormonal states of the animal. The following hypotheses were tested.

1) The autocrine/paracrine regulators in conditioned medium from hepatocytes from fed or fasted rats do not alter ATP CL activity in an identical manner.

2) Streptozocin diabetes alters the production of or response to hepatocyte autocrine/paracrine regulators.

3) Streptozocin diabetes does not alter apparent ATP CL activity in hepatocytes from rats in a short period (3 days), but does after 7 days.

4) EGF activates ATP CL in a time and dose dependent manner.

5) The effect of EGF on ATP CL is dependent on the age of the rat.

6) The mechanism of the effect of autocrine/paracrine regulators is to change the subcellular location of ATP CL.
CHAPTER II
LITERATURE REVIEW

OVERVIEW OF LIPOGENESIS

The process of lipogenesis is vital for maintenance of metabolic homeostasis in the whole animal. The synthesis of lipid is finely regulated, and there is an intricate balance of production, storage and utilization of lipid among tissues, especially liver, adipose tissue and muscle. The storage of lipid as fat provides an extremely efficient means of conserving energy until it is needed. It is more practical to store excess energy as fat at 9 Kcal per gram rather than as glycogen at 4 Kcal per gram. Glycogen also is associated with large amounts of water and in order to store the same amount of energy in glycogen as fat the body would have to carry substantially more weight, although fat does store water in the form of metabolic water released when the fat is oxidized via β-oxidation. Fat serves as an effective thermal insulator and protects vital organs as well. Lipids are prominent components of cell membranes, and the appropriate ratio of cholesterol and phospholipids preserves the integrity of the structure and function of
the cell membranes. Membrane lipids are involved in action of ion channels, nutrient transporters, and signalling of integral receptors upon ligand binding (4). Fatty acylation of enzymes is considered a putative regulatory mechanism. The hydrolysis of phosphatidylinositol contained in cell membranes by phospholipases is involved in transmittance of second messengers to generate various hormone receptor actions (4). Specific lipids are essential in myelination of nerves and functioning of lung surfactant (4). Novel synthesis of cholesterol derivatives may be required in order for DNA to replicate (4). Clearly, the availability of lipids is critical for proper cellular functioning as well as for other roles in the whole body.

Liver is the principal organ regulating overall lipid metabolism. Liver rapidly responds to nutritional state and to subsequent changes in insulin and glucose availability. Glucose is directed to the blood as it is hydrolyzed from glycogen for use at other tissues in the fasted state, and in the fed state glucose is stored in glycogen, and there is a seemingly unlimited capacity to synthesize fatty acids. Non-lactating pigs and ruminants synthesize the majority of fat in the adipose tissue (5). Conversely, fat is generated mostly in the liver in birds while man, rats and mice synthesize fat in both adipose tissue and liver (5). Liver is capable of rapid metabolic
conversion as fasted animals are refed (6). The portal circulation seems to be primarily responsible for the rapid response since the insulin and glucagon secreted from the pancreas reach the liver prior to peripheral blood (6). Under fasting conditions glucagon elevates intracellular cAMP levels which leads to acceleration of glycogenolysis, gluconeogenesis, ureogenesis and ketogenesis (6). Similar metabolic conditions are prevalent in diabetes mellitus. Long chain acyl CoA levels are high in prolonged starvation and diabetes, but are reduced upon high carbohydrate refeeding or provision of insulin (7). A rapid activation of fatty acid synthesis follows high carbohydrate refeeding in vivo as well as in murine livers perfused with glucose (7). Long chain fatty acids have been proposed to be "acute regulators" of lipid synthesis in the liver (8). In vivo, nutritional conditions which give rise to high levels of circulating fatty acids are starvation, high fat feeding and diabetes when the rate of hepatic lipid synthesis is depressed (8). As plasma insulin levels are elevated exogenously or endogenously, lipogenesis is elevated. Increasing blood levels of fatty acid levels experimentally demonstrates that inhibition by the long chain fatty acids is exerted by reducing acetyl CoA carboxylase activity, impairing citrate transport from mitochondria to cytosol and inactivation pyruvate dehydrogenase (8). Forty-eight hours
after refeeding, hepatic lipogenic enzyme activities are high as is acetate incorporation to palmitate and oleic acids (7). During this period there is a concomitant accumulation of saturated and monounsaturated fatty acids as well as triglycerides in the liver (7). The elevated levels of triglyceride synthesis may account for the lipemia seen with high carbohydrate refeeding.

The carbon source for fatty acid synthesis is acetyl CoA which ultimately originates from the cytosolic conversion of glucose to pyruvate. Pyruvate enters the mitochondria for eventual production of citrate which is transported to the cytosol as substrate for ATP CL generating acetyl CoA for either fatty acid or cholesterol synthesis. The committed step in fatty acid synthesis is the acetyl CoA carboxylase reaction yielding malonyl CoA to be incorporated to long chain fatty acids through a series of sequential reactions performed by fatty acid synthetase. De novo fatty acids can follow one of several paths. The fatty acid can be oxidized for energy in liver, heart, skeletal or adipose tissue (9). Alternatively, the fatty acid could become a "complex lipid" as triglycerides are formed and stored in the liver or adipose tissue or associated with lipoproteins and transported from liver to adipose tissue. The fatty acid also could undergo further metabolism to comprise cellular and organelle membranes.
Regulation of lipogenesis is attained at two levels: long term and short term (10). The mechanism of long term regulation will be discussed first as it was initially considered to be the only means of regulation. Gibson et al. (10) maintained that 5 enzymes were susceptible to alteration of synthesis and degradation rates to achieve adaptation. The activities of glucose-6-phosphate dehydrogenase, ATP CL, acetyl CoA carboxylase, fatty acid synthetase and malic enzyme declined "in unison" upon starvation and were similarly elevated together with refeeding (10). The suppression of enzyme activities took place over several hours and were consistent with disappearance of antibody detection of enzymes in the control over the same time period. The change in synthetic rate of the enzymes was found to be the major determinant of "coordinate adaptation" of enzymes (10). Refeeding also induced overall protein synthesis as insulin stimulates release of liver mRNA, and additional rRNA is also synthesized (10). However, recent discoveries concerning regulation of lipogenic enzyme activities complicate these earlier findings. Some of the reduction in activities may be accounted for by subcellular relocalization of the enzymes during nutritional states (11-13). Phosphorylation states of the lipogenic enzymes were found to change upon different hormonal treatment, although the significance has
not been entirely determined (14). Another parameter to be considered is the potential change in antibody reactivity as the enzymes undergo conformational changes due to change in phosphorylation state or subcellular location.

Long term adaptations of the lipogenic enzymes are mostly due to nutritional and hormonal control of enzyme quantities. Short term control involves modulation of lipogenic enzyme activities by substrate and allosteric effectors and interconversion between the phosphorylation states of the enzymes (15). The short term control mechanisms allow for "greater range in fluctuation of lipogenesis" (15). Long term adaptation requires greater than six hours to take effect whereas short term regulation occurs in less than 15 to 30 minutes (16). It was recently shown that treatment of hepatocytes with several growth factors altered lipogenic enzyme activity within 5 minutes (17). Insulin and glucagon affect lipogenesis within 30 minutes of addition: insulin stimulating and glucagon inhibiting lipogenesis (18). Insulin also antagonizes the inhibition produced by glucagon (18). Glucagon depresses glycolysis thus limiting the availability of fatty acid substrates; pyruvate, lactate and citrate levels decline as a consequence (16). The addition of pyruvate and lactate to isolated hepatocytes only partially relieves the low lipogenic rate though levels of citrate return to normal
Therefore it was inferred that glucagon is effective at a step after citrate formation. Insulin does not have the inverse effect on the metabolites: lactate, pyruvate and citrate levels remain stable (16). Insulin as well as glucagon were thought to exert their effects on the enzymes of the lipogenic pathway itself (16).

Allosteric effectors of lipogenic enzyme activities potentially play a role in regulation of lipid synthesis. The key allosteric regulators involved are malonyl CoA, long chain CoA esters, acetyl CoA, fructose, fructose-1,6-bisphosphate and citrate (19). Under fed conditions where the insulin/glucagon ratio is high and cAMP levels are low fructose-1,6-bisphosphate activates pyruvate kinase, pyruvate inhibits the kinase that inactivates pyruvate dehydrogenase, citrate inhibits phosphofructokinase and activates acetyl CoA carboxylase ("coordinating glycolysis and lipogenesis") and malonyl CoA inhibits carnitine acyl transferase I (19). Under fasted conditions with glucagon elevating cAMP levels, the main allosteric effectors are acetyl CoA which activates pyruvate carboxylase and long chain CoA esters which inhibit acetyl CoA carboxylase (19). The level of free fatty acids and long chain CoA esters seems to be pivotal for hepatic lipogenesis. Liver must be capable of disposing of these free fatty acids and long
chain CoA esters in order to participate in lipid synthesis (19).

The activation or deactivation of key regulatory enzymes by phosphorylation/dephosphorylation can coordinately alter enzyme activities (20). Insulin, in fed conditions, in most cases promotes enzyme dephosphorylation which activates the enzymes involved in glucose utilization and storage of an adequate supply of glucose (20). There are rises in the activities of phosphofructokinase, pyruvate kinase, pyruvate dehydrogenase, HMG CoA reductase, α-glycero-phosphate-acyl transferase and glycogen synthetase (19). The effect of insulin on cellular signalling is not clear, though it may act through the phosphatidylinositol pathway activating protein kinase C and specific uncharacterized proteases (21). On the other hand, glucagon favors phosphorylated forms of enzymes in the fasted state. Phosphorylation of lipogenic enzymes leads to deactivation while activating the enzymes involved in glucose production such as glycogen phosphorylase, fructose-bis-phosphatase and phosphorylase kinase (19). Glucagon acts on liver to activate adenylate cyclase, increasing intracellular cAMP levels triggering cAMP dependent protein kinase which phosphorylates certain enzymes. It is impossible to isolate any one mechanism and describe it as the only causative regulator of short term control. Instead allosteric effectors, substrate
availability and covalent modification (phosphorylation state) of enzymes all participate in the regulation of lipogenesis. During prolonged nutritional or hormonal conditions, long term control through alteration of enzyme synthetic rates is also involved.

Both ATP CL and acetyl CoA carboxylase are subject to SER/THR phosphorylation (22-24). Several studies showed that glucagon and insulin phosphorylated the same residues on ATP CL which is in contrast to their inverse effects on activity (22-24). However, most of the procedures used only purified enzyme treated with the hormones which may not be representative of the in vivo action. Allred et.al. (14) found that an increase in phosphorylation of purified acetyl CoA carboxylase by an endogenous protein kinase from rat liver homogenates paralleled a reduction of activity. Roehrig et. al. (1) showed that reduction in ATP CL activity upon phosphorylation could only be detected when assayed at low CoA levels. Thus the results obtained may likely be dependent on the system used and the conditions under which the enzyme activities are measured.

The capacity for lipogenesis in diabetic liver is extremely blunted. The diabetic animal is constantly in a metabolic situation which resembles the fasting state. Diabetes mellitus is actually a "heterogenous collection of disorders" (25). Type I (IDDM) diabetes is characterized
by impairment of insulin production while type II (NIDDM) is more a development of "profound insulin resistance with alterations in the kinetics of insulin secretion" (25). The focus of this brief review will be on IDDM since it relates more closely to the experiments included in this dissertation. The initial aberration in IDDM is loss of insulin production from the pancreatic \( \beta \)-cells. This condition can be mimicked by tail vein injections of streptozocin or alloxan into rats which destroys the \( \beta \)-cells of the islets, and the animals are rendered diabetic (25). Similar results can be attained by treatment with insulin antibodies, but the effect is short-lived and less consistent. Genetic models for IDDM have also been bred, for example, the BB/Wistar rat, but they are more expensive to use than chemically inducing \( \beta \)-cell destruction.

The activities of the lipogenic enzymes in the liver of streptozocin or alloxan diabetic rats have been reported to be as low as 10% of normal (9). The low levels of fatty acid and cholesterol synthesis in diabetic rats can be ameliorated by infusion of insulin (9). Plasma insulin levels are 4 fold lower in normal fasting animal than diabetic fed animals (26). There is also a rise in plasma non-esterified fatty acids (NEFA) and blood ketone bodies without change in triglyceride levels (26). As insulin levels normally decline during fasting, adipose tissue
undergoes lipolysis of NEFAs which are transported to the liver and oxidized to ketones. Severe IDDM can yield a 3 to 10 fold increase in blood levels of NEFAs, ketones and triglyceride (26). There is a rise in triglycerides as well as NEFAs and ketones in diabetes since insulin also regulates lipoprotein lipase activity on endothelial cell surface proximal to adipose tissue. Because lipoprotein lipase is low, triglycerides can not be hydrolyzed (26). The hepatic storage of triglycerides drops since insulin is required for their synthesis and with the continual flux of NEFAs from adipose tissue, there is a development of fatty liver, hyperlipemia along with exaggerated ketogenesis (27). Glucose oxidation also declines in diabetes and is thought to contribute to the "marked derangement" of fatty acid and cholesterol synthesis (27). Glucose can be oxidized through the TCA cycle or through the hexose monophosphate shunt which provides an adequate supply of NADPH for lipogenesis. Some abnormality in shunting of glucose to hexose monophosphate was proposed to contribute to reduction of lipogenesis in diabetes (28). When hexose monophosphate shunt was stimulated independent of glycolysis with exogenous addition of NADP, there was a dramatic rise in the rates of fatty acid and cholesterol synthesis, enough to compensate for lipogenesis in diabetic livers (29). Once lipid synthesis is restored, fatty acid is predominantly
made and incorporated into phospholipids and triglycerides with less regeneration of cholesterol (29).

The metabolism of glucose and lipids is severely and permanently altered in IDDM. Low levels of insulin preclude the ability of liver to store and utilize glucose, and hyperglycemia ensues. Tissues which were dependent on insulin to upregulate glucose transporters to deliver glucose can no longer get an adequate supply of glucose as their energy source and must adjust to using fatty acid and ketones for energy (i.e. muscle). Other tissues which are not dependent on insulin for glucose delivery have difficulty utilizing the glucose since insulin may be required to activate the enzymes involved. Thus, reduction in insulin levels affects glucose and lipid metabolism at multiple levels. Although lipogenic enzyme activities decline in diabetes, recovery is rapid upon reintroduction of insulin. It could be assumed that the abnormality in diabetes is not directly in the functioning of enzymes, but rather in regulation of intracellular signalling.

**ATP CITRATE LYASE**

**Role of ATP citrate lyase in fatty acid synthesis**

The carbons for fatty acid synthesis are derived from acetyl CoA. The acetate originates primarily from glucose which forms cytosolic pyruvate through glycolysis in non-ruminants. A difficulty is that pyruvate dehydrogenase
which converts pyruvate to acetate is confined to the mitochondria. Pyruvate must therefore enter the mitochondria and be converted to acetyl CoA which subsequently could be transported directly or indirectly to the cytosol through simple diffusion or facilitated transport on an acetate-carnitine carrier or by using citrate in an indirect manner, first transporting citrate across the mitochondria then cleaving citrate with ATP CL liberating acetyl CoA into the cytosol (9). The latter proved to be the mechanism for producing acetyl CoA in the cytosol in non-ruminants since the mitochondrial membrane is impermeable to acetyl CoA, and the carnitine acyl CoA transporter does not carry acetyl-carnitine (30). Acetyl CoA can arise from the action of acetate thiokinase which is found both in the cytosol and the mitochondria (31). In non-ruminants, however, there would be relatively little free acetate.

Brady and Gurin were the first to recognize the importance of citrate in fatty acid synthesis in pigeon liver (32). Then several years later ATP CL was isolated and found abundantly in chicken and rat liver although its precise significance was not clear (31). Acetate thiokinase was believed to be the enzyme responsible for supplying the cytosol with acetyl CoA (31). Kornacher and Lowenstein suggested an association between ATP CL activity and the
ability of liver to synthesis lipids under different physiological conditions (33).

The conversion of citrate to acetyl CoA and OAA was primarily believed to be merely the reverse reaction of citrate synthase under different availability of substrates (30). ATP CL was classically considered to be strictly an irreversible reaction (34). Inoue et. al. discovered that the reaction was slightly reversible since minute amounts of $^{32}$Pi were incorporated into ATP, and $^{14}$C-acetyl CoA produced small amounts of $^{14}$C-citrate upon incubation with purified ATP CL (35).

Fatty acid synthesis is extremely energy costly and requires an ample supply of NADPH. ATP CL not only synthesizes acetyl CoA for fatty acid and cholesterol synthesis, but the OAA produced aids replenishment of cytosolic NADPH. Cytosolic NAD:malate dehydrogenase converts the OAA to malate which is subsequently decarboxylated by malic enzyme regenerating not only NADPH but, also pyruvate which is recycled. NADPH is derived from three cytosolic sources in the non-ruminant. In addition to malic enzyme, the majority of NADPH is supplied from the shunting of glucose to the hexose monophosphate shunt. A cytosolic form of NADP-dependent isocitrate dehydrogenase is a minor source for NADPH in non-ruminants. Ruminants differ from non-ruminants in their source of NADPH as they
rely on NADP-dependent isocitrate dehydrogenase (36). ATP CL also indirectly contributes NADPH to the available pool as previously discussed.

**Distribution of ATP citrate lyase**

Significant amounts of ATP CL have been found in tissues of most non-ruminant animals which synthesize fat, such as liver, kidney, heart, spleen, brain and testicles as well as adipose tissue of rodents and the liver and hearts of birds (37). Ruminants do not depend on citrate to supply acetate since rumen microbes contribute significant amounts of acetate to the GI tract. It was previously thought that microbial acetate production was the sole source of fat in bovine, however lactate can also give rise to acetate in bovine adipose (36). The only way lactate can be converted to fatty acid is via the citrate cleavage:malic enzyme pathway (36). Although the activities of the lipogenic enzymes in ruminants are considerably lower than in non-ruminants, they are sufficiently active to convert lactate to fatty acid. The activity of ATP CL in liver and adipose is substantially higher in veal calves than in ruminating calves, probably because the plasma glucose and insulin levels in veal calves are comparatively high (38). The main site of fatty acid synthesis in sheep is adipose tissue; fatty acid synthesis in the liver is nominal in comparison since its main function in ruminants is gluconeogenesis.
The rate of lipogenesis in liver and adipose tissue of sheep increased substantially upon feeding high carbohydrate diets or direct glucose infusion into the abomasum which is accompanied by substantial activation of ATP CL and malate dehydrogenase activities (40). ATP CL has been measured in other rumen tissues such as brain, heart, rumen mucosa and kidney (41-44).

A distinct citrate lyase (CL) enzyme occurs in certain bacteria. The obligate aerobe, Rhodoturula gracilis can store fat (45). It can even develop a condition similar to obesity in animals. CL is an adaptive enzyme in certain bacterial systems depending on the substrates present in the media. Activity is elevated when glucose, citrate or succinate are available and depressed by ethanol or glycerol (45). The bacterial enzyme, however, is not ATP or CoA dependent (45). High quantities of CL were detected in Penicillium spiculospulum which synthesizes and abundance of lauryl CoA (45). However, most aerobes have an intact TCA cycle which can metabolize citrate without requiring a specific citrate cleaving enzyme. Anaerobes though, have an incomplete TCA cycle and must catabolize citrate to pyruvate, acetate and CO₂ by the enzymes CL and oxaloacetate decarboxylase. The anaerobes have to rid themselves of citrate to maintain the proper redox balance as well. Therefore, the regulation of CL in anaerobes is much more
controlled than in aerobic bacteria. Yeast also contain a form of ATP CL with enzymatic properties similar to the mammalian enzyme (46).

**Characteristics and kinetics of ATP citrate lyase**

The reaction catalyzed by ATP CL is one of the most complex biochemical reactions. Three substrates are required (citrate, CoA and ATP) as well as the metal ion, Mg++, yielding four products (acetyl CoA, OAA, ADP and Pi). There is a high specificity for all reactants (47). Citrate can not be mimicked by (-) hydroxy citrate or a stereo selective inhibition of the enzyme occurs. The terminal carboxyl groups of citrate appear indistinguishable, but ATP CL accurately distinguishes them, such that the methyl group of acetyl CoA is derived from the methylene carbon of citrate (48). ATP is vital as well; CTP, UTP, TTP or CTP are not adequate substitutes. Pantetheine can not replace CoA either (48). There are distinct and specific binding sites which exist for each reactant (49). Mg++ is also an absolute requirement for the reaction.

ATP CL is extremely unstable with a half-life of 48 hours in vivo (49). The instability may be attributed to the presence of 45 to 60 sulfhydryl groups of which 6-8 are considered to be reactive (50). Mg-citrate provides some inherent protection, but ATP CL activity is destroyed by sulfhydryl oxidizing agents such as air and oxidative
glutathione (50). The addition of dithiothreitol or reducing agents (β-mercaptoethanol) can reverse the oxidative events (50).

Eukaryotic ATP CL has a MW of 440 KD which includes four presumably identical subunits each of 110 to 120 KD as determined by both sedimentation equilibrium and SDS-PAGE methods (47,35). ATP CL itself is phosphorylated inherently by its reaction when incubated with ATP and Mg++ resulting in two moles phosphate bound per mole of ATP CL. Even though ATP CL is composed of four identical subunits, their conformational arrangement allows only two subunits to be phosphorylated. Walsh and Spector proposed that the reaction occurred as an ordered sequence of partial reactions (51). The mechanistics of the reaction have not been completely resolved. Though it is considered to catalyze two contiguous half reactions (52,53). An inherent thiokinase catalyzes the following reaction:

\[
\text{citrate + ATP + CoA} \rightarrow (3s)\text{-citryl-CoA + ADP + Pi}
\]

This is succeeded by an inherent acetyl CoA lyase reaction:

\[
(3s)\text{-citryl-CoA} \rightarrow \text{acetyl CoA + OAA}
\]

The reaction is initiated upon addition of ATP to the enzyme producing a phospho-enzyme. When \(^{32}\text{P-ATP}\) is incubated with ATP CL in the absence of citrate of CoA, two moles of \(^{32}\text{Pi}\) are covalently bound per mole enzyme (34). The phospho-enzyme form is considerably more stable than the native
form. Once the phospho enzyme is formed and citrate is introduced, \(^3\)\(^2\)Pi is released in an equilibrium ratio of citrate to bound Pi (54). Actually, a citryl-P may be an intermediate which is non-covalently attached to ATP CL. Subsequently, a Pi is liberated leaving a covalent bond between citrate and the enzyme. CoA then acts on the citrylated enzyme to form non-covalently bound citryl CoA which then dissociates to yield free enzyme, acetyl CoA and OAA. The postulated intermediates, ATP CL≈Pi and ATP CL≈citrate have been directly isolated, but the complexity of the ATP CL obstructs isolation of citryl-CoA and citryl-Pi intermediates. The proposed mechanism is represented here:

\[
\begin{align*}
E + ADP\approx Pi & \rightarrow E\approx Pi + ADP \\
E\approx Pi + citrate & \rightarrow E...citryl\approx Pi \\
E...citryl\approx Pi & \rightarrow C\approx citrate + Pi \\
E\approx citrate + CoA & \rightarrow E...citrylCoA \\
E...citryl CoA & \rightarrow E + acetyl CoA + OAA
\end{align*}
\]

The optimum pH of the reaction is pH 8.7. The change of free energy for the overall reaction is \( O \) (55):

\[
\text{Citrate}^{3-} + \text{CoASH} \rightarrow \text{acetyl CoA} + \text{OAA}^{2-} + \text{H}_2\text{O} \\
F=+7600
\]

\[
\text{ATP} + \text{H}_2\text{O} \rightarrow \text{ADP}^{3-} + \text{Pi}^{2-} + \text{H}^+ \\
F=-7600
\]
It would appear that with no change in free energy of the overall reaction that it would be a freely reversible reaction. This is not the case since the ATP CL reaction consequently utilizes the energy from the terminal pyrophosphate bond of ATP to eventually create the thiol ester bond in acetyl CoA (48). The precise mechanism which ATP CL undergoes is not completely agreed upon (52). Plowman and Cleland (55) support an ordered mechanism, while Inoue et. al. (35) prefer the ping-pong mechanism where ATP binds followed by the release of ADP, then citrate reacts. The approach implemented in studying the mechanism must be taken into account. Studies of initial velocity with isotope exchange at equilibrium demonstrate sequential kinetics. Various partial reactions are done step-wise so intermediates can be identified under controlled conditions (56).

