

**GLYCEROLIPID METABOLISM IN MAMMALIAN TISSUES**

By

Douglas P. Lee

A thesis submitted to the Faculty of Graduate Studies  
in partial fulfillment of the requirements for the degree  
of Doctor of Philosophy

Department of Biochemistry and Medical Genetics

University of Manitoba

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**Glycerolipid Metabolism in Mammalian Tissues**

**BY**

**Douglas P. Lee**

**A Thesis/Practicum submitted to the Faculty of Graduate Studies of The University**

**of Manitoba in partial fulfillment of the requirements of the degree**

**of**

**Doctor of Philosophy**

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*To my family,  
Mom, Dad and Tracy*

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## TABLE OF CONTENTS

<b>LIST OF FIGURES</b>	vi
<b>LIST OF TABLES</b>	viii
<b>LIST OF ABBREVIATIONS</b>	ix
<b>ABSTRACT</b>	xi
<b>1. INTRODUCTION AND LITERATURE REVIEW</b>	<b>1</b>
<b>1.1 The Biological Membrane</b>	<b>1</b>
1.1.1 Structure and function of the biological membrane	1
1.1.2 Membrane lipid	4
1.1.3 Membrane protein	15
<b>1.2 The <i>de novo</i> Biosynthesis of Glycerolipids in Mammalian Tissues</b>	<b>16</b>
1.2.1 Introduction	16
1.2.2 1-Acyl- <i>sn</i> -glycerol-3-phosphate (lysophosphatidate)	17
1.2.3 Phosphatidate	21
1.2.4 Cytidine diphosphate 1,2-diacyl- <i>sn</i> -glycerol (CDP-diacylglycerol)	22
1.2.5 1,2-Diacyl- <i>sn</i> -glycerol	23
1.2.6 Phosphatidylcholine	25
1.2.6.1 CDP-choline pathway	28
1.2.6.2 Methylation of phosphatidylethanolamine	32
1.2.7 Phosphatidylethanolamine	34

1.2.7.1	CDP-ethanolamine pathway	36
1.2.7.2	Decarboxylation of phosphatidylserine	37
1.2.8	Triacylglycerol	38
1.2.9	Phosphatidylserine	39
<b>1.3</b>	<b>The Direct Acylation of Glycerol</b>	<b>40</b>
1.3.1	The direct acylation of glycerol	40
1.3.2	Monoacylglycerol	41
<b>1.4</b>	<b>The Catabolism of Glycerolipids in Mammalian Tissues</b>	<b>42</b>
1.4.1	Phospholipases	42
<b>1.5</b>	<b>Lands Pathway</b>	<b>45</b>
1.5.1	Phospholipid acyl composition	45
1.5.2	Deacylation / reacylation cycle	46
<b>1.6</b>	<b>Research Aims and Hypothesis</b>	<b>49</b>
<b>2</b>	<b>MATERIALS AND METHODS</b>	<b>51</b>
<b>2.1</b>	<b>Materials</b>	<b>51</b>
<b>2.2</b>	<b>Methods</b>	<b>53</b>
2.2.1	Cell culture	53
2.2.2	Determination of choline uptake and phosphatidylcholine biosynthesis	53
2.2.3	Choline kinase assay	54
2.2.4	CTP: phosphocholine cytidyltransferase assay	55

2.2.5	CDP-choline: diacylglycerol cholinephosphotransferase assay	55
2.2.6	Measurement of arachidonate release	56
2.2.7	Phospholipase A <sub>2</sub> assay	57
2.2.8	Preparation of subcellular fractions	57
2.2.9	Acyl-CoA: 1-acyl- <i>sn</i> -glycerol-3-phosphate acyltransferase assay	58
2.2.10	Glycerol: acyl-CoA acyltransferase assay	59
2.2.11	Glycerokinase assay	59
2.2.12	Pulse chase analysis	60
2.2.13	Separation of lipids	60
2.2.14	Lipid determination	61
2.2.15	Solubilization and purification of glycerol: acyl-CoA acyltransferase	61
2.2.16	Synthesis of [ <sup>125</sup> I] labeled 12-[(4-azidosalicyl)amino]-dodecanoyl- CoA	62
2.2.17	Photoaffinity labeling	65
2.2.18	Gel electrophoresis	65
2.2.19	Production of antibody	66
2.2.20	Immunoprecipitation of glycerol acyltransferase	67
2.2.21	Statistical analysis	67

<b>3</b>	<b>EXPERIMENTAL RESULTS</b>	<b>68</b>
<b>3.1</b>	<b>Studies on Phosphatidylcholine Metabolism in Human Endothelial Cells</b>	<b>68</b>
3.1.1	Effect of phosphocholine on phosphatidylcholine biosynthesis in human umbilical vein endothelial cells	68
3.1.2	Effect of phosphocholine on choline containing metabolites	70
3.1.3	Effect of phosphocholine on choline uptake	72
3.1.4	Effect of phosphocholine on ATP-induced arachidonate release	74
<b>3.2</b>	<b>Studies on the Deacylation / Reacylation cycle</b>	<b>77</b>
3.2.1	Gel filtration chromatography	77
3.2.2	Gel activity assay	78
<b>3.3</b>	<b>Studies on the Direct Acylation of Glycerol</b>	<b>80</b>
3.3.1	Confirmation of monoacylglycerol production	80
3.3.2	The direct acylation of glycerol	82
3.3.3	Inhibition of glycerokinase	85
3.3.4	Attenuated glycerol-3-phosphate pathway and monoacylglycerol production	85
3.3.5	Effect of glycerol concentration of the direct acylation pathway	88
3.3.6	Characterization of glycerol: acyl-CoA acyltransferase	91
3.3.7	Purification of glycerol: acyl-CoA acyltransferase	95
3.3.8	Photoaffinity labeling glycerol: acyl-CoA acyltransferase	99

3.3.9	Immunoprecipitation of glycerol: acyl-CoA acyltransferase	103
3.3.10	Sequence analysis	105
<b>4</b>	<b>DISCUSSION</b>	<b>107</b>
<b>4.1</b>	<b>Studies on the Modulation of Phosphatidylcholine by Phosphocholine</b>	<b>107</b>
<b>4.2</b>	<b>Studies on the Deacylation / Reacylation Cycle</b>	<b>110</b>
<b>4.3</b>	<b>Studies on the Direct Acylation Pathway</b>	<b>111</b>
<b>5</b>	<b>SUMMARY</b>	<b>116</b>
<b>6</b>	<b>REFERENCES</b>	<b>118</b>

## LIST OF FIGURES

Figure 1.	The fluid mosaic model of the eukaryotic plasma membrane	3
Figure 2.	General structure of a phosphoglyceride molecule	6
Figure 3.	Five major types of phospholipids found in mammalian tissues	7
Figure 4.	Structures of plasmanylcholine and plasmenylcholine	8
Figure 5.	Sphingosine, sphingomyelin and cerebroside	10
Figure 6.	Structure of cholesterol	11
Figure 7.	Pathways for the biosynthesis of phosphoglycerides	20
Figure 8.	Pathways for the biosynthesis of phosphatidylcholine	27
Figure 9.	Pathways for the biosynthesis of phosphatidylethanolamine	35
Figure 10.	Sites phospholipase action	44
Figure 11.	Deacylation / reacylation cycle	48
Figure 12.	Structure of ASD-CoA	64
Figure 13.	Effect of phosphocholine on phosphatidylcholine biosynthesis	69
Figure 14.	Effect of phosphocholine on choline uptake	73
Figure 15.	Effect of phosphocholine on ATP-induced arachidonate release	75
Figure 16.	Pulse-labeling and chase analysis on glycerol metabolism	84
Figure 17.	Effect of attenuated glycerol-3-phosphate pathway on glycerol metabolism	87
Figure 18.	Effect of exogenous glycerol concentration on the direct acylation pathway	90

Figure 19.	Effect of pH and temperature on glycerol acyltransferase activity	93
Figure 20.	Kinetic analysis of glycerol acyltransferase	94
Figure 21.	Gel filtration analysis of solubilized microsomes	97
Figure 22.	Identification of glycerol acyltransferase by photoaffinity labeling and gel electrophoresis	98
Figure 23.	Effect of azidosalicylamino-dodecanoyl-CoA on glycerol acyltransferase	101
Figure 24.	Immunoprecipitation of solubilized glycerol acyltransferase	104
Figure 25.	Amino acid sequence alignment of glycerol acyltransferase with myoglobin	106
Figure 26.	Effect of phosphocholine on phosphatidylcholine metabolism	109
Figure 27.	Pathways for glycerol metabolism	115

## LIST OF TABLES

Table 1.	Phospholipid composition of some mammalian tissues	13
Table 2.	Lipid composition of some biological membranes	14
Table 3.	Radioactivity of aqueous choline-containing metabolites	71
Table 4.	Effect of phosphocholine on phospholipase A <sub>2</sub> activity	76
Table 5.	Identification of monoacylglycerol	81
Table 6.	Glycerol acyltransferase activity in microsomal rat and porcine tissue	92
Table 7.	Detergent solubilization of glycerol acyltransferase	96
Table 8.	ASD-CoA photoinactivation of glycerol acyltransferase	102

## LIST OF ABBREVIATIONS

ADP	adenosine diphosphate
AMP	adenosine monophosphate
ASD	12-[(4-azidosalicyl)amino]-dodecanoic acid
ATP	adenosine triphosphate
CaLB	calcium binding
cDNA	complementary deoxyribonucleic acid
CDP-	cytidine diphospho-
CHAPS	3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate
CMP	cytosine monophosphate
CoA	coenzyme A
cPLA <sub>2</sub>	cytosolic phospholipase A <sub>2</sub>
CTP	cytosine triphosphate
Da	Dalton
dpm	disintegrations per minute
EDTA	ethylenediaminetetraacetic acid
FPLC	fast protein liquid chromatography
GTP	guanosine triphosphate
h	hour
IgG	immunoglobulin G
K <sub>m</sub>	Michaelis-Menten coefficient

min	minute(s)
mRNA	messenger ribonucleic acid
PAGE	polyacrylamide gel electrophoresis
PAP	phosphatidate phosphohydrolase
PKC	protein kinase C
SDS	sodium dodecyl sulfate
<i>sn</i>	stereospecifically numbered
TLC	thin-layer chromatography
Tris	tris (hydroxymethyl)aminomethane
$V_{\max}$	maximal velocity
vol	volume
v/v	volume per volume
w/v	weight per volume

## ABSTRACT

The biological membrane surrounds all cells and delineates intracellular compartments. Lipids form building blocks of the biological membrane, and phosphatidylcholine is the principle lipid in the mammalian membrane. In this study, phosphatidylcholine metabolism and the direct acylation of glycerol for lipid biosynthesis were investigated.

