REGULATION OF POLYGLYCEROPHOSPHOLIPID BIOSYNTHESIS IN THE RAT HEART

by
Philip Cheng

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REGULATION OF POLYGLYCEROPHOSPHOLIPID BIOSYNTHESIS IN THE RAT HEART

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DEDICATION

To Miranda:

her love, support and encouragement during times of stress and deadlines through this and other projects allowed me to persevere.

And to

Mom and Dad:

for the many sacrifices they have made in order for me to achieve what I have.

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I would like to thank my supervisor and "mentor" Dr. Grant M. Hatch who instilled in me a love for science and who taught me, by his example, to always put people first. I remain forever indebted.

I am grateful to my supervisory committee, Dr. Archibald McNicol, Dr. Gilbert Arthur and most all Dr. Patrick C. Choy, who has pointed me to the right direction and who taught me what it means to be a decent human being.

Lastly, I want to thank Dr. Shu Guang Cao for his enthusiasm, support and many late night discussions which I have benefited from them immensely.

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ABBREVIATIONS

ATP	Adenosine-5'-triphosphate
AMP	Adenosine-5'-monophosphate
CDP	Cytidine-5'-diphosphate
°C	Degrees Celsius
Ci	Curie
CO_2	Carbon dioxide
$CoCl_2 \cdot H_2O$	Cobalt chloride
cpm	Counts per minute
CTP	Cytidine-5'-triphosphate
DDW	Double-distilled water
dpm	Disintegrations per minute
EDTA	Ethylenediaminetetraacetic acid
g	Gram
h	Hour
HCl	Hydrochloric acid
HPLC	High pressure liquid chromatography
KH_2PO_4	Potassium phosphate monbasic
M	Molarity of solution
$MgCl_2$	Magnesium chloride anhydrous
$MgCl_2 \cdot 6H_2O$	Magnesium chloride
min	Minute
mL	Milliliter
L	Liter
N	Normality of solution

N_2	Nitrogen
NH_4OH	Ammonium hydroxide
nm	Nanometer
nmole	Nanomole
O_2	Oxygen
p	Statistical probability
pO_2	Partial pressure of oxygen
pH	Powers of hydrogen
P_i	Phosphate
pmol	Picomole
PP_i	Inorganic phosphate
SD	Standard Deviation
TBA	Tetrabutylammonium
Tris	Trizma base
µ	Micro
µg	Microgram
vol	volume

ABSTRACT

Polyglycerophospholipids are a special class of phosphoglycerides which include phosphatidylglycerol, cardiolipin, bis(monoacylglycero)phosphate and bisphosphatidic acid. In this class of phosphoglycerides, two or more glycerol moieties are present joined by phosphodiester linkage with two, three or four fatty acyl chains esterified to the glycerol moieties. Cardiolipin is a major phospholipid component of the heart comprising approximately 15% of the entire phospholipid mass of this organ. Cardiolipin was the first polyglycerophospholipid ever discovered and was isolated from bovine heart by Mary Pangborn in 1942. In the heart, cardiolipin is synthesized exclusively in the inner membrane of the mitochondria via the CDP-diacylglycerol pathway. Cardiolipin is essential for membrane integrity in the mitochondria as well as maintaining the activity of several enzymes including cytochrome *c* oxidase, an intrinsic protein in the electron transport complex. Thus, cardiolipin is important for mitochondrial energy metabolism.

The effect of reduction of high energy nucleotide level on the biosynthesis of cardiolipin was unknown. Hence, the effect of hypoxia on cardiolipin and phosphatidylglycerol (the immediate precursor to cardiolipin) biosynthesis in isolated rat hearts perfused in the Langendorff mode was investigated. Hearts were pulsed-labeled for 60 min with 0.1 mM [1,(3)-^{3}H]glycerol in Krebs Henseleit buffer under control or hypoxic conditions. Radioactivity incorporated into phosphatidylglycerol and cardiolipin were reduced 88% ($P<.05$) and 79% ($P<.05$), respectively, in hypoxic hearts compared to controls. In other experiments, hearts were pulse-labeled for 15 min with 1.4 mM [^{32}P]P_i in Krebs Henseleit buffer saturated with 95% oxygen and 5% carbon dioxide and subsequently perfused for 60 min under control or hypoxic conditions. The radioactivity incorporated into CDP-diacylglycerol, phosphatidylglycerol, and cardiolipin were reduced

61% ($P<.05$), 71% ($P<.05$) and 70% ($P<.05$), respectively, in the hypoxic hearts compared to controls indicating a decreased formation of CDP-diacylglycerol in the hypoxic heart. The activities of the enzymes involved in cardiolipin biosynthesis were unaltered indicating no compensatory response of the enzymes in the CDP-diacylglycerol pathway. Furthermore the cardiac pool sizes of cardiolipin, phosphatidylglycerol, and CDP-diacylglycerol between hypoxic and control hearts were unchanged. However, cardiac ATP and CTP levels were decreased 94% ($P<.05$) and 92% ($P<.05$), respectively, in hypoxic hearts compared to controls. It is postulated that the biosynthesis of the cardiac polyglycerophospholipid cardiolipin may be inhibited by a decreased ATP and CTP level in the heart.

The reacylation of lysophospholipids back to their parent molecules is important for attaining the appropriate fatty acyl composition in many phospholipids and for preventing the accumulation of arrhythmia generating lysophospholipids in the heart. Acyl-coenzyme A:1-acylglycerophosphorylglycerol acyltransferase, the enzyme that reacylates lysophosphatidylglycerol to phosphatidylglycerol, was not characterized in the heart. In this study, the presence of acyltransferase activity for reacylation of lysophosphatidylglycerol to phosphatidylglycerol was examined in rat heart subcellular fractions. The specific activity of acyl-Coenzyme A:1-acylglycerophosphorylglycerol acyltransferase in rat heart subcellular fractions was in the order of microsomal>mitochondrial>cytosol. However, the mitochondrial and cytosolic activities were likely due to contamination with microsomal particles. The activity in the microsomal fraction was found to have a pH optimum in the alkaline range. However, significant enzyme activity was observed at physiological pH. With oleoyl-Coenzyme A as substrate, the enzyme had a preference for the acyl moiety in lysophosphatidylglycerol substrates in the order of myristoyl>palmitoyl>oleoyl>stearoyl. High concentrations of

lysophosphatidylglycerol (0.1 mM or greater) were inhibitory. The apparent K_m's for 1-palmitoylglycerophosphorylglycerol and oleoyl-Coenzyme A were 9.4 and 7.1 μM, respectively. The enzyme was heat labile as pre-incubation at 55°C for 1 min abolished 65% of the activity. The results demonstrate the presence of an active *in vitro* microsomal acyl-Coenzyme A:1-acylglycerophosphorylglycerol acyltransferase activity in the rat heart which is capable of catalyzing lysophosphatidylglycerol acylation to phosphatidylglycerol.

INTRODUCTION

I. Structure and Assembly of Biological Membranes:

The biological membrane is a ubiquitous and universal structure in all living organisms. The surface of bacteria, plants, and animals is covered by a membrane that allow substances to enter and exit the cell in a selective and controlled manner. This membrane encapsulates the cytoplasm and is responsible for forming the numerous intracellular organelles in which essential functions are carried out. These intracellular organelles include the nucleus, mitochondria, endoplasmic reticulum, lysosome, Golgi apparatus, chloroplasts and vacuoles. The membrane provides compartmentation for the cell which differentiates the eukaryotes from the prokaryotes (lacking intracellular compartments) (Prescott *et al.* 1990). In addition, the membrane serves as a barrier to the movement of molecules and ions between the various compartments within the cell. The membrane surrounding these organelles also ensures that only certain types of chemical reactions occur in that particular organelle. This is achieved by orienting membrane bound enzymes in a manner that allows for maximum effective interaction with their substrates. An example of this is cytochrome *c* oxidase associated with oxidative phosphorylation in the mitochondria (Robinson 1993).

The biological membrane contains a variety of lipids. Technically the word lipid (Gk. *Lipos*, fat) is a collective term for three very important classes of biomolecules: phospholipids, glycolipids, and cholesterol. Phospholipids are the most abundant of all lipids. They are essential biomolecules important for all living cells. They function as structural components of cell membranes as well as a selective barrier as mentioned above.

This extraordinary capability of phospholipids is achieved by the presence of both a hydrophilic ("water-loving", or polar) head group and a hydrophobic ("water-hating", or nonpolar) tail region. Thus, phospholipids are amphipathic. When phospholipid molecules are surrounded by an aqueous medium, they tend to aggregate so that the tails are buried and the polar heads are exposed (Alberts *et al.* 1994). It is the combination of hydrophobic interaction, and Van der Waals attractive forces that stabilizes and mediate the molecular interactions in the biological membrane (Stryer 1988). Moreover, the amphipathic nature of the phospholipids which allow the spontaneous formation of the bilayer membrane structure to form the effective barrier for all living cells is illustrated by the "Fluid Mosaic Model" (Figure 1).

II. Introduction to Glycerophospholipids.

By definition, phospholipids are lipids that contain a phosphate. They are usually referred to generically as PHOSPHOLIPIDS, but strictly speaking the term phospholipids encompass a rather broad range of molecules (Figure 2). There are three main types of phospholipids.

1. Sphingomyelin

The first type are the sphingolipids which are derived from a sphingosine backbone rather than a glycerol backbone. Sphingosine is a complex C_{18} unbranched alcohol with a *trans* double bond and an amino group. Within the sphingolipids, there are three major families: sphingomyelin, cerebrosides, and gangliosides. However, only sphingomyelin is considered a phospholipid since it contains a phosphate group (Figure 3). Sphingomyelin

consists of a phosphocholine head group attached to an acylated sphingosine (i.e. ceramide). Sphingomyelin is one of the major phospholipids of mammalian cells. The highest concentration of sphingomyelin is in myelin sheaths that surround nerve cells (Stryer 1988).

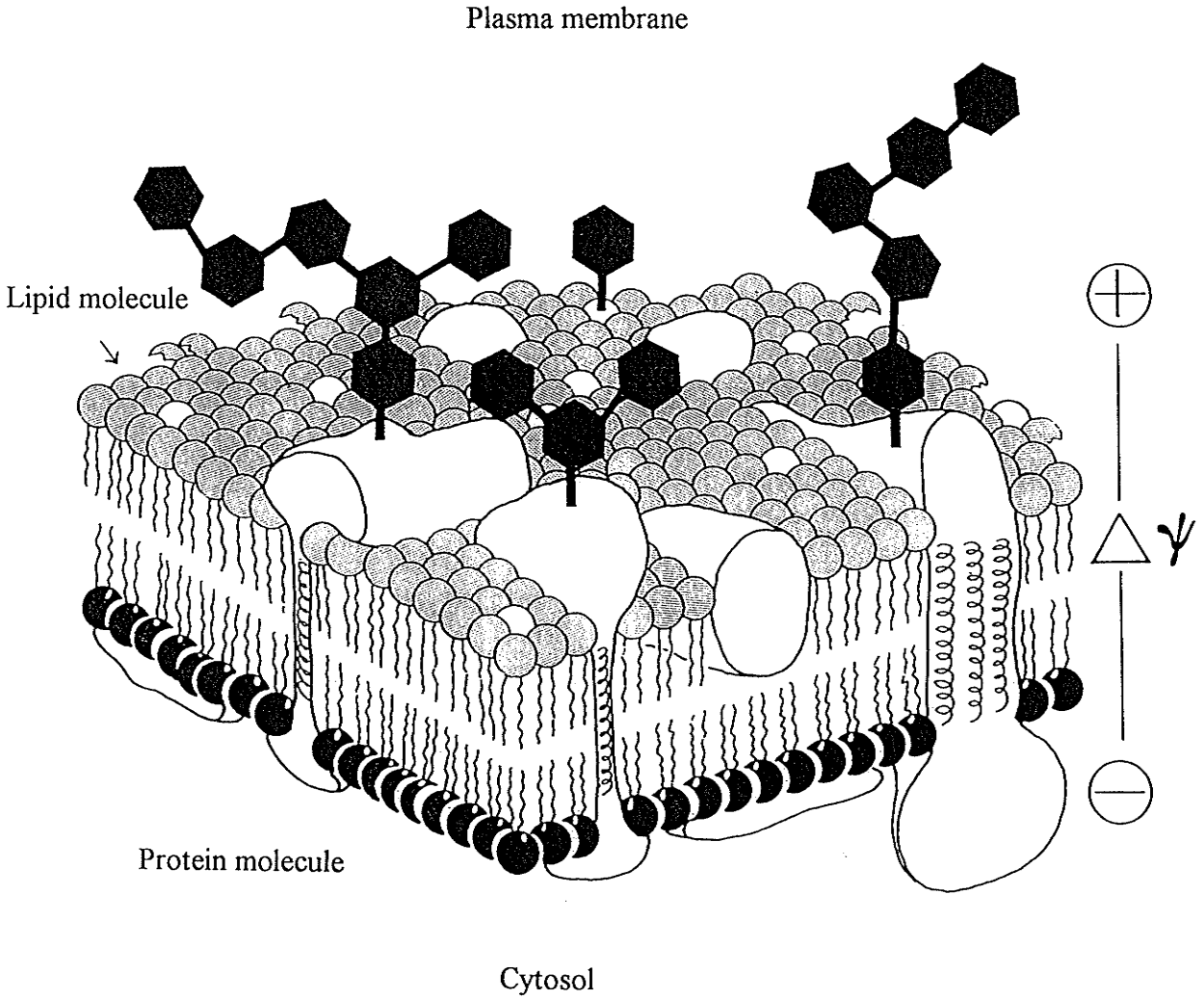


Figure 1: Fluid mosaic model of the eukaryotic plasma membrane.

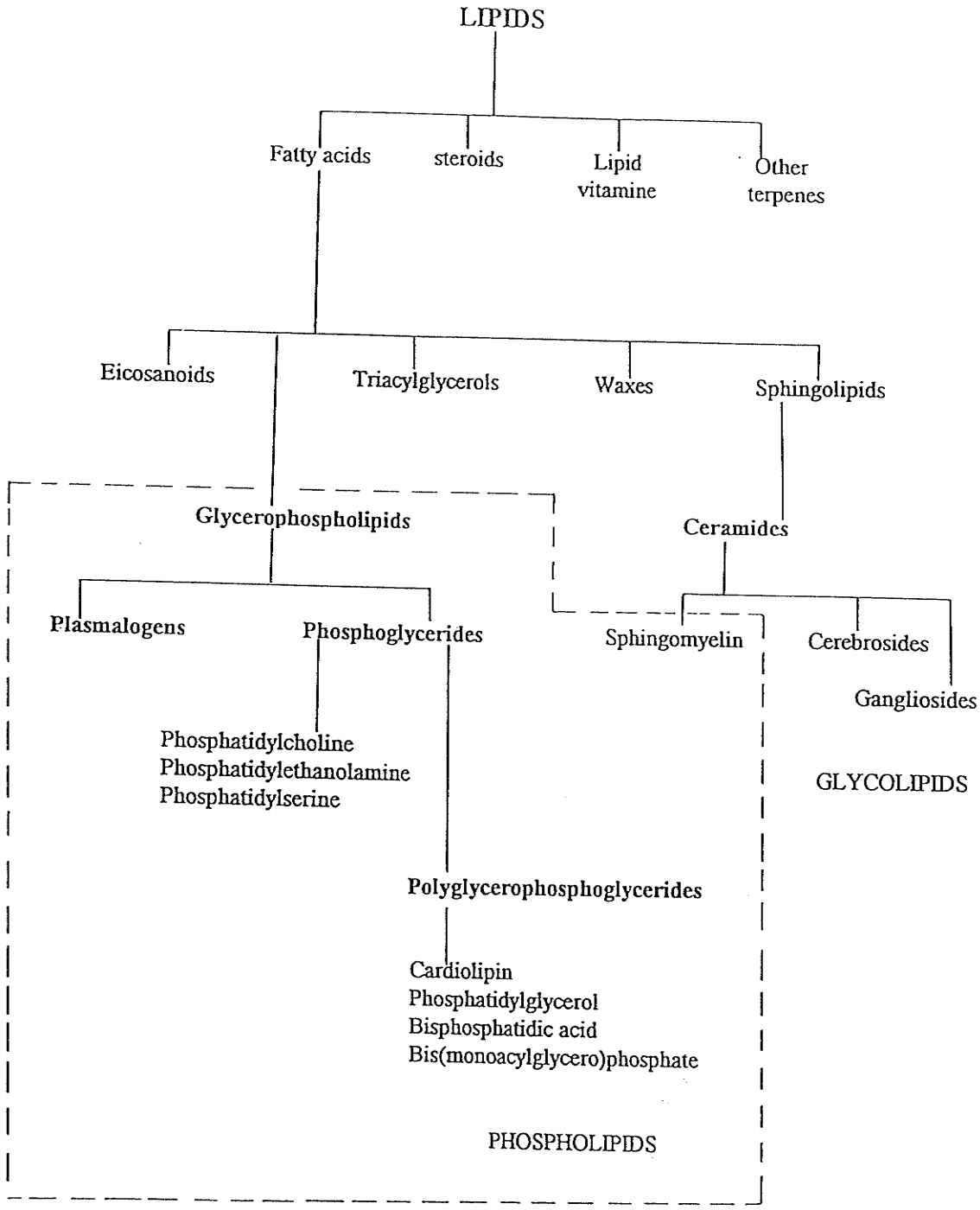


Figure 2: The various classes of lipids.

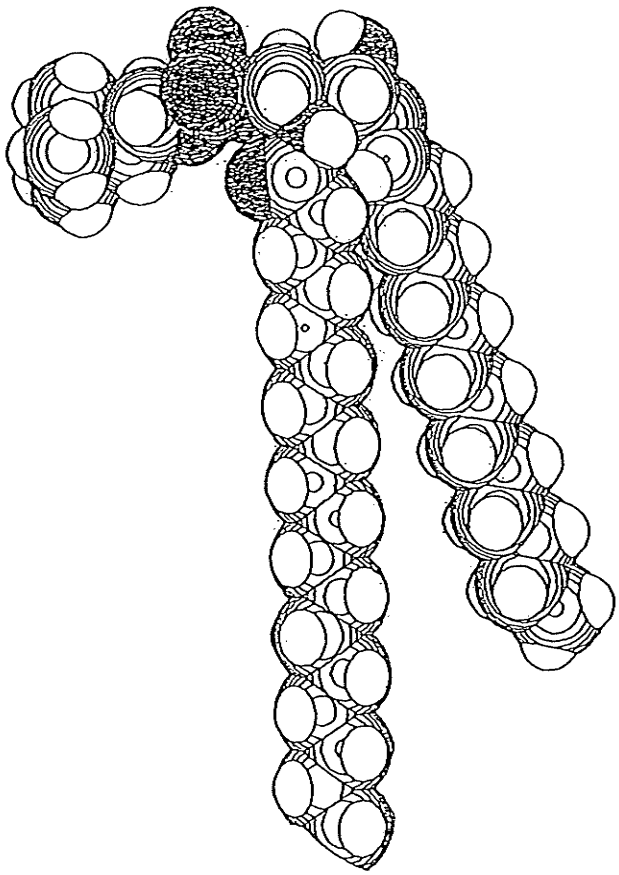


Figure 3: The structure of sphingomyelin.