The ATP CL reaction which is composed of a thiokinase reaction and an acyl CoA lyase reaction is not destroyed by limited proteolysis by trypsin (52). Singh et. al. (57) suggested that trypsin digestion may yield distinct fragments which catalyze specific partial reactions. Purified ATP CL consists of three major bands on SDS-PAGE gels which are identical to short-term treatment with trypsin: 110 to 120 KD, 71 KD and 55 KD. Treatment of ATP CL with trypsin not only preserves activity, but maintains
phosphorylation activity, immuno-reaction, sedimentation and has the same Km for citrate. Though the sensitivity to heat destruction increases. Fragments of 68 to 71 KD and 55 to 53 KD are produced by trypsin or chymotrypsin digestion. Subtilisin further degrades the larger, 70 KD fragment to 37 KD and 25 KD fragments, and ATP CL activity is destroyed (52). The acyl CoA lyase activity resides in the larger 70 KD fragment. Lill et. al. reason that the proteolysis targets the primary region which separates the acyl CoA and part of the thiokinase which reside in the 70 KD fragment from the residual thiokinase, housed in the 53 KD fragment (52). Bloxam et.al. maintained that the assumption could be made that trypsin proteolysis attacks a "hinge region" separating the acyl coA lyase and thiokinase domains (58). However, two polypeptide chains compose other known thiokinases (58). So, it is feasible for proteolysis to occur at a hinge region between acyl CoA and one of the polypeptide chains of the thiokinase (58). The significance of the fragments can be extrapolated to be involved with the location of catalytic sites and sites susceptible to phosphorylation by hormone stimulation (59).

ATP CL contains distinct phosphorylation sites. The inherent autophosphorylation produced by indigenous thiokinase activity is referred to as the catalytic phosphorylation (35,23). It is alkali stable, but acid
labile. The structural phosphorylation site arises from hormonal stimulation and conversely is acid stable, but alkali labile (23). There have been various reports of reversible structural phosphorylation by both insulin and glucagon in both hepatocytes and adipocytes (60,61). A precise function for these structural phosphorylations has not been elucidated though it has been suggested that it may play a role in signalling protein degradation or translocation to mitochondrial membrane (62). There is a putative interaction of limited proteolysis of specific fragments with intact enzyme activity and phosphorylation/dephosphorylation involved in regulation of enzyme activity (61,62). The proteolytic fragments cannot be isolated chromatographically under non-denaturing conditions which suggests a strong association by non-covalent forces (62). In addition, a large conglomerate form of ATP CL was found which is dissociated by Mg++ and probably does not exist in vivo (63).

Regulation of ATP citrate lyase

ATP CL conforms to the overall regulation of lipogenesis and its enzymes as they are subject to nutritional and hormonal control (10). Activity is low in fasting and diabetic state, while refeeding high carbohydrate or administration of insulin replenishes the activity or elevates it further (10). Enzyme activity in diabetic and
starved animals can decline to levels as low as 10 to 20% of controls. Well fed or starved rats refed high carbohydrate diets with minimal fat have an incredible capacity to synthesize fat (9). Long term regulation or adaptation by increasing the enzyme synthesis is accepted as the main mechanism of control (10). It is apparent that ATP CL is quite inducible as are all the lipogenic enzymes (10). As a result, there is a coordinate decline in lipogenic enzyme activities with fasting followed by a subsequent rise in activities upon refeeding. The modification of lipogenic enzyme activities has been attributed to a change in net protein synthesis and degradation. The administration of puromycin and actinomycin D in conjunction with refeeding starved rats prevented the rise in activities of acetyl CoA carboxylase, fatty acid synthetase and ATP CL (10). This was interpreted as proof that new protein synthesis was responsible for the change in enzyme activities (10). Taking into consideration rate equations and steady state, it was statistically determined that protein synthesis rate was responsible for modification of lipogenic enzyme activities by certain nutritional factors since the degradation rate remained constant (10). However, these studies only measured the cytosolic fraction. Often times administration of protein synthesis inhibitors to rats will suppress their appetites and desire to feed,
which could complicate the interpretation. Cycloheximide, another protein synthesis inhibitor used in later studies to validate regulation by protein synthesis, elevates the cellular cAMP levels which can alter the second messenger system turning on protein kinase activity (64). Adaptive changes in enzyme activity occur over the course of several hours (8 hours). Liver mRNA for ATP CL specifically peaks (25 fold) at 15 hours after refeeding a fasted rat (65). Sue et al. (65) said this indicated ATP CL was regulated by pre-translational control, however, their method of measuring mRNA levels was not conventionally accepted. Peripheral venous blood insulin levels rise coordinately with enzyme activity, thus portal insulin levels are considered as the primary signal (10). Rat liver mRNA was found to be increased in response to insulin concentration (66). During the refeeding phase free ribosomes are repleted first, then ribosomes bound to intracellular membranes increase as ribosomal RNA levels rise (67). Short term regulation was always assumed to play an insignificant or minor role in the control (66,67).

Liver must tightly coordinate the utilization and production of glucose upon nutritional and hormonal stimulus. There is evidence that glycolytic reactions and gluconeogenic reactions seem to be compartmentalized within liver acinar region (68,69). A theory was presented for the
existence of "coordinate zonation" of carbohydrate and lipid metabolism in the liver parenchyma (69). Gluconeogenesis is localized in the periportal zone while glycolysis is localized in the perivenous zone. Logically, lipogenic enzymes are localized with glycolysis so that the acetyl CoA derived from glucose can be efficiently utilized for lipid synthesis (70). ATP CL activity is predominantly in the perivenous area and is approximately double that in the periportal area in fed animals (70). This "gradient" is diminished upon starvation, but enhanced up to 2.8 fold by refeeding.

Hormonal regulation of ATP CL is not restricted to insulin and glucagon. Thyroxine restored the ATP CL activity in adipose, adrenals and testes of hypophysectomized rats; activity was correlated with decline of steroid synthesis as well (71). The results supported the suggestion of involvement of ATP CL in lipogenic control in testes and adrenals which is similar to that in liver, mammary and adipose and also that regulation of ATP CL activity is important in tissues which actively synthesize steroids.

ATP CL, as well as the other lipogenic enzymes, is sensitive to the type of carbohydrate available (33). Glucose and fructose feeding elevated liver ATP CL activity more than the other lipogenic enzymes. Fructose has a
greater effect on ATP CL activity than glucose (33). Fructose may be a more readily metabolizable carbohydrate source. Alternatively, high fat diets prevent the induction of lipogenic enzymes. Fat-free diets lead to the accumulation of fatty acids which are generated by glucose conversion to acetyl CoA producing an abundance of palmitate (16:0), stearate (18:0), palmitoleate (16:1) and oleate (18:1). The lipid composition of liver reflects the dietary fat source within 2 days of feeding. Essential fatty acids diminish concomitantly as fat-free diets are fed extensively, and symptoms of an essential fatty acid deficiency can develop (72). If essential fatty acids or are supplemented, liver lipogenic enzyme activities decline (72). The induction of ATP citrate lyase activity by dietary carbohydrate can be prevented by feeding PUFAs in conjunction with high carbohydrates (72). The effect of PUFA on ATP CL occurred in absence of a change in the protein degradation rate which led Schwartz and Abraham (73) to propose that the regulation of ATP CL by PUFA was by converting an active form of the enzyme to an inactive form.

Early studies could not distinguish between the primary alterations caused directly by insulin, and secondary effects of insulin in altering carbohydrate intermediates. Rats which were rendered diabetic began to be used as a research tool to study these effects of insulin. Kornaker
and Lownestein showed that diets high in fructose as well as administration of insulin, but not glucose alone induced ATP CI and fatty acid synthesis in livers of diabetic rats (33). They concluded that insulin functions indirectly to improve glucose utilization and that ATP CI is dependent on the efficiency of carbohydrate metabolism and not on insulin's direct action on ATP CI. In homogenates of liver from streptozocin induced diabetic rats, insulin was obligatorily required to accommodate induction of all lipogenic enzymes by high carbohydrate diet (33).

Investigators began to use cell culture systems which could better control and distinguish between primary and secondary hormonal effects. Goodridge et.al. in 1974, used chick embryo hepatocytes cultured with serum to measure the incorporation of $^{14}$C-glucose into fatty acids and the relation to activities of malic enzyme, fatty acid synthetase and ATP CI activities (74). The half-life of ATP CI in culture is 18 hours as compared to 24 hours in vivo (74). ATP CI activity in newly cultured cells drops rapidly to 60% of initial activity and remains constant there after. Addition of insulin resulted in 2.4 fold induction 24 hours after addition (74). Another study demonstrated that addition of glucose or insulin or both activated ATP CI in hepatocyte cultures, but activation was blocked by addition of cycloheximide (75). It was assumed
that these results confirmed the belief that long term adaptation of enzyme amounts is regulatory for ATP CL. There are complications in these interpretations which were not considered. First of all, cultures of hepatocytes relied on serum which contain many unidentified factors which could have interfered with the action of insulin, and serum could contain unknown amounts of insulin. Secondly, cycloheximide elevates intracellular cAMP levels (64). Either of these complications could result in misleading interpretations.

Treatment of cultured hepatocytes with glucagon, cAMP or free stearate inhibited lipogenesis and all lipogenic enzyme activities (76). Glucagon, dibutyryl cAMP, or theophylline rapidly inhibited ATP CL activity (76). When freshly isolated hepatocytes were treated with glucagon fatty acyl CoA was produced (74). Therefore, glucagon treatment was considered to contribute to the existence of short term control in both cultured and freshly isolated hepatocytes.

In 1968, Foster and Srere did a series of experiments in which they concluded that ATP CL is not involved in primary regulation of lipogenesis (77). In light of Srere's pioneering work with ATP CL, this was the accepted theory for many years in spite of several equally attractive alternative interpretations of the data. His conclusions were based on the premise that ATP CL activity does not
change to the same extent or correlate well with fatty acid synthesis. Inhibition of fatty acid synthesis in alloxan diabetic rats occurred prior to change in ATP CL activity. He claimed that high carbohydrate, low fat diets altered ATP CL activity without any significant change in fatty acid synthesis (77). However, it has been shown multiple times since that high carbohydrate diets do alter rate of fatty acid synthesis (78). Srere et al. added back purified ATP CL to liver homogenates and measured no effect on the rate of fatty acid synthesis. These results are not necessarily conclusive due to inherent limitation of the model. Purified enzyme may not behave the same as it does in vivo and regulatory regions may be hidden upon conformational change caused by purification. Modification of the enzyme could be prohibited or altered upon purification. Also, optimum substrate levels rather than physiologically relevant substrate levels were used. Since ATP CL was considered to be a cytosolic enzyme, most early studies used only the cytosolic fraction to measure ATP CL (31,34,77). Janski and Cornell have shown that ATP CL may also exist in a form which is associated with the mitochondrial membrane (79).

Interest in the regulation of ATP CL and its contribution to lipogenic control was reignited with the discovery that ATP CL could be phosphorylated at a
structural site distinct from the phosphorylation of its catalytic site as an intrinsic part of the enzyme reaction (22,23). The catalytic phosphorylation occurs on a histidyl or glutamyl residue (22,23). NMR spectroscopy detection of the acid labile catalytic site confirmed the phosphorylation to be an a histidyl residue (80). The labeled histidyl peak disappeared rapidly upon addition of substrate citrate and CoA to ATP CL. It is generally believed that a potential of four possible phosphorylations on different sites can occur: two on the catalytic region and two in the structural region (22,23). Glucagon treatment promotes phosphorylation of the structural or regulatory site at a SER as determined by NMR spectroscopy (80). The SER phosphorylation is alkali labile. Unlike the catalytic site, the location of the structural site is in a peripheral domain of the protein (81,35). It is considered that the SER in the regulatory site can not be phosphorylated until ATP CL is activated by phosphorylation of the catalytic sites (35,81). Phosphorylation of a structural or regulatory site was demonstrated in vivo and in vitro purified enzyme systems (35,81). Ragnathan et. al. assumed that there were no distinguishing characteristics between the phospho-and dephospho-ATP CL since there were no measurable differences in pH optimum, reaction kinetics, stability or susceptibility to inhibitors (61). However, the experiment
was performed with purified enzyme and the dephospho-form was attained by treatment with a phosphatase. Either form may not be truly representative of the native enzyme which exists in vivo.

ATP CL in most tissues treated with glucagon were phosphorylated at the structural site (82-84). Since cAMP treatment yields similar results, activation of cAMP-dependent-protein kinase is considered to be the vehicle (83,84). Yet, the phosphorylation of ATP CL is delayed and occurs much more slowly than the phosphorylation of other enzymes by glucagon, so it was considered to contribute to long term regulation (82-84). Later, insulin was also found to induce phosphorylation of ATP CL, which further complicated understanding the potential significance for control by phosphorylation events. The structural sites phosphorylated by insulin, glucagon and cAMP-dependent protein kinase were initially considered to be identical (85,86). Insulin invokes the activation of a kinase which is not dependent on cAMP and is referred to as citrate lyase kinase (87). It also has the distinct ability to phosphorylate not only SER residues, but THR residues contrary to cAMP-dependent protein kinase which can only act on SER (87). A synergistic reaction was discovered between cAMP-dependent protein kinase and CL kinase when the incubation reaction was extended (87). It seemed that cAMP-
dep-PK facilitated phosphorylation by CL kinase (87). Trypsin treatment was used to produce two peptides, A and B, then kinase activity was evaluated (88). Peptide A contained only a SER phosphorylation site, while peptide B housed both a SER and THR phosphorylation site. Pucci et al. mapped three phosphorylation sites in adipose tissue ATP CL on two peptides which existed in vivo (87). These sites were analogous to those obtained by trypsin digestion. They showed that cAMP-dep-PK phosphorylates on SER on peptide A and insulin phosphorylated only peptide B (88). However, these phosphorylation events did not follow a time course which could contribute to short term phosphorylation of ATP CL (88). Belsham, et al. isolated an insulin-directed phosphorylation of a 22 KD peptide as well as ATP CL in rat epididymal pad. Apparent short term control was at play as addition of anti-insulin serum reversed the phosphorylation within 15 minutes. This was significant because it opened the door for consideration of possible short term hormonal regulation by insulin through rapid alteration of phosphorylation state (89). The 22 KD peptide was speculated to be a general protein phosphatase inhibitor as it exhibited similar physical properties. Insulin has been reported to stimulate phosphorylation and dephosphorylation concomitantly of several enzymes (ATP CL, ribosomal protein S6, insulin receptor and acetyl CoA carboxylase) (87).
The insulin stimulated phosphorylation of ATP CL is more complex than it originally appeared. In a series of pulse/chase experiments with $^{32}$P-phosphate, Ramakrishna et. al. (88) found that even though there was an overall increase in phosphorylation of ATP CL by insulin it did not occur equally between peptides A and B. Peptide A was increased 5 to 6 fold, while peptide B declined to levels of 30% of the control and mostly at THR residues (88). The rise in peptide A phosphorylation without change in peptide B occurred upon insulin treatment of 3T3 L-1 cells, hepatocytes and fat pad pieces (87). Conversely, β-adrenergic stimulation resulted in phosphorylation of both peptides A and B (90). A specific THR phosphatase seems to be activated by insulin which is responsible for greater turnover of phosphate on peptide B, thus continually incorporating more unlabeled phosphate and peptide A phosphorylation is more representative of radioactivity in the ATP pool (87-90). The effect of insulin was not reconstructed by addition of isoproterenol (91). Ramakrishna referred to this as a "second site regulation" which accounts for insulin's ability to phosphorylate both peptides A and B where phosphorylation of THR on peptide B causes a conformational change rendering peptide A more susceptible to phosphorylation by a constant activity of cAMP-dependent protein kinase (88).
ATP CL was historically considered to be strictly a cytosolic enzyme since it is involved in lipogenesis which occurs in the cytosol. Then, in 1980 Janski and Cornell discovered an association of ATP CL to outer mitochondrial membrane in isolated rat hepatocytes (79). The phenomena was also seen in purified ATP CL added to isolated mitochondria although the low activity measured was considered insignificant (61). Mitochondrial ATP CL can be obtained by digitonin treatment of isolated hepatocytes where 25% of the activity can be released upon such treatment (79). The localization of ATP CL to the mitochondria was also subject to dietary alteration. Only 76% of ATP CL activity in fasted rats was found in the cytosol when compared to the relative 100% in fed controls (13). This phenomenon is known as "ambiguity." Hexokinase is the prototype ambiquitous enzyme involved in subcellular translocation between mitochondria and cytosol (92). Hexokinase is released from the mitochondria as substrate is replenished (92). However, the subcellular localization of ATP CL does not seem to be affected by its substrates, but rather the bound form is stabilized by intracellular Mg++ levels. Though in the absence of Mg++ in incubation mixes, addition of ATP, citrate and CoA can liberate the mitochondrial bound ATP CL (93). Thus it was suggested that Mg++ may play a role in securing ATP CL to the mitochondria.
The hypolipidemic agent 5-(tetradecycloxy)-2-furoic acid which lowers cellular CoA concentrations (94) causes an increase in bound ATP CL, though the acid stable phosphorylation declined (93). Glucagon lowered the amount of bound enzyme with an increase in acid stable phosphorylation (93). This was thought to signify either that the bound ATP CL was less susceptible to phosphorylation or that the phospho-enzyme was less capable of binding the mitochondria (93). It was also thought that cellular CoA levels may have been partially responsible for regulating binding of ATP CL to the mitochondria (93). Roehrig et. al. (1) found that at low CoA levels phosphorylation of ATP CL by cAMP dependent protein kinase, caused a change in enzyme activity with constant Mg++ levels. Others have shown that ATP CL may be associated with the microsomal fraction which increases upon fasting (83,95), although this may have been due to simple contamination of the microsomal fraction by ruptured outer mitochondrial membrane. It is feasible that association of ATP CL provides an efficient means of utilizing acetyl CoA that is transported from mitochondria to cytosol to be available for fatty acid synthesis (79). This would be especially beneficial in the fasted condition with low availability of substrate for fatty acid synthesis. Although a precise significance for the existence of
phosphorylated or mitochondrially bound forms of ATP CL has not yet been revealed, there seems to be a putative complex interaction between the substrates, subcellular localization, phosphorylation state and other unknown variables. The disagreement among various reports may be because a uniform experimental system has not been absolutely established. Treatments were performed in vivo or in hepatocytes, tissue homogenates, cell lines, fat tissues, epididymal tissue and reconstructed cell systems using purified enzyme. The regulation of ATP CL is not likely to be maintained or remain consistent throughout all these treatments and may not be representative of the actual regulatory mechanism(s).

The response of lipogenic enzyme activity and lipogenesis to alteration of nutritional states is so dramatic that the elucidation of factors involved in such control is of much interest and is actively pursued by many. A variety of other enzyme modifications typically involved in regulation of ATP CL has been examined. Substrates of the reaction were found to offer protection from thermal and proteolytic degradation of purified enzyme (96). Citrate, CoA and 2'-phosphoryl nucleotides such as NADPH were protective at physiological concentrations, although they act at different sites on the enzyme (96). It seemed that their binding allowed for conformational change to occur
which let ATP CL be proteolytically nicked without sacrificing activity, then the new form was less accessible to further proteolysis (96). It was theorized that attaining a new conformational state could involve coordination between acetyl CoA production and availability of reducing equivalents for lipogenesis (96). A small peptide was isolated in liver which protected the ATP CL activity that remained after primary proteolysis from further digestion by ubiquitous exoproteases (59).

Calcitonin administration to rats in vivo causes an elevation of hepatic cytosolic Ca++ levels and leads to an increase in ATP CL activity as well as stimulating lipogenesis in liver and release of free fatty acids into serum (97). The effect peaks within thirty minutes. It is possible that intracellular Ca++ levels through calcitonin may be involved in short term regulation of ATP CL (97).

Acetylation/deacetylation is another mechanism of enzyme modulation. This system exists in bacteria. A bacterial ligase was found to catalyze the acetylation of ATP CL (45). The ligase may be complexed with ATP CL which retain its activity. A deacetylase seems to hydrolyze the complex and inactivate ATP CL (45).

CELL DENSITY DEPENDENCE

Historically the cell concentration at which hepatocytes were incubated, cultured or assayed was not of any concern.
Consequently, many inconsistencies in metabolic as well as growth measurements arose. Cells in culture demonstrate "density dependent regulation" of growth as they proliferate until a specified "saturation density" is reached (98). The phenomena was attributed to contact inhibition where cells multiplied until they touched and presumably generated a signal which arrested their proliferation. An alternative explanation was that the phenomenon was due to depletion of substantial substrates in the media. It was assumed that limiting factors such as low molecular weight nutrients or macromolecular serum factors were imperative to cell growth (99). The absence or depletion of these factors resulted in cessation of cell division by arresting cells in a quiescent phase of the cell cycle (99). The addition of fetal calf serum (FCS) or bovine serum albumin (BSA) was discovered to be an absolute requirement for sustaining cell growth in culture (100). Supplementation of serum factors and hormones to cell cultures generated changes detected in nutrient transport, levels of intracellular cyclic nucleotides and synthesis of RNA and proteins. DNA synthesis was augmented about 15 hours later (101).

Cells have only three states of existence from which to choose. Cells can be quiescent, undergo proliferation or differentiate to assume a specific function (102). Most cells exist in a non-proliferative G₀ stage where they are
quiescent, but can perform differentiated functions (102). There are four main stages of the cell cycle. Gap 1 or $G_1$ varies from 8 to 12 or 15 hours in length. DNA is replicated in the next phase, the S stage (for synthesis) which takes from 4 to 6 hours. A second gap phase, $G_2$, of about 6 hours follows the S stage. Finally, mitosis facilitates cell division in the M stage. Nutrients, hormones, growth factors and ions are required for the cell to traverse the cell cycle. The $G_0/G_1$ transition is the variable parameter between cell types or cell condition and is the regulatory phase. Biochemical conditions conducive for proliferation are reestablished during the $G_0/G_1$ phase. Several events are involved in stimulation of the transition from $G_0$ to $G_1$: induction of specific gene expression, modification of enzyme activity, change in cell maintenance of RNA and protein synthesis rates, attainment of proper metabolic state and acquisition of nutrients (102). In order for the cell to enter the $G_0/G_1$ transition several events are obligatory. Cells must acquire "competence" or the capability to respond to serum growth factors. Mitogenic signals stimulate the cell to be competent, such as platelet derived growth factor (PDGF) on fibroblasts. Only after this signal can cells go through a progression where they can respond to other serum factors, such as epidermal growth factor (EGF) and insulin in fibroblasts. During the
progression of the cells through G₁ biochemical and metabolic adaptations occur which eventually lead to irreversible commitment of cells to traverse through S, G₂, and M. The cell cycle is shown in Figure 1.