The inhibition of phosphatidylcholine biosynthesis by extracellular phosphocholine was studied in human umbilical vein endothelial cells. The activities of the enzymes in the CDP-choline pathway were not altered, but the intracellular phosphocholine pool was significantly reduced. The reduction was caused by competitive inhibition of choline uptake by phosphocholine. Phosphocholine also caused a limited stimulation of arachidonate release from phosphatidylcholine, and the release was potentiated by ATP. These studies clearly demonstrate that exogenous phosphocholine has the potential to modulate phosphatidylcholine metabolism in mammalian tissues.

The acylation of glycerol-*sn*-3-phosphate is regarded as the first committed step for glycerolipid biosynthesis. The direct acylation of glycerol in mammalian tissues has not been previously demonstrated. In this study, lipid biosynthesis in myoblast and hepatocyte cells was reassessed by conducting pulse-chase experiments with [1,3-<sup>3</sup>H] glycerol. The results suggested that a portion of labeled glycerol was directly acylated to form monoacylglycerol and subsequently diacylglycerol and triacylglycerol. This pathway became prominent when the glycerol-3-phosphate pathway was attenuated and when the exogenous glycerol

concentration was elevated. The present study indicates the existence of a novel lipid biosynthetic pathway that may be important during hyperglycerolemia produced in diabetes or other pathological conditions.

Glycerol: acyl-CoA acyltransferase, the enzyme which directly acylates glycerol is located in the microsomal fraction of tissue homogenate. It was identified as an 18 kDa protein after purification by FPLC gel filtration, photoaffinity labeling and gel electrophoresis. The purification was confirmed by immunoprecipitation studies, and sequence analysis of the protein identified the acyltransferase as similar to myoglobin. These studies suggest that myoglobin in the pig heart may be modified and subsequently translocated to the membrane where enzyme activity is then conferred.

# **1 INTRODUCTION AND LITERATURE REVIEW**

## **1.1 The Biological Membrane**

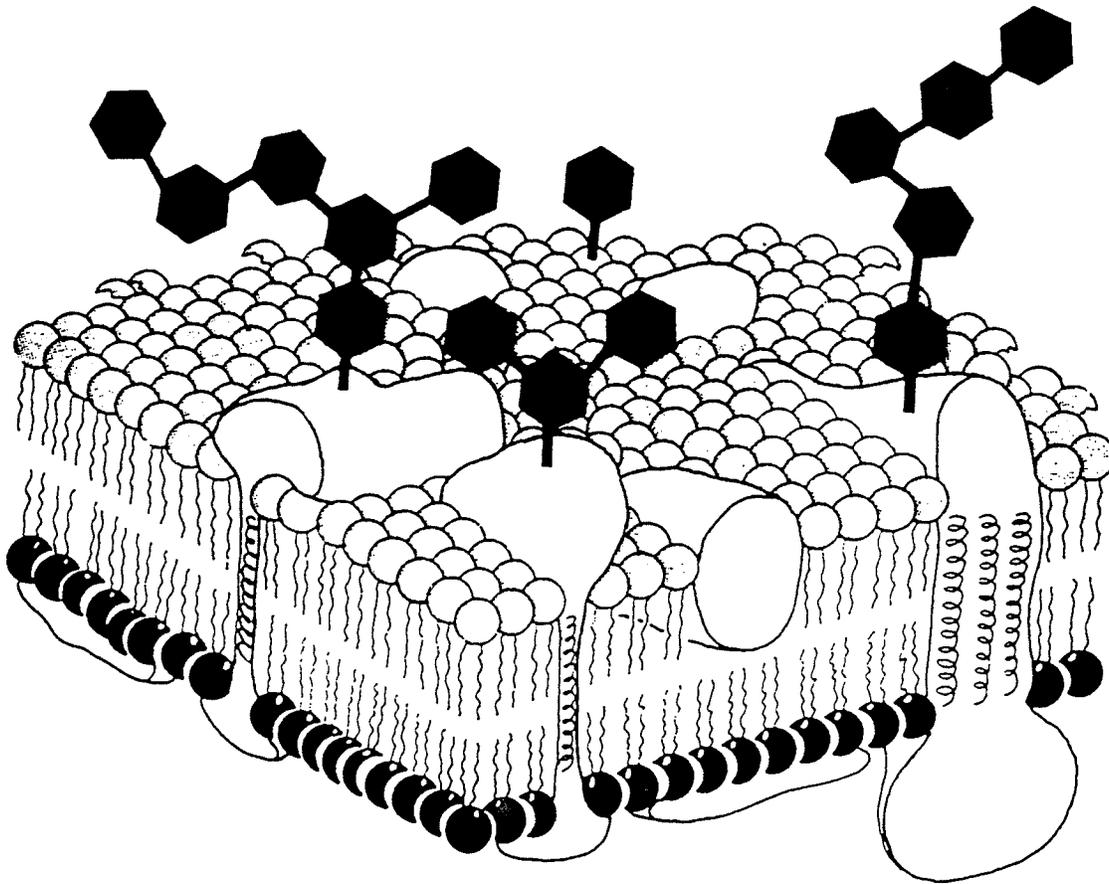
### **1.1.1 The structure of the biological membrane**

The biological membrane is essential to all cellular life (1). The function of the biological membrane is to provide a permeability barrier and also a matrix for the association of membrane protein. The plasma membrane is a biological membrane which surrounds all cells. It provides a selective semi-permeable barrier to regulate the transport of compounds such as nutrients, metabolic precursors and salts. The intracellular membrane delineates subcellular compartmentalization, and the enclosed organelles are vital to cellular processes. The Golgi body, endoplasmic reticulum, nuclear membrane, lysosomes and mitochondria are all delineated by membranes. The biological membrane also functions in the transmission of inter- and intra-cellular signals. The activation of a specific membrane protein or the hydrolysis of lipid, generate signals which mediate a wide variety of cellular responses (2).

The biological membrane is composed predominantly of lipid and protein but may also contain carbohydrate. The membrane is organized as a lipid bilayer with the membrane protein imbedded in the bilayer or associated peripherally (Figure 1). The structure of the biological membrane is described in the fluid mosaic model originally proposed by Singer and Nicolson (3). In this model, the lipid is arranged with the hydrophobic portion orientated toward the interior of the bilayer. The hydrophilic portion of the lipid is orientated toward

the aqueous phase. Membrane carbohydrate is localized exclusively on the extracellular side of the plasma membrane either in the form of glycolipid or glycoprotein. In isolation, the bilayer membrane exists either in a viscous gel or a fluid liquid crystalline state. Under physiological conditions, most if not all lipid membrane is in the liquid crystalline state. Fluidity of the membrane is dependent on the nature of the acyl-chain region comprising the hydrophobic domain of the membrane. In the plasma membrane, fluidity is also dependent on the cholesterol content of the membrane. Movement of lipid or membrane protein in the transverse plane of the bilayer is thermodynamically unfavorable and thus would not spontaneously occur. Movement of lipid or membrane protein along the plane of the membrane is generally unrestricted. The lipid raft is an area on the membrane where movement of either the lipid or membrane protein is laterally constrained allowing for specialization of areas on the lipid membrane (4-6). The lipid raft may be enriched in (glyco)sphingolipid, cholesterol, specific membrane proteins or glycosylphosphatidylinositol-anchored proteins.

In summary, lipid performs a variety of biological functions related to membrane structure, cellular signaling, fluidity and permeability. In the following sections, the structure, composition and interaction of membrane lipid and protein will be discussed.



**Figure 1.** The fluid mosaic model of the eukaryotic plasma membrane depicting lipid, protein and carbohydrate moieties.

### 1.1.2 Membrane lipid

The biological membrane contains a large variety of lipids which may be categorized according to their molecular structure. The glycerol-based phospholipid (phosphoglyceride) is the predominant form of lipid in the mammalian membrane. The sphingosine-based lipid which includes sphingomyelin and glycosphingolipid, is also a major constituent of the membrane along with cholesterol, which functions as a key modulator of membrane fluidity.

The general structure of a phosphoglyceride is shown in Figure 2. The *sn*-1 and *sn*-2 hydroxyl groups of the glycerol backbone are esterified with fatty acids. The 1,2-*sn*-diacylglycerophospholipid is the predominant form of phosphoglyceride found in mammalian tissues. The acyl chains constitute the lipoidal moiety and may vary in length and degree of unsaturation. Typically, the acyl chains are of medium and long chain lengths ( $C_{16}$ - $C_{20}$ ). The acyl group is usually saturated at the *sn*-1 position and unsaturated at the *sn*-2 position. The hydroxyl group of the glycerol moiety at the *sn*-3 position is linked to a phosphate group which in turn is linked to the hydroxyl group of choline, ethanolamine, inositol, serine, glycerol or phosphatidylglycerol. The linkage of these polar head-groups to the glycerol backbone of the lipoidal moiety forms the major phospholipids, e.g. phosphatidylcholine, phosphatidylethanolamine, phosphatidylinositol, phosphatidylserine, phosphatidylglycerol and cardiolipin (Figure 3).

Phospholipids are also classified according to the chemical linkages at the *sn*-1 position. The ether-linked phospholipids include 1-alkenyl-2-acyl-glycerophosphocholine (plasmerylcholine), 1-alkyl-2-acyl-glycerophosphocholine (plasmanylcholine) and their ethanolamine-containing analogues. The ether-linked phospholipid is a minor constituent of

most mammalian membranes but may be abundant in the mitochondria of certain electrically active tissues (7) and in circulating cells such as neutrophils and macrophages (8). The structures of plasmenylcholine and plasmanylncholine are depicted in Figure 4.