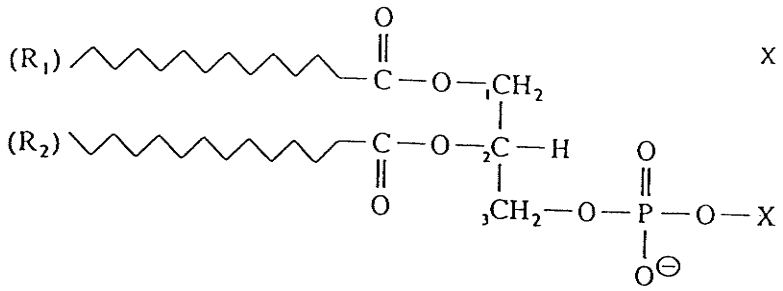
2. Phosphoglycerides

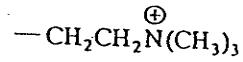
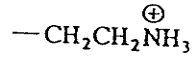
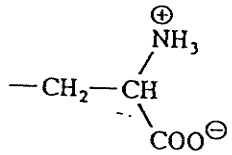
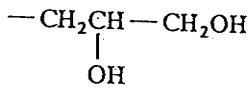
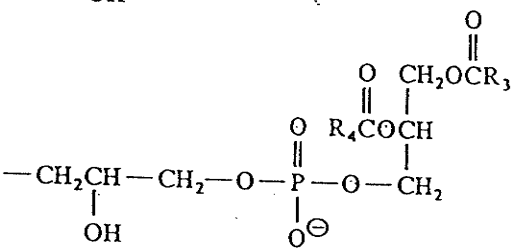
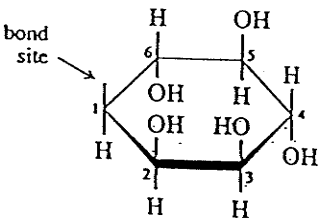
Phosphoglycerides comprise the second, and major, type of phospholipid. They consist of one or two fatty acyl chains esterified to a glycerol backbone at the C_1, C_2 or both positions of the carbon. An alcohol head group is linked to the inorganic phosphate occupying the C_3 position (Table 1). Phosphoglycerides are classified according to the various head groups esterified to the phosphate. In the majority of phosphoglycerides, the fatty acyl chain esterified to the C_1 position of the glycerol backbone is usually saturated. While the fatty acyl chain occupying the C_2 position often has one or more *cis*-double bonds i.e. unsaturated (Figure 4). Each *cis*-double bond creates a small kink in the fatty acyl tail of the phospholipid. This influences the ability of phospholipid molecule interactions with one another, thus affecting the fluidity of the biological membrane (Alberts *et al.* 1994). In the absence of an alcohol head group attached to the phosphate group, the phosphoglyceride is phosphatidic acid. Phosphatidic acid is only present in trace amounts in animal tissues. It is a key intermediate compound for the biosynthesis of the major phosphoglycerides as well as the polyglycerophosphoglycerides (Stryer 1988).

3. Plasmalogens

Not all phospholipids have their fatty acyl chain linked to the glycerol by ester bond as described above. Plasmalogens, the final class of phospholipid, differ from the phosphoglycerides in that they contain an unsaturated ether esterified to the C_1 position of the glycerol backbone (Gurr and James 1971). They are more prevalent in animal tissue, such as heart and brain (Hadly 1985). The more common forms are plasmenylcholine and plasmenylethanolamine (Stryer 1988).

Table 1: Classification of major phosphoglycerides.



Precursor of X (HO — X)	Formula of X	Name of resulting glycerophospholipid family
Water		Phosphatidate
Choline		Phosphatidylcholine (Lecithin)
Ethanolamine		Phosphatidylethanolamine (Cephalin)
Serine		Phosphatidylserine
Glycerol		Phosphatidylglycerol
Phosphatidyl-glycerol		Diphosphatidylglycerol (Cardiolipin)
myo-Inositol		Phosphatidylinositol

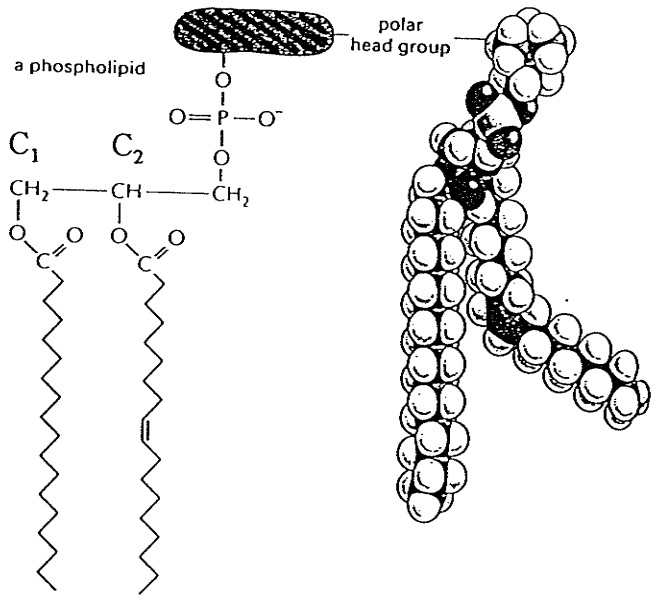


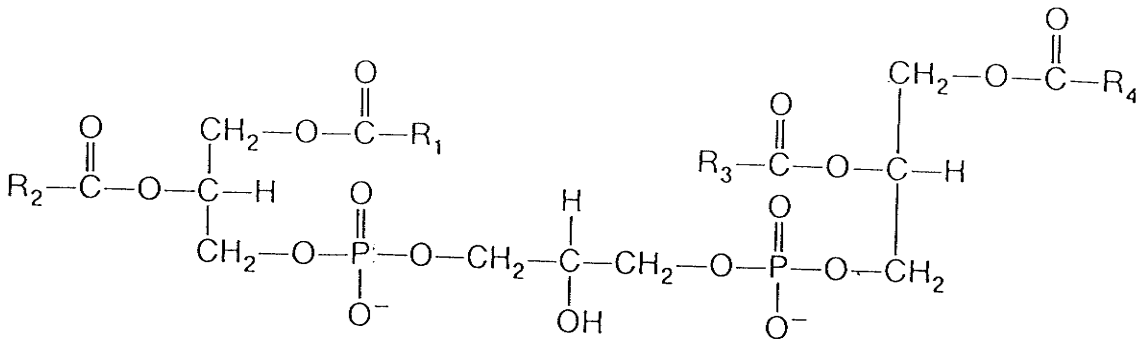
Figure 4: Structure of a phosphatide with C_1 saturated and C_2 unsaturated fatty acid chains.

III. Introduction to Polyglycerophospholipids.

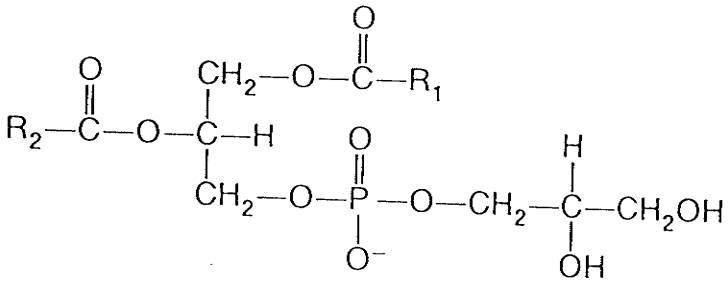
Polyglycerophospholipids are phosphoglycerides that have more than one glycerol backbone and consist of two or more glycerol moieties that are joined by phosphodiester linkage with two, three, or four long chain fatty acyl groups esterified to the C_1 and C_2 positions of the hydroxyl group on the glycerol moieties (Hostetler 1982). Polyglycerophosphoglycerides are an important class of phosphoglycerides that include cardiolipin, phosphatidylglycerol, bis(monoacylglycero)phosphate (Hostetler 1982; Vance 1985), and bisphosphatidic acid (van Blitterswijk and Hilkmann 1993). Polyglycerophospholipids are widely distributed in nature and are found in plants, microorganisms, and animals (Hostetler 1982). The structural formula of the four major polyglycerophosphoglycerides are shown in Figure 5.

1. Bis(monoacylglycero)phosphate

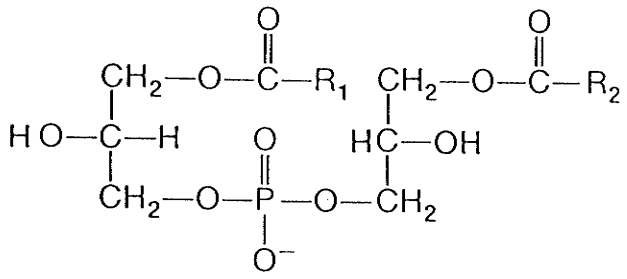
Bis(monoacylglycero)phosphate, also known as lysobisphosphatidic acid, was first isolated from pig lung (Body and Gray 1967). Bis(monoacylglycero)phosphate is found only in trace amounts in most mammalian tissues. However the most abundant source of bis(monoacylglycero)phosphate is in the alveolar macrophage in which it constitutes 14-18% of the total lipid phosphorus (Huterer and Wherret 1979). The functional role of bis(monoacylglycero)phosphate remains unclear. However, it has been speculated that it may serve to stabilize the lysosomal phospholipid bilayer against the potentially lytic endogenous phospholipases since this polyglycerophospholipid was shown to be quite resistant to degradation by lysosomal phospholipases (Hostetler 1982).



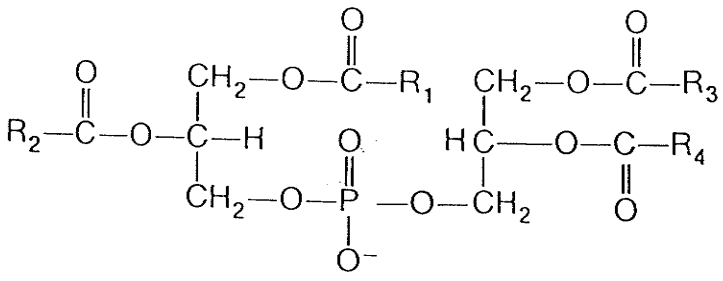
Cardiolipin



Phosphatidylglycerol



Bis(monoacylglycero)phosphate



Bisphosphatidic acid

Figure 5: The structural formula of four polyglycerophospholipids.

2. Bisphosphatidic acid

This uncommon polyglycerophospholipid was first described in the 1970's as a minor membrane component in BHK cells and bacteria (Brotherus and Renkonen 1974; McAllister and De Siervo 1975). However, its function was unknown at that time. The novel feature of bisphosphatidic acid in cell signalling was only recently described (van Blitterswijk and Hilkmann 1993). Bisphosphatidic acid was produced from a condensation reaction of diacylglycerol and phosphatidylglycerol by phospholiplase D-mediated transphosphatidylation. This serendipitous discovery has identified for the first time a physiological role for phospholipase D (van Blitterswijk and Hilkmann 1993).

3. Phosphatidylglycerol

Phosphatidylglycerol was first discovered and isolated from alga, *Scendesmus* (Maruo and Benson 1957). In 1962 Haverkate and coworkers finally confirmed the structure of phosphatidylglycerol from *Bacillus cereus* by testing its susceptibility to phospholipases (Haverkate *et al.* 1962). In the rat heart phosphatidylglycerol was shown to comprise approximately 1-1.5% of the total phospholipid phosphorus mass (Poorthuis *et al.* 1976; Hatch 1994). Although phosphatidylglycerol represents only a minor component of tissue phospholipid, the localization of phosphatidylglycerol and the enzymes which catalyze its biosynthesis are ubiquitous. The largest concentration of phosphatidylglycerol is located in the lung and alveolar type II cells which represent 2.2-5.5% and 4.2-10.4% of the total phospholipid, respectively (Poorthuis *et al.* 1976; Manson and Williams 1980). In the 1960's Kennedy and coworkers elucidated the biosynthesis of phosphatidylglycerol in membrane preparations from chicken liver (Kiyasu

et al. 1963). The *de novo* biosynthesis of phosphatidylglycerol is depicted in Figure 6. Phosphatidylglycerol phosphate is formed from the condensation reaction between glycerol-3-phosphate and CDP-diacylglycerol. This reaction is catalyzed by phosphatidylglycerolphosphate synthase (E.C. 2.7.8.5). Phosphatidylglycerolphosphate is then rapidly dephosphorylated by phosphatidylglycerolphosphate phosphatase (E.C. 3.1.3.27) to produce phosphatidylglycerol.

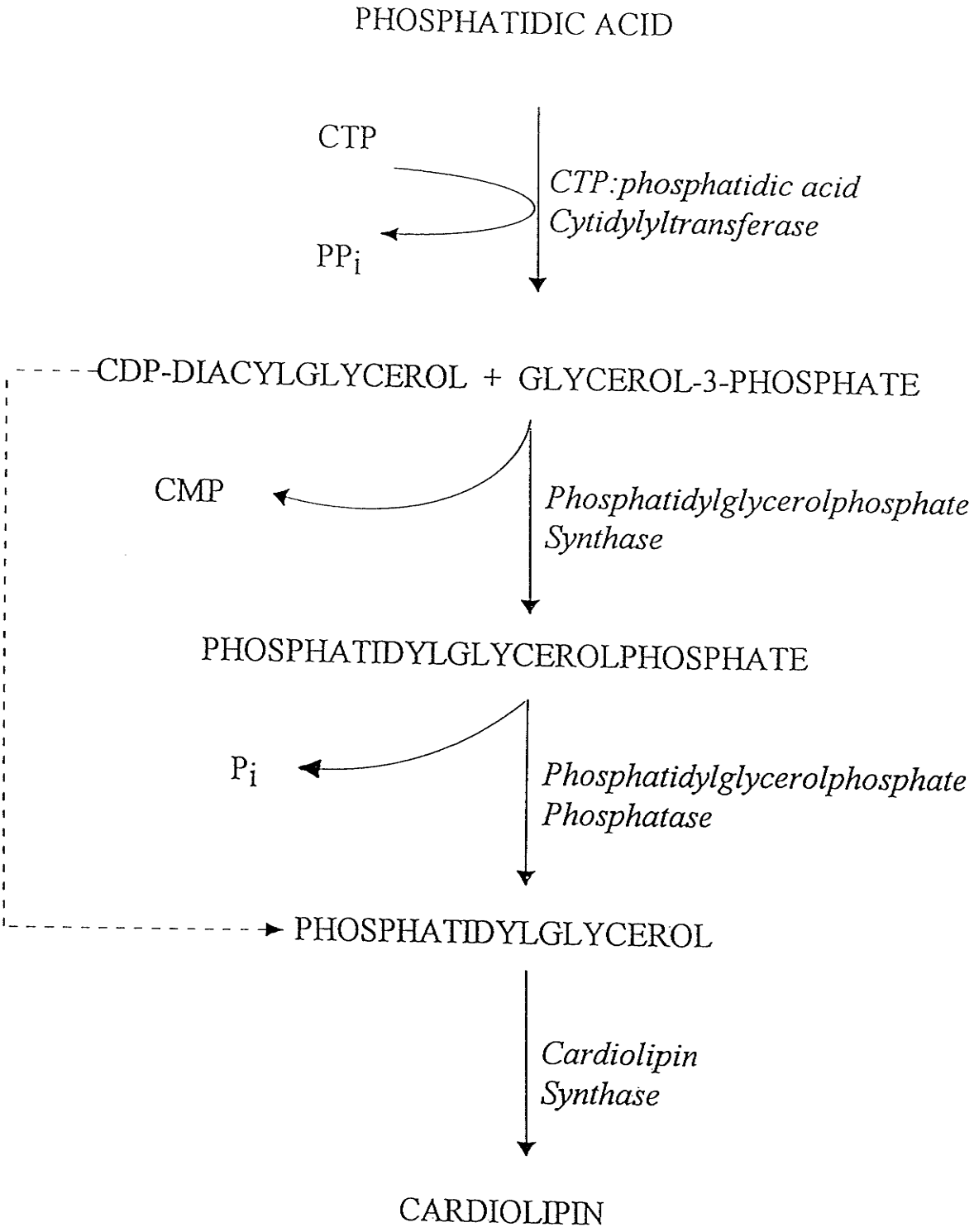


Figure 6: CDP-diacylglycerol pathway for *de novo* cardiolipin biosynthesis.