The density dependence of cells also is apparent with hepatocytes in suspension under conditions of short term incubation. It was primarily assumed that as cell concentration of hepatocytes per ml increased there would be a concomitant rise in production of pyruvate for additional fatty acid synthesis so that on a per cell basis rates would be constant over a wide range of cell densities (103). Thus, fatty acid synthesis was believed to increase proportionally to cell concentration. However, Benyen and Geelen (104) described the previous results as being a function of the experimental parameters. Fatty acid synthesis was elevated during the 30 to 60 minute time period under all conditions which were evaluated. The reasoning for using this time period was to compensate for the well known lag period for of 15 to 30 minutes, which presumably allows accumulation of pyruvate and lactate required for fatty acid synthesis (103,16). The requirement for pyruvate and lactate production by the cell can be substantiated by addition of exogenous pyruvate and lactate which abrogate the lag phase essential for activation of lipogenesis (16). It was noted that the relation of
Figure 1: Stages of the cell cycle.

- **G₀**: Quiescent phase
- **G₁**: Gap 1 (prior to DNA synthesis)
- **S**: DNA synthesis (replication of DNA)
- **G₂**: Interphase between DNA synthesis and mitosis
- **M**: Mitosis (nuclear and cytosolic division to produce two daughter cells)
pyruvate accumulation with cell concentration disappears at higher cell concentrations (105). Other parameters such as decline of epinephrine and time lapsed after kill may contribute as well. Although fatty acid synthesis during the 30 to 60 minute time period is proportional to cell concentration, synthesis during the 0 to 30 minute time period declines inversely with rise in cell concentration (105).

Jurin and McCune (105) examined the effect of cell density on several metabolic parameters in isolated rat hepatocytes. They found lactate and pyruvate levels declined with increasing cell density. High cell density depressed production of citrate, acetoacetate, β-hydroxy butyrate and acetyl CoA. There was also a decline in synthesis of proteins, fatty acids and cholesterol. Supplying lactate and pyruvate to low and high density hepatocytes doubled rate of fatty acid synthesis. The addition of glucose did not alter fatty acid or cholesterol synthesis. It is important to realize that the results attained were not due to depletion of oxygen or substrates or to drastic alterations in physical environment of the cell. ATP levels remain constant between high and low densities and there is no change in pH which remained at 7.4. This consistency in ATP level was confirmed by Voss et al. (106).
Tissues which are highly proliferative in vivo synthesize cholesterol at rapid rates i.e. developing brain, intestinal crypt cells, skin, testes, regenerating liver and hepatomas (107). Much of the regulatory events in cell proliferation occur in the G₁ period of the cell cycle and many growth factors alter or modulate the transition. Cholesterol synthesis presides in the G₁ phase as well (107). There is much speculation that sterol synthesis is a prerequisite before cells can synthesize DNA. Blocking cholesterol synthesis precipitate G₁ arrest in most cell lines. The peak activity of HMG CoA reductase, the regulatory enzyme in cholesterol synthesis is prevalent during G₁ (107). Thus, there is precedence for proposing that enzymes involved in production of intermediate metabolites, especially during the process of lipogenesis, participate in complex events which regulate cell growth and division.

The activity of HMG CoA reductase has also been reported to be subject to regulation by cell density (108). Several mammalian cell types cultured at high density led to a decrease in HMG CoA reductase activity relative to normal in vivo levels in parallel with the decline in sterol and fatty acid synthesis as well as eventual lack of DNA synthesis (108). All enzyme activities were not universally affected by cell density as the activities of NADH
dehydrogenase and 5'-nucleotidase were unaffected by cell density changes. It was concluded that the effect was specific for lipogenic enzymes, although a comprehensive measurement of all enzymes was not done. These results, however do give support for regulation of lipogenesis in concordance with cell growth and division (109). Also supporting this relationship, sterol synthesis has a definite cell cycle specificity, primarily occurring in G\textsubscript{1} phase prior to DNA synthesis (107-109). Cholesterol is a main component of cell membranes and probably is vital for achieving correct properties of membrane fluidity. Addition of compounds which repress HMG CoA reductase activity (25-hydroxy cholesterol or compactin) potently inhibit cell growth in culture, though exogenous cholesterol or mevalonate can restore cell growth (108). Mevalonate is a product of the HMG CoA reductase reaction and seems to be an important metabolic branch point to several pathways which produce products that function in control of cell metabolism (108). Mevalonate eventually gives rise to cholesterol, dolichol, CoQ and isopentenyl adenosine. Other investigators found LDL uptake by cultured hepatocytes was affected by cell density (110). LDL binding, uptake and degradation decline with high cell density. Insulin and compactin, which elevates the number of LDL membrane receptors, were considered as potential mediators of LDL
metabolism. Insulin showed no consistent effect on LDL uptake under varying cell density while compactin augmented LDL uptake and metabolism at both low and high densities, but was more effective at low cell density. The cell density effect on LDL uptake was more prominent than treatment with insulin or compactin.

Jurin and McCune (105) searched for an extracellular signal which could mediate the cell density effect. Exchange of cell free CM in a cross over experiment using CM from high cell density and low cell density incubations of hepatocytes showed high density CM caused an obvious inhibition of fatty acid and cholesterol synthesis of cells incubated at low cell density. The converse was not true, there seemed to be no effect of low density CM on high density hepatocytes. Voss et.al. (3) further examined the components of the CM from high and low density cells. Hepatocytes at intermediate cell densities were treated with low or high density CM. ATP CL activity which supplies acetyl CoA for fatty acid and cholesterol synthesis was inhibited by factor(s) from high density CM and conversely low density CM contained factor(s) which activated the enzyme. Both the activator and inhibitors were >10 KD in size, the inhibitor(s) was heat labile and the activator(s) was heat stable. The CM was further characterized by separating the peptide components. There were distinct
profiles of peptide factors from high and low density CM (111). Several peptide bands were present in low density CM which were absent in high density CM and vice versa. Also, of the peptide bands which were present at both densities there were differences in relative abundance of certain bands. These peptide factors were of large enough MW to assume they are not merely a conglomeration of polyamines or other small nutrients which was a suggested concern in the study of CM factors (112). It is feasible for these various peptides to be involved in the autocrine/paracrine regulation in response to cell density.

The concept of cells intrinsically possessing the capability of regulating their own growth and metabolism through production of autocrine/paracrine factors was first ascribed to transformed cells (113). It was proposed that cancer or tumorigenic cells lost the ability to respond to external signals or were overridden by production of their own factors which promoted uncontrolled growth and proliferation (113). This could be achieved by simultaneous expression of a growth factor and its receptor by the tumor cell (114). However, it is currently recognized that "peptide regulatory factors" which gave short range action may be involved in or promote the regulation of normal cell differentiation and/or proliferation (114). Growth factor
receptors are also susceptible to regulation by cell density (115). Generally an increase in cell density is accompanied by a diminution of growth factor binding. The effect of cell density on growth factor binding is distinct from down regulation of receptors induced by preponderance of growth factor or receptor transmodulation caused by receptor phosphorylation (115). The decreased binding of growth factors to their receptors at high cell density is mostly due to disappearance of the number of high affinity receptors (115). Hepatoma cultures have been used to generate evidence for autocrine regulation of uncontrolled proliferation. mRNA for IGF-I and its receptor were detected in all cell lines while PDGF mRNA and mRNA for its receptor were only present in some cell lines (116). Existence of PDGF mRNA is curious since normally only mesenchymal-derived cells have the capacity to express PDGF. Concomitant existence of PDGF and IGF would provide an ideal growth situation for cells. Two types of factors are required to support proliferation (116). Cells must become competent to enter G_1 phase; this can be achieved by PDGF and fibroblast growth factor (FGF). Other factors must instigate progression through the G_1 phase into the S phase, such as insulin-like growth factor (IGF) and EGF.

Liver is one of the few tissues which retains the potential to regenerate under certain insults.
Theoretically it would seem that hepatocytes would readily proliferate in culture, however this is not so. Many growth factors and cell conditions which readily provided mitogenic signals to other cell types failed to stimulate hepatocytes. High percentages of BSA or FCS are normally conducive to cell division, but this is not so with hepatocyte cultures (117). The enigma was relieved when it was discovered that hepatocytes cultured at low cell density and obligatorily supplemented with EGF and insulin could be persuaded to proliferate (117,118). It was postulated that cell density exerted its effect by permitting cells to traverse the G₀ quiescent state and enter G₁, and that the S phase was promoted by insulin and EGF. Finally low cell density was also required for cells to undergo mitosis (118). The cell density effect was considered to be mediated by a soluble factor since co-cultures of low and high density hepatocytes prevented low density cells from leaving the S stage and entering M phase (118). These findings were attributed to the production of a soluble inhibitor. Later work by Jurin and McCune (105) and Voss et. al. (3) which were previously discussed support this postulation as well.

Hepatocytes in culture can not be insured to be actual hepatocytes unless they retain activity which resembles in vivo functionality. It must be ascertained that parenchymal tissue is being generated instead of fibroblast contaminants
One criteria for validating the existence of parenchymal tissue is presence of glucose-6-phosphatase activity and absence of glutamyl transpeptidase which is high in damaged liver (119). Hepatocytes must also be able to accumulate fat (119). Many primary cultures of hepatocytes retain their ability to synthesize IGFs (120). Nakamura et.al. (121) showed "reciprocal modulation" of growth and differentiation in certain culture conditions of primary hepatocytes. Two responses are considered to be elicited by alteration of cell density. One response is concerned with growth and affects parameters of DNA synthesis, G-6-P dehydrogenase activity, transport of 2-amino-isobutyric acid and synthesis of protein and cholesterol; all are elevated in conditions of low cell density. The other response entails expression of hepatocyte specific characteristics such as activity of tyrosine amino transferase, serine dehydratase, malic enzyme and normal synthesis of triglycerides; all of which prevail in high cell density conditions. The effect of cell density was thought to be due to cell surface components inherently involved in cell-cell contact messages since isolated/separated membranes added to cultures at low cell density could mimic high density conditions (121). However, the specific component was not characterized or scrutinized and there was no consideration of the involvement of
extracellular matrix components producing the effect. Excessive amounts of pyruvate and lactate in culture media of primary hepatocytes also caused an effect similar to that of insulin and EGF (122).

One objective in the study of hepatocyte proliferation has been to find the factor(s) responsible for inducing regeneration of liver which occurs dramatically after hepatectomy. Several groups have isolated several crude factors which behave as hepatotropic growth factors. Nakamura et. al. (123) found a 27 KD cationic peptide in platelets of hepatectomized rats which was heat and acid labile and inactivated upon treatment with dithiothreitol (DTT), the sulfhydryl reducing agent. They named it hepatocyte growth factor (HGF), though it was formerly called hepatotropin. It exerted its maximal effect at nanogram quantities. It behaves as a typical growth factor: effective at nanomolar quantities, high specificity and affinity for putative receptors. Serum from hepatectomized rats contained 5 times the amount of HGF activity compared to controls (123). Russell et. al. (124) showed that the ability of serum factors to initiate DNA synthesis in primary cultures of rat hepatocytes is exclusive to rat serum; human and other mammalian serums are ineffective which may indicate that HGF is exclusively present in rat serum. The factor they isolated which initiated hepatocyte
proliferation had a MW of 56 KD and was contained in platelets.

The other end of the spectrum in regulation of hepatocyte growth has also been represented. Several putative inhibitory factors which act to prevent DNA synthesis in cultured hepatocytes have been isolated. These inhibitory factors are found in both serum and in liver itself. The concept of hepatic proliferation inhibitors was introduced by McMahon et. al. (125) upon isolation and purification of a 36 KD factor from rat liver which depressed liver epithelial cell proliferation. Two unidentified growth inhibitors secreted by the BRL liver cell line were found which were 56 KD and a dimer of two 21 KD subunits (126). They also found two other distinctly different inhibitors produced by virally transformed BRL cells. Each inhibitor seemed to possess specificity for cell type from which it originated. It was suggested that these self-regulatory inhibitors may contribute to inhibition of cell growth and division once high cell densities are achieved. Another uncharacterized inhibitor which inhibits G_1-S transition in hepatocytes was isolated from both serum and liver cytosol (126). Actually proteolysis of a high MW form of the uncharacterized inhibitor produced an active low MW form. It was proposed that this "enzymatic transformation" of the high MW inhibitor may occur by
integral membrane-bound proteases located in liver cell membrane and could possibly represent a regulatory mechanism for hepatocyte proliferation (126).

The intricate interaction of factors which inhibit or promote cell growth and proliferation and which predominates under certain conditions is not well understood. Much effort is being concentrated on the purification and characterization of hepatic growth regulators. The capacity of liver to regenerate in response to a variety of signals is a fascinating aspect of liver growth regulation. Apparently, both systemic and autocrine regulation are involved.

**EPIDERMAL GROWTH FACTOR**

Epidermal growth factor (EGF) was the first growth factor discovered to alter lipogenesis (127). Subcutaneous infusion of EGF to neonatal mice and rats led to formation of fatty livers and stunted normal growth (127). Older control animals underwent whole body accretion when treated with EGF, but there was no abnormal accumulation of liver lipids. The fatty livers developed in neonatal animals were exclusively due to an elevation of triglycerides. Neonatal serum was also hyperlipemic with an increase in all serum lipids. The investigators postulated that the effect of EGF on hepatic lipid metabolism, transport and elevation of triglycerides could be a result of a rise in uptake and
release of triglycerides by liver, an increase in triglyceride synthesis, a decreased oxidation of triglycerides or an abnormality in formation of VLDLs.

A substance contained in extracts from mouse submaxillary glands which when injected into neonatal mice caused "precocious eyelid opening" and tooth eruption was first described as EGF (128). Human milk, saliva and urine contain an abundance of EGF (50 - 100 ng/ml), but only small quantities are present in the serum (≈ 1ng/ml) although α-adrenergic antagonists cause release of EGF from submaxillary glands into the plasma (128). EGF elicits a mitogenic response in many epidermal and epithelial cells (129). EGF is a small molecular weight, heat stable peptide of about 6 KD. The sequence of EGF is highly conserved as it is very similar in mice and humans and shares common antigenicity and biological activity (128). Three intramolecular disulfide bonds are absolutely essential for EGF activity (129). Two EGF peptides are complexed with two binding proteins (29.3 KD each) in a high MW form of ≈ 74 KD in mouse submaxillary gland (129). Human urogastrone, the hormone which inhibits gastric acid secretion, was found to be identical to EGF (129).

The cellular events propagated by EGF are all mediated through its specific integral cell membrane receptor (129). Simple membrane binding may be sufficient for some events
such as cell transport, but receptor-ligand internalization may be required as is the case for DNA synthesis (129). Plasma membrane receptors for EGF have been detected in liver, placenta, skin and chondrocytes of several mammalian species, but are non-existent in hemopoietic cells (130). HeLa cells, Chang liver cells and A-431 cells (human epidermoid carcinoma line) bind an abundance of EGF (131). EGF binds its glycoprotein receptor in a rapid and reversible manner. Interaction of EGF with the 170 KD EGF receptor generates phosphorylation of tyrosine (TYR) residues (131). Several cellular proteins are phosphorylated 3-4 fold within one minute of EGF binding (132). The EGF receptor also undergoes autophosphorylation which presents a putative mechanism for receptor activity (132). The EGF-sensitive phosphorylation seems to be distinct from the cAMP or Ca++ systems which work through phosphorylation of serine or threonine (SER/THR) residues (132). The EGF receptor was the prototype for receptor-mediated endocytosis. Diffuse EGF receptors cluster upon binding of EGF, undergo energy-dependent endocytosis, then are slowly degraded by lysosomes though many of the EGF receptors could be recycled to the surface (129). The 170 KD EGF receptor is a single peptide with the external domain housing EGF-binding affinity linked through a transmembrane
domain to the intracellular domain containing the TYR protein kinase (129).

A minimum time period of 5 hours in cell culture in the presence of EGF is required to substantiate both early and late cell cycle events of $G_1 - S$ transition (133). This is unlike most growth factors which are transiently required at either the initiation or the progression phase (133). Several EGF receptor-mediated events are thought to be essential for mitogenesis (133). EGF receptor clustering/endocytosis and autophosphorylation are necessary, and the Na+/K+ pump may also contribute (133). However, none of the events are mutually exclusive; instead a complex interaction of signals are needed to allow the $G_1 - S$ transition (133).

The EGF receptor demonstrates cross-reactivity with several compounds which are competitive for EGF binding such as TGF-$\alpha$ and vaccinia virus (129). Although there is only a 22% homology between TGF-$\alpha$ and vaccinia virus with EGF, their binding affinities to the EGF receptor are equivalent to that of EGF itself (129). Cells transformed with the sarcoma virus synthesize "EGF-like peptides" referred to as SGFs which competitively bind the EGF receptor (134). All these factors generate comparable intracellular signals upon interaction with the EGF receptor.
The functioning of the EGF receptor is subject to alteration by other growth factors by means of transmodulation. Transmodulation is distinct from receptor down regulation resulting from excessive amount of EGF. Transmodulation refers to the process of a different growth factor altering the affinity of a receptor for its ligand (115). Treatment of cells with phorbol ester, a protein kinase C activator, results in a decline in the affinity of EGF receptor for EGF (135). PDGF mediates its response through protein kinase C as do two factors secreted into the media by virally transformed cells; all of these reduce sensitivity of the EGF receptor to EGF (136). Transforming growth factor-β (TGF-β) inhibits EGF induced mitogenesis in cultures of rat hepatocytes (137). TGF-β causes a measurable increase in association of EGF with EGF receptor, therefore its effect must be exerted at an unknown post-receptor event. Norepinephrine alone induces DNA synthesis in cultured hepatocytes, but addition of EGF is strongly synergistic (138). Norepinephrine reduces EGF receptor binding to EGF while it enhances EGF's effect on DNA synthesis. The EGF receptor can also be phosphorylated on SER and THR residues by protein kinase C which may account for transmodulation produced by growth factors which are protein kinase C dependent (139). Although EGF receptor works through a protein kinase C-independent pathway,
phosphorylation of SER or THR sites on the EGF receptor may alter its capability to bind ligands and relay intracellular signals.

Certain oncogene products mimic the EGF receptor action. The protein product of v-erb-B which is activated by the virus which causes avian erythroblastosis is a "truncated EGF receptor" (140). The v-erb-B protein behaves as a constitutively activated EGF receptor. The majority of the extracellular domain of the EGF receptor is absent and a short sequence on the carboxy terminus where the TYR kinase activity resides is missing. However, the v-erb-B protein possesses an "intrinsic protein tyrosine kinase activity" (140). The sarcoma growth factors (SGFs) which are released into the cell culture medium of cells transformed by murine sarcoma viruses bind the EGF receptor and propagate mitogenesis (134). The neu oncogene product, a tyrosine kinase produced by the virus contributing to neuroglioblastomas, is divergent from the EGF receptor having only 50% homology. Although, it cross-reacts with EGF receptor antibodies and hybridizes with the v-erb-B transcript. The neu oncogene product does not produce any as yet detectable biological effect (138).

A number of uncharacterized peptides are phosphorylated upon stimulation of the EGF receptor TYR kinase (132). One peptide which is phosphorylated is identical to the peptide
which is phosphorylated by the src protein (132). Glucose-6-phosphate dehydrogenase, the rate limiting enzyme in the hexose monophosphate shunt is specifically phosphorylated by the sequence of intracellular signalling events produced by EGF in A-431 cells (140). Unfortunately, the effect of the phosphorylation on the activity of this enzyme was not determined in this study.

A large amount of liver can be surgically removed or chemically damaged, and complete regeneration will occur within 11 days. Unexpectedly, primary cultures of liver cells do not readily undergo proliferation even in the presence of 10% FCS (118). Optimal conditions of low cell density, presence of insulin and EGF are required for induction of mitogenesis (118). The search for a "hepatotropic factor" responsible for inducing hypertrophy of liver under regenerative conditions has not yet been successful. EGF alone does not appear to be a prospect, although serum levels of EGF rise dramatically after partial hepatectomy, up to 33 fold (141). EGF probably is absolutely required to sustain the regenerative process. Addition of EGF to hepatocytes collected at various times after partial hepatectomy greatly simulates DNA synthesis at all times as compared to controls (142). However, binding of EGF to its receptor could only be detected at the 4 hour period, but EGF receptor binding disappeared
completely by 12 and 24 hours, implying that EGF is involved in establishing cell commitment to DNA synthesis very rapidly after hepatectomy and that continual occupation of the EGF receptor is not necessary once the mitogenic signal has been generated (142). The requirement for extended binding of EGF to normal cells affecting mitogenic stimulation and G1/S transition seems to be altered in the liver cells from hepatectomized rats such that EGF only exerts its effect in the early events of mitogenesis.

Liver in vivo has numerous EGF receptors, yet it is non-proliferative unless in is injured. Hepatocytes cultured from hepatectomized rats have low levels of EGF receptors, but addition of EGF causes a drastic increase in mitogenesis. There are high and low affinity EGF receptors, and a theory exists which says that the high affinity EGF receptors may be prevalent under normal non-proliferative conditions and are responsible for maintaining the differentiated state by clearing EGF from the plasma. The low affinity EGF receptors on the other hand, may contribute to the proliferative state (141). This is feasible since high affinity EGF receptors are reduced in conjunction with the proliferative response to hepatectomy (141).

"EGF-induced DNA synthesis" in hepatocytes is dependent on age of the rat (143). Hepatocytes from adult rats (2 monthes of age) must go through a 20 hour lag period when
EGF is added immediately upon establishment of cell culture (143). Conversely, a population of approximately 30% stem cells exists in cultures of neonatal hepatocytes which can rapidly respond to EGF with induction of DNA synthesis. Adult hepatocytes exist in a quiescent G₀ stage while the stem cell population in neonatal hepatocyte cultures is in G₁ phase.