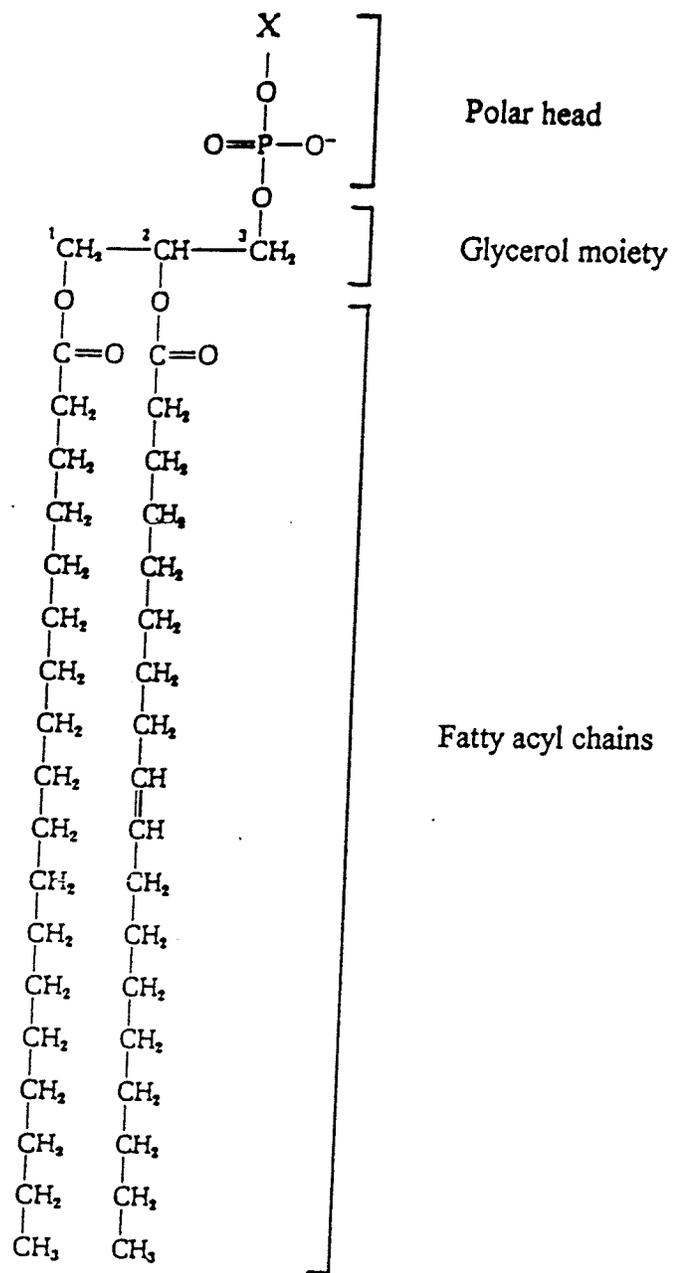
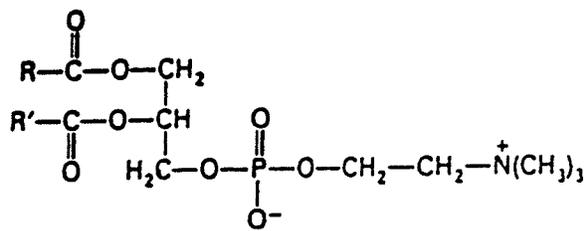
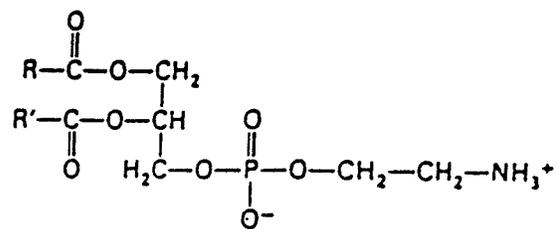


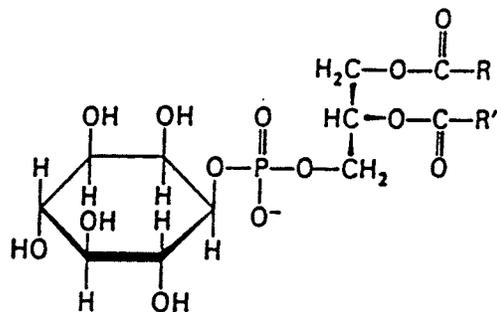
Figure 2. General structure of the phosphoglyceride molecule



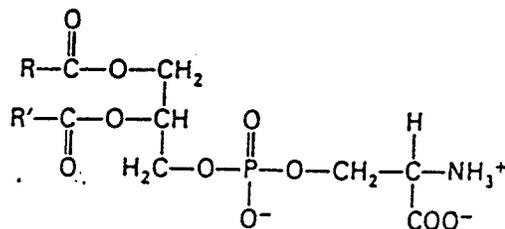
Phosphatidyl choline



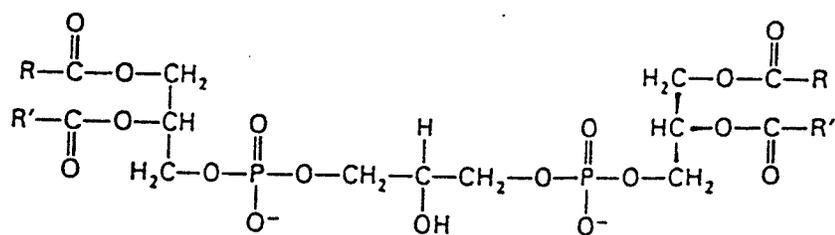
Phosphatidyl ethanolamine



Phosphatidyl inositol

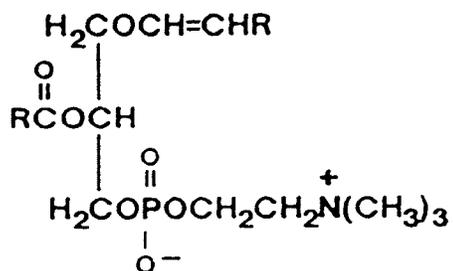


Phosphatidyl serine

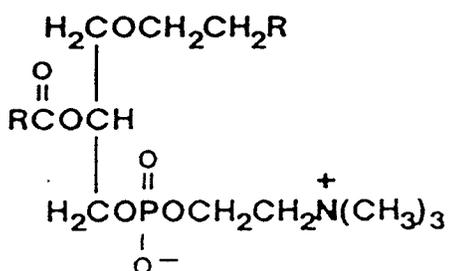


Diphosphatidyl glycerol  
(Cardiolipin)

Figure 3. Five major types of phospholipid found in mammalian tissues. R and R' are acyl groups.



1-alk-1'-enyl-2-acyl-*sn*-glycero-3-phosphocholine  
(plasmenylcholine or choline plasmalogen)



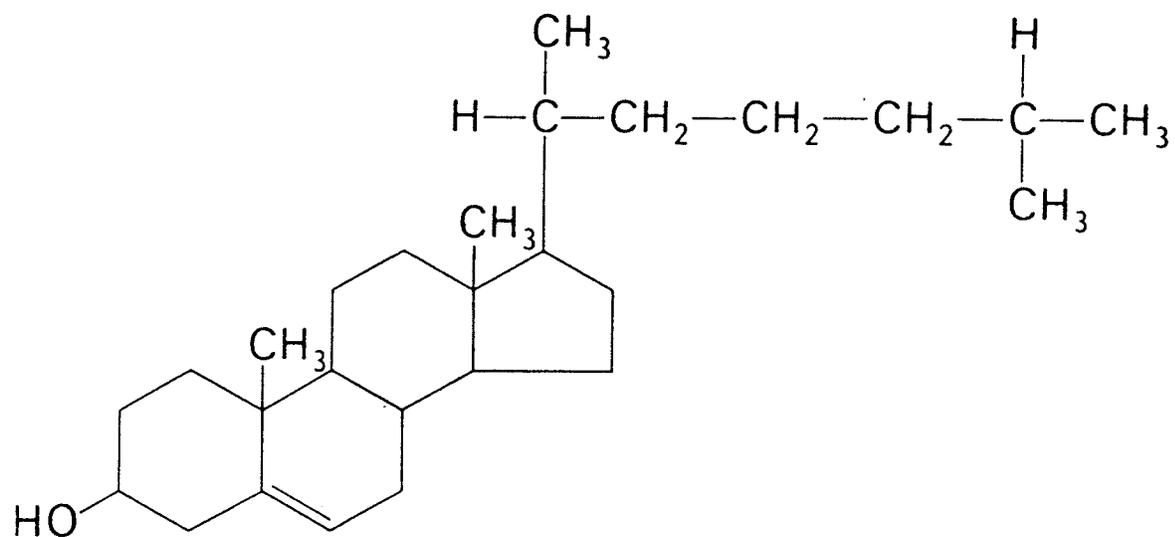
1-alkyl-2-acyl-*sn*-glycero-3-phosphocholine  
(plasmanylcholine)

**Figure 4. Structures of plasmenylcholine and plasmanylcholine.**

Sphingomyelin, a sphingosine-based phospholipid, is predominately found in the plasma membrane of mammalian cells. The terminal hydroxyl group of ceramide is linked to phosphocholine to form sphingomyelin. Ceramide is the N-acylated derivative of sphingosine and is structurally similar to the glycerol backbone of phosphoglyceride. A glycosphingolipid consists of a carbohydrate moiety linked to the terminal hydroxyl group of ceramide. In a cerebroside, the terminal hydroxyl group of ceramide is linked to a single glucosyl or galactosyl residue. More complex glycolipid known as ganglioside contains oligosaccharide chains with one or more residue of N-acetylneuraminic acid. Glycolipid is found on the extracellular leaflet of the plasma membrane and is involved in cellular recognition and adhesion (9). The structures of sphingosine, sphingomyelin and cerebroside are depicted in Figure 5.

Like sphingomyelin, cholesterol is primarily present in the plasma membrane of mammalian cells. The structure of a cholesterol molecule consists of four planar rings (Figure 6). Cholesterol is amphipathic with the C-3 hydroxyl group as the polar head group. The steroid nucleus and the hydrocarbon side chain at C-17 form the non-polar hydrocarbon body. Physical analysis of the membrane indicates that the fluidity and permeability of the membrane is influenced by the cholesterol content (10). In addition to its role as a membrane constituent, cholesterol also serves as a precursor for a variety of products with biological activities. A variety of steroid hormones such as estradiol and the D-vitamins are produced from cholesterol by removal or modification of the side chain at C-17 (10).





**Figure 6. Structure of cholesterol**

Lipid is not uniformly distributed amongst the various organelle membranes. Table 1 and Table 2 list the lipid composition of various mammalian tissues and biological membranes. Phosphatidylcholine and phosphatidylethanolamine are the most abundant phospholipids and appear to have the highest concentration in the mitochondria and the endoplasmic reticulum. Sphingomyelin is present in significant quantities in all the tissues examined but not in the mitochondria or endoplasmic reticulum. Cholesterol is predominately localized to the plasma membrane, although a small amount of cholesterol can be found in the mitochondrial, endoplasmic reticulum, and Golgi membranes (1). Cardiolipin is almost exclusively localized to the inner mitochondrial membrane (11). Studies on the lipid composition of the bilayer leaflets in the plasma membrane indicate an asymmetrical distribution of phospholipids (11). A general feature of the plasma membrane is that phosphatidylinositol, phosphatidylethanolamine and phosphatidylserine are limited to the cytosolic half of the bilayer. The outer layer is predominately composed of phosphatidylcholine, sphingomyelin and glycolipid.

In summary, the lipid composition of a biological membrane is distinct to a particular membrane system. However, the lipid composition of the membrane varies dramatically among different cells and even organelles. In addition, the lipid composition of the same membrane system in different species can also vary significantly. Nevertheless, it is clear that biological membranes are composed of an astonishing variety of lipids.

**Table 1. Phospholipid composition of some mammalian tissues**

Values for phospholipid composition are expressed as percentage of total phospholipid. Adapted from White (12). *n.d.*, not detected.

Tissue	Brain			Heart			Liver		
	Human	Rat	Cow	Human	Rat	Cow	Human	Rat	Cow
Phosphatidylcholine	30	37	30	40	36	42	44	51	56
Lysophosphatidylcholine	n.d.	n.d.	n.d.	4	0.6	n.d.	1	1	n.d.
Phosphatidylethanolamine	36	36	33	26	30	28	28	25	13
Phosphatidylinositol	3	3	6	6	4	4	9	7	8
Phosphatidylserine	18	12	17	3	3	2	3	3	4
Sphingomyelin	13	6	12	5	3	12	5	4	6
Cardiolipin	n.d.	2	0.7	9	11	9	4	5	4
Other	n.d.	n.d.	0.5	7	12	2	7	1	3

**Table 2. Lipid composition of some biological membranes**

Data are expressed as weight percent of total lipid. Adapted from Cullis and Hope (13).  
<sup>a</sup>Human sources; <sup>b</sup>Rat liver.