4. Cardiolipin

Cardiolipin the first polyglycerophospholipid discovered, was isolated from bovine heart by Mary Pangborn in 1942 (Pangborn 1942). Cardiolipin is the principle polyglycerophospholipid in the heart muscle ranging from 9.0% in humans to 14.7% in rats of the total lipid phosphorus mass (Hostetler 1982; Hatch 1994; Poorthuis *et al.* 1976). In eukaryotic systems cardiolipin is synthesized exclusively within the inner leaflet of the inner mitochondrial membrane (Krebs *et al.* 1979) where it comprises about 20% of the phospholipid (Stoffel and Schiefer 1968). The structure of cardiolipin is unique in that it contains three glycerol moieties, two phosphate groups and four unsaturated fatty acyl chains. Generally 77 to 84% of the fatty acid moieties in cardiolipin are linoleic (18:2(n-6)) acid (Rose 1964; Gray 1964). The relative amount of linoleic acid in cardiolipin was shown to increase in skeletal muscle during the postnatal period (~ 40%) and reaches maximal values (~ 90%) during the first two months of life (Bruce 1974). The fatty acyl composition of cardiolipin is thought to be important for its function in the mitochondria. The acyl species are quite symmetrical and are almost exclusively C_{18} chains in both the heart and liver (Schlame *et al.* 1993). However, diets can lead to the incorporation of C_{20} polyunsaturated groups into mammalian cardiolipin without altering the physiological function of cardiolipin to any great extent (Schlame *et al.* 1993; Wolff and Entressangles 1991). In fact, when rats were fed a partially hydrogenated marine oil diet rich in 16:1, 18:1, 20:1 and 22:1 isomeric fatty acids for 10 weeks, they exhibited an increase in 18:2 cardiolipin molecular species in comparison to rats fed a palm oil diet (Hoy and Holmer 1990).

a) Physical and Functional Roles of Cardiolipin in Biological Membranes

It is now generally accepted that biological membranes are structured from a lipid bilayer with proteins intercalated into this continuum as well as bound to the bilayer surface (Singer and Nicolson 1972). The function of intrinsic membrane proteins is clearly affected by the phospholipid in contact with them hence when the fatty acid composition of membrane is altered so that the properties of the bilayer are changed the enzyme activities associated with the membrane are also altered (Overath *et al.* 1975). Thus, there may be specific phospholipid requirements for certain membrane activities. There is much evidence to support the function of intrinsic membrane proteins affected by the lipids in contact with them (Vik *et al.* 1981). For example, hydroxybutyrate dehydrogenase requires phosphatidylcholine for maximal activity (Gazzotti *et al.* 1975). It has long been known that cardiolipin aids in the association and function of several key mitochondrial membrane enzymes (Bell and Coleman 1980). The first of such enzymes is the mitochondrial isoenzyme, creatine kinase. This enzyme is located on the outside of the inner membrane of the mitochondria (Scholte *et al.* 1973). Its association with cardiolipin was demonstrated when adriamycin inhibited the re-binding of the solubilized enzyme to the inner membrane (Newman *et al.* 1981), since adriamycin has been shown to inhibit other cardiolipin-dependent inner-membrane enzymes (Goormaghtigh *et al.* 1986). The mode of action of this drug is to form a specific and tight (Kd = 1 μM) complex with cardiolipin in a molar ratio of 2:1. The consequence of this effect is the impairment of the functioning of the cardiolipin-dependent enzymes (Rietveld *et al.* 1987). Furthermore, the mitochondrial creatine kinase has recently been purified to homogeneity from rat hearts. One of the fragments from the purified enzyme has been identified as the cardiolipin-

binding domain when subjected to cyanogen bromide degradation (Cheneval and Carafoli 1988).

Another inner mitochondrial membrane enzyme enhanced by cardiolipin is cytochrome *c* oxidase (Robinson 1993). This is the terminal oxidase of the respiratory chain. Upon the removal of one or more molecules of cardiolipin, the enzyme activity was lost. However, the activity was reinstated by the addition of exogenous cardiolipin (Vik *et al.* 1981). The phosphate carrier from pig heart mitochondria has revealed a specific and absolute requirement for cardiolipin (Mende *et al.* 1983). Finally, cardiolipin is essential for the proper functioning of the enzymes in the electron transport chain (Ohtsuka *et al.* 1993). Hence cardiolipin is a multi-functional phospholipid essential for cellular oxidative phosphorylation (Cheng and Hatch 1995).

b) Biosynthesis of Cardiolipin

Mammalian mitochondria contain enzymes for the *de novo* synthesis of cardiolipin (Schlame and Halder 1993). A minor pathway for cardiolipin biosynthesis is via the action of a phospholipase D on phosphatidylglycerol (Stanacev and Stukne-Sekalec 1970) and the significance of this pathway is unknown. However, in the rat heart, the majority of cardiolipin is synthesized via the CDP-diacylglycerol pathway (Figure 6). The first step of the reaction is the conversion of phosphatidic acid to CDP-diacylglycerol which requires CTP as co-factor and is catalyzed by the enzyme CTP:phosphatidic acid cytidylyltransferase (E.C. 2.7.7.41) (Poorthuis *et al.* 1976). This is the rate-limiting step of cardiolipin biosynthesis in the heart (Hatch 1994). The subsequent steps involve the condensation of glycerol-3-phosphate with CDP-diacylglycerol to form

phosphatidylglycerolphosphate via the action of phosphatidylglycerolphosphate synthase (E.C. 2.7.8.5) and phosphatidylglycerolphosphate phosphatase (E.C. 3.1.3.27) which rapidly dephosphorylates phosphatidylglycerolphosphate to phosphatidylglycerol as described earlier (Poorthuis *et al.* 1976). Finally phosphatidylglycerol is converted to cardiolipin by a condensation reaction with CDP-diacylglycerol catalyzed by cardiolipin synthase (Hostetler *et al.* 1971). This enzyme has been purified to homogeneity and characterized from rat liver mitochondria (Schlame and Hostetler 1991). The purified cardiolipin synthase requires cobalt as well as the reconstitution by exogenous phospholipids up to 0.1 mM for optimum activity. However, this enzyme is strongly inhibited by its product, cardiolipin (McMurray and Jarvis 1980). Cardiolipin synthase has a molecular weight of 50 Kd as revealed by SDS gel electrophoresis. The pH optimum of this enzyme was in the alkaline range of pH 8-9. The K_m's for its substrates, phosphatidylglycerol and CDP-diacylglycerol, were 45 and 16 μM, respectively (Schlame and Hostetler 1991).

c) Regulation of cardiolipin biosynthesis

Mitochondrial CDP-diacylglycerol hydrolase is a potential regulator of cardiolipin synthesis (Nicolson and McMurray 1984). The physiological role of this degradative enzyme of CDP-diacylglycerol is unknown (Rock and Cronan 1985). However, the enzyme activity of CDP-diacylglycerol hydrolase is several-fold higher than phosphatidylglycerolphosphate synthase (catalyzes CDP-diacylglycerol to phosphatidylglycerolphosphate). Thus, CDP-diacylglycerol hydrolase has the propensity to compete for the small pools of CDP-diacylglycerol. Interestingly, the divalent cations that stimulate cardiolipin synthase are also inhibitory to the hydrolase (Nicolson and

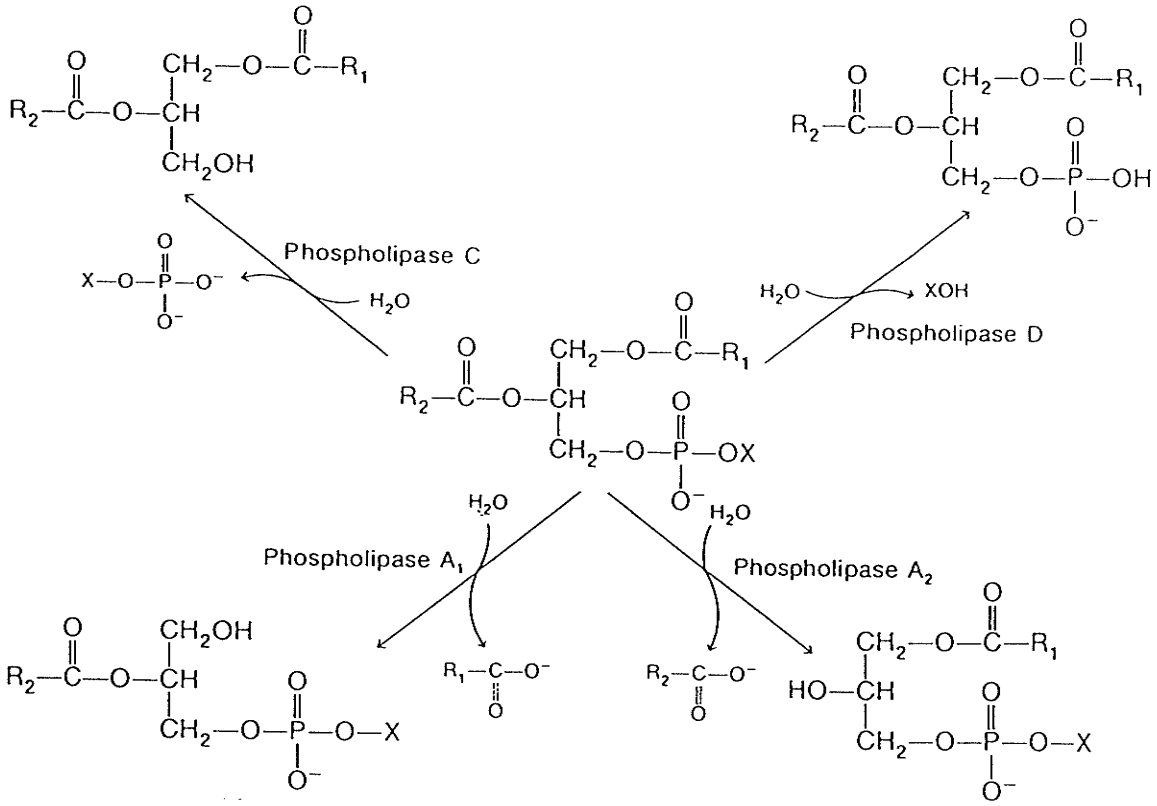
McMurray 1984). Thus, *in vivo* low concentration of cobalt may be rate-limiting for cardiolipin biosynthesis.

The effect of thyroid hormones on the alteration of phospholipid composition of the mitochondrial membrane is well documented (Hoch *et al.* 1981). In rat liver mitochondria, thyroid hormones receptors are thought to be involved in the regulation of mitochondrial oxidative phosphorylation (Sterling *et al.* 1978). It was not surprising to observe the increased levels of cardiolipin content in the exogenous thyroid hormone-treated rat heart and liver (Paradies and Ruggiero 1988; 1990), since the principle subcellular location of cardiolipin in mammalian tissues is the mitochondria (Hostetler 1982). The increased level of cardiolipin was attributed to the increased cardiolipin synthase activity (Hostetler 1991; Cao *et al.* 1995) in the thyroid hormone treated rat. These studies suggest that levels of cardiolipin appear to be regulated to some extent by the effect of thyroid hormone on the activity of cardiolipin synthase.

Little information is available on the catabolism of cardiolipin. It is most likely hydrolyzed by phospholipases C and perhaps to some degree by phospholipases D. Moreover, cardiolipin is also subjected to degradation by phospholipases A, since it has the *sn*-glycero-3-phosphate configuration (Hostetler 1982). In mammalian liver, cardiolipin turnover was found to be a much less rapid than that of other phospholipids (McMurray and Dawson 1969).

IV. Remodeling of phospholipids.

It is well recognized that membrane phospholipids exist in a dynamic flux in which continuous biosynthesis is balanced by degradation. Depending on the cell type, there is a fine enzymatic balance between the catabolic and metabolic enzymes. In naturally occurring phosphoglycerides, the fatty acyl composition may represent a different percentage of the total fatty acid in different glycerolipids, even when these lipids are isolated from the same tissue (Lands and Hart 1964). Phospholipids are well known to undergo a rapid deacylation-reacylation process involving phospholipases and acyltransferases (Irvine 1982). In 1960 William Lands was first to describe the deacylation-reacylation cycle for the modification of fatty acid in phosphatidylcholine (Lands 1960). He proposed that the mammalian tissue contained enzymes that were capable of hydrolyzing as well as reacylating fatty acid on the glycerol backbone of most phospholipids. This may provide a suitable method for modifying preformed phospholipid molecules and adapting their fatty acid composition to the requirement of a particular tissue. Furthermore, this cycle is known to play an important role in the regulation of membrane-bound enzyme activities and in signal transduction (Berridge and Irvine 1984). In principle any ester linkage in phosphoglycerides is susceptible to enzymatic hydrolysis. It is now clear that different enzymes exist for removing the fatty acids from either C_1 or C_2 position of the glycerol backbone. The action of phospholipase A_1 (E.C. 3.1.1.32) hydrolyzes the ester linkage at the C_1 position and phospholipase A_2 (E.C. 3.1.1.4) cleavage at the C_2 position forming 1-acyl lysophospholipid and 2-acyl lysophospholipid, respectively (van den Bosch *et al.* 1965) (Figure 7).



2-acyl lysophospholipid

1-acyl lysophospholipid

Figure 7: Reactions catalyzed by phospholipases.

In the case of cardiolipin biosynthesis, remodelling of the final product is very important since the acyl pattern is not matched by its precursors (Rüstow *et al.* 1989). The newly synthesized cardiolipin was first rapidly deacylated by phospholipase A_2 and subsequently reacylated with the appropriate acyl residue. In addition the pre-existing cardiolipin was relatively resistant to this reaction (Schlame and Rüstow 1990).

Deacylation and reacylation are also extremely important to phosphatidylcholine. Once phosphatidylcholine is made, its fatty acid substituents can be modified by a deacylation and reacylation cycle as was described originally by Lands (Lands 1960). This cycle is responsible for the introduction of polyunsaturated fatty acid into phosphatidylcholine (Vance 1985). Since the liver contains phosphatidylethanolamine species which are not synthesized by the normal cytidine pathway, it was postulated that deacylation and reacylation were involved in the formation of the more highly unsaturated molecular species of phosphatidylethanolamine. *In vivo* experiments showed that 95% of the linoleic acid in 1-stearoyl-2-linoleoyl phosphatidylethanolamine entered the molecule by means of deacylation and reacylation (Åkesson 1970).

1. Deacylation of phospholipids

It has been suggested that deacylation of phosphatidic acid to lysophosphatidic acid is an obligatory event in the synthesis of triacylglcerols (Tzur and Shapiro 1976). It could also operate to prevent the accumulation of phosphatidic acid in membranes (Sturton and Brindley 1980) before it profoundly alters the membrane properties. Experiments with exogenous phospholipase A have shown that generally in mammalian tissues, the saturated fatty acids are esterified of the C_1 position the glycerol backbone

while unsaturated fatty acids are esterified at the C_2 position of phosphoglycerides (Lands 1960).

The action of phospholipase A_2 results in the formation of 1-acyl lysophospholipids (Figure 7). This is a class of phosphoglycerides in which one of the fatty acyl chains is missing at the C_1 position on the glycerol backbone. If the fatty acyl chain at the C_1 position is hydrolyzed by phospholipase A_1, the fatty acyl chain at C_2 may spontaneously migrate back to the C_1 position (Zubay 1988). Lysophospholipids account for only approximately 1-2 % of the total phospholipids in mammalian cells (Zubay 1988) and are tightly controlled by lipid metabolizing enzymes (Weltzien 1979). Although lysophospholipids possess amphipathic property similar to phosphoglycerides, they can only form micelles. The reason being that their polar heads are too large in relation to their single hydrocarbon tails to allow for the lipid bilayer formation (Horton *et al.* 1993). The scarcity of this class of phospholipid is due to their detergent characteristic at concentrations above the critical micellar concentration. At those concentrations lysophospholipids form micelles rather than existing as monomers. Thus, they have the ability to disrupt cellular membranes by intercalation into the membrane matrix and solubilizing the component lipids and proteins (Zubay 1988). In addition, the wedge-shape structure of lysophospholipids when incorporated into the biological membrane might alter the curvature of the membrane, thereby possibly modifying its physical properties and physiological function (Corp *et al.* 1981; Lucy 1970). Lysophospholipids rapidly accumulate within 8 to 10 min after the onset of myocardial ischemia. Thus, there is close correlation between the increased levels of lysophospholipids and the electrophysiological alterations during ischemia (Van der Vusse *et al.* 1992). In search for the critical concentration of free lysophospholipids effects on the heart, media containing

1.2 mM of exogenous lysophospholipids in the presence of albumin induced appreciable electrophysiological alterations in cardiac tissues (Corp *et al.* 1981). Whereas significantly lower concentrations of free lysophospholipids (10-50 μM) elicited profound electrophysiological disturbances (Arnsdorf and Sawicki 1981). Thus, accumulation of lysophospholipids are cytolytic and lysophospholipids have been implicated to promote cardiac arrhythmia's (Kinnard *et al.* 1988).

Recently, phospholipase A_2 has been shown to be secreted from liver during perfusion under physiological and pathophysiological conditions and phosphatidylglycerol is the preferred substrate for this secretory phospholipase A_2 (Hatch *et al.* 1993). Therefore, it is conceivable that lysophosphatidylglycerol accumulation may occur due to the hydrolytic action of phospholipase A_2 on extracellular phosphatidylglycerol.

2. Reacylation of lysophospholipids in the heart

Reacylation of phosphatidylcholine in the heart has been extensively studied by Choy and coworkers (Arthur and Choy 1984). The reacylation of lysophospholipids by acyltransferases provides a number of essential functions in the cell. It is part of the mechanism for the remodelling of the fatty acyl chains to maintain the asymmetry of cellular phospholipids and it also may be crucial in preventing the accumulation of these cytolytic compounds as described previously. The subcellular localization of the acyltransferases has been the subject of considerable debate. Most of the enzyme activity has been shown to exist in the microsomal fraction in most tissues, its presence in other subcellular fractions has only been recently suggested (Arthur *et al.* 1987a). Although the presence of acyl-CoA:1 alkenyl-glycero-3-phosphocholine acyltransferase and acyl-CoA:1

acyl-glycero-3-phosphocholine acyltransferase were assayed in the microsomal fractions of the guinea-pig heart, their activities were demonstrated in the mitochondrial fraction as well by utilizing different acyl donors, since it was postulated that these enzymes are highly acyl-specific (Arthur *et al.* 1987a).

a) Fatty acyl chain donor

The key ingredient for lysophospholipid reacylation is the universal acyl group carrier, acyl-coenzyme A. Coenzyme A was discovered and characterized in 1945 by Fritz Lipmann, who observed that a heat stable cofactor was required in many enzyme catalyzed acetylations (Lipmann 1945). The " A " in the name stands for acetylation. This molecule consists of a β-mercaptoethylamine unit which has a high reactive terminal sulfhydryl group, a pantothenate unit, adenine and ribose-3'-phosphate (Stryer 1988). This carrier mediates the interchange of activated groups (i.e. fatty acyl chains) in a wide variety of biochemical reactions. It is the sulfhydryl group of the β-mercaptoethylamine moiety of Coenzyme A that is directly involved in the enzymatic reactions. Biosynthesis of Coenzyme A occurs in the mammalian liver where pantothenic acid serves as the precursor. Pantothenic acid is found in natural products and must be supplied in the diet. The reaction involves several key enzymes to form the end product Coenzyme A (Zubay 1988).

Before the acyl-Coenzyme A can be used as a fatty acyl chain donor, fatty acids must be "activated". The fatty acids taken into cells are activated in the cytoplasm by reaction with coenzyme A and ATP to yield fatty acyl-Coenzyme A, PP_i and AMP in a reaction catalyzed by acyl-Coenzyme A synthases (Gurr and James 1971; Zubay 1988).