A potential mechanism for the effect of EGF on mitogenesis during regeneration is translocation of EGF to the nucleus. Raper et al. (144) discovered a significant decline in a degradation product of EGF in the bile and observed that a lot of EGF remained in the liver. They hypothesized that during liver regeneration, EGF is preferentially translocated to the nuclei rather than the lysosomes during the "pre-S phase" (beginning at 2 to 14 hours, peaking at 20 hours followed by maximal mitosis at 24 hours). These findings conform to previous observations of the extreme rise in EGF levels in plasma of hepatectomized rats, especially at 6-18 hours post-operative occurring simultaneously with decline and eventual disappearance of EGF receptors several hours prior to onset of DNA synthesis in regenerating liver (141). Furthermore, the periportal cells which have the highest activity of DNA synthesis during regeneration take up over 90% of the EGF (141).
EGF also affects several metabolic processes which act in a shorter time period than DNA synthesis. EGF can activate glycolysis with increased lactate production, stimulate uptake of glucose and K+ and enhance protein and RNA synthesis (128). EGF seems to be capable of inducing specific lipogenic effects in hepatocytes as well (128). After the pioneering work of Heimburg et al. in 1965 demonstrating the induction of fatty livers in neonates by administration of EGF (127), the involvement of EGF in the potential regulation of lipogenesis was not pursued until recently. The putative significance of lipogenesis in cell division/proliferation suggests that growth factors could also alter lipogenic processes. EGF acutely stimulated de novo fatty acid synthesis in isolated rat hepatocytes (145, 146). In accordance with a rise in fatty acid synthesis in hepatocytes from fasted-high carbohydrate refeed rats there was a concomitant increase in the phosphorylation of ATP CL and acetyl CoA carboxylase in a manner similar to insulin (146). The EGF stimulated phosphorylation site of acetyl CoA carboxylase differed from the cAMP dependent phosphorylation site and did not alter activity (146). The phosphorylation site of ATP CL was reported to be on the same residue as the phosphorylation site of cAMP dependent protein kinase, however, the activity of ATP CL was not measured. ATP CL could have a special
significance as a target for growth factors since it supplies substrate for both fatty acid and cholesterol synthesis necessary for new lipids for membrane biogenesis, and its stimulation may be a prerequisite for proliferation. Interaction of EGF with EGF receptors also propagates the phosphorylation of glucose-6-phosphate dehydrogenase, the rate limiting enzyme for the hexose monophosphate shunt which provides sufficient NADPH for use in fatty acid synthesis (132). EGF also stimulates production and release of prostaglandin E$_2$ and prostaglandin F$_2$\alpha into hepatocyte culture medium (147). Exogenous addition of these prostaglandins promotes DNA synthesis. Again, this implies the existence of autocrine regulation of hepatocytes through the ability of EGF to produce these prostaglandins.

**OTHER GROWTH FACTORS AND LIPOGENESIS**

Insulin is well known for its role as the primary regulator of glucose homeostasis. Insulin not only effects intermediary metabolism but can also function as a growth factor. Propagation of most cell types in culture necessarily requires the presence of insulin, although it alone is not sufficient. It has long been assumed that any growth response produced by insulin was accomplished through its cross-reactivity with the IGF-I receptor at supraphysiological levels. However, it is now believed that the insulin receptor itself may be capable of producing
cellular growth responses (145). It is reasonable for the insulin receptor to be capable of signalling cell growth since it has an inherent TYR kinase activity which is activated upon ligand binding (146). Many growth factors and oncogene protein products which activate DNA synthesis/proliferation possess TYR kinase activity. Although the mechanism remains elusive, it is widely believed that the TYR kinase-related events are fundamental in cell proliferation.

Insulin also plays a major regulatory role in the control of lipogenesis. Physiological conditions which result in low insulin levels such as fasting and diabetes result in low rates of lipid synthesis. However, upon refeeding or exogenous administration of insulin the rate of lipid synthesis rises. It has been suggested that insulin is involved in the long term adaptation by modifying the rate of synthesis of lipogenic enzymes. Fatty acid synthesis, however, can be stimulated by the addition of insulin to isolated hepatocytes with in 30 minutes (16). Insulin can acutely shift the phosphorylation state of several lipogenic enzymes, though the significance to activity is not well understood.

The research of Chen (107-108) showed that inhibition of cholesterol synthesis precluded the synthesis of DNA. An increasing number of researchers are becoming proponents
of the hypothesis that there is a putative requirement for synthesis of new lipids for membrane biogenesis requisite to cell division. The cell membrane is composed mostly of cholesterol and phospholipids. Cellular homeostasis is insured by proper composition of the cell membrane. An appropriate amount of cholesterol and phospholipids must exist to maintain efficient ion exchange, membrane fluidity and ultimately signalling of integral cell membrane receptors (4). Newly synthesized cholesterol is transported to the cell membrane within 10 minutes to 1 hour by an unknown mechanism (148). As previously discussed, the cell density effect where cell density is inversely proportional to lipogenic rates has implications for autocrine/paracrine factors contributing to the regulation of lipogenesis as well as DNA synthesis (105,3). Thus, it is logical to expect growth factors to affect various aspects of lipogenesis.

The rate of fatty acid synthesis in isolated rat hepatocytes was also found to be stimulated by the phorbol ester PMA, an analogue of diacylglycerols which activate protein kinase C, EGF and the 2.5S subunit of nerve growth factor (NGF) which promotes extension of long axonal processes in cells of neural origin and has an affinity for the insulin receptor (145,149). The effect of 2.5S NGF and EGF was not altered by exogenous addition of lactate or 2-
chloropropionate. Providing lactate alleviates relying on glycolysis to produce pyruvate as the carbon source for new fatty acid synthesis and 2-chloropropionate acts on pyruvate dehydrogenase to allow a greater availability of acetyl CoA. Therefore, the induction of fatty acid synthesis by 2.5S NGF and EGF was not only due to increased production of carbon source through glycolysis and/or pyruvate dehydrogenase activity, but rather EGF and 2.5S NGF, acting at the insulin receptor, could act specifically on the lipid synthesis pathway probably through modification of the lipogenic enzymes (145,149). Other growth factors which were examined (bombesin, substance P and transferrin) had no effect and FGF, a mitogen for mesodermal-derived cells, acted to inhibit fatty acid synthesis (145,149). Coincidentally, the receptors of both EGF and insulin have intrinsic TYR kinase activity which the receptors of the other growth factors tested lacked. It is reasonable to speculate that DNA synthesis and lipogenesis could be concordantly regulated through activation the TYR kinase activity. On the other hand, PMA works through protein kinase C activation to stimulate fatty acid synthesis and protein kinase C phosphorylates only the SER/THR residues and not the TYR residues. Though it is possible for protein kinase C and TYR kinase both to activate indirectly a common mediator of lipogenesis (149).
Insulin shares considerable sequence homology with IGFs although there are distinct receptors for each (150). The IGFs stimulate glucose uptake and lipid synthesis in adipose tissue and also have an anti-lipolytic effect similar to that of insulin (150). IGF-I also induces the differentiation of porcine pre-adipocytes and there is a two-fold stimulation of ATP CL activity in the process (151).

Platelets synthesize, store and release considerable quantities of PDGF which is mitogenic to mesenchymal-derived cells (152). PDGF is thought to contribute to normal growth of wound healing and the aberrant process of atherogenesis (153). PDGF is believed to initiate the proliferation of smooth muscles at "the site of endothelial cell injury" leading to accumulation of lipid, most of which is cholesterol ester attained from LDL (152). Cholesterol ester and phospholipid formation and DNA synthesis have a dose dependent response to PDGF in skin fibroblasts (152). Cholesterol ester and phospholipid syntheses gradually rise after introduction of PDGF during G1 phase of the cell cycle several hours before DNA synthesis occurs (152). The effect of PDGF is much more pronounced in normal skin fibroblasts as compared to those from mutant fibroblasts of familial hypercholesterolemia which lack LDL receptors and meet the cellular requirement for cholesterol entirely through de
novo synthesis (154). Not only does PDGF elevate cholesterol ester synthesis but it also stimulates cholesterol synthesis in skin fibroblasts from familial hypercholesterolemic patients (155). Smooth muscle cells can increase cholesterol synthesis in response to PDGF as well (155). It seem that the proliferative effect PDGF produces in wound healing and atherogenesis may entail dual simulation of lipid metabolism and DNA synthesis.

Hyperlipidemia is a consequence of infection caused by secretion of the cytokine tumor necrosis factor (TNF) by invasive microorganisms (156). TNF is a small peptide capable of destroying tumors in vivo. Adipose tissue lipoprotein lipase, which hydrolyses triglycerides contained in the lipoproteins releasing free fatty acids to the adipose tissue, is inhibited by TNF (156). The hyperlipidemia is confounded by the ability of TNF to stimulate fatty acid and sterol synthases in the liver. In vivo infusion of TNF produced a chronic effect such that fatty acid and cholesterol synthesis are elevated with in 90 minutes and 7 hours respectively and high levels are retained out to 16 hours. Alteration in HMG CoA reductase by TNF is responsible for the increase in cholesterol synthesis (157). The mechanism of TNF's action on the liver is unknown. The activities of acetyl CoA carboxylase and fatty acid synthetase were not modified by 90 minutes after
TNF injection to rats (157). However, both enzyme activities were elevated in liver at the 7 hour time period. The effect apparently was not exerted by changing phosphorylation state of the enzymes, but rather by changing citrate levels at 90 minutes and acyl CoA levels by 16 hours. Citrate and acyl CoA behave as metabolic effectors increasing lipogenic enzyme activities (157).

Blocking mitochondrial transport of citrate inhibits DNA synthesis and prevents proliferation of lymphoma cell cultures presumably because G₂/G₀ or G₁/S can not be traversed (2). The effect was attributed in part to the lack of acetyl CoA produced from citrate transport to cytosol available for synthesis of cholesterol, and cholesterol derivatives which are necessary to signal onset of DNA synthesis. Rao and Coleman (2) postulated that if production of acetyl coA by ATP CL becomes a limiting factor, cholesterolgenesis is diminished and the cell can not undergo DNA synthesis and division.

The effect of other growth factors on different aspects of lipogenesis was the impetus for the examination of the effect of TGF-β on ATP CL activity by Reinhart and Roehrig (158). TGF-β is considered to be a fundamental regulator of cell growth as it is highly conserved and potent at extremely small concentrations (159). Receptors of TGF-β are expressed ubiquitously in all cell types examined (159).
It is thought to be a "bi-functional regulator" of growth since its action in a particular cell is dependent not only on cell type, but on presence and interactions with other growth factors (160). TGF-β was originally named for its ability in conjunction with TGF-α to reversibly transform non-neoplastic murine fibroblasts accompanied by loss of density-dependence and induction of anchorage independent growth in culture (159). However, TGF-β can also act as an anti-proliferative factor in certain transformed cells and many epithelial cells including cultures of hepatocytes (141). The production of various extra-cellular matrix components can be induced by TGF-β in conjunction with simultaneous production of inhibitors of extracellular proteases (159). Despite much effort the receptor mechanism of TGF-β has not yet been elucidated. No inherent receptor TYR kinase activity has been detected though protein G activation has been considered a good possibility (161). TGF-β acts on several metabolic parameters such as stimulation of glucose and amino acid transport, elevation of prostaglandin synthesis and augmentation of glycolysis (159). The half-life of TGF-β in plasma is 2.2 minutes though fasting and partial hepatectomy reduced its clearance by the liver (159). Almost all of the TGF-β in plasma is extracted by liver, slowly degraded in lysosomes and excreted into bile (162). TGF-β was found to have a
transient and dose dependent effect on ATP CL activity in isolated rat hepatocytes (158). During the first 30 minutes of incubation with nM concentrations of TGF-β, ATP CL activity was rapidly activated and gradually declined to levels below the controls. Further addition of TGF-β at 30 minutes resulted in pronounced inhibition of enzyme activity. The interaction of TGF-β with other growth factors and autocrine/paracrine factors has a complexity which is not easily understood and seems to be unpredictable based on the limited evidence presently available.

The evidence for alteration of lipogenesis by growth factors is substantial. The requirement of cholesterol and its derivatives prior to DNA synthesis, the ability of various growth factors to modify different cell cycle stages and lipogenic enzymes, and the capacity for density-dependence of cells which appear to be regulated by the synthesis and secretion of endogenous growth factors coupled with density dependent changes in lipid synthesis rate seem to support the hypothesis. Growth factors act on cells to rapidly modify lipogenic enzyme activity and also effect of the synthetic rate of the enzymes. A number of regulatory devices can be used. The various growth factors could conceivably exert a "coordinate regulation" through a common cellular signal (163). The effect of many of the growth factors is mediated through TYR autophosphorylation of their
receptors and possible by TYR phosphorylation of other intracellular proteins. A change in the phosphorylation state of ATP CL, acetyl CoA carboxylase and HMG CoA reductase has been detected which could potentially alter their activities. A "common metabolic effector" could be produced in response to growth factor binding although this is not likely since changes in metabolic effectors could be readily detected (163). The lipogenic enzymes are capable of subcellular translocation under various conditions. Growth factors could activate a distinct protease and limited proteolysis of the lipogenic enzymes could specifically change enzyme activities. The mechanisms involved in growth factor stimulation of proliferation have not been elucidated, but it is suspected that the same mechanisms may also alter lipogenesis.
CHAPTER III
MATERIALS AND METHODS

Animals

Male, Sprague-Dawley rats were obtained from Harlan Co. (Indianapolis, Indiana) and weighed 150-170 grams upon arrival. The rats were paired in galvanized, wire bottom, mesh cages with ad libitum access to water and semi-purified, high carbohydrate diet (Table 1). A 7-10 day acclimation period was allowed prior to experimental use. Rats were maintained on a 7 PM to 7 AM light cycle and all experiments were performed at 9 AM +/- 1 hour during the dark cycle or active period of the rats.

In experiments requiring the use of fasted, fed and refed rats, the rats were randomly assigned to each group and singly caged. All groups received water ad libitum. Animals in the fasted group were deprived of food for 48 hours, the refed group was fasted for 48 hours, then refed 48 hours before being sacrificed.

Rats were rendered diabetic by administering injections of streptozocin (a generous gift from Upjohn, Kalamazoo, MI) by tail vein. Animals were randomly assigned to the
Table 1. Diet composition.

<table>
<thead>
<tr>
<th>Component</th>
<th>% by weight</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dextrose</td>
<td>58</td>
</tr>
<tr>
<td>Vitamin-free casein</td>
<td>21</td>
</tr>
<tr>
<td>Celufil-non nutritive bulk</td>
<td>13</td>
</tr>
<tr>
<td>Salt mix USP XIV</td>
<td>4</td>
</tr>
<tr>
<td>Vitamin Diet Fortification Mixture (^b)</td>
<td>2</td>
</tr>
<tr>
<td>Mazola corn oil</td>
<td></td>
</tr>
</tbody>
</table>

\(^a\) Dry ingredients, excluding vitamins were purchased from the U.S. Biochemical Corporation, Cleveland, Ohio.

Vitamins and corn oil were refrigerated prior to use. Dry ingredients were stored in a low moisture environment.

Twenty kg quantities of the diet were prepared in a Hobart industrial mixer.

\(^b\) Contents (g/kg mixture) were 5.0 (1000 lu/g) alpha tocopherol, 45.0 L-ascorbic acid, 75.0 choline chloride, 3.0 D-calcium pantothenate, 5.0 inositol, 2.25 menadione, 4.5 niacin, 5.0 P-aminobenzoic acid, 1.0 pyridoxine HCl, 1.0 riboflavin, 1.0 thiamin HCl, 900,000 units vitamin A acetate, 100,000 units calciferol (D\(_3\)), .02 biotin, .09 folic acid, .00135 vitamin B. The mixture was titrated in dextrose.
diabetic or control group and fasted with ad libitum access to water for 24 hours before injecting 65 mg/kg body weight streptozocin (dissolved in citrate buffered saline at pH 4.5). Injection volumes were kept low (≤ .3 ml) to minimize discomfort of the animal. Control groups were injected with citrate buffered saline at pH 4.5 alone. All rats were lightly anesthetized with diethyl ether immediately before the tail vein injections. Following the 24 hour fast, rats were either reintroduced to the high carbohydrate diet for 1 day then switched to Purina Rodent Chow (#5002) pellets for 48 hours before being sacrificed or refed the high carbohydrate diet for 7 days before being sacrificed.

Hepatocyte Isolation

Hepatocytes were prepared according to the method of Berry and Friend (164) as modified by Seglen (165) and Geelen (166). The technique was also modified slightly by eliminating the addition of bovine serum albumin (BSA) to buffers (167). We found BSA to contain variable cytotoxic constituents between lots. Elimination of BSA improved the consistency of hepatocyte preparations considerably. The HEPES buffers were prepared previously in concentrated solutions and frozen in aliquots, then diluted and gassed continuously with 95% O₂ : 5% CO₂ (flow rate of 6 L/min.). The HEPES buffers and Krebs Ringer buffer were adjusted to
pH 7.4 with 30% KOH. Hepatocytes were isolated in 20 to 30 minutes and experiments completed within 1 1/2 hours.

A ventral, lateral midline incision was made into the peritoneal cavity of a deeply ether anesthetized rat. The cavity was cleared of the viscera and the hepatic portal vein was cannulated with a blunted 16-gauge needle, tied securely with unwaxed dental floss and perfused with HEPES (-Ca++) buffer (non-recirculating) by a Cole Parmer, model 7565, peristaltic pump, maintained at 37°C in a water bath. The aorta and inferior vena cava were severed to exsanguinate the rat and permit unobstructed perfusion, and the heart was snipped. The liver was then removed from the peritoneal cavity and mounted on a wax ball in the perfusion apparatus: the buffer flow rate was adjusted to 40 ml/min. Once the liver effluent was clear of blood, HEPES (+ Ca++) containing 30 mg/100 ml Collagenase Type II (Sigma Chemical Co., St. Louis, MO) to degrade the connective tissue matrices was recirculated continuously until the liver became swollen.

The perfused liver was transferred to a petri dish containing Krebs Ringer buffer (supplemented with 1.4 mg/ml Bacitracin from Sigma Chemical Co., St. Louis, MO). The liver lobes were sliced open with a scalpel and parenchymal cells were lightly shaken out. The hepatocytes were rinsed three times with Krebs Ringer, collected by spinning the cells down on a low speed Dynac table top centrifuge. The
supernatant containing dead cells and blood contaminants was discarded, and the pellet of hepatocytes was resuspended in Krebs Ringer buffer. Cell concentration and viability were determined with a standard hemocytometer (American Optical Co., Buffalo, NY) using a Spencer light microscope. Hepatocytes were counted and the viability was assessed by the trypan blue dye exclusion method as described by Seglen (165). Only preparations with a viability of > 83% were used. Hepatocytes were adjusted to a concentration of $1 \times 10^7$ cells/ml before subsequent experiments. Samples of hepatocyte preparations and Krebs Ringer buffer were stored at $-20^\circ C$ for protein determinations (168) which were done as a validation of the optically estimated cell concentrations by the method of Uhal and Roehrig (169). An average of the two cell concentration techniques was used for subsequent calculations.

**Conditioned Media Preparation**

Freshly isolated hepatocytes were adjusted to cell concentrations of $1 \times 10^5$ cells/ml for low density and $1 \times 10^7$ cells/ml for high density conditioned medium (CM) with Krebs Ringer buffer. Hepatocytes at low and high densities were incubated in a Dubnoff metabolic shaking incubator at $37^\circ C$ with 95% $O_2$ : 5% $CO_2$ (flow rate of 6 L/min.). Initially, a time course was done at 0, 10, 30, and 60
minutes to determine the maximal effect of CM on ATP CL which was found to be at 30 minutes. All other experiments used media from 30 minute incubations. The cells were removed from the media immediately following the incubation period by low speed centrifugation at 132XG by a Dynac table top clinical centrifuge for two minutes. The pelleted cells were discarded, and the supernatant collected as CM and stored at -20°C. CM from several different preparations was pooled in equivalent volumes before use to diminish inherent variability between hepatocyte preparations.

Measurement of Lactate Dehydrogenase Activity

Lactate dehydrogenase (LDH) activity was measured (170) in low and high density CM collected from hepatocytes to insure that the integrity and viability of the cells remained intact and constant during the incubation periods. Hepatocytes were incubated at high and low densities for 0, 10, 30 and 40 minutes in duplicate. One set of samples was immediately sonicated on ice (Branson Ultrasonic Oscillator, model 185, microtip, setting 6) to release the intracellular enzymatic content of the whole cells. The other set of samples was centrifuged at a low speed for 2 minutes and the supernatant of cell-free CM was sonicated as above. LDH activity of both whole cells and cell-free CM was measured spectrophotometrically. The LDH activity of the CM was compared to that of whole cells to analyze the
percentage of activity as a measure of contamination of cell breakage and intracellular constituents in the cell-free CM. The percentage of LDH in the cell-free CM was low (2-3%) and constant over the incubation time.

**Growth Factor and CM Treatment**

Freshly isolated hepatocytes (1 x 10^7 cells/ml) were treated with 1 ml of pooled, cell-free high or low density CM or exogenous nanomolar concentrations of EGF (Sigma Chemical Co., St. Louis, MO). Duplicate samples were adjusted to a final volume of 3 ml with Krebs Ringer buffer and a final concentration of 1 x 10^6 cells/ml. The samples were incubated in a Dubnoff metabolic shaking water bath supplied with 95% O₂ : 5% CO₂ at flow rate of 6 L/min. Time response curves for ATP CL activity were established for hepatocytes treated with either CM or EGF at time intervals of 0, 5, 10, 20 and 40 minutes. Dose response curves for ATP CL activity were obtained for each growth factor separately. EGF was supplemented at 10^-1, 10^-1, 10^-1, 10^-9 and 10^-8 M, then incubated for the predetermined time for optimal activity. Samples were immediately transferred to ice and sonicated (Branson Ultrasonic Oscillator, model 185, microtip, setting 6) for 15 seconds to terminate the incubation period. Enzyme activity of ATP CL was subsequently measured spectrophotometrically.
ATP Citrate Lyase Activity

The activity of ATP (pro-3S)-lyase (EC 4.1.3.6.), also referred to as citrate oxaloacetate lyase or citrate cleavage enzyme, was detected by the method of Osterland and Bridger (171) using a Guilford updated Beckman D. U. spectrophotometer (except the concentration of β-mercaptoethanol and Trizma base were tripled in the reaction mixture). The enzymatic action of ATP CL produces oxaloacetate which can then be coupled to the malate dehydrogenase reaction which converts NADH to NAD. NAD production is concomitant with a decline in absorbance at 340 nm which can be detected on the spectrophotometer. The ATP CL reaction is measured by the addition of 10-25 μl liver sample to 1 ml of reaction mixture composed of the following: 10 mM ATP, 20 mM citrate, 0.2 mM coenzyme A (CoA), 9.4 nM NADH, 10 mM magnesium chloride (MgCl₂), 3 units malate dehydrogenase and 0.6 M pH 7.4 Tris chloride with 30 mM β-mercaptoethanol. Duplicate samples were measured, and the average value was used in calculating the mean and standard error of the mean among experiments. Activity was reported as change of NADH concentration per mg cellular protein or change of concentration of NADH per $10^3$ cells.
Liver Homogenate Preparation

Fed, fasted and/or refed rats received a blow to the head and were immediately decapitated. An incision was rapidly made into the peritoneal cavity; livers were quickly removed and placed on ice. Livers were weighed and added to the ice cold homogenate buffer (0.3 M mannitol, 10 mM EDTA) containing the protease inhibitors Na-tosyl-L-lysine chloro-methyl ketone (TLCK) and Na-tosyl-L-proline chloro-methyl ketone (TPCK) at 1 mg/L, at a ratio of 1 gram liver : 5 volumes homogenate buffer. All subsequent steps remained at 0-4°C on ice. Livers were homogenized with a motor-driven Potter Elvehjem homogenizer. The homogenate was then centrifuged at 500xG for 5-10 minutes in a Servall Refrigerated Automatic centrifuge. The resulting pellet of cell debris and nuclei was discarded, and the supernatant was recentrifuged at 10,000xG for 10 minutes or put in a high speed microfuge for 2 minutes, which sediments the mitochondria down in the pellet with the rest of the cytosolic fraction remaining in the supernatant which can then be further fractionated.