Lipid	Erythrocyte <sup>a</sup>	Myelin <sup>a</sup>	Mitochondria <sup>b</sup> (inner and outer membrane)	Endoplasmic reticulum <sup>b</sup>
Cholesterol	23	22	3	6
Phosphatidylethanolamine	18	15	35	17
Phosphatidylcholine	17	10	39	40
Sphingomyelin	18	8	-	5
Phosphatidylserine	7	9	2	5
Cardiolipin	-	-	21	-
Glycolipid	3	28	-	-
Others	13	8	-	27

### **1.1.3 Membrane protein**

Membrane proteins are classified into two categories. The peripheral or extrinsic membrane protein is associated with the hydrophilic part of the membrane that has limited interaction with the hydrophobic core of the lipid bilayer. The association of the peripheral protein with the membrane may occur by association with another membrane protein, electrostatic interaction or perhaps with the polar head group of acidic phospholipids. The peripheral protein can be removed from the membrane by treatment with salt (14). In the second category of membrane protein, the intrinsic or integral membrane protein is associated with the membrane by interaction with the hydrophobic core of the lipid bilayer. The integral membrane protein can be further categorized into simple or complex protein. The simple integral membrane protein spans the membrane through the hydrophobic core only once, whereas the complex integral membrane protein may pass the membrane multiple times. The integral membrane protein is hydrophobic in the region which spans the membrane and generally hydrophilic in the region which interacts with the aqueous environment. The majority of integral membrane proteins can be extracted from the native membrane by treatment with detergents such as Triton X-100 or Chaps (14).

## 1.2 The *de novo* Biosynthesis of Glycerolipids in Mammalian Tissues.

### 1.2.1 Introduction

Studies on lipid biosynthesis date back to the 1950s when most of the pathways were elucidated largely through work conducted in Eugene Kennedy's laboratory (15, 16). Since then, a considerable amount of knowledge has been gained on the enzymes which catalyze the lipid biosynthetic reactions and factors which regulate lipid biosynthesis. Most of the lipid biosynthetic enzymes are associated with the membrane, and consequently the initial studies were hampered by difficulties in purifying the enzymes. To compound this difficulty, kinetic analysis of the enzymes has not been straightforward since many of the substrates and products are insoluble in aqueous solutions. In the last few decades, in part due to advancement in technology and the wide availability of nucleotide and amino acid sequence information, great strides in the understanding of these enzymes at the molecular level have been made. In particular, the sequence information obtained from lipid biosynthetic enzymes purified from prokaryotes and yeast have provided the means to search the expressed sequence tag database for the mammalian homolog. Using this approach, several enzymes including acyl-CoA:1-acyl-*sn*-glycerol-3-phosphosphate acyltransferase (Section 1.2.4), CDP-choline: 1,2 diacylglycerol cholinephosphotransferase (Section 1.2.7.1) and CDP-diacylglycerol synthase (Section 1.2.5) have been cloned.

While a major function of the lipid is to form the building blocks of the biological membrane, a small population of lipids has been implicated as signaling molecules acting either as intracellular second messengers or as extracellular agonists that modulate cell

function (17, 18). It is speculated that within the cell, separate lipid pools exist for participation in various biological functions (19, 20). It is clear that the signaling and modulation of cell function by lipids are important physiological aspects of cellular function. A review on these topics however, is beyond the scope of this thesis. The following sections will instead focus on the *de novo* biosynthesis of phosphoglycerides in mammalian tissues.

### **1.2.2 1-Acyl-*sn*-glycerol-3-phosphate (lysophosphatidate)**

The acylation of glycerol-3-phosphate represents the first committed step in glycerolipid biosynthesis (Figure 7). Glycerol-*sn*-3-phosphate is synthesized from the phosphorylation of glycerol in a reaction catalyzed by glycerokinase (EC 2.7.1.30). Alternatively, glycerol-*sn*-3-phosphate is produced from the reduction of the glycolytic intermediate dihydroxyacetone-3-phosphate in a reaction catalyzed by dihydroxyacetone-3-phosphate dehydrogenase (EC 1.1.1.94). The acylation of glycerol-*sn*-3-phosphate is catalyzed by acyl-CoA: glycerol-*sn*-3-phosphate acyltransferase (glycerol-3-phosphate acyltransferase) (EC 2.3.1.15), resulting in the production of 1-acyl-*sn*-glycerol-3-phosphate (lysophosphatidate) (21). The enzyme exhibits the lowest specific activity of all enzymes in the glycerol-3-phosphate pathway suggesting that this step may be rate limiting (22, 23).

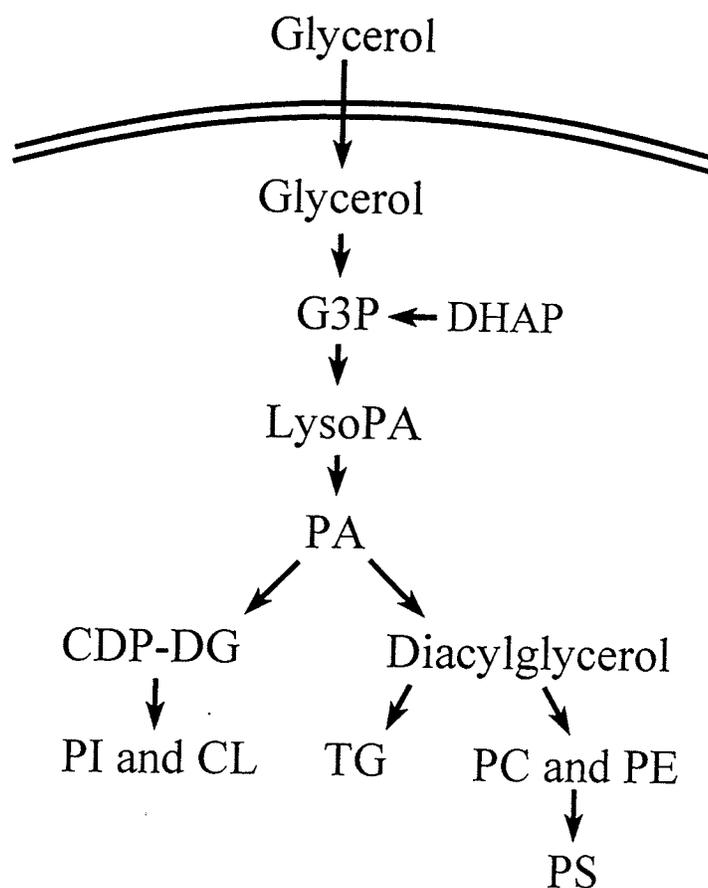
A partial purification of the acyltransferase from rat liver microsomes (24) and a full purification from rat liver mitochondria have been reported (25). Two isoenzymes have been identified based on differences in their pH optima,  $K_m$  values, sensitivity to heat and N-ethylmaleimide, subcellular localization and  $Mg^{+2}$  (26). The microsomal enzyme is N-ethylmaleimide sensitive and does not display any preference for saturated or unsaturated

acyl-CoAs. In contrast, the mitochondrial enzyme is insensitive to N-ethylmaleimide and exhibits substrate preference for saturated acyl-CoAs. Both acyltransferases may be regulated via a dephosphorylation / phosphorylation mechanism. A tyrosine kinase has been purified from adipose tissue which reversibly inactivated the microsomal acyltransferase (27). The mitochondrial acyltransferase was inactivated by an AMP-activated kinase (28).

Due to difficulties in reproducing the reported purification procedure (24), the amino acid sequence of the microsomal acyltransferase is not yet available. In contrast, the mitochondrial acyltransferase has been cloned from the mouse and rat liver (29, 30). Alignment of amino acid sequences from various acyltransferases has revealed several regions of strong homology. Several amino acid residues have been identified in the mitochondrial acyltransferase which are critical for catalysis (31). Studies on the expressed recombinant mitochondrial acyltransferase indicate that the enzyme is an integral membrane protein with two transmembrane domains. The N and C termini are orientated toward the inner surface of the mitochondrial outer membrane while the internal domain of the protein is exposed to the cytosol. Transcription of the mitochondrial acyltransferase is modulated during adipocyte differentiation (32, 33), by insulin treatment and in fasted mice re-fed with a high carbohydrate diet (29). Indirect evidence indicates that the majority of lysophosphatidate produced from the mitochondrial acyltransferase is used for cardiolipin biosynthesis although a small amount of lysophosphatidate can move to other cellular sites for conversion to other glycerolipids (34).

In an alternative pathway, lysophosphatidate is formed by the acylation of dihydroxyacetone-phosphate and reduction of the newly formed 1-acyl-dihydroxyacetone-3-

phosphate to lysophosphatidate (35). The acyl-CoA: dihydroxyacetone-3-phosphate acyltransferase (EC 2.3.1.42) activity is found in microsomes and peroxisomes. The microsomal activity is thought to arise from glycerol-3-phosphate acyltransferase since both glycerol-3-phosphate and dihydroxyacetone-3-phosphate are mutually competitive. In addition, the enzyme activities display similar pH optima, acyl-CoA specificity and sensitivity to heat, N-ethylmaleimide, trypsin and detergent (36). The peroxisomal enzyme activity is distinct from the microsomal enzyme activity and the former is required for plasmalogen biosynthesis (36). 1-Acyldihydroxyacetone-3-phosphate reductase (EC 1.1.1.101), which catalyzes the formation of lysophosphatidate, is enriched in peroxisomes but is also found in microsomes (37).



**Figure 7. Pathways for the biosynthesis of phosphoglycerides.** DHAP, dihydroxyacetone-3-phosphate; G3P, glycerol-3-phosphate; LysoPA, lysophosphatidate; PA, phosphatidate; CDP-DG, cytidine diphosphate-diacylglycerol; PI, phosphatidylinositol; CL, cardiolipin; TG, triacylglycerol; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PS, phosphatidylserine.

### 1.2.3 Phosphatidate

Phosphatidate is currently believed to occupy a central branch point in the lipid biosynthetic pathways (21). It is converted to CDP-diacylglycerol which serves as the precursor for the biosynthesis of the acidic phospholipids such as phosphatidylinositol and cardiolipin (Figure 7) (38). Alternatively, phosphatidate is dephosphorylated to produce diacylglycerol which is a precursor of triacylglycerol, phosphatidylcholine and phosphatidylethanolamine.