Three different kinds of synthases are known, they vary according to the chain length of the fatty acids that they activate (Wakil 1970). Once the fatty acyl donor has been formed, it can then be used in reacylation. As illustrated in Figure 8, the reacylation of a lysophospholipid begins with the nucleophilic attack on the acyl-Coenzyme A molecule by a lysophospholipid giving rise to a tetrahedral intermediate. The reacylated phospholipid is formed followed by the expulsion of the Coenzyme A molecule which will be recycled (Zubay 1988).

b) Reacylation of lysophosphatidylglycerol

Lysophosphatidylglycerol formed from the action of phospholipase A_2 can be reacylated back to the parental phosphatidylglycerol by the action of acyl-Coenzyme A:1-acylglycerophosphorylglycerol acyltransferase. Benjamin Wittels first described this reaction using rat liver microsomes in the early 1970's (Wittels 1973). *Lyso* compounds are probably formed as intermediates and these may be reacylated to the diacyl phosphoglycerides by transfer of fatty acid from acyl-Coenzyme A, thereby accounting for the fatty acid "turnover" of phosphoglycerides in nature. However, it is not clear at this point whether acyltransferases plays a role in the regulation of the lysophospholipid levels in tissues.

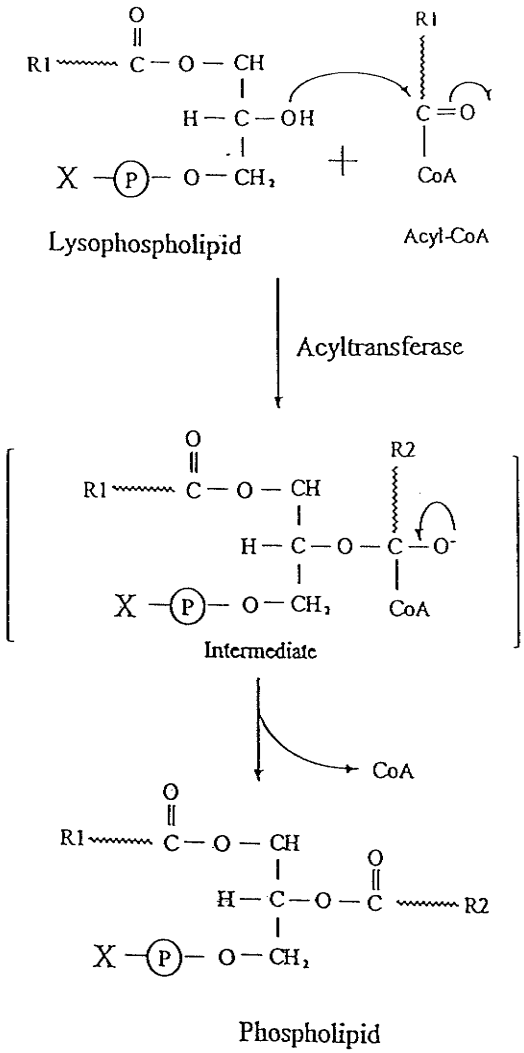


Figure 8: Basic mechanism of lysophospholipid reacylation.

RESEARCH AIMS

Phosphatidylglycerol and cardiolipin are the principle polyglycerophospho-glycerides in the mammalian heart (Hostetler 1982). The regulatory mechanisms which control phosphatidylglycerol and cardiolipin biosynthesis are largely unknown. At present the regulatory mechanisms that govern cardiolipin biosynthesis in the heart have received little attention. The myocardium derives most of its energy from mitochondrial oxidative phosphorylation under normal physiological conditions (Taegtmeyer 1985). When available oxygen is limited, as in the case of myocardial hypoxia, myocardial creatine phosphate levels fall precipitously followed by a gradual decline of the high-energy phosphate stores (Braunwald and Sobel 1988; Feinstein 1962). ATP is required for the amination reaction in CTP production, consequently the loss of ATP would reduce the production of CTP (Stryer 1988). CTP is a required co-factor in the CDP-diacylglycerol pathway, however the effect of a reduction in cardiac high energy stores on cardiolipin biosynthesis in mammalian heart was unknown.

Phospholipase A activity directed towards phosphatidylglycerol in the subcellular fractions of the rat heart was recently demonstrated (Hatch *et al.* 1995; Cao and Hatch 1995). This activity could potentially hydrolyze existing pools of phosphatidylglycerol, thus causing an intracellular accumulation of lysophosphatidylglycerol. Therefore it is conceivable that the heart possesses the ability to reacylate lysophosphatidylglycerol back to the more stable and parental phosphatidylglycerol. The objective of this thesis was to investigate cardiac phosphatidylglycerol and cardiolipin biosynthesis under hypoxic conditions and to examine the ability of the heart to reacylate lysophosphatidylglycerol to phosphatidylglycerol.

1. The regulation of phosphatidylglycerol and cardiolipin biosynthesis in the hypoxic rat heart

The effect of reduced cardiac energy status on phosphatidylchloine biosynthesis was demonstrated in the isolated perfused hamster heart (Hatch and Choy 1990). To examine if cardiolipin biosynthesis was regulated by the cardiac energy status, the isolated rat heart was perfused in the Langendorff mode under both control and hypoxic conditions with radioactive glycerol or P_i. Cardiac perfusion under hypoxic conditions was employed as a model to reduce the high energy stores in the rat heart. This is a highly reproducible and stable method useful in metabolic labelling studies (William and Kobayashi 1984). Furthermore, the isolated rat heart perfusion under hypoxic conditions is an established model for the overall oxygen delivery to below the critical level required to support the metabolic ATP demands of the tissue (de Leiris *et al.* 1984).

2. The reacylation of lysophosphatidylglycerol to phosphatidylglycerol

Lysophospholipids perturb the cellular membrane integrity and are cytolytic at high concentrations (Zuby 1988). It is therefore important for cells to undergo reacylation of lysophospholipids to prevent their accumulation. Furthermore reacylation of lysophospholipids provides a functional mechanism to remodel the fatty acid composition to the requirement of a particular tissue (Gurr and James 1971). Thus, acyl Coenzyme A: 1-acylglycerophosphorylglycerol acyltransferase for the acylation of lysophosphatidylglycerol was characterized in the rat heart. The goal of this study was to investigate the presence of acyl Coenzyme A: 1-acylglycerophosphorylglycerol acyltransferase activity in the rat heart. The fully characterized acyl Coenzyme A: 1-

acylglycerophosphorylglycerol acyltransferase will aid in our understanding of its functional role in the metabolism of phosphatidylglycerol in the mammalian heart.

METHODS AND MATERIALS

I. Materials

1. Experimental Animals

Male Sprague Dawley rats (100-220 g, body weight) were used throughout the study. Rats were maintained on Purina rat Chow, and tap water *ad libitum*, in a light- and temperature controlled room. Treatment of animals conformed to the Guidelines of the Canadian Council on Animal Care.

2. Chemicals

[1,(3)-^{3}H]Glycerol, [^{32}P]P_i, and [U-^{14}C]glycerol-3-phosphate were obtained from Amersham, Oakville, Ontario, Canada. [1-^{14}C]oleoyl-Coenzyme A was obtained from Dupont, Mississauga, Ontario. Ecolite^{TM} scintillation cocktail and thin layer plates (silica gel 60, 0.25 mm thickness) were obtained from CanLab Division of Baxter Co., Winnipeg, Manitoba, Canada. Sucrose, ATP, CTP, phosphatidic acid, lysophosphatidic acid, CDP-diacylglycerol, phosphatidylglycerol, cardiolipin, bovine serum albumin, and Trizma base were purchased from Sigma Chemical Co., St. Louis, MO. Malachite green base was generously donated by Dr. G. Arthur. Oleoyl-CoA, CDP-diacylglycerol were obtained from Serdary Research Laboratories, Englewood Cliffs, New Jersey. Lysophospholipids were products of Avanti Polar Lipids, Alabaster, Alabama. Beckman Ultrasphere-ODS C_{18} reverse-phase high performance liquid chromatography column was purchased from Beckman Instruments, Mississauga, Ontario, Canada. All other chemicals and solvents were of analytical grade, and obtained from either Fisher Scientific, Edmonton, Alberta, Canada; Sigma Chemical Co., St. Louis, MO, U.S.A.; or CanLab Division of Baxter Co., Mississauga, Ontario, Canada.

II. Methods

A. Effect of Hypoxia on Cardiolipin Biosynthesis.

1. Perfusion of isolated rat hearts in the Langendorff mode

Male Sprague Dawley rats were sacrificed by decapitation. The heart was quickly removed and cannulated via the aorta using a modified syringe needle (18 gauge). The remaining blood in the coronary circulation was removed by injecting the heart with 5 mL of Krebs Henseleit buffer (Krebs and Henseleit 1932) using a 5 cc syringe. Buffer was prepared fresh by combining 100 mL of solution A (contained 70.1 g/L sodium chloride, 21.0 g/L sodium bicarbonate, and 9.91 g/L dextrose), 10 mL of solution B (contained 3.55 g/100 mL potassium chloride, 2.94 g/100 mL magnesium sulfate, and 1.63 g/100 mL sodium phosphate, monobasic), 5 mL of solution C (contained 3.37 g/100 mL calcium chloride, dihydrate), and doubled-distilled water (DDW) was added to a final volume of 1 L in a volumetric flask. Immediately after, the heart was placed on the perfusion apparatus (Figure 9) and perfused in the Langendorff mode (Langendorff 1895) for 10 min to allow for stabilization. After which, 12.5 mL of Krebs Henseleit buffer containing either radioactive or non-radioactive compounds at various timed intervals was perfused through the heart. All perfusions were performed at 37°C with a flow rate of 2.5 mL/min. The viability of the heart under these conditions could be maintained for up to 4 hr of perfusion (Arthur and Choy 1984). Subsequent to perfusion, 10 mL of air was injected into the heart to remove the residual radioactive or non-radioactive perfusate that remained in the coronary circulation.

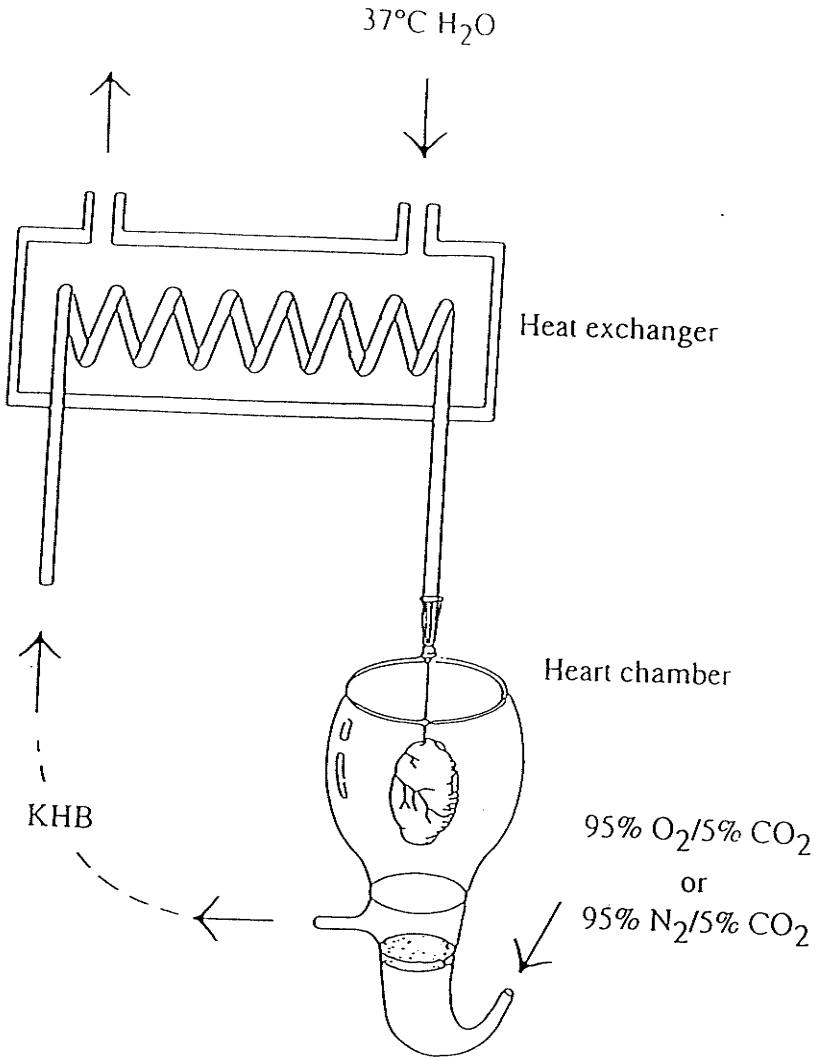


Figure 9: Retrograde perfusion apparatus.

Krebs Henseleit Buffer flows through a heat exchanger, is warmed to 37°C, and subsequently into the heart via the aorta in a retrograde manner. The buffer is collected in the heart chamber and recirculated. The pool of buffer in the heart chamber is saturated with either 95% oxygen / 5% carbon dioxide (control) or 95% nitrogen / 5% carbon dioxide (hypoxic).

2. Radioactive labelling studies in the isolated perfused heart

Rat hearts were perfused with 12.5 mL Krebs Henseleit buffer containing 0.1 mM [1,(3)-^{3}H]glycerol (2.5 μCi/mL) for 60 min in continuous pulse-labeling experiments. The concentration of glycerol (0.1 mM) used throughout this experiment was representative of the physiological plasma concentration in rats (Robinson and Newsholme 1969). The perfusate was saturated either with 95% O_2 / 5% CO_2 (control) or 95% N_2 / 5% CO_2 (hypoxic). The pO_2 of the O_2 saturated buffer was 69.6±2.0 kPa while the pO_2 for the nitrogen gas saturated buffer was 2.9±0.7 kPa (Hatch and Choy 1990). In the pulse-chase experiments, the heart was perfused for 15 min with Krebs Henseleit buffer containing 1.4 mM [^{32}P]P_i (67 μCi/mL) under control condition and subsequently chased with Krebs Henseleit buffer (1.4 mM P_i) for another 60 min under either control or hypoxic conditions.

3. Isolation of radioactive containing metabolites

Subsequent to perfusion, the hearts were weighed (typically 0.30-0.50 g) and homogenized by a 20 sec burst of a Polytron® Homogenizer (Kinematika, Lucerne, Switzerland) in 5 mL of chloroform:methanol (2:1, by vol). Tissue left on the homogenizer probe was removed by homogenization with another 5 mL of chloroform:methanol (2:1, by vol). The two 5 mL homogenates were combined and centrifuged at full speed for 10 min in a bench top centrifuge (Model TJ-6, Beckman Instruments, Mississauga, Ontario, Canada) to pellet debris. After centrifugation, the homogenate was decanted into a 16 x 125 mm screw-cap tube. Addition of 5 mL of 0.73% sodium chloride to the homogenate resulted in the formation of organic and

aqueous phases. The tube was vortexed and centrifuged at full speed for 10 min in a bench top centrifuge causing a biphasic separation. The upper aqueous phase was removed by aspiration and the remaining organic layer was further washed with 5 mL of theoretical upper phase (chloroform:methanol:0.9% sodium chloride, 3:48:47, by vol). The tube was vortexed and centrifuged as described above. The resulting upper aqueous phase and interphase was removed and the remaining organic phase was dried down under a stream of nitrogen gas. The dried organic lipid residue was resuspended in 100 μL chloroform/methanol (2:1, by vol). Thin layer chromatography plates treated with 0.4 M boric acid were used to resolve individual phospholipid species. Briefly, the thin layer plates were sprayed with a 0.4 M solution of boric acid and dried over night (Poorthius et al. 1976). Before application of the lipid sample, the thin layer plates were heat activated for 1 hr at 145°C. The thin layer plate was allowed to cool then 25 μL of the resuspended lipid sample was placed on the plate. The thin layer plate was subjected to a two-dimensional separation. In the first dimension, the plate was developed in a solvent system containing chloroform:methanol:NH_4OH:DDW (70:30:2:3, by vol). The second dimension, at 90° to the first separation, was performed in a solvent system containing chloroform:methanol:DDW (65:35:5, by vol). The thin layer plate was allowed to dry overnight and the separated phospholipids were visualized by exposing the plate to iodine vapor. Figure 10 illustrates the typical phospholipid migration pattern in a the two-dimensional separation system. Corresponding radioactive phospholipids of interest were removed and placed in 6 mL scintillation vials. Radioactivity of [^{32}P] and [^{3}H] incorporated phospholipids were determined immediately and 48 hours, respectively, after the addition of 5 mL of the Ecolite™ scintillation cocktail. The radioactivity was determined in a Beckman Model LS 3801 liquid scintillation counter. However the radioactive CDP-diacylglycerol was resolved by a one-dimensional separation system

containing chloroform:methanol:acetic acid:DDW (50:24:4:8, by vol) (Holub and Piekarski 1976). A 10 µL sample of the organic phase was placed on a thin layer plate with 25 µL of 2 mM CDP-diacylglycerol standard. The CDP-diacylglycerol band was identified by exposing the thin layer plate to iodine vapor and the corresponding silica gel was removed and placed into a 6 mL scintillation vial and radioactivity determined as described above.