Subcellular Fractionation

Once the liver homogenate was prepared and the cytosol and mitochondrial fractions separated, the outer and inner mitochondrial membranes were separated by the method of Greenawalt (172), with minor modifications by increasing the speed of centrifugal separation to 10-12,000xG. The
cytosolic fraction was further separated to collect the microsomes by ultracentrifugation (Beckman Ultracentrifuge, model L) at 105,000xG for 1 hour. All steps were performed at 0 to 4°C. The cytosolic, outer mitochondrial membrane, mitoplasts and microsome fractions were assayed for ATP CL activity and monoamine oxidase activity (173) to test the purity of the preparation and subcellular localization of ATP CL. An aliquot of each fraction was boiled with SDS and β-mercaptoethanol for subsequent SDS PAGE.

**SDS Polyacrylamide Gel Electrophoresis**

Liver homogenates and subcellular fractions were qualitatively separated by electrophoresis on 7 1/2% SDS polyacrylamide gels. Samples of each (1 ml) were treated with 24 μl β-mercaptoethanol (2%), 180 μl 30% SDS (3%) and 75 μl 0.005% Bromophenol blue dye in order to denature and visualize the proteins. All samples were boiled for 4 minutes in a boiling water bath, then several grains of sucrose were added to increase the density of the samples. Running buffer was added to the electrophoretic apparatus and 20 to 50 μl of samples were loaded. The gel was electrophoresed over night at 7.5 mAmps (~20 volts). Electrophoresis was terminated as the dye front approached the bottom of the gel. The gel was either subjected to Western blotting or standard molecular weights were determined after the gels were rinsed with 50% methanol/10% acetate (15 min.), dyed with 0.6% comassie brilliant blue R
250 (in former solution), rinsed again for 15 minutes followed by an overnight rinse with 10% methanol/10% acetate. The standard gel was dried on a gel dryer and photographed.

**Western Blot Procedure**

Separated proteins from the previous SDS PAGE were transferred to nitrocellulose using a Western blot apparatus. 80 % Towbins (25mM Tris, 192 mM glycine): 20% methanol buffer facilitated the transfer (Biorad transblot cell). The Western blot procedure was performed at .5 amps for four hours. The nitrocellulose was allowed to dry for at least two hours before treating it with polyclonal rabbit anti-rat antibody to ATP CL coupled to visualization with alkaline phosphatase (kit from R&D Systems). The nitrocellulose was blocked for 1 hour with 1% gelatin in immunoblot buffer (10 mM Tris, .9% NaCl, pH 7.6) with constant agitation as were all steps. The ATP CL antibody at 1:20,000 in 1% gelatin buffer was incubated with the nitrocellulose for 3 hours. The blot was then rinsed 2 times in immunoblot buffer as were all subsequent steps. The blot was incubated with anti-IgG coupled to biotin at 1:500 in 1% gelatin buffer for 2 hours followed by incubation with avidin conjugated alkaline phosphatase at 1:1000 in 1% gelatin buffer. Finally the blot was visualized by a mixture of 60 μl p-nitroblue tetrazolium (50mg/ml in 70% N,N-dimethyl formamide) and 5-bromo-4-
chloro-3-indolyl phosphate p-toluidine salt (50 mg/ml in N,N-dimethyl-formamide) added to 10 mls of alkaline phosphatase buffer (100 mM Tris, pH 9.5, 100 mM NaCl, 5 mM MgCl₂). The reaction was terminated by rinsing with deionized water. The nitrocellulose blot was dried and subjected to laser densitometry on an LKB 2222-020 Ultrascan XL to detect protein bands which were reactive to the ATP CL antibody.
CHAPTER IV
RESULTS AND DISCUSSION

The process of cell growth and proliferation requires biogenesis of additional membranes. Cholesterol and phospholipids are the main lipid constituents of cell membranes, and de novo synthesis of new lipids is probably required for membrane biogenesis. It has been proposed that new lipid synthesis is a prerequisite for cell division (4). Activation of lipid synthesis then should be an early event in cell proliferation. ATP CL produces acetyl CoA as substrate for cholesterol and phospholipid synthesis. Therefore, ATP CL activity should be modified under conditions which alter cell growth.

ATP CL activity was measured in freshly isolated rat hepatocytes which underwent short term incubation (≤60 minutes). Isolated hepatocytes (Table 2) were used in lieu of liver homogenates because the cell membranes must remain intact for interaction with extracellular regulatory factors and hormones (174). Experimental parameters are displayed in Table 2. Growth factors and probably autocrine/paracrine regulators exert their effects on a cell through binding to
Table 2. Experimental Parameters.

<table>
<thead>
<tr>
<th>Experiment</th>
<th>n</th>
<th>Rat wt.(g)</th>
<th>Hepatocyte viability(%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>FED/FASTED CM</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Assay$^1$</td>
<td>3</td>
<td>255 ± 45</td>
<td>93 ± 4</td>
</tr>
<tr>
<td>Fed CM$^2$</td>
<td>6</td>
<td>289 ± 54</td>
<td>90 ± 2</td>
</tr>
<tr>
<td>Fst CM$^3$</td>
<td>6</td>
<td>235 ± 33</td>
<td>91 ± 2</td>
</tr>
<tr>
<td>DIABETES 3 DAY</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Diab Asy$^4$</td>
<td>3</td>
<td>242 ± 15</td>
<td>81 ± 1</td>
</tr>
<tr>
<td>Diab CM$^5$</td>
<td>3</td>
<td>242 ± 15</td>
<td>81 ± 1</td>
</tr>
<tr>
<td>Cont Asy$^6$</td>
<td>3</td>
<td>291 ± 5</td>
<td>84 ± 2</td>
</tr>
<tr>
<td>Cont CM$^7$</td>
<td>3</td>
<td>220 ± 9</td>
<td>82 ± 2</td>
</tr>
<tr>
<td>DIABETES 7 DAY</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Diab Asy$^4$</td>
<td>3</td>
<td>194 ± 33</td>
<td>80 ± 1</td>
</tr>
<tr>
<td>Diab CM$^5$</td>
<td>4</td>
<td>203 ± 6</td>
<td>80 ± 1</td>
</tr>
<tr>
<td>Cont Asy$^6$</td>
<td>4</td>
<td>315 ± 13</td>
<td>84 ± 3</td>
</tr>
<tr>
<td>Cont CM$^7$</td>
<td>4</td>
<td>291 ± 28</td>
<td>84 ± 2</td>
</tr>
<tr>
<td>EGF/AGE</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>8 week$^8$</td>
<td>4</td>
<td>242 ± 21</td>
<td>86 ± 3</td>
</tr>
<tr>
<td>10 week$^9$</td>
<td>4</td>
<td>276 ± 13</td>
<td>84 ± 1</td>
</tr>
<tr>
<td>12 week$^{10}$</td>
<td>3</td>
<td>226 ± 13</td>
<td>86 ± 2</td>
</tr>
<tr>
<td>14 week$^{11}$</td>
<td>3</td>
<td>339 ± 8</td>
<td>85 ± 4</td>
</tr>
<tr>
<td>8 wk dens$^{12}$</td>
<td>3</td>
<td>236 ± 12</td>
<td>85 ± 2</td>
</tr>
</tbody>
</table>

Values are means ± standard deviations.

1 Assay = ATP CL activity in hepatocytes from fed rats
2 Fed CM = CM collected from hepatocytes from fed rats
3 Fst CM = CM collected from hepatocytes from fasted rats
4 Diab Asy = ATP CL activity in hepatocytes from diabetic rats
5 Diab CM = CM collected from hepatocytes from diabetic rats
6 Cont Asy = ATP CL activity in hepatocytes from control rats
7 Cont CM = CM collected from hepatocytes from control rats
8 8 week = ATP CL activity in hepatocytes from 8 week rats
9 10 week = ATP CL activity in hepatocytes from 10 week rats
10 12 week = ATP CL activity in hepatocytes from 12 week rats
11 14 week = ATP CL activity in hepatocytes from 14 week rats
12 8 wk dens = ATP CL activity in hepatocytes from 8 week rats at different cell densities
integral membrane receptors which then relay intracellular signals upon binding.

Several of the experiments involved collection of conditioned media (CM). CM was made by incubating isolated hepatocytes for specific time periods (0, 15, 30 and 60 minutes), removing the cells by low speed centrifugation (in the pellet) and collecting the remaining supernatant as the cell free CM. CM was collected from hepatocytes incubated at high cell density \(10^7\) cells/ml) and low cell density \(10^5\) cells/ml). The CM from at least 4 separate preparations were pooled and frozen in aliquots for storage at -20 C until the day of use. Freshly isolated hepatocytes were prepared and adjusted to an intermediate density \(10^6\) cells/ml) then incubated with the appropriate thawed pooled CM for appropriate time periods.

Hepatocytes incubated at different densities were not treated with any exogenous hormones or metabolites. Therefore, the hepatocytes themselves were producing and secreting factors into the CM which can act on the hepatocytes themselves to alter ATP CL activity. Such regulation is typical of autocrine/paracrine control. Autocrine regulation involves production of a regulator, hormone or growth factor by a cell which then acts on that particular cell itself to alter some cellular parameter. Paracrine regulation is similar to autocrine regulation
except that the factor is produced by one cell and targets a neighboring cell. Hepatocytes incubated at low cell density apparently secrete an activator(s) which acts as an autocrine/paracrine regulator of ATP CL activity (3). Conversely, hepatocytes incubated at high cell density secrete an inhibitor(s) of ATP CL activity (3). The objective of the experiments reported here was to determine to what extent ATP CL was regulated by autocrine/paracrine or endocrine regulation under several dietary and hormonal conditions. This work can be divided into four studies which are briefly described in the following paragraphs.

In the first study, the putative autocrine/paracrine regulators contained in CM from 48 hour fasted rat hepatocytes were compared to those in CM collected from fed rat hepatocytes. Freshly isolated hepatocytes from fed rats at an intermediate density were incubated with high and low density CM from fed and fasted hepatocytes. The effect of the CM factors on ATP CL activity was then measured.

In the second study, a preliminary assessment of a potential mechanism of the effect of ATP CL was made using an alternative set of experiments performed in an attempt to distinguish the subcellular localization of ATP CL among liver homogenates from fed, fasted (48 hours) or refed (48 hours) rats. In the first case, Greenawalt's buffer containing protease inhibitors was used to separate the cytosol, outer mitochondrial membrane, mitoplasts and
microsomes by differential centrifugation. In the second case, the cytosolic and non-cytosolic fractions, minus the nuclei, were similarly separated from fed and fasted (48 hours) rat liver homogenates, but only with the use of .3M mannitol in absence of protease inhibitors and differential centrifugation. Each fraction was subjected to 7.5% SDS PAGE, Western blotted then treated with an avidin/biotin conjugated system using alkaline phosphatase color reaction to visualize ATP CL antibody reactivity. The relative densities of the antibody reactive bands were measured on a densitometer.

The third study focused on possible abnormal autocrine/paracrine regulation of ATP CL in hepatocytes from diabetic rats. High and low density CM was collected from streptozocin induced diabetic or normal control rats. Freshly isolated hepatocytes from diabetic rats were treated with control CM, and freshly isolated hepatocytes from control rats were treated with high and low density CM from hepatocytes from diabetic or control rats. The activation or inhibition of ATP CL activity was comparatively evaluated.

In the fourth study, the ability of EGF to regulate ATP CL activity in intermediate density hepatocytes was examined in rats of various ages (8, 10, 12 and 14 weeks) to test for the ability of EGF to alter ATP CL as a function of age. Freshly isolated hepatocytes were supplemented with EGF at
nanomolar concentrations ranging from $10^{-2}$ to $10^{-8}$ M, incubated for 5 minutes and dose response curves were determined for each age group. Time response curves to EGF ($10^{-9}$ M) were generated for hepatocytes from each age group at 0, 5, 10, 20 and 40 minute time periods, then compared to controls without EGF supplementation.

**FED & FASTED CM STUDY**

In vivo, hepatic lipogenesis and enzyme activities are depressed by fasting for 48 hours (10). Previous studies demonstrated a definite decline in ATP CL activity with increasing cell density of hepatocytes (3). The fasted hepatocytes were shown to have a blunted activity regardless of cell density (3). Even at low cell density which usually causes a marked induction of enzyme activity, ATP CL activity in fasted hepatocytes was extremely retarded. Hepatocytes derived from refeed animals showed an exaggerated activation of ATP CL over a range of cell densities, but the effect was most prominent in lower cell densities (3). The density effect was specific for ATP CL and not universal for all cell parameters since activity of malic enzyme and ATP concentration remained constant over various cell densities (3, 105). Cell free CM collected from hepatocytes at low ($10^5$ cells/ml) or high ($10^7$ cells/ml) cell densities which was reintroduced to freshly isolated hepatocytes at an intermediate cell density ($10^6$ cells/ml) can mimic the
activation and inhibition of ATP CL activity, respectively (3).

The ability of components of CM to alter enzyme activity or cellular functioning is referred to as autocrine/paracrine regulation. At least one of the components of low density CM (LD CM) which behaves as an activator(s) of ATP CL was found to be peptide(s) of > 10 KD molecular weight and was heat stable (3). The inhibitor(s) contained in the high density CM (HD CM) was also determined to be > 10 KD molecular weight, but was heat labile (3). Furthermore, upon destruction of the inhibitor(s), heat stable activation of ATP CL could then be detected in freshly isolated hepatocytes treated with boiled HD CM. This insinuated that the inhibitor was more potent than the heat stable activator present in the HD CM. Later, the peptide components of the HD CM and LD CM were separated by SDS PAGE (111). The peptide profiles in the HD CM and LD CM were distinguishably different. Several peptides were unique to LD CM and absent from HD CM. Conversely, several peptides existed in HD CM which were absent from LD CM. Many peptide bands were common to both HD CM and LD CM. Of these common peptides, several varied in their relative abundance between LD CM and HD CM.

The effect of fasting on the production of putative autocrine/paracrine regulators in LD CM and HD CM was examined in the following experiments. Hepatocytes from
fed and 48 hour fasted rats were incubated at low and high cell densities for time periods of 0, 15, 30 and 60 minutes in order to collect cell free conditioned media. LD CM and HD CM from hepatocytes form fed and fasted rats were then reintroduced to freshly isolated hepatocytes at an intermediate density and incubated for 10 minutes at 37°C followed by sonication and measurement of ATP CL activity.

Data from experiments using fed LD CM and HD CM reproduced results which were consistent with previous studies with LD CM activating ATP CL activity and HD CM being inhibitory. ATP CL was maximally activated 2.5 fold by 30 minute LD CM (Figure 2). HD CM reduced activity to 50% of 0 control by 15 minutes conditioning with slight increase by 30 and 60 minute conditioning. LD CM from fasted hepatocytes lost its capacity to elevate ATP CL activity and actually behaved in an inhibitory manner, reducing it to 60% of 0 control by 30 minute conditioning (Figure 3). ATP CL activity was inhibited by HD CM from fasted rats similar to fed HD CM, although the curve was shifted slightly and was not as inhibitory as fed HD CM as maximal inhibition was attained by 30 minute conditioning only to 80% of 0 control values. Fasted LD CM also decreased ATP CL activity to 50% of control 0 values within 30 minutes. It is unusual that LD CM from fasted hepatocytes is more inhibitory than HD CM. The inhibitory effect of fed HD CM and fasted HD CM disappeared by 60
Figure 2: Activity of ATP CL in hepatocytes from fed rats treated with CM from fed rats. Values ± SEM are percentage of control ATP CL activity in hepatocytes at time 0 in absence of CM for 3 experiments. O is freshly isolated hepatocytes treated with low density CM (LD CM) pooled from 3 hepatocyte preparations. • is freshly isolated hepatocytes treated with high density CM (HD CM) pooled from 3 hepatocyte preparations. Hepatocytes were incubated at a final concentration of 10^6 cells/ml in 3 ml total volume (2 ml Krebs Ringer + 1 ml CM). There was a statistically significant effect of CM type (P > 0.06), but no time effect (P > 0.1) as determined by two-way ANOVA on Minitab.
Figure 3: Activity of ATP CL in hepatocytes from fed rats treated with CM from fasted rats. Experimental details are identical to those in Figure 2. The effect of CM type from hepatocytes from fasted rats on ATP CL activity in hepatocytes from fed rats was not significantly different (P > 0.1) as determined by two-way ANOVA on Minitab.
minute conditioning; however, this was not the case with the fasted LD CM as the inhibition was more pronounced. Three-way ANOVA (SAS) was done to evaluate the effects of nutritional state, density of CM (HD or LD) and time on ATP CL activity. Although there was not a statistically significant time effect, there was a definite interaction of nutritional state with density of the CM (P=0.02). It is difficult to test statistically values which vary over time. The effect tested may not be statistically significant, yet it could have profound physiological importance or significance. Many cellular events show transient activation, or peak at vital time periods or have pulsatory activity. All of these are extremely difficult to assess statistically. The results have implications for altered hepatic autocrine/paracrine regulation under fasting conditions. It may be that fasting not only deprives cells of substrate, reduces insulin levels thus leading to a decline in protein synthesis with increased protein degradation, but also produces autocrine/paracrine mediators which prevent cells from undergoing metabolic processes which could be detrimental to long term survival.

It appears that the presence of putative autocrine/paracrine factors in CM contribute to the regulation of ATP CL activity. Different regulatory factors possibly in combination with changes in relative abundance of shared factors probably exist in fasted CM versus fed CM
since ATP CL activity is altered differently. LD CM from fasted and fed hepatocytes alter ATP CL activity in opposite fashions. Fasted LD CM lacks the stimulatory properties of fed LD CM and fasted LD CM even is inhibitory. It could be assumed that hepatocytes produce different autocrine/paracrine regulators under conditions of low cell density in fed state compared to the fasted state. The inhibitor in HD CM was previously found to be more potent than the activator. Thus it is possible that an inhibitor(s) is simply produced and secreted into LD CM in the fasted state in addition to other LD CM constituents. On the other hand, there could be a deficiency in the production of the activator(s) accompanied by a change in abundance of factors. If the enzyme is altered by phosphorylation or a change in subcellular location, the way it responds to autocrine/paracrine regulation may therefore be modified.

The mechanism(s) of these autocrine/paracrine factors is unknown. It can be speculated that their action could be facilitated through changes in subcellular location or phosphorylation state of the enzyme. Autocrine production of growth factors can regulate cell growth and metabolism in culture (113-116). Many growth factors rapidly activate TYR kinase activity which is intrinsic to growth factor receptors, however, the chain of events following has not been fully explained. Cell density has been found to alter
the binding of several growth factors to their receptors (115). Binding of EGF, TGF-β and FGF to specific receptors was inversely reduced as cell density increased (115). It seems that if the autocrine regulators produced by the hepatocytes incubated at different cell densities were growth factors, the capacity of the hepatocytes to respond to the factors would be diminished at high cell densities. Whatever the uncharacterized factors may be, the regulation of growth and metabolic functions including the activity of at least one lipogenic enzyme is acutely sensitive to autocrine/paracrine factors which vary with cell density.

The reduced lipid synthesis under fasting conditions was historically attributed to degradation of the lipogenic enzymes which were believed to exist only in the cytosol (77). The amount of enzyme present regulated by balance of protein synthesis versus degradation was considered to be the only means of altering lipogenic enzyme activity (7). However, Janski and Cornell (13) reported apparent mobilization of ATP CL from cytosol to mitochondria in liver during prolonged fasting. Allred et.al. (12) discovered that the total amount of acetyl CoA carboxylase in liver homogenates did not change in response to fasting, but rather there was a redistribution of the enzyme from the cytosol to the mitochondria, where it resided with extremely low activity levels. Similar results were obtained for ATP CL activity and location during fasting. Larger amounts of
ATP CL were associated with liver mitochondria in fasted state in comparison to fed or refed states (13). The enzyme's apparent Km for ATP, citrate and CoA was lower when associated with the mitochondria, although it is possible that the Vmax is also lowered (175). Based on analysis of the apparent Km's and the concentration of the substrates for ATP CL, only citrate and CoA levels in cell could become prohibitive under certain physiological conditions (175). It may be that the availability of CoA and citrate are sometimes limiting for optimal ATP CL activity. The lower Km of mitochondrial ATP CL for its substrates may prevent imbalance of citrate levels in the cytosol during fasting when cytosolic ATP CL may be inhibited (176).

The phosphorylation states of ATP CL and acetyl CoA carboxylase change under different hormonal stimuli (83-85). Although the phenomena has been widely reported, most researchers do not detect alteration of enzyme activity by change in phosphorylation state for ATP CL. Acetyl CoA carboxylase was reported to be phosphorylated by an endogenous protein kinase in liver (14). This phosphorylation was correlated with lower activity. Furthermore, activity was restored upon removal of the phosphate by addition of phosphorylase phosphatase (14). Roehrig (1) showed that affect of phosphorylation on ATP CL activity is sensitive to levels of CoA. The phosphorylated form of ATP CL was inhibited only at low CoA levels in
analogy with the effect of phosphorylation of pyruvate kinase in the presence of fructose-1,6-bisphosphate (1).

The effect of cell density on a variety of metabolic and growth parameters has been well recognized. Synthesis of fatty acids, cholesterol and proteins were reduced in hepatocytes at high cell densities as well as rates of accumulation of metabolites: pyruvate, lactate, citrate, acetyl CoA, acetoacetate and β-hydroxybutyrate (105). Hepatocytes will not proliferate in culture unless they are plated at low cell density and incubated with growth promoters as is the case with most cell types (143).

**SUBCELLULAR DISTRIBUTION**

Lipogenesis occurs exclusively in the cytosol, and it has usually been assumed that the enzymes contributing to lipid synthesis were located only in the cytosol. Synthesis and degradation of the lipogenic enzymes were believed to be the sole form of regulation of fatty acid synthesis until it was proposed that the total cellular amount of enzymes is not necessarily indicative of activity (34). There was a realization that other modes of regulation may occur. Substrate levels and metabolic effectors could also contribute to regulation. For example, excessive levels of citrate stimulated enzymes which were pertinent to fatty acid synthesis (33). Lipogenic enzymes have also been shown to be regulated by covalent modification. More recently evidence has been presented to suggest that total enzyme
activity could be altered by changes in subcellular distribution or compartmentation (11,79). ATP CL was found to be associated with mitochondria in isolated rat hepatocytes which were treated with digitonin to promote release of cytosolic enzymes and markers (79). Under these conditions approximately 25% of the ATP CL activity was released as a mitochondrial enzyme (79). The addition of the substrates for ATP CL (CoA, citrate and ATP) enhanced release of ATP CL from "non-cytosolic compartments." However addition of physiological levels of MgCl$_2$ blocked the effect of the substrates without directly effecting release of ATP CL into the cytosol. Another lipogenic enzyme, acetyl CoA carboxylase was found in an inactive form associated with the outer mitochondrial membrane (11). The mitochondria contained generous amounts of acetyl CoA carboxylase although the activity was nominal (20 fold less active) compared to the cytosolic acetyl CoA carboxylase (11).