Phosphatidate is synthesized *de novo* from the acylation of lysophosphatidate in a reaction catalyzed by acyl-CoA: 1-acyl-glycerol-*sn*-3-phosphate acyltransferase (lysophosphatidate acyltransferase) (EC 2.3.1.51). The enzyme is located in the mitochondrial and microsomal fractions, but enzyme activity has also been detected in the plasma membrane (39). The LPAAT- $\alpha$  and LPAAT- $\beta$  cDNAs have been isolated from human brain and leukocyte cDNA libraries (40) and encoded for separate isoforms of lysophosphatidate acyltransferase. Lysophosphatidate- $\alpha$  acyltransferase mRNA has been detected in all tissues but with higher expression in immune cells, epithelium and skeletal muscle (41). Lysophosphatidate acyltransferase- $\beta$  mRNA was expressed in most human tissues but with higher expression in the heart, liver and pancreas (40, 42). From the ubiquitous expression of the lysophosphatidate acyltransferase in all tissues, it is clear that phosphatidate is an important intermediate in lipid biosynthesis. The role of the enzyme in signal transduction cannot be excluded since the over-expression of either isoenzyme is correlated with the enhancement of a cytokine-induced signaling response (40).

The LPAAT- $\alpha$  gene has been mapped to the class III region of the human major

histocompatibility complex in the chromosome band 6p21.3 (43). The expressed LPAAT- $\alpha$  displayed intermediate activity with arachidonyl-CoA ( $C_{20:4}$ ) and the highest activity with palmitoyl-CoA ( $C_{16:0}$ ). Studies using confocal immunofluorescence microscopy indicate that the isoenzyme is localized on the endoplasmic reticulum. In contrast, the LPAAT- $\beta$  cDNA obtained from a human heart library (42) has been mapped to chromosome 9, region q34.3. The expressed recombinant protein exhibited higher activity towards arachidonyl-CoA than stearoyl-CoA ( $C_{18:0}$ ) or palmitoyl-CoA (42).

#### **1.2.4 Cytidine diphosphate 1,2-diacyl-*sn*-glycerol (CDP-diacylglycerol)**

CDP-diacylglycerol is produced from phosphatidate and CTP in a reaction catalyzed by CDP-diacylglycerol synthase (EC 2.7.7.41). In mammalian tissues, CDP-diacylglycerol is produced as a precursor for phosphatidylinositol and cardiolipin synthesis (38). Since the concentration of CDP-diacylglycerol is much lower than phosphatidate, CDP-diacylglycerol synthase is thought to be the rate-limiting enzyme in phosphatidylinositol and cardiolipin synthesis (38). It appears that the phosphatidylinositol pool is tightly regulated, since the overexpression of CDP-diacylglycerol synthase and phosphatidylinositol synthase in COS-7 cells did not result in elevated phosphatidylinositol levels (44). Two isoforms of CDP-diacylglycerol synthase have been cloned from various sources (45, 46). Studies employing fluorescence *in situ* hybridization indicate that the genes encoding CDP-synthase, CDS1 and CDS2 are localized to chromosomes 4q21.1 and 20p13, respectively (47). Subcellular fractionation studies indicate that the majority of CDP-diacylglycerol synthase activity is localized in the endoplasmic reticulum, but activity has also been detected in the

mitochondrial, nuclear and plasma membranes (48, 49). It is postulated that the enzyme associated with the endoplasmic reticulum is for the synthesis of phosphatidylinositol whereas the mitochondrial enzyme is for the synthesis of cardiolipin (44).

Although CDP-diacylglycerol synthase has been purified and cloned from bacteria, yeast and *Drosophila*, purification of the enzyme from mammalian sources has not been achieved (50). Studies on the crude microsomal preparation indicate that the enzyme requires  $Mg^{2+}$  for activity and is stimulated by GTP. The enzyme is inhibited by CDP-diacylglycerol and inactivated by non-ionic detergents such as Triton X-100 (50). In contrast, the mitochondrial enzyme is not inactivated by non-ionic detergents nor stimulated by GTP. The two isoenzymes are separable by ion-exchange chromatography and display different kinetic properties. These studies indicate that the mitochondrial and microsomal CDP-diacylglycerol synthases are separate and distinct proteins.

*In vitro* studies of microsomal CDP-diacylglycerol synthase indicate that the enzyme has little or no selectivity for the acyl composition in phosphatidate. In contrast, structural studies on CDP-diacylglycerol isolated from mammalian tissues show an abundance of stearate at the *sn*-1 position and arachidonate at the *sn*-2 position (51). The mechanism for CDP-diacylglycerol to acquire its specific fatty acid composition is not clear.

### 1.2.5 1,2-Diacyl-*sn*-glycerol

Diacylglycerol is produced from phosphatidate in a reaction catalyzed by phosphatidate phosphatase (EC 3.1.3.4) (Figure 7). Diacylglycerol is also synthesized from the acylation of monoacylglycerol which occurs readily in the enterocytes of the small

intestine and liver (52). The reaction is catalyzed by acyl-CoA: monoacylglycerol acyltransferase (EC 2.3.1.22). The enzyme is also active in adipose tissue (53) and cardiomyocytes (54). Diacylglycerol is acylated to form triacylglycerol in a reaction catalyzed by diacylglycerol: acyl-CoA acyltransferase (EC 2.3.1.20). Alternatively, it is also the precursor for phosphatidylethanolamine and phosphatidylcholine biosynthesis. Diacylglycerol is also an important signal transduction molecule which activates protein kinase C. The signaling diacylglycerol is produced in a phospholipase C mediated hydrolysis of phosphatidylinositol-4,5-bisphosphate (55). Alternatively, the diacylglycerol may be produced in a sustained manner via the catabolism of phosphatidylcholine catalyzed by phospholipase D (EC 3.1.4.4) and phosphatidate phosphatase (17).

At least two types of phosphatidate phosphatases exist in mammalian tissues (56). The type I phosphatidate phosphatase is  $Mg^{2+}$ -dependent and inactivated by N-ethylmaleimide (56, 57). The enzyme translocates from the cytosol to the endoplasmic reticulum on stimulation by fatty acids or acyl-CoAs (58). It is stimulated by glucagon, glucocorticoid, cAMP, growth hormone and inhibited by insulin. The enzyme is displaced from the membrane by okadaic acid suggesting that it may be regulated by a protein kinase (58). Its regulation and subcellular localization indicate that the type I phosphatidate phosphatase is for triacylglycerol and phospholipid biosynthesis (58). At present, the microsomal phosphatidate phosphatase of mammalian cells has not yet been purified or identified at the molecular level.

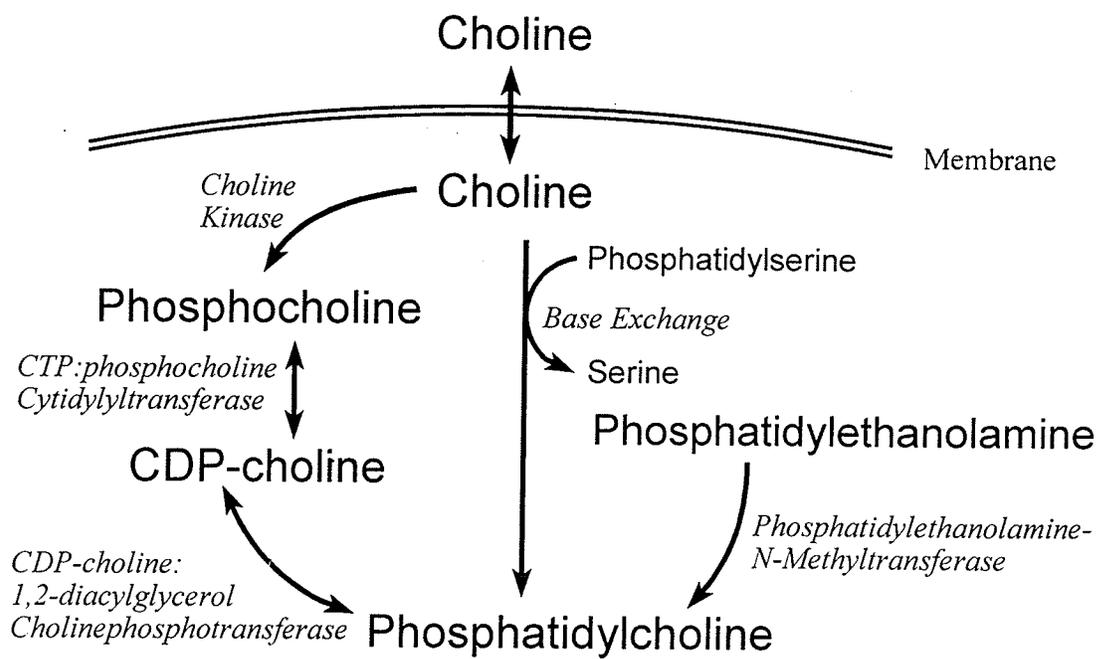
The reaction catalyzed by type II phosphatidate phosphatase is important in signal transduction (59). The type II phosphatidate phosphatase is localized to the plasma

membrane, not dependent on  $Mg^{2+}$  for activity and not inactivated by N-ethylmaleimide (60). Several investigators have reported on the purification and characterization of type II phosphatidate phosphatase (61-63). The enzyme is a 35 kDa glycoprotein (59) and also catalyzes the dephosphorylation of ceramide-1-phosphate, lysophosphatidate and sphingosine-1-phosphate (64). Sequence analysis of the purified type II phosphatidate phosphatase cDNA sequences indicates the existence of at least two isoenzymes in mammalian tissues (65). Expression of the enzyme in a human prostatic adenocarcinoma cell line has been shown to be modulated by androgens (66). Computer modeling predicts that the type II phosphatidate phosphatase is a channel-like integral membrane protein with six transmembrane domains (67). Surprisingly, the type II phosphatidate phosphatase sequence is highly homologous to proteins with alternate functions. The type II phosphatidate phosphatase shares 48.1% and 34.4% identity with *Drosophila* Wunen and rat Dri 42 proteins which participate in germ cell migration and epithelial differentiation, respectively (65).

### **1.2.6 Phosphatidylcholine**

Several metabolic pathways exist for phosphatidylcholine biosynthesis (Figure 8). The CDP-choline pathway was first described by Kennedy and co-workers (15, 16). In this pathway, choline is transported across the membrane into the cell via a choline transporter. Choline is then converted to phosphocholine by choline kinase (EC 2.7.1.32). Phosphocholine is subsequently converted to CDP-choline in a reaction catalyzed by CTP: phosphocholine cytidyltransferase (phosphocholine cytidyltransferase) (EC 2.7.7.15). The condensation of CDP-choline with diacylglycerol catalyzed by CDP-choline:

diacylglycerol cholinephosphotransferase (cholinephosphotransferase) (EC 2.7.8.2) produces phosphatidylcholine and CMP. A small amount of phosphatidylcholine can be produced through the base exchange pathway, but the process is generally considered as a minor pathway. In the liver, a significant amount of the phosphatidylcholine is synthesized by the progressive methylation of phosphatidylethanolamine. The transfer of methyl groups from S-adenosylmethionine is catalyzed by phosphatidylethanolamine N-methyltransferase (EC 2.1.1.17). The major pathways for phosphatidylcholine biosynthesis in mammalian tissues will be further examined in the following sections.