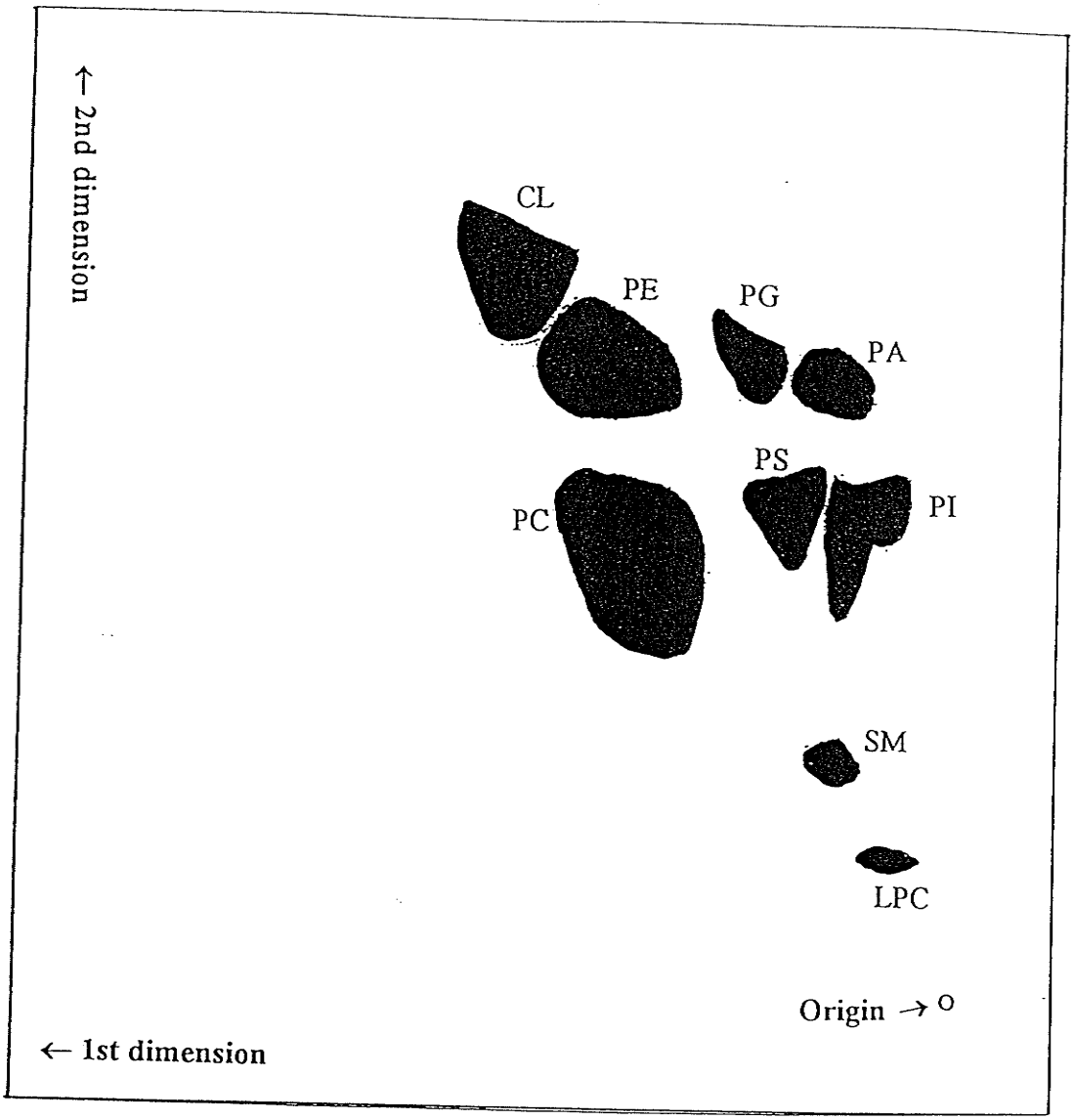


Figure 10: Two dimensional thin layer chromatogram of rat heart phospholipids. Abbreviations used: CL, cardiolipin; PG, phosphatidylglycerol; PE, phosphatidylethanolamine, PC, phosphatidylcholine, PS, phosphatidylserine; PI, phosphatidylinositol, SM, sphingomyelin; LPC, lysophosphatidylcholine.

4. Determination of cardiolipin, phosphatidylglycerol and CDP-diacylglycerol pool sizes

In phospholipid mass determination experiments, 35 μL of the resuspended residue was placed on borate treated thin layer plates and developed in the two-dimensional chromatography system described above (Poorthuis *et al.* 1976). Silica gel corresponding to the phospholipids were identified by exposure to iodine vapor and removed from the plate. The phosphorus content of the phospholipid were determined via two methods:

a) Phospholipid mass determination by the Rouser method (Rouser *et al.* 1966). Basically the selected silica gel fractions were placed in 13 x 100 mm test tubes. 450 μL of 70 % perchloric acid was added to each sample tubes as well as the standard tubes which contain 0 to 200 nmoles of potassium phosphate. The reaction tubes were heated to 180°C for 60 min and allowed to cool before 2.5 mL water was added. To each of the reaction tubes, 0.5 mL of 2.5 % ammonium molybydate and 0.5 mL of 10 % ascorbic acid were added. The tubes were incubated in a water bath for 15 min at 95°C. After cooling, the silica gel was separated from the samples by centrifugation. The samples and blank were transferred to plastic disposable cuvette, and the absorbence were taken at 820 nm. The values were then converted to μg of phosphorus using a factor derived from a standard curve prepared using potassium phosphate.

b) Sensitive malachite green phosphorus determination method (Zhou and Arthur 1992). The separation of CDP-diacylglycerol from a 15 μL aliquot of the organic phase, without standard CDP-diacylglycerol, was performed as described above. Silica gel corresponding CDP-diacylglycerol bands were removed and placed in test tubes. 400-1000 μL of perchloric acid was added to each tube containing the silica gel as well as the

standard tubes. The tubes were covered by placing a sheet of glass over the openings and heated to 180°C in a heating block until the mixtures became clear (indication of completed digestion). The tubes were cooled and centrifuged. 25-100 μL of the centrifuged liquid fractions was placed into clean tubes and DDW was added to bring the final volume to 200 μL. Subsequently, 1 mL of Working solution I was added to the tubes using a repeater pipette. The Working solution I contained: 3 volumes of 0.3 % malachite green, 1 volume of 4.2 % ammonium molybydate, and 0.0016 volume of 4 % Tween 20. After the addition of the Working solution I, the tubes were vortexed and incubated at room temperature of 30 min. The absorbence of the samples was taken at 660 nm against DDW as blank.

5. Mitochondrial fractionation of the heart by differential-centrifugation

Crude mitochondrial fractions were prepared for assay of enzyme activities of the following enzymes: CTP:phosphatidic acid cytidylyltransferase, phosphatidylglycerolphosphate synthase, phosphatidylglycerolphosphate phosphatase, and cardiolipin synthase. The animal was sacrificed by decapitation and the heart was quickly removed and perfused with Krebs Henseleit buffer saturated with either 95% O_2 / 5% CO_2 or 95% N_2 / 5% CO_2. Subsequent to perfusion, a 10% homogenate was prepared by homogenizing the heart (Polytron® 20 sec burst) in homogenizing buffer containing 5 mM Tris-HCl, 0.25 M sucrose, 0.145 M sodium chloride, pH 7.4. For cardiolipin synthase assays, the heart was homogenized in 10 mM Tris-HCl, 0.25 M sucrose, 2 mM EDTA, pH 7.4. The homogenate was then centrifuged for 10 min at 1,000 x *g* (Sorvall RC-5 Superspeed refrigerated centrifuge with SS-34 rotor). The resulting pellet was discarded and the supernatant was centrifuged at 12,000 x *g* for 15 min. The resulting pellet was

resuspended in homogenizing buffer (0.5-1.0 mL) using a Dounce tissue grinder (15 hand strokes) and was designated the mitochondrial fraction.

6. Protein determination of the crude mitochondrial fraction

Protein concentration of the crude mitochondrial fraction was determined by a modified method of Bradford (Bradford 1976). A 5 μL aliquot of the crude mitochondrial fraction was incubated with 5 mL of DDW:Bio Rad Protein Determination reagent (4:1, by vol). The mixture was vortexed and incubated at room temperature for 5 min. Absorbance was measured at 595 nm against a blank. Fatty acid free albumin (1 mg/mL) was used as a standard.

7. Assay of enzyme activities involved in cardiolipin biosynthesis

a) CTP:phosphatidic acid cytidylyltransferase

CTP:phosphatidic acid cytidylyltransferase was assayed by determining the conversion of [5-^{3}H]CTP to [5-^{3}H]CDP-diacylglycerol (Hatch 1994). Test tubes (16 x 100 mm) treated with dimethyldichlorosilane 2% in 1,1,1,-trichloroethane were dried overnight at 142°C and were used as the reaction tubes. The reaction mixture contained 50 mM Tris-maleate, pH 6.5, 10 mM [5-^{3}H]CTP (specific activity, 5,739 dpm/nmol), 0.1 mg of mitochondrial protein, 0.5 mM PA dissolved in 0.15 M Triton X-100. Homogenizing buffer was added to yield a final volume of 90 μL. The reaction was initiated by the addition of 10 μL of 0.2 M $MgCl_2 \cdot 6H_2O$. The mixture was incubated at 30°C for 5 min and the reaction was terminated by the addition of 0.5 mL of 0.1 M HCl in

methanol. To the mixture was added 1 mL of chloroform and 1.5 mL of 1.0 M $MgCl_2$. The suspension was vortexed and the mixture was centrifuged at full speed for 10 min in a bench top centrifuge. The aqueous phase was removed by suction and 500 µL of the organic phase was placed in a 6 mL scintillation vial. The organic phase in the vial was evaporated to dryness at 60°C in a water bath. Then 5 mL of scintillation cocktail was added to the vial and the radioactivity determined in a Beckman Model LS 3801 liquid scintillation counter with internal standards.

b) Phosphatidylglycerolphosphate synthase

Phosphatidylglycerolphosphate synthase activity was assayed by determining the conversion of [U-^{14}C]glycerol-3-phosphate to phosphatidyl[U-^{14}C]glycerol-3-phosphate (Carman and Belunis 1983). The reaction mixture contained 50 mM Tris-HCl, pH 7.0, 10 mM β-mercaptoethanol (prepared fresh), 0.5 mM [U-^{14}C]glycerol-3-phosphate (specific activity, 11,794 dpm/nmol), 2 mM CDP-diacylglycerol (dipalmitoyl) dissolved 10 mM Triton X-100 and 50-100 µg of mitochondrial protein to a final volume of 90 µL. The reaction mixture was initiated by the addition of 10 µL of 0.15 M $MgCl_2 \cdot 6H_2O$. The mixture was then incubated at 30°C for 10 min. 0.5 mL of 0.1 M HCl in methanol was added to terminate the reaction. To the mixture was added 1 mL of chloroform and 1.5 mL of 1 M $MgCl_2$. Radioactivity in the organic fraction was determined as described for CTP:phosphatidic acid cytidylyltransferase.

c) Phosphatidylglycerolphosphate phosphatase

Phosphatidylglycerolphosphate phosphatase activity was assayed by determining the conversion of phosphatidyl[U-^{14}C]glycerol-3-phosphate to phosphatidyl[U-^{14}C]glycerol (MacDonald and McMurray 1980). The reaction mixture contained 50 mM Tris-maleate, pH 6.5, 10 mM β-mercaptoethanol, 0.5 μM phosphatidyl[^{14}C]glycerol-3-phosphate (specific activity, 15,000 dpm/nmol) and 0.1 μg of mitochondrial protein to a final volume of 90 μL. The reaction mixture was incubated at 37°C for 5 min and was terminated by addition of 2 mL of chloroform:methanol:HCl (100:100:0.6, by vol). This was followed by the addition of 1 mL of chloroform and 1.5 mL of 0.73% sodium chloride to cause biphasic separation. The tube was vortexed and centrifuged at full speed for 10 min in a bench top centrifuge. The resulting aqueous phase was removed and the organic phase was dried under nitrogen gas. The residue was resuspended in 25 μL of chloroform:methanol (2:1, by vol), and 20 μL was placed onto a 0.5 N oxalate-treated thin layer plate (Stahl 1969). Oxalate plates were prepared by immersing the thin layer plate in a solution of 0.5 N oxalic acid and were dried over night before use. This treatment is to ensure clear separation of phosphatidylglycerolphosphate and phosphatidylglycerol on the thin layer plate. Standard phosphatidylglycerol (3 μL) was placed on the thin layer plate with the sample. The solvent system used for development of the thin layer plate was chloroform:methanol:HCl (87:13:0.2, by vol). Iodine vapor was used to visualize the separated phospholipids. The band corresponding to phosphatidylglycerol was removed and the radioactivity determined.

d) Cardiolipin synthase

Cardiolipin synthase activity was assayed by determining the conversion of phosphatidyl[U-^{14}C]glycerol to [^{14}C]cardiolipin (Schlame and Hostetler 1992). The reaction mixture contained 0.3 mM EDTA dissolved in 45 mM Tris-HCl, pH 8.5, 50 μM CDP-diacylglycerol, 8 μM phosphatidyl[U-^{14}C]glycerol (specific activity, 45,000 dpm/nmol), and 0.1 mg of mitochondrial protein to a final volume of 90 μL. The reaction was initiated by the addition of 5 mM $CoCl_2 \cdot H_2O$ and incubated at 37°C for 120 min. The reaction was terminated by the addition of 3 mL of chloroform:methanol (2:1, by vol). This mixture was then decanted into a screw cap tube (16 x 125 mm) and 1 mL of chloroform followed by 1 mL of water were added and immediately mixed. The mixture was centrifuged at full speed in a bench top centrifuge for 10 min. The upper aqueous phase was removed by suction and the organic phase was evaporated to dryness under nitrogen gas. The residual lipid sample was resuspended in 25 μL of chloroform:methanol (2:1, by vol), and 20 μL was placed onto a 0.5 N oxalate treated thin layer plate. Standard phosphatidylglycerol (3 μL) and cardiolipin (2 μL) were placed on the thin layer plate with the sample. The same solvent system used for isolation of phosphatidylglycerol in the phosphatidylglycerolphosphate phosphatase assay was employed. The thin layer plate was exposed to iodine vapor in order to visualize the separated phospholipids. The silica gel containing the cardiolipin fraction was removed and the radioactivity was determined.

8. Determination of adenosine-5'-triphosphate and cytidine-5'-triphosphate

Subsequent to perfusion under either control or hypoxic conditions the heart was weighed and homogenized in 5 mL chloroform:methanol (2:1, by vol). Tissue left on the homogenizer probe was removed by another 5 mL of chloroform:methanol. The two 5 mL homogenates were combined and centrifuged at full speed for 10 min in a bench top centrifuge to pellet debris. After centrifugation, the homogenate was decanted into a 16 x 125 mm screw-cap tube. Addition of 3 mL of water to the homogenate resulted in the formation of organic and aqueous phases. The tube was vortexed and centrifuged at full speed for 10 min in a bench top centrifuge. The resulting upper aqueous phase was collected in a 16 x 125 mm test tube. The organic phase was washed twice with 3 mL of water and the aqueous phase pooled. The pooled aqueous phase was dried down under air (24-48 h) and the residue was resuspended in 200 µL of water. The sample was diluted either 20 x (for ATP determination) or 5 x (for CTP determination) with TBA buffer. TBA buffer contained 0.03 M KH_2PO_4, 0.02 M tetrabutylammonium phosphate in 19 % acetonitrile, pH adjusted to 2.65 using H_3PO_4. 20 µL of the diluted sample was injected into an Ultrasphere-ODS C_{18} reversed phase HPLC column for the detection of either ATP or CTP. In an isocratic system, TBA was used to elute nucleotide from the column. The flow rate was set at 2 mL/min and 1.2 mL/min for ATP and CTP, respectively. Nucleotides were detected by an ISCO® UA-5 Absorbance / Fluorescence Detector, wavelength used was 254 nm at ambient temperature. Under these conditions the retention time for ATP and CTP was 4.4 and 5.7 min, respectively. The numerical data was generated by a Beckman 450 Data System / Controller.

B. Reacylation of Lysophosphatidylglcerol in the Rat Heart

a) Subcellular fractionation of the heart

Male Sprague Dawley Rats were sacrificed by decapitation and the hearts quickly removed, and perfused for 2.5 min in the Langendorff mode described in Part A:1. with an ice cooled buffer containing 0.25 M sucrose, 0.145 M NaCl, 10 mM Tris-HCl, pH 7.4 (homogenizing buffer), to remove blood. A 10% homogenate was prepared in buffer and centrifuged for 10 min at 1,000 x *g* (Beckman J2-H with JA-20 rotor). The resulting supernatant was centrifuged at 12,000 x *g* for 10 min. The resulting pellet was resuspended in 5 mL of homogenizing buffer by 15 strokes of a hand-held Dounce (loose fitting) tissue homogenizer and again centrifuged at 10,000 x *g* for 10 min. The resulting pellet was resuspended in 1 mL homogenizing buffer with a (tight fitting) Dounce tissue homogenizer and used as the source of mitochondrial fraction for assay of enzymes. The post-mitochondrial supernatant was centrifuged for 60 min at 100,000 x g (Beckman Ultra centrifuge with 70.1 Ti rotor) and the resulting pellet resuspended in 0.5 mL of homogenizing buffer with a (tight fitting) Dounce tissue homogenizer and used as the source of microsomal fraction. The supernatant from this centrifugation was used as the source of cytosol. All subcellular fractions were aliquot into 1.5 mL cryo-tubes and immediately placed in -150°C liquid nitrogen cryogenic container until use. Marker enzyme analysis revealed that the mitochondrial fraction was contaminated with 10 % microsomal particles and microsomal fraction with 5 % mitochondrial particles (Hatch and Choy 1987).

b) Enzyme assay of acyl Coenzyme A:1-acylglycerophosphorylglycerol acyltranferase

Acyl Coenzyme A:1-acylglycerophosphorylglycerol acyltranferase activity was determined in rat heart cytosolic, mitochondrial and microsomal subcellular fractions. In 16 x 100 mm test tubes the assay mixture contained 50 mM Tris-HCl (pH 9.0), 65 μM of lysophospholipids, 50 μg protein, 60 μM of [1-^{14}C]oleoyl-Coenzyme A (specific activity 2,300 dpm/nmol) and DDW to a final volume of 0.7 mL. The enzyme assay was incubated for 30 min at 25°C and the reaction was terminated by the addition of 3 mL chloroform:methanol (2:1, by vol). 0.8 mL of 0.9% potassium chloride was added to facilitate phase separation and the tubes were vortexed and centrifuged at full speed in a bench top centrifuge. The aqueous fraction was removed by suction and the organic fraction dried under nitrogen gas and resuspended in 25 μL of chloroform:methanol (2:1 v/v) and 20 μL placed onto 0.5 N oxalate-treated thin-layer plate with phosphatidylglycerol standard. The plate was developed in a solvent system containing chloroform:methanol:HCl (87:13:0.2, by vol). The plate was stained with iodine vapor and silica gel corresponding to phosphatidylglycerol was removed and [^{14}C]phosphatidylglycerol was determined by a Beckman Model LS 3801 liquid scintillation counter with internal standard.

9. Statistical determination

All results are depicted as mean ± standard deviation (number of experiments) unless otherwise indicated. Student's t-test was used for the determination of statistical significance. The level of significance was defined as $P<0.05$.

RESULTS

Part I: Effect of Hypoxia on Phosphatidylglycerol and Cardiolipin Biosynthesis

1. Continuous pulse labelling studies

To determine if the biosynthesis of phosphatidylglycerol cardiolipin was affected by hypoxia, rat hearts were perfused in the Langendorff mode (Langendorff 1895) (Figure 9) with 0.1 mM [1,(3)-^{3}H]glycerol (2.5 μCi/mL) for 60 min in a continuous pulse-labeling experiment under control or hypoxic conditions. Subsequent to perfusion, hearts were homogenized in 10 mL of chloroform:methanol (2:1, by vol). 100 μL of the homogenate was taken to determine the total radioactivity incorporated into the hearts. Total radioactivity incorporated into the hearts remained the same for both control and hypoxic hearts (Table 2). The homogenates were then evaporated under a stream of nitrogen gas. The dried residues were resuspended in 100 μL of chloroform:methanol (2:1, by vol); 10 μL was taken to determine the radioactivity incorporated into the organic fraction. In contrast to homogenates, the radioactivity incorporated into the organic fraction was significantly reduced by 78% ($P>0.05$) in control hearts compared to hypoxic hearts (Table 2). Radioactivity incorporated into cardiolipin and phosphatidylglycerol were also significantly reduced by 79% ($p<.05$) and 88% ($p<.05$), respectively, in the hypoxic hearts compared to controls (Figure 11). Thus, hypoxia caused an apparent reduction in biosynthesis of phosphatidylglycerol and cardiolipin. A problem with continuous pulse-labeling experiments is that changes in the radioactivity incorporated into metabolites may be due to isotope dilution or altered uptake of radioactive precursors (Whitehead and Vance 1981).