Immunoquantitation, measurement of relative abundance of the ATP CL antibody reactive bands by laser densitometry, was employed to determine the relative abundance of ATP CL associated with intracellular compartments during different nutritional states relative to enzyme activity. Rats were fasted for 48 hours or fasted 48 hours followed by refeeding for 48 hours or were maintained in the fed state. Rats from
each group were decapitated, and the livers were quickly removed and homogenized in either Greenawalt's buffer or .3M mannitol. Subcellular fractions were isolated by differential centrifugation as discussed in methodology section. Samples were immediately subjected to 7.5% SDS PAGE followed by Western blotting onto nitrocellulose, then immunodetection using rabbit anti-rat ATP CL antibody. Initial experiments were performed to ensure that the bands which corresponded to the molecular weights of ATP CL were not the result of non-specific interaction of the avidin-based detection system with biotin containing enzymes. The relative amounts of antibody reactive bands were measured by densitometry. Molecular weights were determined from the standard molecular weight markers seen in (Figure 4). The experiment was performed in two different ways and different subcellular fractions were obtained in each. The procedure using Greenawalt's buffer allowed separation of cytosol, microsomes, mitoplasts and outer mitochondrial membrane. Only the cytosolic and non-cytosolic fractions minus the nuclei were collected from livers homogenized in .3M mannitol. The two procedures yielded different results.

The significance of the bands must be kept in perspective. All amounts of reactive bands are relative only to the separation of bands in that particular gel lane and are reported as percent of total. Direct comparison of the amount of one band between lanes would be invalid since
Figure 4: Standard curve for molecular weight markers on 7.5% SDS-PAGE. Samples (20 ul) were applied to 7.5% gels, run at 7.5 mamps, then stained with coomassie blue. The points represent the relative migration difference for each band and the best fit line was generated by Minitab with $R^2 = 0.94$. The standard sample contained phosphorylase b (130 KD), BSA (75 KD), albumin (50 KD), carbonic anhydrase (39 KD), soybean trypsin inhibitor (27 KD) and lysozyme (17 KD).
they are reported in relative percents pertaining only to that lane, although the relative percents of one band between lanes could be evaluated. As can be seen plates 1 and 2, ATP CL antibody reacted with numerous bands during the course of the western blotting procedure. Either the antibody specificity is low or there was an abundance of ATP CL degradation products which were used for immunization. Only putative ATP CL bands which were detected in other labs were examined in order to reduce the complexity to interpreting the confusing multitude of bands. The disadvantages and problems with this technique will be discussed later.

ATP CL is a tetramer of four presumably identical subunits of 120 KD (57). Other investigators have reported extreme sensitivity of this enzyme to proteases, and treatment of ATP CL purified from rat liver with the common proteases subtilisin, trypsin and chymotrypsin yielded peptides of 70 KD, 68 KD and 53 KD (52). The 68 KD fragment was further degraded only by subtilisin to two peptides of 37 KD and 25 KD. Therefore, only the 120 KD, 70 KD, 68 KD, 53 KD, 37 KD and 25 KD bands were evaluated. Acyl CoA lyase activity resides in the 70 KD, 68 KD and (37 KD + 25 KD) fragments (52). The 53 KD fragment alone was inactive for either partial reaction of ATP CL; acyl CoA lyase or thiokinase (52). However, complete ATP CL activity was restored upon combination of the 53 KD fragment with 70 KD,
Plate I. Photograph of ATP CL immunoblot of fed, fasted and refed rat liver fractions homogenized by Greenawalt's procedure.

Lane 1. Cytosol from fed rats
Lane 2. Cytosol from fasted rats
Lane 3. Cytosol from refed rats
Lane 4. Standard molecular weight markers
Lane 5. Microsomes from fed rats
Lane 6. Microsomes from fasted rats
Lane 7. Microsomes from refed rats
Lane 8. Outer membranes from fed rats
Lane 9. Outer membranes from fasted rats
Lane 10. Outer membranes from refed rats
Lane 11. Standard molecular weight markers
Lane 12. Mitoplasts from fed rats
Lane 13. Mitoplasts from fasted rats
Lane 14. Mitoplasts from refed rats
Plate II. Photograph of ATP EL immunoblot of fed, fasted and re-fed rat liver fractions homogenized by 0.3 M mannitol procedure.
Lane 1. Total homogenate from fed rat 1
Lane 2. Total homogenate from fed rat 2
Lane 3. Cytosolic fraction from fed rat 1
Lane 4. Cytosolic fraction from fed rat 2
Lane 5. Non-cytosolic fraction from fed rat 1
Lane 6. Non-cytosolic fraction from fed rat 2
Lane 7. Total homogenate from fasted rat 1
Lane 8. Total homogenate from fasted rat 2
Lane 9. Cytosolic fraction from fasted rat 1
Lane 10. Cytosolic fraction from fasted rat 2
Lane 11. Non-cytosolic fraction from fasted rat 1
Lane 12. Non-cytosolic fraction from fasted rat 2
68 KD or (37 KD + 25 KD) fragments (52). It was inferred that the peptides involved in acyl CoA lyase activity and partial, incomplete thiokinase activity are housed in the 70 KD and 68 KD fragments while the 53 KD fragment is a partial thiokinase.

The fractionation procedure using the Greenawalt's buffer will be discussed first. Figure 5 depicts the ATP CL antibody reactive bands in the cytosol of fed, fasted and refed rat livers. The 120 KD monomer was detected only in the fed and fasted cytosolic fractions and was absent from the refed cytosol. None of the various cytosols contained any of the 70 KD fragment. The largest reaction of ATP CL antibody with the enzyme was with the 68 KD fragment in all three nutritional states with the 68 KD fragment being most abundant in the fed cytosol. Small amounts of 53 KD fragment were detected in the fed cytosol with more in both the fasted and refed cytosols. It would seem unusual for considerable amount of the 37 KD fragment to be present without detection of any 25 KD fragment since they are presumably the products of proteolysis of the 68 KD fragment. Although in the procedure used here, the 25 KD could have been further degraded as an abundance of small molecular weight bands are present.

The outer mitochondrial membrane (Figure 6) from fed liver contained 120 KD, 53 KD and 70 KD fragments (in order of amount of antibody reaction). There were less of these
Figure 5: Immun quantitation of ATP CL in the cytosol of liver isolated by Greenawalt's procedure. Values are from homogenates pooled from 3 rats each from fed, fasted or refed rats, boiled in SDS and β-mercaptoethanol, electrophoretically separated on 7.5% SDS-PAGE and Western blotted with rabbit anti-rat antibody to liver ATP CL. ATP CL antibody reactive bands were quantitated by laser densitometry.
Figure 6: Immunoquantitation of ATP CL in outer mitochondrial membrane isolated by Greenawalt's procedure. Details are same as for Figure 5.
in the fasted and refeed states. Complete ATP CL activity can theoretically only be restored when 70 KD, 68 KD or (37 KD ± 25 KD) fragments are combined with the 53 KD fragment. Since none of the 68 KD fragment was present in the fed or fasted outer membrane fraction and relatively small amounts of 70 KD fragment were detected, the 70 KD fragment could be considered limiting in restoring ATP CL activity to the fed and fasted outer membrane fractions, but the 53 KD fragment may be limiting in the refeed. The 37 KD and 25 KD fragments alone were predominant in refeed and fasted outer membrane fractions respectively, therefore most of the fragments were possibly inactive.

The mitoplasts (Figure 7) were composed of a majority of the 120 KD monomer with smaller amounts of the other fragments and absence of the 68 KD fragment. The refeed mitoplasts were especially enriched with the 120 KD monomer. The microsomes (Figure 8) from fed fasted and refeed livers contained only degradative fragments (68 KD and 37 KD) with an absence of any 53 KD fragment which should prohibit reconstruction of ATP CL activity. However, when the ATP CL activity was directly measured in each fraction, activity was present in the microsomes from all three states (Table 3). Most of the activity in the fed, fasted and refeed livers was measured in the mitoplasts which is in agreement with the high amount of the 120 KD monomer detected by the ATP CL antibody. The detection of ATP CL activity and ATP
Figure 7: Immunoquantitation of ATP CL in mitoplasts isolated by Greenawalt's procedure. Details are same as for Figure 5.
Figure 8: Immunoquantitation of ATP CL in microsomes isolated by Greenawalt's procedure. Details are same as for Figure 5.
Table 3. Enzyme Activity in Subcellular Fractions of Liver Homogenates

<table>
<thead>
<tr>
<th>Fraction</th>
<th>ATP CL (nm/m/mg)</th>
<th>MAO (nm/m/mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Greenawalt's Procedure</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cytosol</td>
<td></td>
<td></td>
</tr>
<tr>
<td>fed</td>
<td>5.63</td>
<td>0</td>
</tr>
<tr>
<td>fast</td>
<td>0.75</td>
<td>0</td>
</tr>
<tr>
<td>refed</td>
<td>3.64</td>
<td>0</td>
</tr>
<tr>
<td>Outer Membrane</td>
<td></td>
<td></td>
</tr>
<tr>
<td>fed</td>
<td>7.17</td>
<td>9.9</td>
</tr>
<tr>
<td>fast</td>
<td>3.10</td>
<td>4.6</td>
</tr>
<tr>
<td>refed</td>
<td>1.38</td>
<td>3.6</td>
</tr>
<tr>
<td>Mitoplasts</td>
<td></td>
<td></td>
</tr>
<tr>
<td>fed</td>
<td>16.92</td>
<td>0</td>
</tr>
<tr>
<td>fast</td>
<td>37.20</td>
<td>0</td>
</tr>
<tr>
<td>refed</td>
<td>53.03</td>
<td>0</td>
</tr>
<tr>
<td>Microsomes</td>
<td></td>
<td></td>
</tr>
<tr>
<td>fed</td>
<td>1.95</td>
<td>2.7</td>
</tr>
<tr>
<td>fast</td>
<td>2.53</td>
<td>1.9</td>
</tr>
<tr>
<td>refed</td>
<td>5.97</td>
<td>0</td>
</tr>
<tr>
<td>3M mannitol Procedure</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td></td>
</tr>
<tr>
<td>fed</td>
<td>6.33</td>
<td>*</td>
</tr>
<tr>
<td>fast</td>
<td>5.88</td>
<td>*</td>
</tr>
<tr>
<td>Cytosol</td>
<td></td>
<td></td>
</tr>
<tr>
<td>fed</td>
<td>17.97</td>
<td>*</td>
</tr>
<tr>
<td>fast</td>
<td>4.18</td>
<td>*</td>
</tr>
<tr>
<td>Non-cytosolic fraction</td>
<td></td>
<td></td>
</tr>
<tr>
<td>fed</td>
<td>3.92</td>
<td>*</td>
</tr>
<tr>
<td>fast</td>
<td>8.25</td>
<td>*</td>
</tr>
</tbody>
</table>

Values are means of duplicate determinations for fractions pooled from 3 rats.

* represents omitted measurement of MAO activity
CL antibody reactivity in all subcellular fractions signifies either contamination between fragments or actual existence of ATP CL in all fragments. When monoamine oxidase activity, an outer mitochondrial membrane marker, was determined, contamination was found only in fasted and refed microsomes, but it was considerable (2 to 10 fold of the activity measured) in other subcellular compartments.

In attempt to reduce some of the confounding factors such as the time required to prepare the fractions, which increased the opportunity for proteolysis of ATP CL even though protease inhibitors were present, in the previous experiment, the cytosol was rapidly separated from the non-cytosolic fractions minus the nuclei in .3M mannitol which lacked protease inhibitors. Results previously obtained in this laboratory noted that SDS-β-mercaptoethanol boiled samples of the enzyme stored in the freezer yielded an increasing number of antibody reactive bands after even short storage periods, so care was taken to do the SDS-PAGE immediately.

The total liver homogenate, collected by the .3 M mannitol procedure, not only demonstrated slightly more ATP CL activity (6.3 nm/min/mg in fed versus 5.9 nm/min/mg in fasted), but much more of the 120 KD monomer was detected by ATP CL antibody (Figure 9). Similar amounts of 70 KD and 37 KD fragments were detected in fed and fasted homogenates although the majority of the antibody reacted with the 68
Figure 9: Immunoquantitation of ATP CL in total liver homogenates isolated by differential centrifugation. Values are from 2 livers pooled from fed and fasted rats then homogenized in 5 volumes of 0.3 M mannitol and centrifuged at 500 X G. Subsequent treatment of the samples was the same as for Figure 5.
KD fragment in the fasted liver. The 53 KD fragment existed only in the fed livers. It would appear that ATP CL activity would be much higher in the fed homogenate than in the fasted since there is considerably more 120 KD monomer in the fed liver and a lack of 53 KD fragment in the fasted liver, yet the direct ATP CL activity measurements do not confirm this.

The cytosol of fasted rats (Figure 10) contained much more 120 KD monomer than does the fed cytosol. The percentages of 70 KD, 68 KD and 37 KD fragments were about equivalent, and these made up the overwhelming majority of the ATP CL antibody reactivity. Again, the 53 KD fragment was only found in the fed cytosol. The relative quantities of the bands between the fed and fasted cytosol do not approximate activity level. ATP CL activity in fed cytosol is four times the activity of the fasted cytosol. The fasted non-cytosolic fractions (Figure 11) have over two times the ATP CL activity than the fed. The prevalence of the 120 KD monomer is similar in the fed and fasted non-cytosolic fractions. The predominant ATP CL reactive fragments in the fasted non-cytosolic fraction are the 70 KD and 68 KD bands, while in the fed non-cytosolic fraction it is the 53 KD and 68 KD fragments. The 53 KD fragment was consistently absent from the fasted non-cytosolic compartment. This suggests that additional forms of enzyme regulation such as covalent modification may be at play.
Figure 10: Immunoquantitation of ATP CL in the cytosolic fraction of livers isolated by differential centrifugation. Values are from 2 livers pooled from fed an fasted rats. The cytosolic fraction was isolated upon ultracentrifugation of total homogenates at 105,000 X G, in the supernatant. Subsequent treatment of the samples was the same as for Figure 5.
Figure 11: Immunoquantitation of ATP CL in the non-cytosolic, post-nuclear fraction of livers isolated by differential centrifugation. Values are from 2 livers pooled from fed and fasted rats. The post nuclear fraction is composed of all the material precipitating between 500 and 105,000xG. Subsequent treatment of the samples was the same as for Figure 5.
Caution must be exercised in attempting to interpret these results. Much is inherently unknown with the use of liver homogenates. Many unidentified proteases exist even with addition of protease inhibitors and their means of activation is uncertain. The accuracy of differential centrifugation of the liver homogenate to separate the subcellular fractions may not be similar to the in vivo situation. The forces experienced during centrifugation may disrupt membranes and enzyme associations to the membranes. However, several general conclusions may be made. The results obtained are extremely dependent on the technique used. There was variation in the direct measurement of ATP CL activity and antibody detection and formation of proteolytic degradation products between the two very similar techniques implemented here. Greenawalt's buffer contained two protease inhibitors and HEPES, and the procedure included digitonin treatment of the non-cytosolic fraction. Differential centrifugation with .3 M mannitol in the absence of protease inhibitors only separated the cytosolic and non-cytosolic fractions from the post-nuclear homogenates.

The 120 KD monomer is not the only fragment capable of producing ATP CL activity as noted previously and, since there seemed to be no relation between the relative amount of 120 KD monomer and the ATP CL activity. The degradation products created by endogenous proteases must retain at
least partial activity capable of restoring the full ATP CL activity when combined with each other. These results are in agreement with those of Lill et.al. (52). Using means of direct visualization of ATP CL activity on disc gels (177), it was observed that low molecular weight bands could partake in the ATP CL reaction (178). It was later realized that these small bands were degradation products of ATP CL, formed by endogenous proteases, which retained at least partial activity (52).

The activity of ATP CL in liver is dependent on nutritional condition. The prevalence of activity distributed among the subcellular compartments is also altered. The change in the relative abundance of the 120 KD monomer and proteolytic products is in accordance with change in activity in response to the fed, fasted or refed state. The precise proteolytic fragments which contribute to reconstruction of ATP CL activity were not discernable by these methods. Additional proteolytic fragments were obtained here in comparison to those characterized by Lill et.al. (52) perhaps due to their use of purified ATP CL treated with selected proteases. Liver homogenates contain many unidentified endogenous proteases which may further degrade the fragments previously observed, although partial activity may not be destroyed by the further proteolysis.

Lipid synthesis in the liver is extremely sensitive to the nutritional state of the animal. Lipogenesis is
severely depressed in the fasted condition in accordance with low availability of glucose and a low insulin/glucagon ratio (15). Upon refeeding there is an overshoot in lipid synthesis. An alternative explanation to the regulation of lipid synthesis by change in the quantity of lipogenic enzymes is the control of lipogenic enzyme activity by subcellular relocalization of the enzymes in response to nutritional state. Acetyl CoA carboxylase which exists in a less active form in livers from fasted rats (12) experienced a decline in activity and amount in the cytosol which was compensated for by redistribution of the enzyme to its inactive form on the mitochondria (12). The cytosolic level of acetyl CoA carboxylase increased concomitant with decline in mitochondrial level of the enzyme in liver as a fasted animal is refeed. The trend observed with depression of amount of acetyl CoA carboxylase in the cytosol paralleled an increase in the mitochondrial form under fasting conditions followed by replenishment of enzyme in the cytosol upon refeeding is in agreement with change in the activity of acetyl CoA carboxylase during these nutritional states. Furthermore, analogous results were obtained in livers of alloxan diabetic rats where a constant amount of total acetyl CoA carboxylase existed, but there was a change in subcellular distribution of the enzyme as substantial amount of the enzyme was relocalized from the cytosol to the mitochondria as diabetes developed
This infers potential involvement of insulin in subcellular mobilization of acetyl CoA carboxylase during change in nutritional state. There is also correlation of ATP CL activity with subcellular compartmentation in hepatocytes similar to that of acetyl CoA carboxylase. Activity in the non-cytosolic fraction in livers from fasted rats was twice that of livers from fed rats (13). In addition, CoA levels were shown to affect the subcellular localization of ATP CL (13). Hepatocytes were digitonin fractionated and supplemented with CoA which resulted in "solubilization" of ATP CL from the subcellular organelles and release as a purely cytosolic enzyme (13). The opposite effect was observed with the hypolipidemic agent 5-(tetradecyloxy)-2-furoic acid which produces an appreciable inhibition of fatty acid and cholesterol synthesis through esterification of CoA which suppresses free CoA levels (94). Such treatment caused an increase in association of ATP CL with the non-cytosolic compartments in accordance with a decrease in availability of CoA (93). This may suggest a vital importance for synchronization of CoA levels with ATP CL distribution (93).

Several studies provided evidence for association of the lipogenic enzymes with the outer mitochondrial membrane (79,12). This would be a logical mechanism since the outer membrane of the mitochondria is in contact with the cytosol so that the enzymes would be accessible to signals for
relocalization. Linn and Srere (95) are proponents of microsomal association of ATP CL specifically by binding to the endoplasmic reticulum. ATP CL binding to microsomes was prohibited in the presence of small amounts of CoA and high salt concentration (95). They found essentially no association of ATP CL with the outer mitochondrial membrane. These results must be met with caution, however, since purified ATP CL was added back to isolated fraction of mitochondria, outer membrane and microsomes, and they did not use an outer mitochondrial membrane marker. Since the outer membrane is quite fragile, it might easily rupture during the experiment and because of its size would migrate with the microsomal fraction during centrifugation. This method may not be representative of in vivo ambiguity. If the enzyme undergoes subcellular redistribution upon change in nutritional or physiological state, it would seem likely that some signalling mechanism must be at play which would not be intact once the enzyme is purified or cells are fractionated. They also found no difference in the ability of the phosphorylated form or dephosphorylated form of the enzyme to bind the microsomes (95). In direct contrast to these results, Stralfors (180) showed that stimulation of phosphate incorporation into ATP CL by insulin or isoproterenol, a β-adrenergic agent, was accompanied by immobilization in the non-cytosolic fractions of the enzyme in adipocytes treated with digitonin. Identical treatment
did not affect the distribution of lactate dehydrogenase or hexokinase, therefore the effect seems to be specific for ATP CL. Lin and Bridger (181) refuted this with their findings that dephospho-ATP CL and purified ATP CL phosphorylated by incubation with cAMP dependent protein kinase were distributed to the same degree between the cytosol and the mitochondria.

The significance of the phosphorylation state or subcellular location of several enzymes, including ATP CL, is still not completely clear. Many of the results reported by separate labs are not comparable due to inherent differences in cell isolation, fractionation and conditions of enzyme assays or other methodology. The use of hepatocytes treated with digitonin, fractionation of liver homogenates or isolation/purification/reconstruction experiments probably do not give similar information. Stralfors (180) proposed a more extended effect of the phosphorylation state of ATP CL such that hormone-induced SER phosphorylation may not produce a "rapid metabolic response." Instead, some ATP CL may be bound to intracellular organelles under all physiological conditions. Enzyme modification at multiple levels may be required to integrate cellular signals for regulation. "Mass action" is the simplest means of regulation by availability of substrate (92). Allosteric effectors or binding of ligands to induce conformational changes also affect enzyme
activity. The putative reversible association of enzymes with intracellular membranes leading to change in activity may similarly act through conformational change (79). All of these mechanisms in conjunction with long term changes in enzyme synthesis/degradation may be integrated to control enzyme activity or availability.

DIABETIC STUDY

The diabetic condition leads to aberrant regulation of metabolism. Insulin is not only the major glucoregulator, but also contributes to modification of activity of numerous enzymes. Poor wound healing and proliferative diabetic retinopathy are critical side effects of diabetes. Both involve autocrine production of growth factors to regulate growth and repair in response to systemic factors (182). Hepatic lipogenesis is reduced in diabetes in conjunction with low levels of lipogenic enzyme activity (179). ATP CL activity which is reduced in isolated hepatocytes from diabetic rats can be restored upon addition of insulin. Previous work from this lab (3) has reported the existence of putative autocrine/paracrine regulators produced by hepatocytes and capable of acting upon hepatocytes. These regulators alter ATP CL activity. Examples of abnormal autocrine/paracrine regulation are apparent in diabetes. The functioning of autocrine/paracrine regulation in diabetic liver was examined to investigate possibility that
disregulation may occur in diabetes specifically as it relates to ATP CL activity.

The following experiments were conducted to answer two questions: 1) do hepatocytes from diabetic rats have the capacity to secrete (and/or synthesize) the autocrine/paracrine regulators of ATP CL activity previously described (3) and 2) do hepatocytes from diabetic rats have the capacity to respond to these regulators when they are added to hepatocyte incubation medium? To explore these questions, hepatocytes from diabetic rats were incubated to obtain CM which was tested using hepatocytes from control rats. The hepatocytes from diabetic animals were also incubated with CM from control rats in attempt to answer the second question. The experimental design was as follows. Rats were injected with 65 mg streptozocin/Kg body weight to render the treatment group diabetic with matched age and weight controls receiving saline injections. Two trials were done with a variation in the duration of the experiment being 3 days or 7 days. Some of the variables measured are reported in Table 4. Blood glucose levels in both 3 day and 7 day diabetic animals differed significantly from controls (271 mg% and 444 mg%, respectively versus 157 mg% and 108 mg%). The protocol for induction of diabetes by streptozocin requires 24 hour fasting period prior to injection. The 3 day and 7 day experiments differed in that the 3 day rats received semi-purified, high carbohydrate
Table 4. Experimental Parameters of the Diabetes Study.