**Figure 8. Pathways for the biosynthesis of phosphatidylcholine**

### 1.2.6.1 CDP-choline pathway

The CDP-choline pathway is sometimes referred to as the Kennedy pathway or the *de novo* synthesis pathway. In 1955, Kennedy and coworkers demonstrated that CTP is an essential substrate for the incorporation of phosphocholine into phosphatidylcholine (15). They also demonstrated that CTP: phosphocholine cytidyldyltransferase catalyzes the formation of CDP-choline, and the enzyme is located in the cytosolic and microsomal fractions of guinea pig liver. In a separate report, Kennedy demonstrated that cholinephosphotransferase catalyzes the condensation of diacylglycerol with CDP-choline to form phosphatidylcholine (16).

Choline is an essential nutrient in the diet, and its absence may have profound effects on phosphatidylcholine biosynthesis (68, 69). In the heart, choline is taken up by a saturable mechanism with a  $K_m$  of 0.1 mM (70). Thus, it is possible that the plasma choline concentration (approximately 0.18mM) may provide a mechanism for the regulation of choline uptake in the heart. In other tissues, two types of choline transporters have been identified. One transporter exhibits a relatively high affinity for choline and requires  $\text{Na}^+$  for activity (71). This receptor is associated with the synthesis of acetylcholine in cholinergic synaptosomes. The other receptor is a low affinity,  $\text{Na}^+$ -independent receptor which transports choline into non-cholinergic cells. This receptor is responsible for transporting choline for subsequent phosphorylation by choline kinase and phosphatidylcholine synthesis (71).

Phosphocholine is synthesized from choline and ATP in a reaction catalyzed by choline kinase. The enzyme displays an absolute requirement for  $\text{Mg}^{2+}$ . In most instances,

the enzyme has been recovered exclusively in the high-speed supernatant of tissue or cell homogenates indicating that the enzyme is cytoplasmic in origin (72). In isolated cases, choline kinase activity has also been detected in membrane fractions (73, 74). The enzyme has been purified to homogeneity from numerous sources including the rat kidney, liver and brain (75, 76). In most cases, the purified enzyme also displays significant activity with ethanolamine (77). Immunological studies and chromatographic analysis of choline kinase indicate that the enzyme exists in multiple isoforms (78). This observation is supported by the discovery of several distinct cDNA clones coding for choline kinase (79, 80). Certain chemical carcinogens, growth factors and transfection of cells with the ras/raf oncogene have been shown to elevate choline kinase activity in the cell (77). From these studies, Kiss *et al.* suggest that phosphocholine produced from choline kinase may act as a second messenger in a process leading to the induction of DNA synthesis (81).

Phosphocholine is converted to CDP-choline by CTP: phosphocholine cytidylyltransferase. In the CDP-choline pathway, phosphocholine cytidylyltransferase is the rate-limiting enzyme in phosphatidylcholine biosynthesis. The enzyme is regulated transcriptionally (82, 83) and at the post-translational level. For example, the enzyme is regulated by protein phosphorylation (84), translocation to the membrane (85), alteration in membrane composition (86) and the rate of enzyme turnover (87). The enzyme has been purified to homogeneity from rat liver and cloned (88, 89). Three isoenzymes have been characterized and include  $\alpha$ -,  $\beta$ 1-, and  $\beta$ 2- phosphocholine cytidylyltransferase (90). The  $\alpha$  isoform contains a nuclear localization structure, a catalytic domain, a helical lipid binding domain and a phosphorylation domain. The  $\beta$ 1 and  $\beta$ 2 isoforms also contain the highly

homologous catalytic domain and the lipid binding domain. However, they lack the nuclear localization signal and differ at the C termini (91). The  $\beta$  isoforms are produced from the same gene by alternate splicing of the transcript.

The translocation of phosphocholine cytidyltransferase from the cytosol to the membrane activates the enzyme, and it is regarded as an important mechanism for regulation of phosphatidylcholine biosynthesis. Enzyme translocation is stimulated by choline deficiency (92), diacylglycerol, phorbol ester (93) and anionic lipid (94). Immunofluorescence and electron microscopy studies indicate that the bulk of phosphocholine cytidyltransferase  $\alpha$  is essentially nuclear in origin while some of the enzyme is localized on the endoplasmic reticulum (95). The  $\beta 1$  and  $\beta 2$  isoforms are exclusively confined onto the endoplasmic reticulum (91). The membrane binding domain of phosphocholine cytidyltransferase is an amphipathic helical structure which interacts with lipid and membrane. The domain exerts an inhibitory control over catalytic activity in the absence of lipid but becomes activating in the presence of lipid (96). The soluble pool of phosphocholine cytidyltransferase is highly phosphorylated, and dephosphorylation is concomitant with translocation and activation of the enzyme. It was hypothesized that translocation of phosphocholine cytidyltransferase is regulated by a proline-directed protein kinase (97). Subsequently, mutation analysis on the phosphorylation domain of the enzyme indicates that membrane association is not triggered by dephosphorylation (98). At present, the physiological significance of the phosphorylation domain is not clearly understood but may play a role in coordination of phosphocholine cytidyltransferase with the cell cycle and enzyme stability (99).

Currently, the notion that phosphocholine cytidyltransferase is activated upon translocation to the membrane has been challenged. In a study by Northwood *et al.*, cytidyltransferase redistributed from the nucleus to the endoplasmic reticulum in fibroblasts with the concomitant movement of enzyme activity (100). In addition, DeLong *et al.* reported that cytidyltransferase  $\alpha$  is localized exclusively to the nucleus, and shuttling between the nucleus and cytoplasm does not occur. Rather, previous observations of cytidyltransferase shuttling were due to the non-specific detection of the isoenzymes present in the various subcellular compartments (101). Further studies will be required to settle this controversy.

In the final step for the *de novo* formation of phosphatidylcholine, CDP-choline condenses with diacylglycerol to form CMP and phosphatidylcholine in a reaction catalyzed by cholinephosphotransferase. In this reaction,  $Mg^{2+}$  or  $Mn^{2+}$  are essential cofactors for maximal activity. The analysis of subcellular fractions with marker enzymes indicates that most of the enzyme activity resides on the endoplasmic reticulum (102). Enzyme activity has also been detected in the Golgi, mitochondrial and nuclear membrane fractions (103). Although phosphatidylcholine biosynthesis is predominately regulated by phosphocholine cytidyltransferase, cholinephosphotransferase may also become rate limiting. Phosphatidylcholine biosynthesis may be decreased by limited diacylglycerol availability (104) and by short-term fasting. The fasting causes argininosuccinate accumulation in the liver which in turn directly inhibits cholinephosphotransferase (105). Enzyme activity is also modulated by thyroid hormone (106), short-chain C6-ceramide (107), lysophosphatidylcholine and calcium concentration.

Although a homogenous purification of cholinephosphotransferase has not yet been obtained, the enzyme has been partially purified from the hamster heart (108). In an alternative approach, a 55 kDa protein band was labeled by the photoaffinity probe 3'(2')-O-(benzoyl)benzoyl [<sup>32</sup>P] CDP-choline. The labeled protein was subsequently identified as cholinephosphotransferase after gel electrophoresis and autoradiography (109).

Analysis of the human expressed sequence tag database for the yeast cholinephosphotransferase homolog has resulted in the cloning of two distinct cholinephosphotransferase genes. hCPT1 is positioned to chromosome 12q, and the gene product is specific for CDP-choline (110). Overexpression of hCPT1 in yeast devoid of their own cholinephosphotransferase reconstituted phosphatidylcholine biosynthesis. In contrast, hCEPT1 is found on chromosome 1, and its gene product utilizes both CDP-choline and CDP-ethanolamine for phosphatidylcholine and phosphatidylethanolamine synthesis (111). The choline / ethanolaminephosphotransferase is expressed ubiquitously in all tissues and utilizes a broad range of diacylglycerol (111). Kinetic studies on the expressed enzyme indicate an apparent  $K_m$  of 37  $\mu$ M and a  $V_{max}$  of 10.5 nmol/min/mg for CDP-choline and an apparent  $K_m$  of 101  $\mu$ M and a  $V_{max}$  of 4.35 nmol/min/mg for CDP-ethanolamine when dioleoylglycerol ( $C_{18:1}$ ) is used.

#### **1.2.6.2 Methylation of phosphatidylethanolamine**

Phosphatidylcholine is also synthesized in the liver via the progressive methylation of phosphatidylethanolamine in reactions catalyzed by phosphatidylethanolamine N-methyltransferase (Figure 8). The methylation of phosphatidylethanolamine accounts for 20-