Table 2: Total radioactivity incorporation into rat hearts after perfusion.

The hearts were perfused with 0.1 mM[1,(3)-^{3}H]glycerol for 60 min under control or hypoxic conditions. Subsequently the hearts were homogenized in 10 mL of chloroform:methanol (2:1, by vol). Homogenate (100 μL) was taken for determination of total radioactivity incorporated into the hearts perfused in control and hypoxic conditions. The homogenate was then evaporated under a stream of nitrogen gas. The dried phospholipid residue was resuspended in chloroform:methanol (2:1, by vol); 10 μL was taken to determine the radioactivity incorporated into the organic fraction. Values represent the mean ± SD of three hearts.

	Control	Hypoxic
	(dpm x 10^6/ g heart)	
Total Radioactivity	6.1±0.7	6.4±0.2
Organic Fraction	1.8±0.5	0.4±0.1*

(*P<.05)

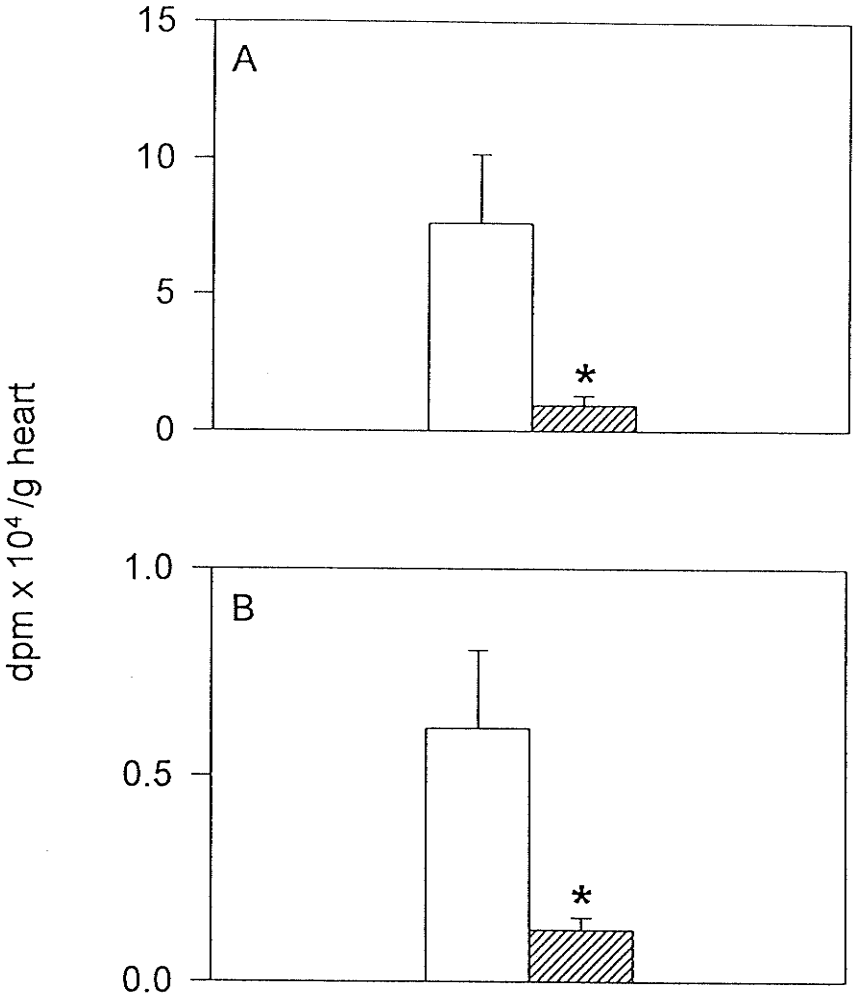


Figure 11: Radioactivity incorporated into cardiolipin and phosphatidylglycerol in hearts perfused with 0.1 mM [1,(3)-^{3}H]glycerol.

Rat hearts were perfused for 60 min with Krebs Henseleit buffer containing 0.1 mM [1,(3)-^{3}H]glycerol under control (□) or hypoxic (▨) conditions. The hearts were homogenized and phosphatidylglycerol and cardiolipin were isolated by thin layer chromatography and the radioactivity associated with (A) phosphatidylglycerol (B) and cardiolipin determined. Vertical bars represent the mean ± SD of at least 3 hearts (*P<.05).

2. Pulse-chase labelling studies

To circumvent the problem a pulse-chase protocol was designed. Rat hearts were pulse-labeled for 15 min with 1.4 mM [^{32}P]P_i (67 μCi/mL) in 95% O_2-saturated Krebs Henseleit buffer and subsequently the radioactivity was chased with non-radioactive Krebs Henseleit buffer (containing 1.4 mM of P_i) for 60 min under control or hypoxic conditions. The radioactivity in both control and hypoxic hearts was 3.1±0.5 x 10^6 cpm/g heart at the beginning of the chase. The radioactivity incorporated into CDP-diacylglycerol, phosphatidylglycerol and cardiolipin were determined at time 0 and 60 min of the chase. Radioactivity incorporated into CDP-diacylglycerol, phosphatidylglycerol and cardiolipin was increased with the time of perfusion in both control and hypoxic hearts (Figure 12A, B, C). The radioactivity incorporated into CDP-diacylglycerol, phosphatidylglycerol and cardiolipin was decreased 61% ($p<.05$), 71% ($p<.05$), and 70% ($p<.05$), respectively, in hypoxic hearts compared to controls (Figure 12A, B, C). These experiments suggested that a reduction in the formation of new CDP-diacylglycerol, phosphatidylglycerol and cardiolipin occurred in the hypoxic heart.

3. Effect of hypoxia on the pool sizes of cardiolipin, phosphatidylglycerol and CDP-diacylglycerol.

To determine if hypoxia affected phosphatidylglycerol and cardiolipin phosphorus mass, rat hearts were perfused under control or hypoxic conditions for 60 min. Subsequently, the phospholipids were extracted and the phospholipid phosphorus determined. As seen in Figure 13 there were no significant changes in the pool sizes of phosphatidylglycerol, cardiolipin and CDP-diacylglycerol between hypoxic hearts and

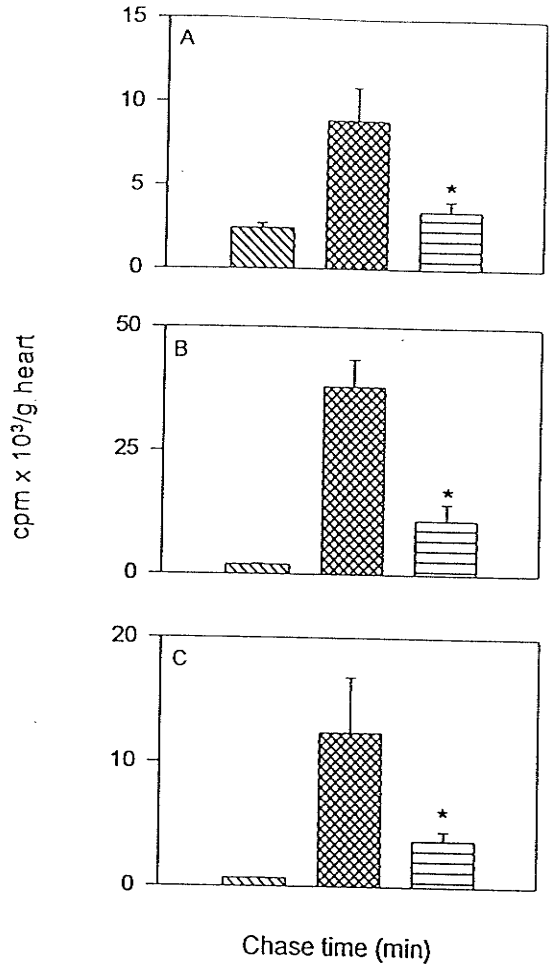


Figure 12: Pulse-chase experiment of 1.4 mM [^{32}P]Pi incorporated into CDP-diacylglycerol, phosphatidylglycerol and cardiolipin.

Rat hearts were pulsed for 15 min with Krebs Henseleit buffer containing 1.4 mM [^{32}P]Pi under control conditions (0, ▨). Subsequently, the hearts were perfused for 60 min with Krebs Henseleit buffer under control (C60, ▩) or hypoxic (H60, ▤) conditions. The hearts were homogenized subsequent to perfusion and various phospholipids were isolated by thin layer chromatography. The radioactivity associated with (A) CDP-diacylglycerol, (B) phosphatidylglycerol, and (C) cardiolipin was determined. Vertical bars represent the mean ± SD of 3 hearts (*P<.05).

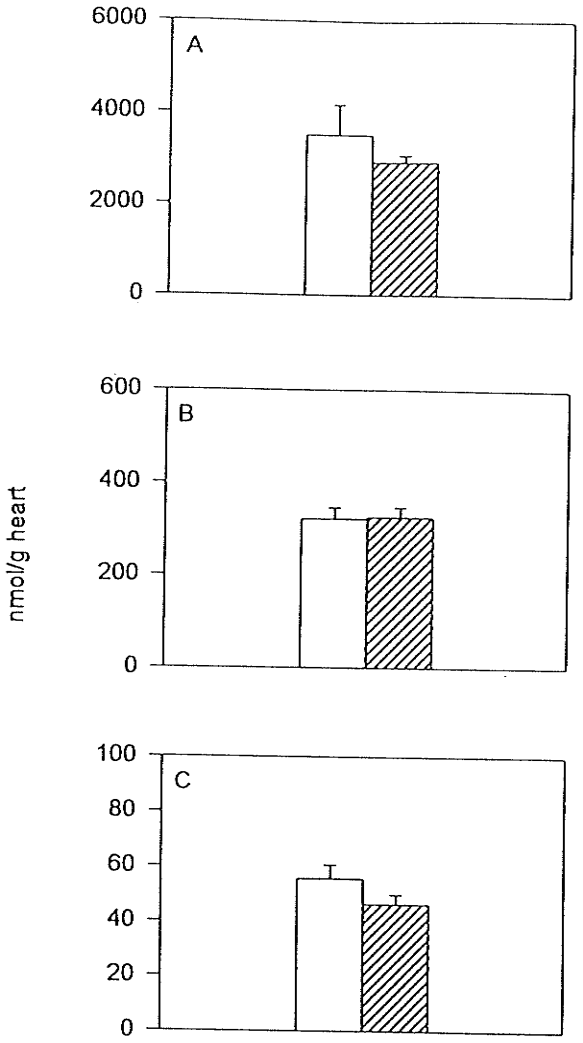


Figure 13: Pool size of cardiolipin, phosphatidylglycerol and CDP-diacylglycerol in control and hypoxic hearts determined in phospholipid phosphorus assay.

Hearts were perfused for 60 min with Krebs Henseleit buffer under control (□) or hypoxic (▨) conditions. The hearts were homogenized subsequent to perfusion and phospholipids were isolated by thin layer chromatography. The pool sizes were determined for: (A) cardiolipin, (B) phosphatidylglycerol, (C) CDP-diacylglycerol. The vertical bars represents the mean ± SD of at least 3 hearts.

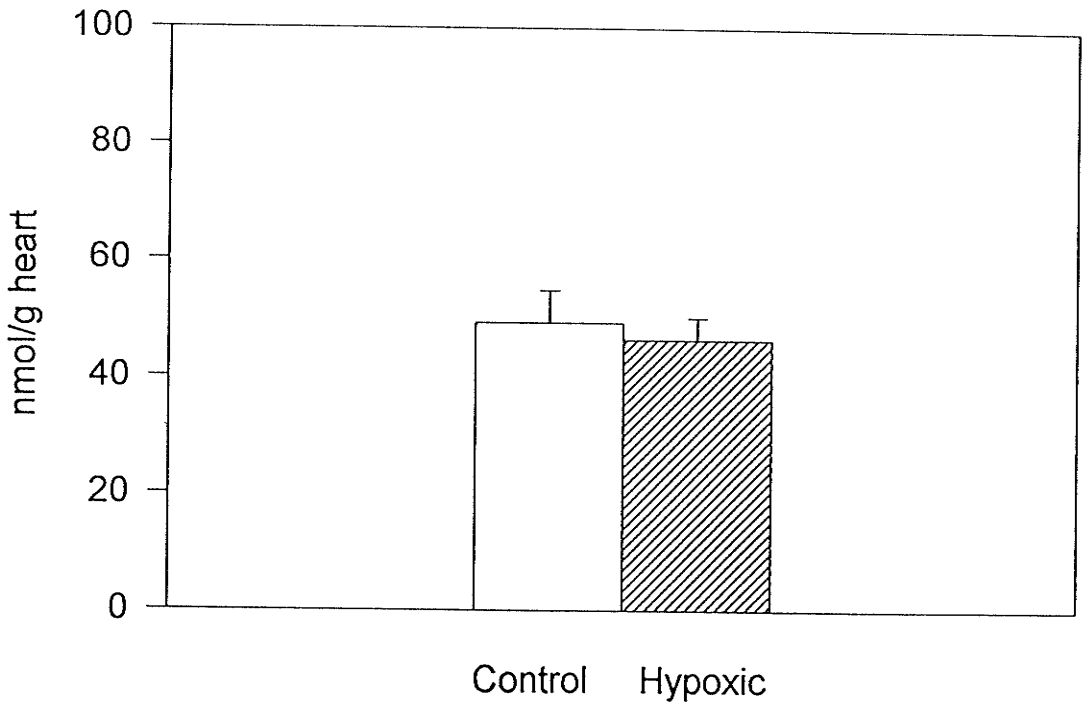


Figure 14: Confirmation of CDP-diacylglycerol pool size in control and hypoxic hearts via malachite green phosphorus assay.

Hearts were perfused for 60 min with Krebs Henseleit buffer under control or hypoxic conditions. The hearts were homogenized subsequent to perfusion and phospholipids were isolated by thin layer chromatography. The pool size was determined by the sensitive malachite green method as described in the Methods.

controls. CDP-diacylglycerol pool size was further confirmed by the more sensitive malachite green phosphorus determination method (Figure 14). Moreover the pool size of other phospholipids were determined in the hearts after perfusion under either control or hypoxic conditions. There was no significant changes in the pool size of other phospholipids (Table 3). Thus, the reduction of radioactivity in phosphatidylglycerol and cardiolipin in the hypoxic heart was not caused by a change in the pool size of phosphatidylglycerol and cardiolipin or its precursors.

4. Effect of hypoxia on the *in vitro* enzyme activities involved in phosphatidylglycerol and cardiolipin biosynthesis

The decrease in radioactivity observed in phosphatidylglycerol and cardiolipin could be due to a change in the activity of one of the enzymes involved in their biosynthesis. Thus, the specific activities of the enzymes involved in phosphatidylglycerol and cardiolipin biosynthesis from control and hypoxic hearts were determined. Hearts were perfused for 60 min under control or hypoxic conditions. Subsequent to perfusion, the hearts were homogenized and the mitochondrial fractions were prepared by differential centrifugation. CTP:phosphatidic acid cytidylyltransferase, phosphatidylglycerolphosphate synthase, phosphatidylglycerol-phosphate phosphatase, and cardiolipin synthase were assayed in the isolated mitochondrial fractions. Enzyme activities between the control and hypoxic hearts were unaltered (Table 4). Thus, the reduction of radioactivity in phosphatidylglycerol and cardiolipin in the hypoxic heart was not caused by a change in the activity of the enzymes involved in phosphatidylglycerol and cardiolipin biosynthesis.

Table 3: Pool size of phospholipids in control and hypoxic hearts.

Hearts were perfused for 60 min. with Krebs Henseleit buffer under control or hypoxic conditions. The hearts were homogenized and the phospholipid pool sizes were determined as described in the Methods. Values represent the mean ± SD of at least three hearts.

Phospholipid	Control	Hypoxic
	(nmole/g heart)	
Phosphatidic acid	37.8±16.2	36.5±3.3
Phosphatidylcholine	7946.1±958.3	6587.5±669.5
Phosphatidylethanolamine	6857.8±138.2	6120.9±452.1
Phosphatidylserine	655.4±71.6	584.4±76.5
Phosphatidylinositol	1076.8±103.3	806.6±44.9
Lysophosphatidylcholine	177.2±31.0	140.5±25.0
Sphingomyelin	144.3±18.5	146.0±13.5

Table 4: *In vitro* enzyme activities involved in cardiolipin biosynthesis.

Rat hearts were perfused for 60 min with Krebs Henseleit buffer under either control or hypoxic conditions. Subsequent to perfusion the hearts were homogenized and the activities of the enzymes involved in phosphatidylglycerol and cardiolipin biosynthesis were determined in isolated mitochondrial fractions prepared from the homogenate. The enzymes were assayed under optimal conditions. Values represent the mean ± SD (number of hearts).

	Activity (pmol/min per mg of protein)	
Enzyme	Control	Hypoxic
CTP:phosphatidic acid cytidylyltransferase	33.1±6.5 (3)	33.5±9.7 (3)
Phosphatidylglycerolphosphate synthase	72.6±9.7 (3)	71.7±13.5 (3)
Cardiolipin synthase	1.0±0.7 (4)	1.1±0.5 (3)
Phosphatidylglycerolphosphate phosphatase	0.70±0.05 (3)*	0.72±0.13 (3)*

* nmol/min per mg of protein.

5. Effect of hypoxia on ATP and CTP level of the heart

In the CDP-diacylglycerol pathway, CTP is a cofactor required for the conversion of phosphatidic acid to CDP-diacylglycerol. Since the continuous-pulse and pulse-chase labelling studies indicated a possible decreased formation of new CDP-diacylglycerol, the levels of cardiac ATP and CTP were determined in the hearts perfused for 60 min under control or hypoxic conditions. Using a C_{18} reverse phase HPLC column, the high energy nucleotides were able to be separated by an isocratic system. The average retention time of this particular system for ATP and CTP was 4.4 and 5.7 min, respectively (Figure 15). The peak areas generated by Beckman 450 Data System/Controller were used to quantify the levels of the high energy nucleotide. It was found that in the hypoxic hearts, ATP and CTP levels were significantly decreased 94% and 92%, respectively, when compared to controls (Table 5). Thus, the large reduction of cardiac CTP may have been responsible for the decreased formation of CDP-diacylglycerol in the hypoxic hearts and the resulting decrease in radioactivity incorporated into phosphatidylglycerol and cardiolipin.