<table>
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<th>Condition</th>
<th>Control</th>
<th>Diabetic</th>
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<tbody>
<tr>
<td>Wt. regain (g)</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>3 day</td>
<td>37.5 ± 9.6</td>
<td>18.8 ± 6.1</td>
<td>.005</td>
</tr>
<tr>
<td></td>
<td>n=12</td>
<td>n=8</td>
<td></td>
</tr>
<tr>
<td>7 day</td>
<td>47.8 ± 7.8</td>
<td>-11.7 ± 15.2</td>
<td>.0001</td>
</tr>
<tr>
<td></td>
<td>n=16</td>
<td>n=6</td>
<td></td>
</tr>
<tr>
<td>Blood glucose (mg%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3 day</td>
<td>157 ± 25</td>
<td>271 ± 11</td>
<td>.025</td>
</tr>
<tr>
<td></td>
<td>n=6</td>
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<tr>
<td>7 day</td>
<td>108 ± 9</td>
<td>444 ± 165</td>
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<tr>
<td></td>
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</tr>
<tr>
<td>Liver wt. (g)</td>
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<td>9.4 ± 0.5</td>
<td>NS</td>
</tr>
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<td>3 day</td>
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<td>n=3</td>
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</tr>
<tr>
<td>Liver (% body wt.)</td>
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<td>4.10 ± .01</td>
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</tr>
<tr>
<td>3 day</td>
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<td>n=3</td>
<td></td>
</tr>
<tr>
<td>Hepatocyte viability (%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3 day</td>
<td>83 ± 2</td>
<td>81 ± 1</td>
<td>NS</td>
</tr>
<tr>
<td></td>
<td>n=6</td>
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<tr>
<td>7 day</td>
<td>84 ± 2</td>
<td>80 ± 1</td>
<td>NS</td>
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<tr>
<td></td>
<td>n=6</td>
<td>n=4</td>
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</tbody>
</table>

Values are mean ± standard deviation.

The differences between the means of the controls and diabetics were analyzed by paired t-test and P values were determined on Minitab.
diet for 24 hours before being shifted to Purina rat chow pellets. In the 7 day experiment, rats received the high carbohydrate diet during the entire refeeding period. The weight regain after fasting varied considerably between diabetic and control groups (3 day 18.8 g versus 37.5 g) (Table 4). The 7 day diabetic group even had negative weight gains (-11.7 g versus 47.8 g for the controls).

ATP CL activity in 0 time control hepatocytes from diabetics and controls was slightly reduced (by ≈25% in 3 day group and 19% in 7 day group) as seen in Table 5, but were not significantly different due to high variability of activity values. The ATP CL activity in the 3 day group is much higher than that of the 7 day group (23.07 nm/min/mg versus 4.64 nm/min/mg controls) probably as a result of 3 day group being in a refed state. ATP CL activity declines in the fasted state while refeeding after fast causes an overshoot in ATP CL activity (15). The ATP CL activity in liver homogenates did not differ between diabetic and control (by 12% in 3 day group) either (Table 5). The ATP CL activity measured in hepatocytes was considered representative of in vivo homogenate activity. In the intact liver there are approximately $10^8$ cells/g. During the experiment the highest cell density used was $10^7$ cells/ml. Most of the time hepatocytes at the intermediate density ($10^6$ cells/ml) was used. ATP CL activity in
Table 5. ATP CL Activity in Liver Homogenates and Hepatocytes from 3 Day Diabetic Study.

<table>
<thead>
<tr>
<th>ATP CL source</th>
<th>Control</th>
<th>Diabetic</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Homogenates</td>
<td>1180 ± 202</td>
<td>1047 ± 66</td>
<td>NS</td>
</tr>
<tr>
<td>(nm/min/g liver)</td>
<td>n=3</td>
<td>n=3</td>
<td></td>
</tr>
<tr>
<td>Hepatocytes</td>
<td>26.9 ± 11.3</td>
<td>19.9 ± 9.8</td>
<td>NS</td>
</tr>
<tr>
<td>(nm/min/mg protein)</td>
<td>n=3</td>
<td>n=3</td>
<td></td>
</tr>
</tbody>
</table>

Values are mean ± standard deviation.

The differences between the means of the controls and diabetics were analyzed by paired t-test and P values were determined on Minitab.
preparations of intermediate density was approximately 3 fold greater than the activity in homogenates and well above that of the high density preparations. This agrees with the findings of Voss et.al. (3) that there is a reduction in ATP CL activity/mg as hepatocyte cell density increases.

Cell free CM was collected from hepatocytes at high \((10^7\) cells/ml) and low \((10^5\) cells/ml) cell densities from diabetic and control. To determine whether the peptides present in CM (111) in these preparations were the result of intracellular protein leakage from damaged cell. LDH activity was measured in CM and in hepatocytes (Table 6) since the presence of the cytosolic enzyme, LDH in the CM infers cell breakage. The effects produced by the cell free CMs could not be attributed to contamination of CM by cytosolic elements since only 2.7% LDH activity was detected in cell free CM, and the value was essentially constant from 0 to 30 minutes (Table 6).

Hepatocytes from 3 day diabetic rats were incubated with 30 minute control CM for up to 20 minutes. ATP CL activity was measured in sonicated hepatocytes after 0, 5, 10 and 20 minutes incubation periods. Variable baseline activity values were obtained in diabetic cells in absence of CM, but ATP CL activity was somewhat stimulated in hepatocytes from diabetic rats treated with LD control CM (Figure 12). HD control CM did not seem to have much effect (Figure 12).
Table 6. Lactate Dehydrogenase Activity in Hepatocytes and CM from 7 Day Diabetic Rats.

<table>
<thead>
<tr>
<th>Incubation time (min.)</th>
<th>CM</th>
<th>Hepatocytes</th>
<th>CM/Hept(%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0.27</td>
<td>16.2</td>
<td>1.7</td>
</tr>
<tr>
<td>10</td>
<td>0.30</td>
<td>10.2</td>
<td>2.9</td>
</tr>
<tr>
<td>20</td>
<td>0.32</td>
<td>12.3</td>
<td>2.6</td>
</tr>
<tr>
<td>30</td>
<td>0.44</td>
<td>13.1</td>
<td>3.4</td>
</tr>
</tbody>
</table>

mean = 2.7

Values are lactate dehydrogenase activity in nm/min/mg protein.

The percent of lactate dehydrogenase activity in CM and hepatocytes from diabetic rats incubated from 0 to 30 minutes was compared and the percent of activity as a ratio of CM/hepatocyte was determined.
Figure 12: ATP CL activity in hepatocytes from 3 day streptozocin diabetic rats treated with CM from 3 day control rats. Values ± SEM for 3 experiments are percentage of ATP CL activity from time 0 control in absence of CM.

-○- is freshly isolated rat hepatocytes from 3 day diabetic rats in absence of additional CM. -●- is freshly isolated hepatocytes from 3 day diabetic rats treated with LD CM from control rats pooled from 3 hepatocyte preparations. -▲- is freshly isolated rat hepatocytes from 3 day diabetic rats treated with HD CM from control rats pooled from 3 hepatocytes preparations. There were no statistically significant effects (P > 0.1) of media (HD CM or LD CM) or time as determined by two-way ANOVA, repeated measures, on SAS.
LD diabetic CM elevated activity in control hepatocytes by 2 to 3 fold. Cells from diabetic rats did not seem as capable of responding to LD control CM (elevated only 1.5 to 2 fold). The action of HD diabetic CM on control hepatocytes varied little from diabetic cells incubated with HD control CM, producing congruent curves (Figure 13). A different perspective is seen in Figure 14; ATP CL activity in control hepatocytes was activated similarly by LD diabetic CM and LD control CM (up to 5 fold). On the other hand, the cells from diabetic rats were not activated by LD control CM to the same extent as were control hepatocytes (2 fold maximum). Incubation of HD control CM with either control or diabetic hepatocytes did not have much effect on ATP CL activity (Figure 15). Due to restrictions inherent in the techniques of statistical analysis ATP CL activity in hepatocytes from 3 day diabetic and control rats had to be considered separately. Three-way ANOVA, repeated measures (SAS), on treatment of hepatocytes from control rats with CM from diabetic rats did not show significant effects of density of the CM (LD or HD), media type (control or diabetic) or time (all with $P > 0.1$). ATP CL activity in hepatocytes from diabetic rats treated with CM from control rats was analyzed by two-way ANOVA, repeated measures (SAS), for significant effects of density of CM or time. There were no statistically significant effects of either (both with $P > 0.1$). Since there were no statistically
Figure 13: ATP CL activity in hepatocytes from 3 day control rats treated with CM from 3 day streptozocin diabetic rats.  
-○- is freshly isolated hepatocytes from 3 day control rats in absence of additional CM.  -●- is freshly isolated hepatocytes from 3 day control rats treated with LD CM from 3 day diabetic rats pooled from 3 hepatocyte preparations.  
-△- is freshly isolated hepatocytes from 3 day control rats treated with HD CM from 3 day diabetic rats pooled from 3 hepatocyte preparations. Details and statistics are same as described for Figure 12.
Figure 14: Effect of LD CM from 3 day streptozocin diabetic and control rats on ATP CL activity in hepatocytes from 3 day streptozocin diabetic and control rats. — is freshly isolated hepatocytes from 3 day control rats treated with LD CM from 3 day control rats pooled from 3 hepatocyte preparations. — is freshly isolated hepatocytes from 3 day diabetic rats treated with LD CM from 3 day control rats pooled from 3 hepatocyte preparations. — is freshly isolated hepatocytes from 3 day control rats treated with LD CM from 3 day diabetic rats pooled from 3 hepatocyte preparations. There were no statistically significant effects (P > 0.1) of media (HD CM or LD CM), type (diabetic or control CM) or time as determined by three-way ANOVA, repeated measures, by SAS. Details are same as described for Figure 12.
Figure 15: Effect of HD CM from 3 day streptozocin diabetic and control rats on ATP CL activity in hepatocytes from 3 day streptozocin diabetic and control rats. -○- is freshly isolated hepatocytes from 3 day control rats treated with HD CM from 3 day control rats pooled from 3 hepatocyte preparations. -●- is freshly isolated hepatocytes from 3 day diabetic rats treated with HD CM from 3 day control rats pooled from 3 hepatocyte preparations. -▲- is freshly isolated hepatocytes from 3 day control rats treated with HD CM from 3 day diabetic rats pooled from 3 hepatocyte preparations. There were no statistically significant effects (P > 0.1) of media (HD CM or LD CM), type (diabetic or control CM) or time as determined by three-way ANOVA, repeated measures, by SAS. Details are same as described for Figure 12.
significant effects, trends in alteration of ATP CL activity will be considered and discussed.

Unexpectedly, the ATP CL activity in neither control or diabetic hepatocytes was inhibited by HD control CM or HD diabetic CM in contrast to previous results (3). It is possible that hepatocytes from refed rats may produce different autocrine/paracrine regulators than do hepatocytes from fed rats. Insensitivity to inhibitory autocrine/paracrine factors after a period of refeeding would be consistent with the observation of substantially increased multinucleate cells in livers from refed rats (178). It is also possible that the enzyme activity has not fully recovered to levels of fed hepatocytes even 7 days after fasting. The activities of several enzymes were previously shown to remain slightly elevated out to 5 days following fasting (183).

The 7 day trial yielded somewhat different results than did the 3 day trial. Baseline activity values for hepatocytes from 7 day diabetic rats demonstrated transient activation of ATP CL with up to 4.5 fold induction within 5 minutes, followed by a rapid decline in activity to 0 time control levels at 10 minutes which then remained steady through 20 minutes (Figure 16). Incubation of hepatocytes from diabetic rats with LD control CM activated ATP CL approximately 10 fold within 5 minutes, then rapidly declined to 4 to 5 fold by 10 minutes and 3 fold by 20
**Figure 16**: ATP CL activity in hepatocytes from 7 day streptozocin diabetic rats treated with CM from 7 day control rats. There were no significant effects ($P > 0.1$) of media or time as determined by two-way ANOVA, repeated measures, by SAS. Details are same as for Figure 12.
minutes. Again HD control CM did not seem to have much effect on cells from diabetic rats; there was little difference between the groups except for slight, transient activation at 10 minutes. In absence of added CM, ATP CL activity increased 3 fold within 5 minutes in the 7 day control group, then rose slightly and remained steady.

LD diabetic CM elevated activity in control hepatocytes to a great extent, 5 fold with in 5 minutes to 8.5 fold within 20 minutes (Figure 17). HD diabetic CM stimulated activity in control hepatocytes to 2 fold within 5 minutes and was constant through 20 minutes, however the activity was reduced in comparison with baseline levels in control hepatocytes. Comparison of the ability to activate ATP CL activity between LD control CM and LD diabetic CM (Figure 18) shows that LD diabetic CM and LD control CM eventually, by 20 minutes augmented ATP CL activity in control hepatocytes (≈9 fold). However, there were differences in the rapidity of stimulation of enzyme activity. LD control CM stimulated activity dramatically within 5 minutes, whereas the activation by LD diabetic CM was gradual (Figure 18). Hepatocytes from diabetic rats responded to LD control CM by extensive activation (10 fold) of ATP CL with in 5 minutes followed by decline to 2 fold at 20 minutes (Figure 18).
Figure 17: ATP CL activity in hepatocytes from 7 day control rats treated CM from 7 day streptozocin diabetic rats. There were significant effects of media ($P > 0.01$) and time ($P > 0.01$) as determined by two-way ANOVA, repeated measures, by SAS. Details are same as for Figure 13.
Figure 18: Effect of LD CM from hepatocytes from 7 day streptozocin diabetic and control rats on ATP CL activity in hepatocytes from 7 day streptozocin diabetic and control rats. There were no significant effects of media (HD CM or LD CM), type (diabetic or control) or time as determined by three-way ANOVA, repeated measures, by SAS. Details are as described for Figure 14.
The HD diabetic CM and the HD control CM gave different results relative to previous studies which showed consistent inhibition of ATP CL activity by HD CM (< 100%). Control hepatocytes incubated with HD diabetic CM had the most steady activity curves, activating ATP CL 2 fold then remaining steady in comparison with addition of HD control CM which gave erratic activities between 3.5 fold and 6 fold with in the 20 minute incubation period (Figure 19). Hepatocytes from diabetic rats also reacted peculiarly to HD control CM; there was a 3.5 fold activation of ATP CL at 10 minutes, but all other times approximated the 0 control level (Figure 19). Statistically analysis will be done in the same manner for the 7 day diabetic experiment as is was for the 3 day diabetic experiment: activity of ATP CL in hepatocytes from diabetic and control rats will be considered separately. Treatment of hepatocytes from 7 day control rats with CM from 7 day diabetic rats was analyzed by three-way ANOVA, repeated measures (SAS), to test for effects of density of CM, media type and time. There were significant effects of all three (all at P > 0.01). Conversely, two-way ANOVA, repeated measures (SAS), showed no statistically significant effects of density of CM or time (both with P > 0.1) on ATP CL activity in hepatocytes from 7 day diabetic rats created with CM from control rats.

HD diabetic CM was boiled to test for existence of heat labile peptide inhibitors which were found in HD CM in
Figure 19: Effect of HD CM from 7 day streptozocin diabetic and control rats on ATP CL activity in hepatocytes from 7 day diabetic and control rats. There were no significant effects of media (HD CM or LD CM), type (diabetic or control) or time as determined by three-way ANOVA, repeated measures, by SAS. Details are as described for Figure 15.
previous work (111). When hepatocytes from diabetic rats were treated with boiled HD control CM there was a slight inhibition of ATP CL at 10 minutes (Figure 20). The reverse situation occurred in the previous study; HD CM inhibitory factor(s) was found to be heat labile. Not only was the inhibition of ATP CL activity alleviated upon boiling of HD CM, but activating factor(s) which was masked by the inhibitor further activated the ATP CL. Thus the inhibitor(s) was assumed to be more potent than the activator(s) (3). Analogous results were attained with treatment of hepatocytes from control rats with boiled HD diabetic CM (Figure 21). When boiled HD diabetic CM was added to hepatocytes from control rats the activity of the enzyme rose steadily from 2 fold to 5 fold, well above control levels of 3.5 fold maximum (Figure 21). However, ATP CL activity in hepatocytes from control rats was activated by unboiled HD control CM and boiled HD control CM was inhibitory upon boiling. The statistical significance of the effects of boiling HD CM and time were analyzed by two-way ANOVA, repeated measures (SAS). Neither boiling HD CM from control rats nor time had a significant effect on ATP CL activity in hepatocytes from 7 day diabetic rats (both with P > 0.1). However, there was a statistically significant effect of boiling HD CM from diabetic rats on ATP CL activity in hepatocytes from 7 day
Figure 20: Effect of boiled HD CM from 7 day control rats on ATP CL activity in hepatocytes from 7 day streptozocin diabetic rats. -O- is freshly isolated hepatocytes from 7 day diabetic rats in absence of any additional CM. - ● - is freshly isolated hepatocytes from 7 day diabetic rats treated with HD CM from control rats which was boiled for 30 minutes. - △ - is freshly isolated hepatocytes from 7 day diabetic rats treated with HD CM from control rats which was not boiled. There were no significant effects (P > 0.1) of boiling HD CM or time as determined by two-way ANOVA, repeated measures, by SAS. Details are same as for Figure 12.
Figure 21: Effect of boiled HD CM from 7 day streptozocin diabetic rats on ATP CL activity in hepatocytes from control rats. -○- is freshly isolated hepatocytes from 7 day control rats in absence of any additional CM. -●- is freshly isolated hepatocytes from 7 day control rats treated with HD CM from diabetic rats which was boiled for 30 minutes. -↑- is freshly isolated hepatocytes from 7 day control rats treated with HD CM from diabetic rats which was not boiled. There was a significant effect of boiling HD CM (P > 0.04), but not time (P > 0.1) as determined by two-way ANOVA, repeated measures, by SAS. Details are same as for Figure 12.
control rats (with P=0.03), although there was no time effect (P > 0.1).

Several possible explanations could apply here. First of all, the hepatocyte cell density used to collect CM could have been inaccurate, although CM was pooled from several cell incubations which should have prevented this. Yet, if the cell density was sufficiently overestimated, production and secretion of inhibitory factors into the medium under high cell density incubation could have deviated sufficiently to produce this result. Considering cell contact as a likely factor involved in inhibitory effect of high cell density (184), diminished cell density could prevent appropriate contact inhibition signals. High cell density factors are appreciably more susceptible to reduction of cell density than are low cell density factors since cell contact is probably not involved in the production of activators from cells at low densities (184). Another possibility is that these control rats were not identical to control rats used in the previous experiments. That would require that the rat strain acquired from the vendor deviated enough to produce changes in lipid metabolism which has been noted (178). Alternatively, and most likely, a rebound effect following fasting/refeeding cycle may be at play, such that metabolism is still equilibrating 7 days post-fast. Differences in 3 day and 7 day hepatocyte functioning clearly arise from differences
in refeeding stage of the animal. ATP CL activities are dramatically elevated upon refeeding then presumably return gradually to normal levels within a few days. Thus the results of 3 day and 7 day trials may not be comparable.

All the data must also be considered in conjunction with CM peptide factor profiles which were performed on 3 day diabetic and control media (111). Distinct peptides were present in LD and HD control CM which were absent from LD and HD diabetic CM (≈57.8 and 32 KD) and vice versa (≈79.4 and 20.4 KD). Two peaks were more prevalent in LD and HD diabetic CM than in LD and HD control CM (≈55.2 and 27.9 KD). Although none of these peptides were further isolated or characterized for inhibitory or stimulatory properties, they may be representative of distinct autocrine/paracrine regulators which could be involved in regulation of ATP CL and warrant further examination. Enzyme activity may be affected not only by presence of distinct factors in CM, but also by abundance or disappearance of factors which may be present ubiquitously.

Despite difficulties inherent in the comparison of the 3 day and 7 day experiments, a few general speculations can be made. The 3 day diabetic trial supports the notion that HD diabetic CM has less inhibitory capability than HD control CM (which behaves similarly on control and diabetic hepatocytes). There was a trend for LD diabetic CM and LD control CM to activate ATP CL in control hepatocytes in a
similar manner, while diabetic hepatocytes inherently are not as capable of responding to the activator(s) endogenous to the LD control CM.

The 7 day diabetic trial yielded different results. The HD diabetic CM and the HD control CM gave such variable activity values that much of the data is inconclusive. This may have been a result of highly compromised hepatocytes after 7 days of uncontrolled diabetes resulting in high blood glucose levels. It was strange that neither diabetic or control HD CM were inhibitory to ATP CL activity. In fact, hepatic ATP CL was activated by both HD CMs, which is in direct conflict with previous results (3). LD CM from hepatocytes from diabetic and control rats produced expected activation of ATP CL. There was a trend for LD diabetic CM and LD control CM to activate ATP CL in control hepatocytes. ATP CL activity in hepatocytes from control rats abruptly rose (≈10 fold) in response to LD control CM while LD diabetic CM produced a more gradual elevation of activity. ATP CL activity transiently soared in cells from diabetic rats treated with LD control CM, but plummeted thereafter. It can be generally concluded that hepatocytes from diabetic rats are aberrant in their response to autocrine/paracrine mediators and differ in production and secretion of these mediators under varying cell densities.

The insulin deficiency characteristic of diabetes affects multiple levels of regulation from blood glucose
regulation to alteration of mechanisms of cell growth and metabolism. Insulin is thought to be necessary for protein synthesis and may play a role in Ca++ activated intracellular events (185). Insulin also participates in maintenance of growth and repair. Long term insulin deprivation may modify or compromise the ability of cells to maintain homeostasis. Autocrine/paracrine regulation may contribute significantly to cellular maintenance. Several consequences of diabetes involve cell growth which has gone awry. Proliferative diabetic retinopathy is a considerable concern for diabetics as is poor wound repair. There may be an imbalance of the intricate composition of factors present in the local cellular environment which is not conducive to normal cellular growth and repair (182). This effect may be attributed to abnormality in production of autocrine/paracrine regulators by diabetic cells. Wound healing is dependent on a progression of events which involves interaction of various systemic factors with cells at the site of a wound for autocrine/paracrine production of growth factors, i.e. PDGF (153). The inappropriate proliferation of retinal capillaries is present in 25% of diabetics (182). It has not been definitely attributed to change in any systemic factor, though IGF-I levels may be important (182). It is possible that interaction of autocrine/paracrine factors with systemic factors is altered, thus giving rise to retinopathy (182).
The regulation of hepatic growth is complex and many aspects are unresolved. However, liver is one of the few tissues that retains the capacity to regenerate. In vitro, it is obligatory for hepatocytes to be plated at a low cell density in order to proliferate under optimal conditions (118). Autocrine regulatory factors are thought to be synthesized in response to conditions which are conducive to proliferation (113). Short term incubations of hepatocytes produce distinct autocrine/paracrine factors dependent on cell density (3,105).