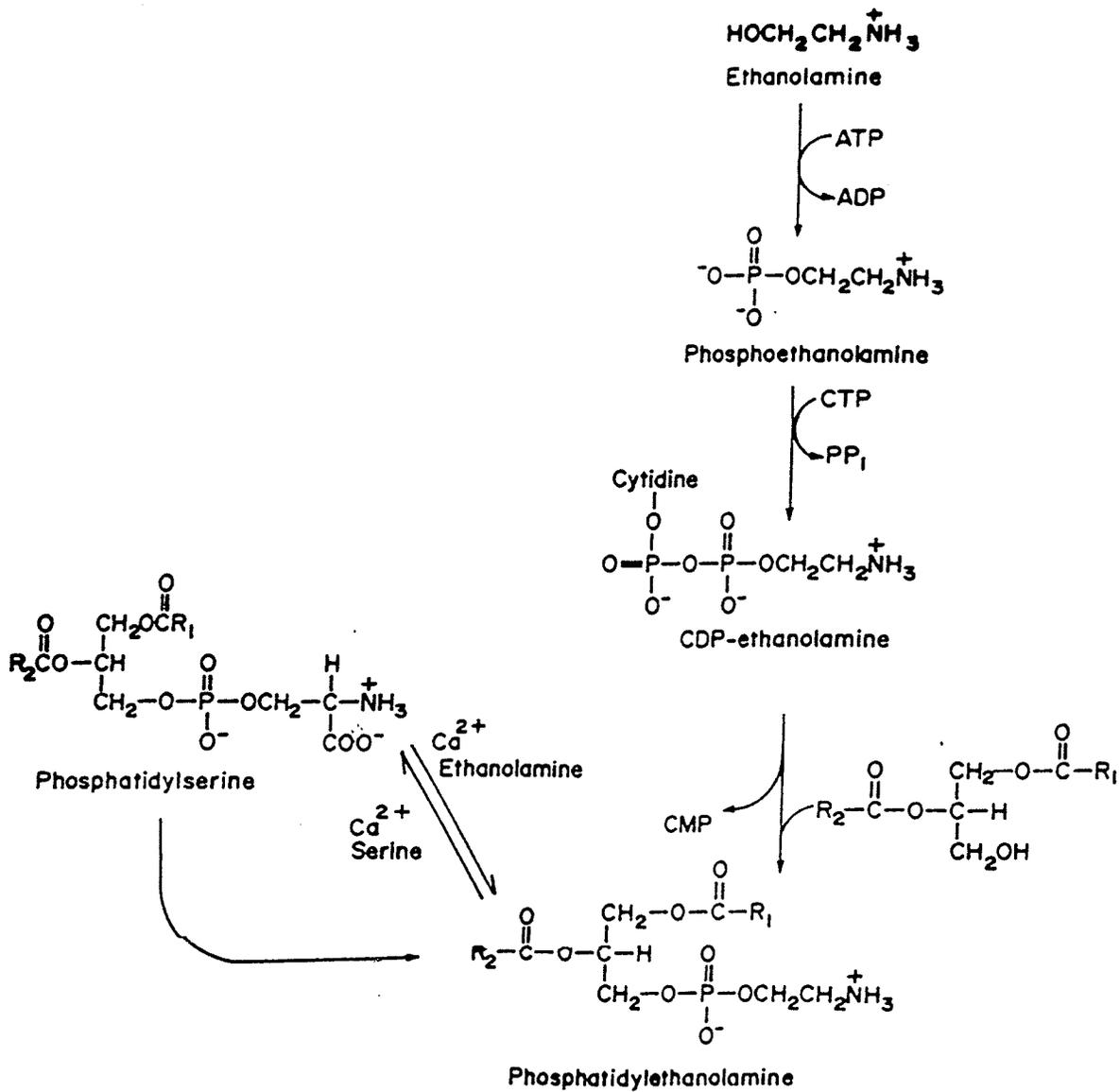
40% of phosphatidylcholine synthesized in the liver (112). The pathway is not essential for growth or development but is required for phosphatidylcholine synthesis when dietary choline is deficient (113). Expression of phosphatidylethanolamine methyltransferase in Chinese hamster ovary cells defective in *de novo* synthesis of phosphatidylcholine at the restrictive temperature failed to prevent cell death (114). It was postulated that insufficient phosphatidylcholine was produced by the methylation pathway to maintain the necessary phosphatidylcholine levels required for cellular replication (115). Phosphatidylcholine from the methylation pathway was found to contain a diverse array of long chain polyunsaturated species and a high amount of arachidonate. In contrast, newly formed phosphatidylcholine from the CDP-choline pathway contains mostly medium length saturated acyl chains (116). The molecular distinction of phosphatidylcholine produced from the methylation and CDP-choline pathways may reflect on functional differences of the two pathways in the liver. For example, an inverse relationship between phosphatidylethanolamine methyltransferase activity and cell proliferation has been well established (117, 118). Thus, it is speculated that phosphatidylethanolamine methyltransferase may function as a suppressor of hepatocyte growth and transformation (119).

Two isoforms of the enzyme, PEMT1 and PEMT2, have been characterized (120). PEMT1 is localized on the endoplasmic reticulum, and PEMT2 is localized on the endoplasmic reticulum-like membrane fraction that sediments with mitochondria after centrifugation (120). Knock out studies have demonstrated that both isoforms are encoded by the *Pempt* gene (113). Both isoforms catalyze the methylation of phosphatidylethanolamine, phosphatidylmonomethylethanolamine, and

phosphatidyl dimethylethanolamine.

### 1.2.7 Phosphatidylethanolamine

Phosphatidylethanolamine makes up about 20-30% of the total phospholipid content in most mammalian tissues. Most of the phosphatidylethanolamine is produced via the CDP-ethanolamine pathway (Figure 9) (121). In this pathway, ethanolamine is converted to phosphoethanolamine by ethanolamine kinase (EC 2.7.1.82). Phosphoethanolamine is subsequently converted to CDP-ethanolamine in a reaction catalyzed by CTP: phosphoethanolamine cytidylyltransferase (phosphoethanolamine cytidylyltransferase) (EC 2.7.7.14). The condensation of CDP-ethanolamine with diacylglycerol catalyzed by CDP-ethanolamine: diacylglycerol ethanolaminephosphotransferase (ethanolaminephosphotransferase) (EC 2.7.8.1) produces phosphatidylethanolamine. A small amount of phosphatidylethanolamine is synthesized via the base exchange pathway, but this process represents a minor pathway. Phosphatidylethanolamine is also produced from the decarboxylation of phosphatidylserine. The major pathways for phosphatidylethanolamine biosynthesis in mammalian tissues will be further examined in the following sections.



**Figure 9.** Pathways for the biosynthesis of phosphatidylethanolamine.  $R_1$  and  $R_2$  are acyl groups.

### 1.2.7.1 CDP-ethanolamine pathway

Ethanolamine is converted to phosphatidylethanolamine after three sequential reactions in a pathway similar to the CDP-choline pathway. Ethanolamine is phosphorylated to phosphoethanolamine in a reaction catalyzed by ethanolamine kinase. Although many choline kinases also contain ethanolamine kinase activity, several ethanolamine-specific kinases have been identified (122, 123). Two distinct ethanolamine kinases have been purified to homogeneity from the rat liver (124). Ethanolamine kinase I is a 36 kDa protein and displays no choline kinase activity. Ethanolamine kinase II is a 160 kDa protein and displays different kinetic properties from ethanolamine kinase I. The human cDNA for an ethanolamine-specific kinase has been recently obtained from searching the expressed sequence tag data base (125). The cDNA encodes a protein with a predicted molecular size of 49.7 kDa. Overexpression of the cDNA in COS-7 cells resulted in a 170-fold increase in ethanolamine specific enzyme activity.

Phosphoethanolamine is converted to CDP-ethanolamine in a reaction catalyzed by CTP: phosphoethanolamine cytidyltransferase. The conversion of phosphoethanolamine to CDP-ethanolamine is considered as the rate-limiting step of the pathway. The enzyme has been purified from rat liver, and extensive studies confirm that the phosphoethanolamine and phosphocholine cytidyltransferases are separate enzymes (126). In contrast to phosphocholine cytidyltransferase, phosphoethanolamine cytidyltransferase activity is not affected by phospholipid. In addition, the analysis of subcellular fractions with marker enzymes indicates that the enzyme is predominately localized in the cytosolic fraction (127). Immunogold electron microscopy studies revealed that phosphoethanolamine

cytidylyltransferase is actually distributed between the cisternae of the rough endoplasmic reticulum and the cytosol (126). Thus, phosphoethanolamine cytidylyltransferase activity may also be modulated via translocation between the two subcellular compartments. The enzyme however, does not contain the amphipathic  $\alpha$ -helical membrane binding domain found in phosphocholine cytidylyltransferase. Two putative phosphorylation sites on phosphoethanolamine cytidylyltransferase have been identified suggesting that the enzyme may be regulated by phosphorylation (128).

The phosphoethanolamine cytidylyltransferase cDNA has been cloned from rat liver and human glioblastoma cDNA libraries (128, 129). Phosphoethanolamine cytidylyltransferase contains a large repetitive internal sequence in the N- and C- terminal halves of the protein. Both repetitive sequences contain the HXGH motif, the most conserved region of the active domain of other cytidylyltransferases suggesting the existence of two catalytic sites in phosphoethanolamine cytidylyltransferase (128).

In the final step, CDP-ethanolamine is converted to phosphatidylethanolamine in a reaction catalyzed by ethanolaminephosphotransferase. The enzyme has been purified to homogeneity from bovine liver microsomes (130). Concurrent with the cloned choline / ethanolaminephosphotransferase (111), the purified enzyme also displays activity with CDP-choline. The cDNA open reading frame predicts a 46.6 kDa protein containing seven membrane-spanning domains.

#### **1.2.7.2 Decarboxylation of phosphatidylserine**

Phosphatidylethanolamine is also generated via the decarboxylation of

phosphatidylserine in a reaction catalyzed by phosphatidylserine decarboxylase (EC 4.1.1.65). The mammalian enzyme is localized to the outer leaflet of the inner mitochondrial membrane (131). A partial purification of the enzyme from rat liver has been described, although the yield was low, and the preparation was unstable (132). A phosphatidylserine decarboxylase between 60-70 kDa from the plasma membrane of rat liver has also been described (133). The cDNA for phosphatidylserine decarboxylase has been cloned by complementation of a Chinese hamster ovary cell line auxotrophic for phosphatidylserine (134). Transcriptional and translational studies indicate that the *pssC* gene product is converted into mature phosphatidylserine decarboxylase via multiple steps of post-translational processing (135).

### 1.2.8 Triacylglycerol

Diacylglycerol and acyl-CoA is converted to triacylglycerol and CoA by acyl-CoA: diacylglycerol acyltransferase (diacylglycerol acyltransferase) (EC 2.3.1.20). The regulation of diacylglycerol acyltransferase may be important in triacylglycerol biosynthesis during intestinal fat absorption, lipoprotein assembly, the regulation of plasma triacylglycerol concentrations, fat storage in adipocytes, energy metabolism in muscle and in milk production (36). Diacylglycerol acyltransferase activity is localized on the endoplasmic reticulum, and its activity is strongly dependent on  $Mg^{+2}$  concentration (136). The enzyme has been purified to homogeneity and identified as a 60 kDa protein although no amino acid sequence information was reported (137). In a homology search of the expressed sequence tag database using acyl CoA: cholesterol acyltransferase (EC 2.3.1.26), a clone was identified

homologous to the C terminus of the cholesterol acyltransferase (138). Expression of the cDNA did not result in cholesterol acyltransferase production. Rather, the cDNA encodes for an enzyme with acyltransferase activity specific for diacylglycerol. The gene was expressed in all tissues examined, and expression correlated with the differentiation of NIH 2T2-L1 cells into adipocytes (138).

### 1.2.9 Phosphatidylserine

Phosphatidylserine is a major phospholipid in the mammalian plasma membrane making up 5-10% of the total phospholipid pool. As previously mentioned, phosphatidylserine is also an intermediate in the biosynthesis of phosphatidylethanolamine. In mammalian tissues, phosphatidylserine is synthesized in mammalian cells via the base-exchange reactions (139). Phosphatidylserine synthase I (EC 2.7.8.8) uses phosphatidylcholine as a substrate for exchange with serine. Phosphatidylserine synthase II uses phosphatidylethanolamine as the phosphatidyl donor for exchange with L-serine to produce phosphatidylserine. These reactions are energy-independent, require  $\text{Ca}^{2+}$ , and have a slightly alkaline pH optimum. Enzyme activity is detectable in mitochondria (140), plasma membrane (141), microsomes (142) and nuclei (143). Kinetic studies and purification of the enzymes indicate that separate enzymes exist for choline, ethanolamine and serine base-exchange activities. The cDNAs encoding for the enzymes have been obtained in a complementation study with phosphatidylserine auxotrophic Chinese hamster ovary cells (144).