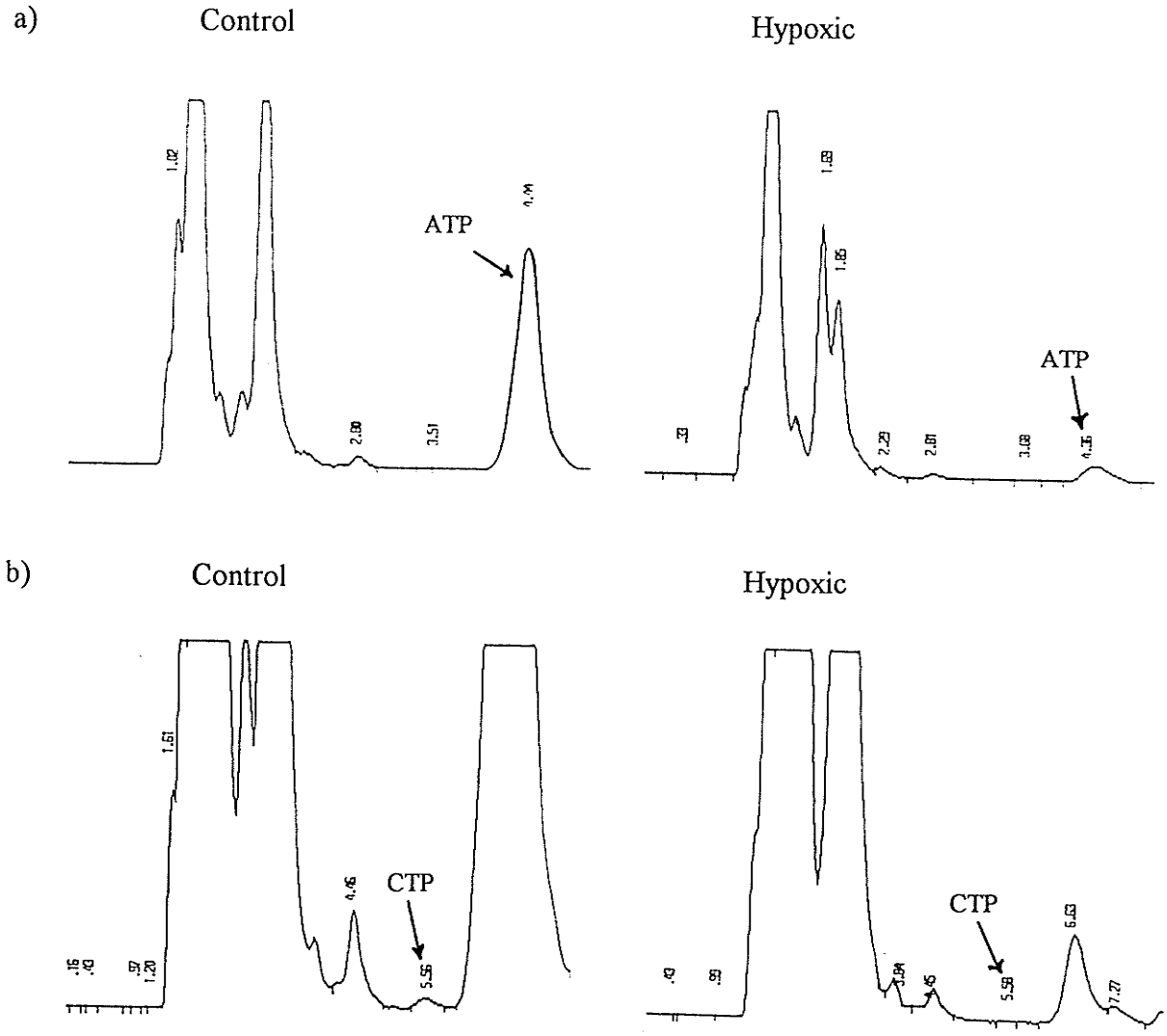


Figure 15: HPLC separation of a) ATP and b) CTP in the isolated perfused rat heart.

Rat hearts were perfused with Krebs Henseleit buffer for 60 min in control or hypoxic conditions. The hearts were homogenized subsequent to perfusion. The high energy nucleotides in the aqueous phase were separated on an Ultrasphere-ODS reverse phase C_{18} HPLC column. The absorbance at 254 nm was recorded.

Table 5: ATP and CTP concentration in control and hypoxic hearts.

Rat hearts were perfused for 60 min with Krebs Henseleit buffer under either control or hypoxic conditions. Subsequent to perfusion ATP and CTP levels were quantified by C_{18} reverse phase HPLC. Values represent the mean ± SD (number of hearts).

	ATP	CTP
	(nmol/ g heart)	
Control	633±110 (3)	5.25±0.85 (3)
Hypoxic	*39±8 (3)	*0.41±0.06 (3)

(*P<.05)

Part II: Reacylation of Lysophosphatidylglycerol to Phosphatidylglycerol in Rat Heart

1. Acyl-Coenzyme A:1-acylglycerophosphorylglycerol acyltransferase localization in the rat heart

Recently, it was demonstrated that the heart contains phospholipase A activity directed towards phosphatidylglycerol in microsomes, mitochondria and cytosol which resulted in the formation of lysophosphatidylglycerol (Hatch *et al.* 1995). This could potentially lead to an accumulation of lysophospholipids in the heart. Thus, the possibility of lysophosphatidylglycerol reacylation to the parent phosphatidylglycerol in microsomal, mitochondrial, and cytosolic fractions was investigated. Utilizing [1-^{14}C]oleoyl-Coenzyme A as fatty acyl donor and 1-palmitoylglycerophosphorylglycerol as fatty acid acceptor, acyl-Coenzyme A:1-acylglycerophosphorylglycerol acyltransferase activity was observed in microsomes, mitochondria and cytosol in an *in vitro* assay (Table 6). The activity was greatest in microsomes followed by mitochondria and cytosol. Marker enzyme studies performed previously indicated that the enzyme activity in the mitochondrial and cytosolic fractions might have been caused by contamination of microsomal particles (Arthur et al 1986). Thus, the enzyme to synthesize phosphatidylglycerol from lysophosphatidylglycerol was found to located in the cardiac microsomal fraction.

Table 6: Subcellular localization of acyl-Coenzyme A:1-acylglycerophosphoryl-glycerol acyltransferase activity in rat heart.

Rat hearts were homogenized and subcellular fractions prepared from the homogenate as described in the Methods. Acyl-Coenzyme A:1-acylglycerophosphorylglycerol acyltransferase activity was assayed in microsomes, mitochondria and cytosol in the presence of 60 μM of [1-^{14}C]oleoyl-Coenzyme A and 65 μM of 1-palmitoylglycerophosphorylglycerol. Each value represents the mean ± SD of at least three hearts.

Subcellular Fraction	Specific Activity of acyl-Coenzyme A:1-acylglycerophosphorylglycerol acyltransferase (nmole/min/mg of protein)
Microsomes	0.556±0.028
Mitochondria	0.036±0.006
Cytosol	0.004±0.003

2. Characterization of acyl-Coenzyme A:1-acylglycerophosphorylglycerol acyltransferase activity in rat heart microsomes

Since the acyl-Coenzyme A:1-acylglycerophosphorylglycerol acyltransferase activity was greatest in the microsomal fraction, all subsequent characterization and analysis of this enzyme was performed using only this fraction.

a) Effect of pH on rat heart microsomal acyl-Coenzyme A:1-acylglycero-phosphorylglycerol acyltransferase

As illustrated in Figure 16, the acyl-Coenzyme A:1-acylglycerophosphorylglycerol acyltransferase exhibited a distinct pH profile. Maximum activity was observed towards alkaline pH. A gradual decrease of activity was observed when the pH value was moving towards neutrality. However, significant activity was still observed at pH 7.5 indicating that the reacylation reaction may occur at physiological pH. The pH optimum in alkaline condition was similar to that previously reported for rat liver microsomal acyl-Coenzyme A:1-acylglycerophosphorylglycerol acyltransferase (Wittels 1973). However, the specific activity observed in the present study was approximately 5-fold higher than previously reported in cardiac microsomal fractions (Wittels 1973).

b) Lysophosphatidylglycerol specificity of rat heart microsomal acyl-Coenzyme A:1-acylglycero-phosphorylglycerol acyltransferase

Numerous studies performed on different acyltransferases in the past have shown that each individual enzyme has its own acyl specificity towards the acyl group in the

lysophospholipid and acyl-Coenzyme A. It was previously demonstrated that hamster heart acyl:Coenzyme A:1-acylglycerophosphorylcholine acyltransferase exhibited substrate specificity (Arthur and Choy 1986) towards the acyl groups. Thus, the specificity of the rat heart microsomal acyl-Coenzyme A:1-acylglycerophosphorylglycerol acyltransferase for various lysophospholipids was investigated. As seen in Table 7, significant acyl-Coenzyme A:1-acylglycero-phosphorylglycerol acyltransferase activity towards various lysophospholipids was observed. Using the concentration of 65 μM of lysophospholipids, the specificity of acyl-Coenzyme A:1-acylglycerophosphorylglycerol acyltransferase for various lysophospholipids was in the order of 1-myristoylglycerophosphorylglycerol > 1-palmitoylglycerophosphorylglycerol > 1-oleoylglycerophosphorylglycerol > 1-stearoyl-glycerophosphorylglycerol. With concentrations of 1-palmitoylglycerophosphorylglycerol at 0.1 mM or greater present in the assay, acyl-Coenzyme A:1-acylglycerophosphorylglycerol acyltransferase activity was attenuated in a concentration-dependent manner (Figure 17). Thus, high concentrations of lysophospholipids were shown to be inhibitory for the enzyme.

c) Kinetic Studies on rat heart microsomal acyl-Coenzyme A:1-acylglycerophosphoryl-glycerol acyltransferase

The apparent K_m's for oleoyl-Coenzyme A and 1-palmitoylglycerophosphoryl-glycerol were determined though the use of the Eadie-Hoffstee plot. It has the advantage over the Lineweaver Burk plot in that data over the whole concentration range are more evenly weighted (Zubay 1988). The activities of the enzyme at different 1-

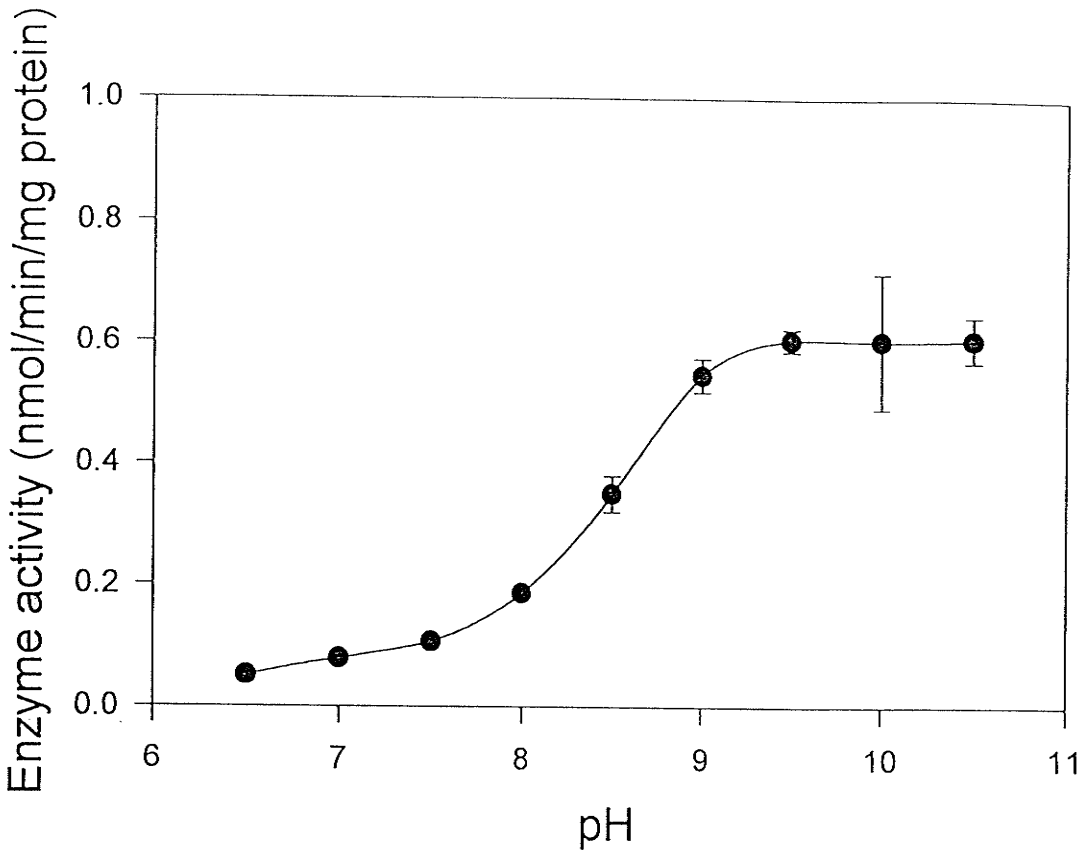


Figure 16: The effect of pH on rat heart microsomal acyl-Coenzyme A:1-acylglycerophosphorylglycerol acyltransferase.

Acyl-Coenzyme A:1-acylglycero-phosphorylglycerol acyltransferase activity in rat heart microsomes was assayed using [1-^{14}C]oleoyl-Coenzyme A and 1-palmitoylglycerophosphorylglycerol as substrate at various pH. Values represent the mean ± SD of at least three hearts.

Table 7: Substrate specificity of rat heart microsomal acyl-Coenzyme A:1-acylglycerophosphorylglycerol acyltransferase for various lysophosphatidylglycerols.

Acyl-Coenzyme A:1-acylglycerophosphorylglycerol acyltransferase activity in rat heart microsomes was assayed in the presence (65 μM) of various lysophosphatidylglycerols with [1-^{14}C]oleoyl-Coenzyme A (60 μM) as described in the Methods. Values represent the mean ± SD of at least three hearts.

	Specific Activity of acyl-Coenzyme A:1-acylglycerophosphorylglycerol acyltransferase
Lysophosphatidylglycerol	nmole/min per mg protein
1-myristoylglycerophosphorylglycerol (14:0)	0.641±0.037
1-palmitoylglycerophosphorylglycerol (16:0)	0.546±0.028
1-oleoylglycerophosphorylglycerol (18:1)	0.414±0.140
1-stearoylglycerophosphorylglycerol (18:0)	0.094±0.019

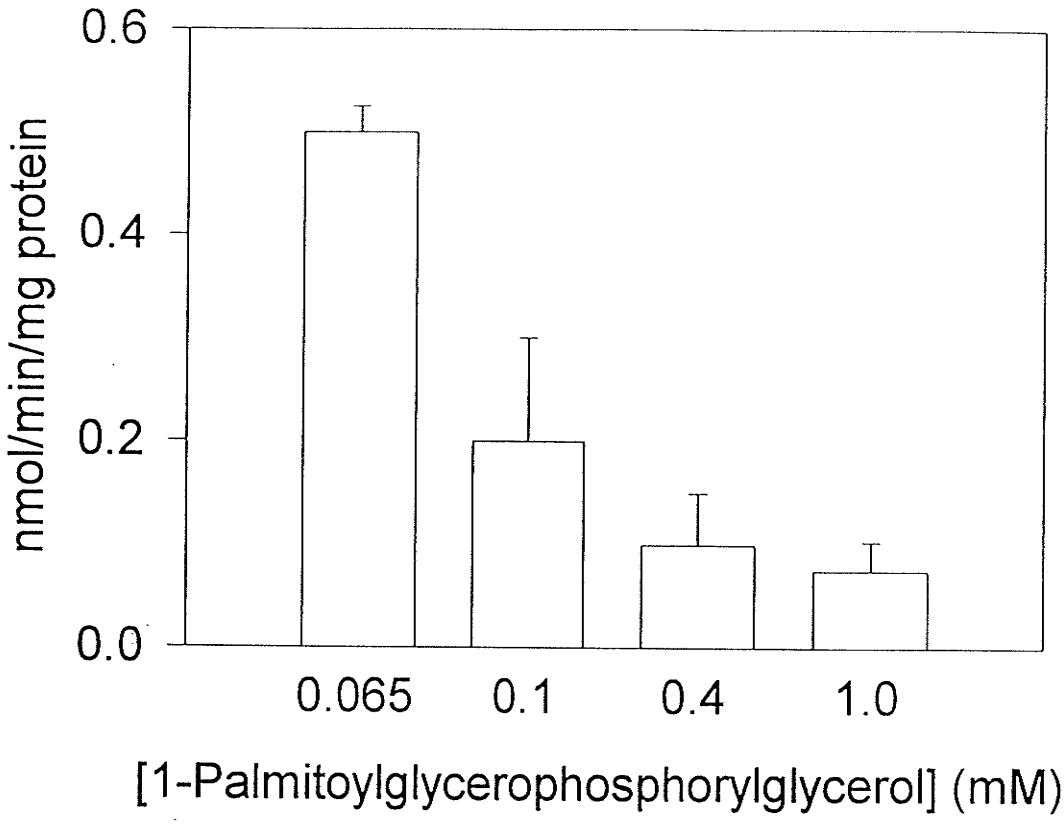


Figure 17: Effect of various concentrations of 1-palmitoylglycerophosphorylglycerol on the activity of rat heart microsomal acyl-Coenzyme A:1-acylglycerophosphoryl-glycerol acyltransferase.

Acyl-Coenzyme A:1-acylglycerophosphorylglycerol acyltransferase activity was assayed in rat heart microsomes in the presence of 0.065-1.0 mM concentrations of 1-palmitoylglycerophosphorylglycerol. Values represent the mean ± SD of at least three hearts.

palmitoylglycerophosphorylglycerol concentrations in the presence of a fixed amount of oleoyl-Coenzyme A were determined. The apparent K_m of acyl-Coenzyme A:1-acylglycerophosphorylglycerol acyltransferase for 1-palmitoylglycerophosphorylglycerol derived from the slope of the Eadie-Hoffstee plot was determined to be 9.4 μM (Figure 18A). The activities of the enzyme at different oleoyl-Coenzyme A concentrations in the presence of a fixed amount of 1-palmitoylglycerophosphorylglycerol were determined and again the apparent K_m of acyl-Coenzyme A:1-acylglycerophosphorylglycerol acyltransferase for oleoyl-Coenzyme A was derived from the slope of the Eadie-Hoffstee plot and determined to be 7.1 μM (Figure 18B).

d) The effect of heat inactivation on acyl-Coenzyme A:1-acylglycerophosphorylglycerol acyltransferase at 55°C

Rat heart microsomal fractions were pre-incubated at 55°C for up to 10 min and subsequently acyl-Coenzyme A:1-acylglycero-phosphorylglycerol acyltransferase activity was determined. 65% of the enzyme activity was lost by pre-incubation at 55°C for only 1 min indicating a rapid inactivation (Figure 19). By 2 min of pre-incubation at 55°C, acyl-Coenzyme A:1-acylglycerophosphorylglycerol acyltransferase activity was almost completely abolished. Thus, the cardiac microsomal acyl-Coenzyme A:1-acylglycerophosphorylglycerol acyltransferase is a heat labile enzyme. All of the above results indicate the *in vitro* presence of an active acyl-Coenzyme A:1-acylglycerophosphorylglycerol acyltransferase activity in the heart microsomal fraction.