The composition of HD CM varies from LD CM in several small peptides which is apparent in the peptide profile of media separated on SDS PAGE (111). The peptide media factors collected from diabetic hepatocytes were different by the presence or absence of several factors in comparison with non-diabetic ones. This difference was accompanied by change in ability of diabetic CM factors to alter ATP CL activity. It seems that not only are the quantity or composition of the autocrine/paracrine regulators modified, but the functioning of the diabetic hepatocytes may be compromised as well. Provision of cholesterol and phospholipids is essential for membrane biogenesis and ultimately for growth and repair processes. Theoretically, the cholesterol and phospholipid must be newly synthesized. A deficiency in cell growth and repair is obvious in diabetes as seen in poor wound healing and development of
retinopathy. The experiments presented here suggest that the capability of the diabetic liver to produce and respond to autocrine/paracrine regulation may also be compromised in diabetes.

**EGF/Age Study**

The first growth factor discovered to affect lipogenesis was EGF. Infusion of EGF into neonatal rats and mice resulted in development of fatty livers accounted for by elevation of triglyceride levels (27). EGF was found to be as effective as insulin in stimulating fatty acid synthesis in isolated rat hepatocytes (145). Holland and Hardie (146) demonstrated phosphorylation of both ATP CL and acetyl CoA carboxylase in response to addition of insulin and EGF in isolated rat hepatocytes. The activity of acetyl CoA carboxylase was unaffected by the phosphorylation and the activity of ATP CL was not measured.

The effect of EGF on activity of ATP CL in isolated rat hepatocytes was examined in this study. The time and dose responses of EGF were determined. In the original design of the experiment ATP CL activity in separate rat hepatocyte preparations was determined with respect to time and dose response without considering the age of the rat. However, the results were widely variable. Upon further examination, it seemed that the age and/or weight of the animal influenced the effect of EGF on the enzyme. Thus, the EGF dose and time effects on ATP CL activity were measured in
hepatocytes from 8, 10, 12 and 14 week old animals. Mouse submaxilllary EGF was incubated with freshly isolated hepatocytes at 37°C from 0 to 40 minutes at concentrations from 0 to 10^{-8} M.

The control values (no added EGF at time 0) were not significantly different as a function of age as determined by one-way ANOVA (Minitab) with P > 0.1 (Figure 22). In Figure 23, the dose response of ATP CL activity to EGF concentrations from 0-10^{-8} M is shown for hepatocytes from 8 week old rats. Activity was increased nearly two fold by 10^{-8} M EGF. This curve follows the expected gradual rise in activity with increasing concentrations of EGF and levelling off at 10^{-8} M EGF. The half maximal effect was between 10^{-12} and 10^{-11} M EGF. These values are consistent with the effects of EGF on lipogenesis reported by Holland and Hardie (146). However, the effect of the concentration of EGF on ATP CL activity was not statistically significant when tested by one-way ANOVA (Minitab) with P > 0.1. The dose response curves for EGF in hepatocytes from 10, 12 and 14 week old rats were considerably more variable and atypical for dose response curves with one or more of the values from each age not fitting the curve (Figure 24). Again, there was not a statistically significant effect of EGF concentration when tested by one-way ANOVA (Minitab) with P > 0.1 in the 10, 12 and 14 week old rats. It appeared
Figure 22: Control values for ATP CL activity in hepatocytes as a function of age. Values are mean of ATP CL activity (nm/min/10^3 cells) in hepatocytes from 8, 10, 12 and 14 week rats in absence of EGF at 5 minute incubation. The effect of age on ATP CL activities in control hepatocytes was not significantly different (P > 0.1) as determined by one-way ANOVA by Minitab.
Figure 23: EGF dose response curve for ATP CL activity in hepatocytes from 8 week old rats. EGF was supplemented to freshly isolated hepatocytes from 8 week old rats at 0 to $10^{-9}$ M. Values ± SEM are percentage of time 0 control in absence of EGF for 4 experiments. The EGF effect was not significant ($P > 0.1$) as determined by one-way ANOVA by Minitab.
Figure 24: EGF dose response curve for ATP CL activity in hepatocytes from 10, 12 and 14 week old rats. Panel A reflects means of 4 experiments from 10 week old rats. Panels B and C reflect means of 3 experiments from 12 and 14 week old rats, respectively. Details are same as for Figure 23. There were no statistically significant effects of EGF concentration or age (P > 0.1) as determined by two-way ANOVA by Minitab.
that the 14 week old rats were less responsive to EGF addition to hepatocytes than were younger rats. However, when the data for all dose response curves from all four age groups combined (n=14) were analyzed by one-way ANOVA (Minitab) there was a significant effect of EGF concentration at P=.05, this was probably due to an increase in number of samples which helped compensate for the high variability among the values. The composite figure which combines all the experiments regardless of age is given in Figure 25.

Time response curves were plotted for 10^{-9} M EGF incubated from 0 to 40 minutes. This concentration of EGF was chosen because it was above the half maximal effect and below the maximal effect. This should have allowed better discrimination of potential EGF time effects than would incubation with maximal EGF. The shapes of the curves were complex. The EGF treated hepatocytes of 8, 10 and 12 week animals exhibited an initial spike in ATP CL activity at 5 minutes which subsequently dropped off, though remained high in comparison with 0 control (Figures 26-29). The spike in ATP CL activity at 5 minutes did not occur in hepatocytes from 14 week old rats. The peak in control activity was at the 10 minute time period and declined thereafter in all but the 14 week animals which had widely variable activity. The EGF effect on ATP CL activity over time was statistically
Figure 25: EGF dose response curve for ATP CL activity in hepatocytes from rats from combined age groups (8, 10, 12 and 14 weeks). Details are as described in Figure 23, except n=14. There was a statistically significant effect of EGF concentration (P > 0.05) as determined by one-way ANOVA by Minitab.
Figure 26: EGF time response curve of ATP CL activity in hepatocytes from 8 week old rats. Hepatocytes were treated with 10⁻⁵ M EGF (---) or control without EGF (---) and incubated for 0, 5, 10, 20 or 40 min. Values ± SEM are the percentage of ATP CL activity in time 0 control in absence of EGF for 4 experiments. There were statistically significant effect of EGF concentration (P > 0.06), time (P > 0.05) and age (P > 0.01) as determined by three-way ANOVA, repeated measures, by SAS.
Figure 27: EGF time response curve of ATP CL activity in hepatocytes from 10 week old rats. Details and statistics are same as in Figure 26.
Figure 28: EGF time response curve of ATP CL activity in hepatocytes from 12 week old rats. Details and statistics are same as in Figure 26 except n=3.
Figure 29: EGF time response curve of ATP CL activity in hepatocytes from 14 week old rats. Details and statistics are same as in Figure 26 except n=3.
evaluated in conjunction with age effect. Three-way ANOVA, repeated measures (SAS) showed statistically significant effects of EGF treatment (P=0.06), time (P=0.05) and there was a strong age effect (P=0.003). The effect of EGF on ATP CL activity was exerted most dramatically in the 8 week hepatocytes. The EGF effect was blunted in the 10 and 12 week animals and essentially absent by 14 weeks. The half maximal effect based on curve fitting was unchanged from 8 to 14 weeks.

An additional experiment was conducted using 8 week old rats to investigate the ability of EGF to alter ATP CL activity under conditions of varying hepatocyte cell densities. EGF dose response curves were generated for hepatocytes at low cell density (10^5 cells/ml) and high cell density (10^7 cells/ml); an intermediate cell density (10^6 cells/ml) was used in the experiments previously discussed. Low density hepatocytes appeared to retain the capacity to respond to EGF. The ATP CL activity per mg protein was very high in low density cells (Figure 30) and the 1/2 Vmax was approximately equal to 10^{-12} - 10^{-11} M EGF which is in agreement with the values for hepatocytes at intermediate density. However, when the data was statistically analyzed by two-way ANOVA, repeated measures (SAS) for effects of EGF concentration and cell density, only the cell density effect
was significant (at $P > 0.01$) with $P=0.12$ for the EGF concentration effect. On the other hand, the activity per mg in high density cells was considerably lower and was unresponsive to changes in EGF concentrations (Figure 30).

The rate of lipogenesis gradually declines as animals approach adulthood (130). Lipids are synthesized rapidly during periods of growth and development as phospholipids and cholesterol are absolutely required for biogenesis of new cell membranes. It is natural to suppose that the lipogenic enzyme activities decrease with age as well. The activity of ATP CL was lower in hepatocytes from 14 week animals than from 8, 10 and 12 week old ones. The capacity of the hepatocytes to respond to EGF by increasing activity of ATP CL also decreased with age.

EGF, a 6.4 KD peptide prevalent in submaxillary glands, shares many properties with insulin in its receptor binding action (128). The liver possesses an abundance of EGF receptors (130). EGF rapidly and reversibly binds its receptor with a high affinity and binding is specific and saturable (128). Another growth factor, TGF-α, also has a high, competitive affinity for the EGF receptor (186). TGF-α is thought to play a fundamental role in embryogenesis and its actions are considered to be mediated through the EGF receptor since no specific receptor for TGF-α has been isolated. The levels of systemic EGF increase during development and subsequently more EGF is stored in the
Figure 30: Effect of cell density on EGF dose response on ATP CL activity in hepatocytes from 8 week old rats. Hepatocytes from 8 week old rats were adjusted to high cell density ($10^7$ cells/ml) ( — ) or low cell density ($10^5$ cells/ml) ( — ) and treated with 0 or $10^{-9}$M EGF. Values ± SEM are a percentage of ATP CL activity in time 0 controls in absence of EGF for 3 experiments. There was a statistically significant effect of cell density ($P > 0.01$), but not dose ($P > 0.1$) as determined by two-way ANOVA, repeated measures, by SAS.
submaxillary gland (130). Levels of EGF in the blood of adult rats is approximately $10^{-7}$ M (143) which is in the range of the level of EGF just shown to affect ATP CL activity in the liver. The ramifications of TGF-α and EGF in postnatal development are not well understood.

Both low affinity and high affinity EGF receptors exist in hepatocyte cultures (115). The EGF receptor population may change with the age of the animal, or there could be interference with EGF receptor binding by onset of production of a factor which inhibits receptor signalling. TGF-β would be a likely candidate since it has fundamental inhibitory properties. The addition of TGF-β to hepatocyte cultures abolishes the mitogenic effect of EGF through receptor transmodulation such that TGF-β reduces the affinity of the receptor for EGF and interferes with proper functioning (138). The high affinity receptors disappear during prolonged culturing of hepatocytes after addition of EGF (119). It has been theorized that the mitogenic response which EGF elicits in cultured hepatocytes is promoted by EGF binding to low affinity receptors (141). This is substantiated by the low number of high affinity receptors which exist in hepatocytes derived from hepatectomized rats which are extremely sensitive to EGF. The effect of EGF on ATP citrate lyase in short term incubations of isolated hepatocytes may be facilitated by
the high affinity EGF receptors which seem to be responsible for transmitting acute, rapid-acting messages (128, 187).

Many growth factor receptors have an inherent TYR kinase activity which is activated upon ligand binding to its specific receptor, such as insulin, PDGF, IGF-I and EGF (133). The TYR kinase is activated rapidly and phosphorylates its intracellular substrates within minutes. Holland and Hardie (146) showed that insulin and EGF activated TYR kinase activity indirectly and lead to phosphorylation of SER residues on ATP CL and acetyl CoA carboxylase as well as ribosomal S-6 protein. The stimulation of fatty acid synthesis by EGF (145) and insulin previously observed was correlated with increasing phosphorylation of the two lipogenic enzymes (146). The activity of purified acetyl CoA carboxylase was not altered by the phosphorylation resulting from EGF action (146). However, it was not considered that purification procedures may have rendered the enzyme active prior to addition of the EGF.

The phosphorylation of ATP CL which could be generated by activation of protein kinase by EGF may explain the mechanism of rapid activation of ATP CL activity in these experiments. ATP CL activity rose dramatically with in 5 minutes of EGF addition in 8 week rat hepatocytes. The rapid activation of a metabolic parameter followed by a transient decline, then a gradual increase is typical of
effects produced when high and low affinity receptors are present simultaneously. The role of phosphorylation in regulation of ATP CL activity will, of course, need to be resolved in order to determine whether EGF affects the enzyme by this means.

Several other growth factors have been shown to stimulate certain aspects of lipogenesis as was discussed previously. ATP CL was transiently activated by TGF-β then gradually declined below control levels over 60 minute incubation of isolated hepatocytes (158). Supplementation of TGF-β at 30 minutes further decreased the activity of ATP CL ruling out the possibility that TGF-β had been destroyed (158). DNA synthesis is inhibited by TGF-β (137). Conversely, EGF stimulates hepatocyte proliferation in absence of serum (143). The optimal conditions for propagating cultured hepatocytes involves addition of nanomolar concentrations of EGF to low density concentration of hepatocytes (143,121). Introduction of EGF to hepatocytes from hepatectomized rats dramatically increased rate of DNA synthesis (143). Apparently, EGF plays an intricate role in the regulation of hepatocyte growth and probably in the acute control of hepatocyte metabolism since EGF rapidly raised ATP CL activity and phosphorylation of two lipogenic enzymes (146).

The mechanism of EGF action has not been established, but the TYR kinase seems to play a pivotal role. The most
likely mechanism for the rapid modification of ATP CL activity would be through a change in phosphorylation state or site of phosphorylation. The action of EGF could occur through a cascade of events initiated by TYR kinase activation of EGF receptor which could phosphorylate protein kinase C or another SER/THR kinase which would then phosphorylate specific enzymes thus altering activity. Alternatively, the TYR protein kinase could activate a protease upon its TYR phosphorylation which could target the enzymes allowing them to be more susceptible to another protein kinase or phosphatase, or the TYR kinase could activate some uncharacterized cascade which ultimately results in some other covalent modification of the enzymes. Although several groups have not been able to correlate change in structural or regulatory phosphorylation sites with enzyme activity, it seems likely that it is of some significance. Perhaps the assay conditions or isolation procedures are not representative of optimal activation in vivo.
CHAPTER V
CONCLUSIONS

The research presented in this dissertation examined the regulation of the lipogenic enzyme, ATP CL, under various conditions which are conducive to alterations in growth control. A coordinate set of biochemical and metabolic changes facilitate cell proliferation. Cell division probably involves not only DNA replication, but membrane biogenesis as well. It has been proposed that de novo synthesis of membrane lipids may be a prerequisite for subsequent DNA synthesis. The main lipid constituents of cell membranes are cholesterol and phospholipids. The stimulation of de novo lipid synthesis would be an early event in the proliferative process.

A few studies have demonstrated the putative significance of lipids is cell proliferation. Inhibition of HMG CoA reductase, which catalyzes the regulatory reaction in cholesterol synthesis, prevents mitogenic stimulation of DNA synthesis (109). A rise in the activity of HMG CoA reductase occurs in concert with cell proliferation (108). The precursor for lipogenesis is acetyl CoA and ATP CL provides acetyl CoA for the synthesis
of cholesterol and fatty acids in the cytosol. It may be necessary for ATP CL to be activated early in proliferation to supply the substrate for subsequent de novo lipogenesis which may be required for cell division to continue.

Growth and metabolism are apparently altered under changes in physiological and nutritional conditions. Prolonged fasting deprives animals of an adequate source of energy and lowers circulating insulin levels. Growth and repair are also compromised in the fasting state. Hepatic lipogenesis, as well as the activity of the enzymes involved, is dramatically reduced by fasting. There is also a decline in lipogenesis with increasing cell density in vitro. ATP CL activity is retarded in hepatocytes incubated at high cell density, while conditions of low cell density stimulate enzyme activity (3). Diabetes is characterized by chronic insulin deficiency and several aspects of growth control are deregulated with diabetes such that poor wound healing and development of retinopathy are critical side effects.

In cell culture, the exogenous addition of growth factors produce a mitogenic response. Several metabolic parameters are altered by these growth factor mitogens. DNA, mRNA and protein synthesis, and glycolysis are often simulated by the action of growth factors. Cultures of hepatocytes can only proliferate in the presence of EGF, under optimal conditions of low cell density.
The regulation of ATP CL was examined under various conditions which alter growth both in vivo and in vitro. The activity of ATP CL was measured in rat hepatocytes isolated from the primary organ regulating lipid metabolism, liver, under various physiological or nutritional states. It was previously found that ATP CL activity is sensitive to regulation by autocrine/paracrine factors produced by hepatocytes (3, 111). Hepatocytes from fed rats produced an activator(s) of ATP CL activity under low cell density conditions and an inhibitor(s) at high cell density (3). In the study presented here hepatocytes which were incubated at both high and low cell density, from fasted rats produced autocrine/paracrine factors which inhibited ATP CL activity. Hepatocytes from fasted rats seemed to be incapable of producing the activator(s) as do fed hepatocytes incubated at low cell density (3).

Subcellular translocation of lipogenic enzymes is considered a putative mechanism of regulation of enzyme activity (13,92). The distribution of ATP CL antibody-reactive bands produced from SDS PAGE of subcellular fractions from liver homogenates from fed, fasted and refed rats, differed with the nutritional state of the animal. ATP CL from liver homogenates seemed to undergo extensive proteolysis, however, many of the degradative products must retain at least partial activity. The extent of proteolysis also varied among nutritional states.
Diabetes is characterized by aberrant production and/or response to insulin. Physiological regulation of many biological functions is compromised in diabetes. A trend was found in diabetes which altered not only the ability of hepatocyte to respond to autocrine/paracrine factors, but also to produce them. ATP CL activity in hepatocytes from diabetic rats was activated and inhibited in a different manner than the activity in hepatocytes from control rats.

EGF is mitogenic for hepatocytes in culture and is absolutely required to support proliferation. Hepatic ATP CL activity was susceptible to regulation by EGF. Hepatocytes were responsive to EGF at nanomolar concentrations which approximates circulating levels of EGF. There was a trend toward a dose response to EGF concentration and a rapid activation of ATP CL activity by EGF was established with activity remaining relatively high over time. Younger animals appeared to have greater sensitivity or greater capacity to respond to EGF than did older animals.

The results reported here provide a starting point for further evaluation of the role of ATP CL in proliferation and growth. Since ATP CL is apparently subject to regulation by a growth factor and autocrine/paracrine factors, there may be implications for ATP CL activity in relevance to growth control. The autocrine/paracrine factors must be isolated and characterized to determine
which peptides are responsible for the inhibition or activation of ATP CL activity. The effect of these factors on other lipogenic and glycolytic enzymes, and metabolites could also be examined. The rate of lipogenesis in response to the autocrine/paracrine factors and other growth factors should be determined and correlated to activation of lipogenic enzymes. The putative mechanisms for regulation, such as SER/THR and TYR phosphorylation and subcellular translocation in response to the factors could also be further considered. It could even be extended to evaluate the effects of interaction of autocrine/paracrine factors and known growth factors on lipogenic events. The ramifications of the role of lipogenesis in proliferation and cell growth provide exciting possibilities for future research and may have profound significance to researchers on many levels.
Appendix A: Liver Perfusion Buffers.

HEPES without Calcium

Stock HEPES

83.0 g NaCl  
5.0 g KCl  
24.0 g HEPES  
60 ml freshly made in NaOH (4.0 g/100 ml)

Dilute to ~400 ml; pH to 7.5; bring to 400 ml.

Freeze in 10 ml aliquots. Dilute 20 ml stock + 5 ml 1 M glucose to 500 ml deionized water.

Gas with 100% oxygen before and during use.

HEPES with Calcium

Stock HEPES

4.0 g NaCl  
0.5 g KCl  
24.0 g HEPES  
0.7 g CaCl₂  
66 ml freshly made in NaOH (4.0 g/100 ml)

Bring to ~100 ml; pH to 7.5; bring to 100 ml.

Freeze in 5 ml aliquots. Dilute 10 ml + 1 ml 1 M glucose to 100 ml with deionized water.

Add 30 mg Sigma Type II collagenase.

Gas with 100% oxygen before and during use.
Krebs Ringer Bicarbonate Buffer

Premix:

100 parts 0.9% NaCl  
4 parts KCl (1.15 g/100 ml)  
1 part MgSO$_4$ (3.82 g/100 ml)  
1 part KH$_2$PO$_4$ (2.11 g/100 ml)

On day of use:

108 ml premix  
3 ml CaCl$_2$·2H$_2$O (1.61 g/100 ml)  
21 ml NaHCO$_3$ (1.3 g/100 ml)

Gas with 95% oxygen/5% carbon dioxide for 5 min.

Add 183.0 mg bacitracin
Appendix B: Protein Determination.

Bradford Method

100.0 mg comassie brilliant blue G-250

Dissolve in 50 ml 95% ethanol, 100 ml 85% (w/v) phosphoric acid. Dilute to 1 L with deionized water.

Filter prepared solution at least 24 hr prior to use.
Appendix C: Subcellular Fraction Isolation Buffer.

Greenawalt's Buffer

- 70 mM sucrose
- 220 mM D-mannitol
- 2 ml HEPES
- 3.6 mg/ml EtOH TPCK
- 3.6 mg/ml EtOH TLCK
- 1 ml/L B-mercaptoethanol

Refrigerate.

Add 0.5 mg/ml BSA on day of use.

Adjust to pH 7.4 with KOH on day of use.
Appendix D: Enzyme Assay Mixtures.

ATP CL

In order of addition for each sample:

0.3 ml (0.6 M) Tris pH 8.4/(30 mM) B-mercaptoethanol
0.1 ml (10 mM) MgCl₂
0.4 ml deionized water
10 mM ATP
20 mM citrate
3 units MDH

0.1 ml (0.2 mM) CoA
0.1 ml (9.4 mM) NADH

Read on spectrophotometer at 340 nm.

LDH

For each sample:

0.9 ml (110 mM) pyruvate and (100 mM) phosphate
0.1 ml (9.4 mM) NADH

Read on spectrophotometer at 340 nm.

MAO

For each sample:

1.0 ml (200 um) KH₂PO₄, pH 7.2 and 10 um benzylation

Read on spectrophotometer at 250 nm.
Appendix E: SDS PAGE Preparation.

Loading Buffer

To 1.0 ml sample, add:

- 180 ul (20%) SDS (3% SDS)
- 24 ul (100%) B-mercaptoethanol (2% B-mercaptoethanol)
- 75 ul Bromphenol blue dye
- Several grains of sucrose

Boil for 4 min.

Running Buffer

- 144.0 g/L glycine
- 30.0 g/L Tris
- 5.0 g/L BME

Dilute 1:5 with deionized water on day of use.

Stacking gel

- 2 ml 30% acrylamide/0.8% Bis
- 2 ml 1.5 M Tris, ph 6.8
- 15.5 ml deionized water

Degas.

- 0.1 ml 20% SDS
- 0.01 ml TEMED
- 0.1 ml 13% ammonium persulfate
7.5% gel

6.7 ml 30% acrylamide/0.8% Bis
3.3 ml 30% acrylamide
10 ml 1.5 M Tris Cl, pH 8.7
19.7 ml deionized water

Degas.

0.1 ml 13% ammonium persulfate
0.2 ml 20% SDS
0.02 ml TEMED


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175. Voss, A. (1984) Ph.D. Dissertation. The Ohio State University, Columbus, OH.