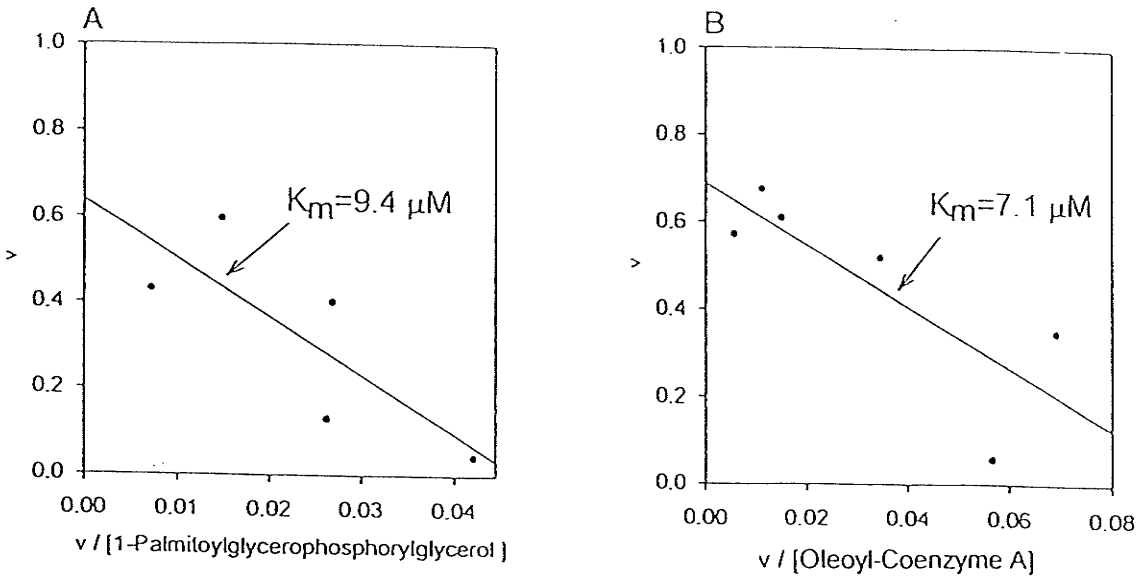


Figure 18: Eadie-Hoffstee plot of rat heart microsomal acyl-Coenzyme A:1-acylglycerophosphorylglycerol acyltransferase.

A) Acyl-Coenzyme A:1-acylglycerophosphorylglycerol acyltransferase activity was assayed in rat heart microsomes in the presence of 60 μM oleoyl-Coenzyme A and 1-65 μ M 1-palmitoylglycerophosphorylglycerol and a graph of activity verses activity/substrate plotted. B) Acyl-Coenzyme A:1-acylglycerophosphorylglycerol acyltransferase activity was assayed in rat heart microsomes in the presence of 65 μM 1-palmitoylglycerophosphorylglycerol and 1-100 μM oleoyl-Coenzyme A and a graph of activity verses activity/substrate plotted. Values represent the mean of at least two hearts.

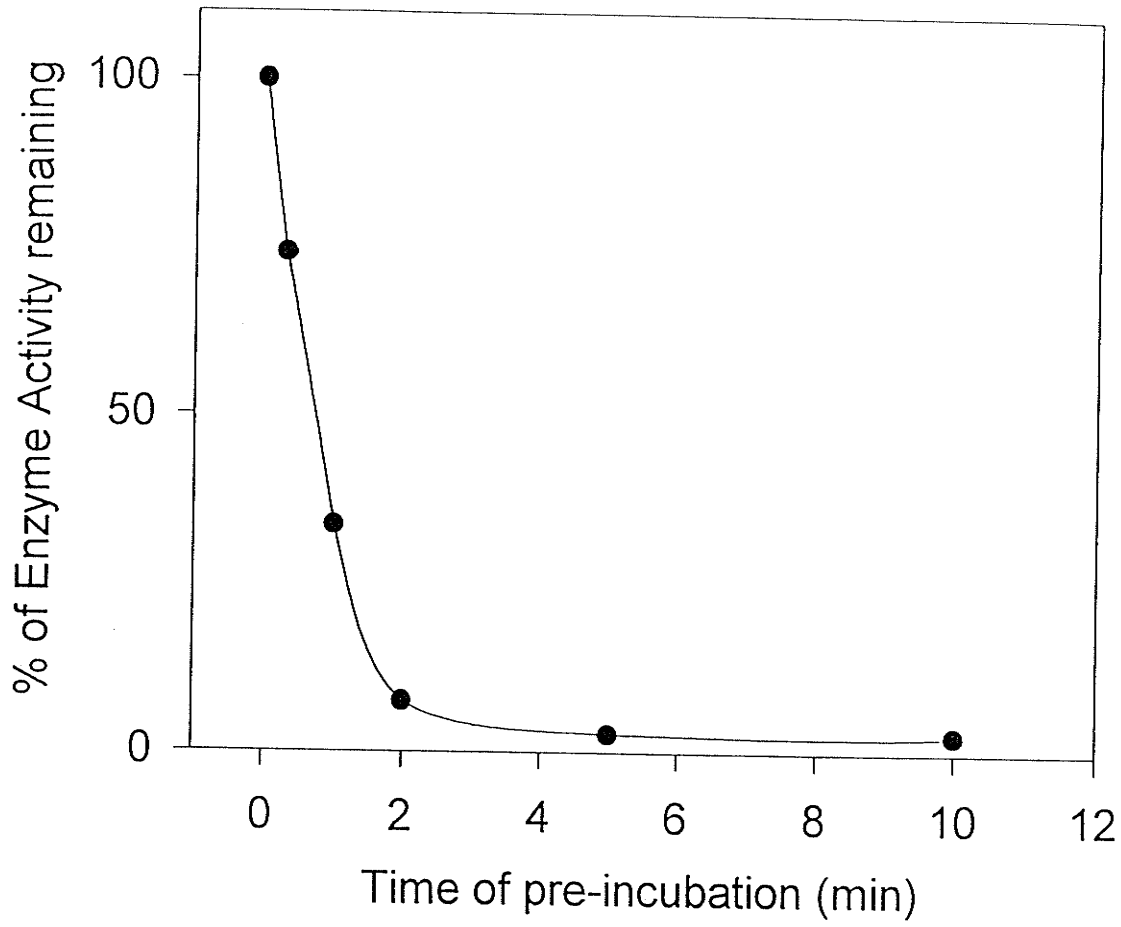


Figure 19: The effect of pre-incubation of microsomal fractions at 55°C on acyl-Coenzyme A:1-acylglycerophosphorylglycerol acyltransferase activity.

Microsomal fractions were pre-incubated for up to 10 min at 55°C and the acyl-Coenzyme A:1-acylglycerophosphorylglycerol acyltransferase activity in rat heart microsomes subsequently determined. Values represent the mean of at least two hearts. The activity at time 0 was 0.55 nmole/min/mg protein.

DISCUSSION

1. The Effect of Hypoxia On Cardiolipin Biosynthesis

The objective of this study was to determine if hypoxia affected cardiolipin biosynthesis in the heart. In order to examine this hypothesis we perfused hearts in the Langendorff mode with radioactive glycerol or P_i under control or hypoxic conditions. Langendorff perfusion is a highly reproducible, stable method which is especially useful in metabolic labeling studies (William and Kobayashi 1984). Furthermore a rapid decrease in the level of high energy nucleotide is achieved by the hypoxic model employed for this study (de Leiris *et al.* 1984). In the continuous pulse-labeling studies, an apparent reduction in the biosynthesis of new phosphatidylglycerol and cardiolipin was observed. In addition, the pulse-chase studies indicated that the radioactivity incorporated into CDP-diacylglycerol, phosphatidylglycerol and cardiolipin was reduced in the hypoxic hearts compared to controls at 60 min of perfusion. Thus, perfusion of the heart under hypoxic conditions inhibits cardiolipin biosynthesis.

The level of cardiac ATP and CTP was reduced by perfusing the isolated rat heart under hypoxic conditions consistent with that of hamster hearts perfused under similar conditions (Hatch and Choy 1990). Indeed, following a 60 min perfusion under hypoxic conditions, we observed a 94% decrease in the cardiac ATP level compared to controls. ATP is required for the amination reaction in CTP production (Stryer 1988), therefore loss of ATP would consequently reduce the biosynthesis of CTP. We observed a corresponding reduction of 92% in the cardiac CTP level in hypoxic hearts compared to controls.

The mechanism of the reduction of cardiolipin biosynthesis under hypoxic conditions was investigated. The results indicated that the activities of the enzymes of the CDP-diacylglycerol pathway were unaltered between the hypoxic and control hearts. This is in contrast to previous studies on phosphatidylcholine biosynthesis in the hamster heart (Hatch and Choy 1990; Choy 1982). In those studies, CTP:phosphocholine cytidylyltransferase activity was increased as a compensatory mechanism for the reduction in cardiac CTP level. The results of the present study suggest that there may not be such a compensatory mechanism for reduced cardiolipin biosynthesis caused by lowered cardiac CTP.

ATP is required to convert glycerol to glycerol-3-phosphate and oxygen is required for oxidative phosphorylation and the conversion of [^{32}P]Pi to [^{32}P]ATP. Thus, the reduction in labeling of phosphatidylglycerol and cardiolipin in the [^{32}P]Pi perfused hearts was not surprising. Depletion of cardiac ATP and hence CTP might also result in a decreased formation of new CDP-diacylglycerol producing less available radioactive CDP-diacylglycerol to form cardiolipin. Indeed, this was observed in the pulse-chase studies. Despite the drastic reduction in the ATP and CTP level of the heart, the pool sizes of CDP-diacylglycerol, phosphatidylglycerol and cardiolipin in the hypoxic hearts were unchanged compared to controls. One possible explanation for this is that a large decrease of phospholipid mass would simply be incompatible with the viability of the heart. Alternatively, the total phospholipid pool may eventually decrease but was not detectable after 60 min of perfusion under hypoxic conditions. Perhaps an alteration in phospholipid mass would be apparent if the hypoxic treatment was prolonged. However perfusion for periods beyond 60 min under hypoxic conditions causes irreversible damage to the heart (Hatch and Choy 1990).

Previous studies have set a precedent for the regulation of cardiolipin biosynthesis under conditions of altered mitochondrial oxidative metabolism. For example, thyroid hormone treatment of rats increased the oxidative activity of heart mitochondria and increased the cardiolipin content in these hearts 51% (Paradies and Ruggiero 1988). In rat liver cardiolipin synthase activity was increased 52% in rats treated with thyroxine (Hostetler 1991). Such an increase in cardiolipin synthase activity has offered a plausible mechanism for the increased cardiolipin observed in the tissues of thyroid hormone-treated animals. The above studies are in contrast to this study utilizing acute hypoxia since thyroid-induced increased oxidative activity appears to be associated with increased cardiolipin biosynthesis. The observed reduction in cardiac phosphatidylglycerol and cardiolipin biosynthesis in hearts perfused under hypoxic conditions is interesting in light of the observation that ultraviolet A radiation inhibits phosphatidylglycerol and cardiolipin biosynthesis in N.C.T.C. 2544 human keratinocytes (Djavaheri-Mergny *et al.* 1994) and decreased ATP levels have been observed in lymphocytes exposed to a ultraviolet A radiation (Marks *et al.* 1990).

2: Reacylation of Lysophosphatidylglycerol in the Rat Heart

The objective of this study was to show that rat heart contains an enzymatic system capable of catalyzing the reacylation of lysophosphatidylglycerol to phosphatidylglycerol via the enzyme acyl-Coenzyme A:1-acylglycerophosphorylglycerol acyltransferase. The results clearly confirm the presence of an *in vitro* acyl-Coenzyme A:1-acylglycerophosphorylglycerol acyltransferase activity in cardiac microsomal fractions. The enzyme displayed a preference for the myristoyl groups of the lysophospholipid over the palmitoyl, oleoyl or stearoyl groups. It would appear that

shorter chain length lysophospholipid substrates were preferred over the longer chain lysophospholipids. However, 1-oleoylglycerophosphorylglycerol proved to be a more preferred substrate than 1-stearoylglycerophosphorylglycerol indicating that specificity of acyl-Coenzyme A:1-acylglycerophosphorylglycerol acyltransferase for its lysophospholipid substrates may also be dictated by the and degree of unsaturation. The presence of an acyl-Coenzyme A:1-acylglycerophosphorylglycerol acyltransferase activity in rat liver, kidney, lung and heart had been reported (Wittels 1973). However, the acyl-Coenzyme A:1-acylglycerophosphorylglycerol acyltransferase activity observed in the present study was more than 5-fold higher than that reported previously for the rat heart enzyme (Wittels 1973). This discrepancy was likely due to the use of higher concentrations (0.1 mM) of 1-palmitoylglycerophosphorylglycerol used in the previous study of which were found to be inhibitory for the cardiac enzyme. The higher lysophospholipid concentration used previously was likely optimal for the rat liver enzyme (Wittels 1973) and was less than ideal for the cardiac enzyme.

Lysophospholipids are known to promote cardiac arrhythmia's (Kinnard *et al.* 1988) owing to their ability to perturb the cellular membrane (Zubay 1988). Phosphatidylglycerol was recently shown to be released from the liver during perfusion (Lekka *et al.* 1993). Thus, a serum source of lysophospholipid is quite conceivable given the fact that serum also contains significant Group-II phospholipase A_2 activity (Vadas and Pruzanski 1989). This serum activity may be dramatically increased in a variety of clinical disorders including septic shock, adult respiratory distress syndrome, psoriasis, renal failure, gout and arthritis (Pruzanski and Vadas 1990). Indeed, liver perfusion studies have indicated that phospholipase A_2 may be released under both physiological and pathophysiological conditions and that this phospholipase A_2 has a preference for

phosphatidylglycerol substrates (Hatch *et al.* 1993). Interestingly, renal failure, septic shock, rheumatoid arthritis and infection have all been associated with cardiac arrhythmia's (Armstrong and Menahem 1993; Rokas *et al.* 1990; Odeh 1993; Charmes *et al.* 1992). Cardiac microsomal membranes are a combination of sarcolemma, Golgi and sarcoplasmic reticulum and significant phosphatidylglycerol is observed in rat heart microsomes (Hatch *et al.* 1995). Acyl-Coenzyme A:1-acylglycerophosphorylglycerol acyltransferase activity appeared to be localized to the microsomal fraction. This is conceivable since the sarcolemma would be the first membrane exposed to lysophospholipid generated from outside the cell and the acyl-Coenzyme A:1-acylglycerophosphorylglycerol acyltransferase activity in this membrane could attenuate the membrane lytic effect of such compounds by resynthesizing them back to phosphatidylglycerol.

The subcellular localization of cardiac acyltransferases has been the subject of considerable debate. In rabbit heart acyltransferase activity directed towards 1-acylglycerophosphorylcholine was reported to occur exclusively in microsomes (Gross and Sobel 1982) or distributed among all subcellular fractions (Needleman *et al.* 1985). In myocytes this activity was observed in microsomal and cytosolic fractions (Severson and Fletcher 1985). The ability to reacylate both lysophosphatidylcholine and lysophosphatidylethanolamine had been reported in guinea pig heart mitochondria (Arthur *et al.* 1987a; Arthur *et al.* 1987b). Cardiac microsomes contain only approximately 27% of the total cardiac phosphatidylglycerol content (Hatch *et al.* 1995). Given the fact that about 70% of cardiac phosphatidylglycerol is localized to the mitochondria (Hatch *et al.* 1995) the ability to reacylate lysophospholipid to phosphatidylglycerol would have been expected to occur in the mitochondrial fraction. However, since the specific activity of acyl-Coenzyme A:1-acylglycerophosphorylglycerol acyltransferase was approximately 10-

fold higher in microsomal fractions compared to mitochondria and the mitochondrial fraction contained 10% microsomal particles it could be argued that mitochondria might be devoid of acyl-Coenzyme A:1-acylglycerophosphorylglycerol acyltransferase activity. It is quite conceivable that acyl-Coenzyme A:1-acylglycerophosphorylglycerol acyltransferase activity may not be required in mitochondria since rat liver cardiolipin synthase, the enzyme which converts phosphatidylglycerol to cardiolipin, did not have a particular molecular species requirement for its phosphatidylglycerol substrate (Schlame *et al.* 1993). Thus, a specific resynthesis of the mitochondrial pool of phosphatidylglycerol used for cardiolipin biosynthesis may not be essential. Since separate pools of phosphatidylglycerol exist in cardiac mitochondria (Hatch *et al.* 1995) and extra-mitochondrial phosphatidylglycerol may be imported into cardiac mitochondria (Cao and Hatch 1995), the role of the microsomal acyl-Coenzyme A:1-acylglycerophosphorylglycerol acyltransferase may be to generate the appropriate acyl composition of phosphatidylglycerol observed in microsomal organelles and mitochondria. If this is the case, then at least a portion of cardiac phosphatidylglycerol synthesized in the microsomes by acyl-Coenzyme A:1-acylglycerophosphorylglycerol acyltransferase may be imported into mitochondria. However, the current study does not directly rule out the possibility of a distinct mitochondrial acyl-Coenzyme A:1-acylglycerophosphorylglycerol acyltransferase.

SUMMARY

In the first part of the study, an intact isolated rat heart was used to study the effect of hypoxia on cardiolipin biosynthesis. Perfusion of the heart under hypoxic conditions resulted in the reduction of newly synthesized cardiolipin. The results provide strong evidence to support that the biosynthesis of cardiolipin is governed by the ATP and CTP level of the heart. In part two of the study, the presence of an active *in vitro* acyl-Coenzyme A:1-acylglycerophosphorylglycerol acyltransferase activity in rat heart microsomes was demonstrated. It is hypothesized that this enzyme may play an important role in the metabolism of lysophosphatidylglycerol in the mammalian heart.

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