### 1.3 The Direct Acylation of Glycerol

#### 1.3.1 The direct acylation of glycerol

The biosynthesis of monoacylglycerol was studied by Kinsella who examined the incorporation of [ $^{14}\text{C}$ ] glycerol into the lipid fraction of bovine mammary cells (145). After the cells were incubated with radiolabeled glycerol, the specific radioactivities of monoacylglycerol, diacylglycerol, triacylglycerol and other various lipids were determined. More than any other lipid, the monoacylglycerol pool was found to have the highest specific radioactivity immediately after pulse-labeling. The labeling profiles clearly indicated monoacylglycerol as an early intermediate in lipid biosynthesis. Since *de novo* monoacylglycerol biosynthesis had not been previously established, the production of monoacylglycerol was instead attributed to the catabolism of the labeled diacylglycerol or lysophosphatidate. Remarkably, little is known about the *in vivo* direct acylation of glycerol.

The acylation of glycerol-3-phosphate has been comprehensively studied (146, 147). To a lesser extent, the direct acylation of glycerol has also been documented (148, 149). Glycerol was found to compete with water as a nucleophile at the active site of the enzyme, resulting in the formation of monoacylglycerol. Monoacylglycerol is also produced from glycerol by a 60 kDa lysophospholipase-transacylase with lysophosphatidylcholine as the acyl donor (149). In each case, a high concentration of glycerol is required to detect product formation. It has been documented that glycerol, ethanol, and several other alcohols form esters with [ $^{14}\text{C}$ ]-palmitate in the presence of adipose tissue microsomes (150). An 85 kDa recombinant phospholipase  $A_2$  was also reported to transfer an acyl group from

phosphatidylcholine to glycerol.

### **1.3.2 Monoacylglycerol**

Monoacylglycerol in mammalian tissues is relatively low in abundance. The role of monoacylglycerol in lipid biosynthesis and in signaling is not fully understood. In enterocytes, the absorbed monoacylglycerol is derived from the hydrolysis of dietary glycerolipids. In hepatocytes, cardiomyocytes and adipocytes, the monoacylglycerol is thought to be derived from the hydrolysis of triacylglycerol (54). In Swiss 3T3 cells, monoacylglycerol is preferentially incorporated into phosphatidylinositol (151).

2-Arachidonylglycerol has recently been identified as the endocannabinoid which binds to the cannabinoid receptors found in nervous tissues and in cells of the immune system (152). The cannabinoid system has been implicated in the regulation of cellular homeostasis (153), vascular health (154) and the immune system (155). It is speculated that the cannabinoid system represents one element of a neurotransmitter system which controls neuronal excitability (156). The importance of this system however is not clear due to a lack of direct evidence for the synthesis, release and effects of endocannabinoids at the junction between nerve cells. Indirect evidence indicates 2-arachidonylglycerol is produced via the catabolism of diarachidonyl-phosphatidylcholine or phosphatidic acid (157, 158).

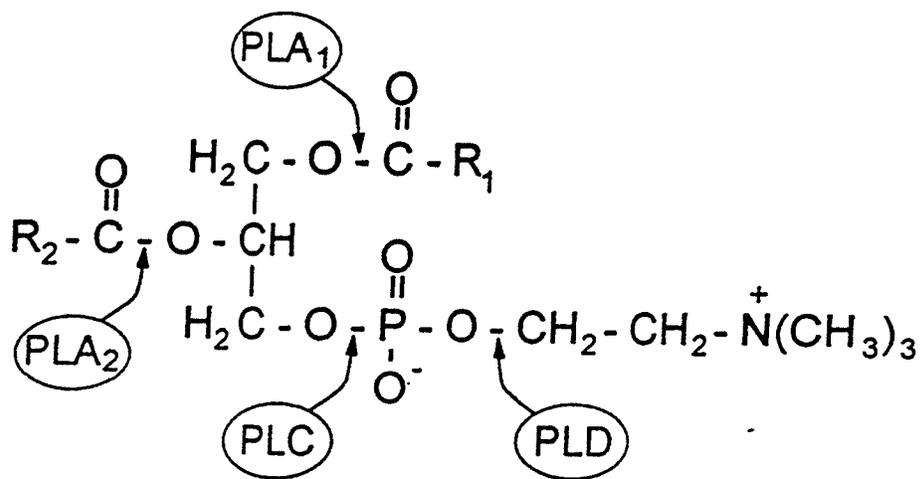
## 1.4 The Catabolism of Phospholipids

### 1.4.1 Phospholipases

Phospholipid is catabolized through the action of various phospholipases. The different types of phospholipases are categorized according to the specific phospholipid bond it hydrolyzes. Phospholipase A<sub>1</sub> (EC 3.1.1.3.2) specifically cleaves the acyl group at the *sn*-1 position, while cleavage of the acyl group at the *sn*-2 position is catalyzed by phospholipase A<sub>2</sub> (EC 3.1.1.4). Phospholipase C (EC 3.1.4.3) and phospholipase D (EC 3.1.4.4) are responsible for cleavage at the phosphate group (Figure 10).

In mammalian cells, the cytosolic phospholipase A<sub>2</sub> (cPLA<sub>2</sub>) has been extensively studied because it represents an attractive therapeutic target. The cPLA<sub>2</sub> preferentially cleaves phosphatidylcholine containing arachidonate at the *sn*-2 position and is a key mediator in arachidonate release. Arachidonate is a precursor of a wide spectrum of pro-inflammatory mediators including prostaglandins, thromboxanes and leukotrienes (159). The enzyme is activated by micromolar amounts of Ca<sup>2+</sup> (160) which causes its translocation to membrane fractions such as Golgi, endoplasmic reticulum and nuclear membranes (161). cPLA<sub>2</sub> is also modulated by receptor activation in response to a wide variety of stimuli including norepinephrine (162), bradykinin (163), cytokine (164) and epidermal growth factor (165). cPLA<sub>2</sub> has been purified and cloned from several sources (166, 167). The nucleotide sequence predicts a protein with a molecular weight of 85.2 kDa, and the gene is expressed in all tissues (168). Structural studies of cPLA<sub>2</sub> have shown that the protein contains a regulatory calcium binding domain (CaLB) at the N-terminus and a catalytic

domain at the C-terminus. The CaLB domain displays a high affinity for  $\text{Ca}^{2+}$  and localizes cPLA<sub>2</sub> to membrane structures (169). The cPLA<sub>2</sub> sequence also contains several consensus phosphorylation sites for both serine / threonine and tyrosine kinases (170). The activation of protein kinase C (171), mitogen activated protein kinase (162) and extracellular signal-regulated kinase 2 (p42 mitogen activated protein kinase) (172) has been correlated with the activation of cPLA<sub>2</sub>. However, the involvement of the protein kinases in cPLA<sub>2</sub> activation is cell and stimuli specific (173).



**Figure 10. Sites of phospholipase action**

## 1.5 Lands Pathway

### 1.5.1 Phospholipid acyl composition

Structural studies of phospholipid molecules in mammalian tissues indicate that the acyl content is highly specific in terms of chain length, position and saturation. As previously mentioned, the acyl moiety esterified to the glycerol backbone is usually saturated at the *sn*-1 position and unsaturated at the *sn*-2 position. For example, in rat liver phosphatidylcholine, 38.4% of the acyl moieties at the *sn*-1 position is occupied by stearate (C<sub>18:0</sub>), and 50% at the *sn*-2 position by arachidonate (C<sub>20:4</sub>) (174). The composition of acyl groups in phospholipids strongly influences the physical properties of membranes. In turn, the fluidity, permeability or bilayer thickness of the membrane may modulate the activities of membrane proteins (175).

As previously discussed, the lipoidal moiety of phosphatidylcholine is synthesized via the glycerol-3-phosphate pathway. Phosphatidate is produced by the sequential action of glycerol-3-phosphate and lysophosphatidate acyltransferases. *In vivo* and *in vitro* studies demonstrated that unlike the endogenous phospholipid pool, newly formed phosphatidate contains predominately monoenoic and dienoic acyl species. It is apparent that cholinephosphotransferase has limited selectivity for 1,2-diacylglycerol substrates with specific acyl composition (111). In addition, the acyl composition of newly formed phosphatidylcholine is similar to the diacylglycerol produced from phosphatidate (176). Rather, the proper acyl composition of phospholipids is acquired in a remodeling process after *de novo* synthesis (177).

### 1.5.2 Deacylation / reacylation cycle

The phospholipid acyl moieties are remodeled in a deacylation / reacylation process sometimes referred to as the Lands pathway (178). For example, phosphatidylcholine is converted to lysophosphatidylcholine by phospholipase A (Figure 11). Subsequently, lysophosphatidylcholine is reacylated back to phosphatidylcholine with the proper acyl chain by acyl-CoA: 1-acyl-*sn*-glycerol-3-phosphocholine acyltransferase (lysophosphatidylcholine acyltransferase) (EC 2.3.1.23). Acyl-CoA: 1-acyl-*sn*-glycerol-3-phosphocholine acyltransferase highly prefers unsaturated acyl-CoA, and acyl-CoA: 2-acyl-*sn*-glycerol-3-phosphocholine acyltransferase highly prefers saturated acyl-CoA (179, 180). Lysophosphatidylcholine acyltransferase has been detected in all mammalian tissues studied (181). The enzyme is localized predominately to the microsomal membrane (182), but activity can also be found in mitochondria and plasma membrane (183, 184). Alternatively, lysophosphatidylcholine can be further catabolized to glycerophosphocholine.

Major advances in delineating the mechanisms which govern phospholipid remodeling and maintenance of the acyl composition have been hindered by difficulties in purification of lysolipid acyltransferases. The difficulty occurs because extrication of the enzyme from its lipid membrane environment leads to irreversible inactivation of the enzyme. The difficulty is compounded by the fact that acyltransferase activity is also inhibited by even low amounts of detergents. Since the solubilization of the enzyme from the membrane in an active form is paramount to its subsequent purification by chromatography, a complete reassessment of detergents for solubilization of lysophosphatidylcholine acyltransferase from microsomal membranes has been conducted

(182). It was found that treatment of microsomes with 1% Chaps and 0.1M KCl results in the solubilization of 25-30% of total enzyme activity.

The purification of lysophosphatidylcholine acyltransferase from the bovine brain and heart has been reported (185, 186). The molecular weight of the proteins were found to be 43 and 64 kDa respectively. The enzyme utilizes a variety of unsaturated long chain acyl-CoA but is specific for arachidonyl-CoA. The enzyme prefers lysophosphatidylcholine, but also uses lysophosphatidylethanolamine, lysophosphatidylserine or lysophosphatidylinositol as the acyl acceptor. In a separate study, lysophosphatidylcholine acyltransferase has also been identified as a 21 kDa protein band by solubilization with high concentrations of oleoyl-CoA and lysophosphatidylcholine (187). The acyltransferase converted oleoyl-CoA and lysophosphatidylcholine into vesicular phosphatidylcholine. The newly formed vesicles contained a substantial amount of enzyme and was subsequently isolated by density gradient centrifugation. The recovered acyltransferase displayed preference for unsaturated acyl-CoAs and lysophosphatidylcholine.

Due to difficulties in reproducing the original purification protocol reported over a decade ago, no further work has been reported on the purified enzymes. Hence, information on the amino acid sequence of the enzyme is not available and attempts to obtain the cDNA have not been reported.

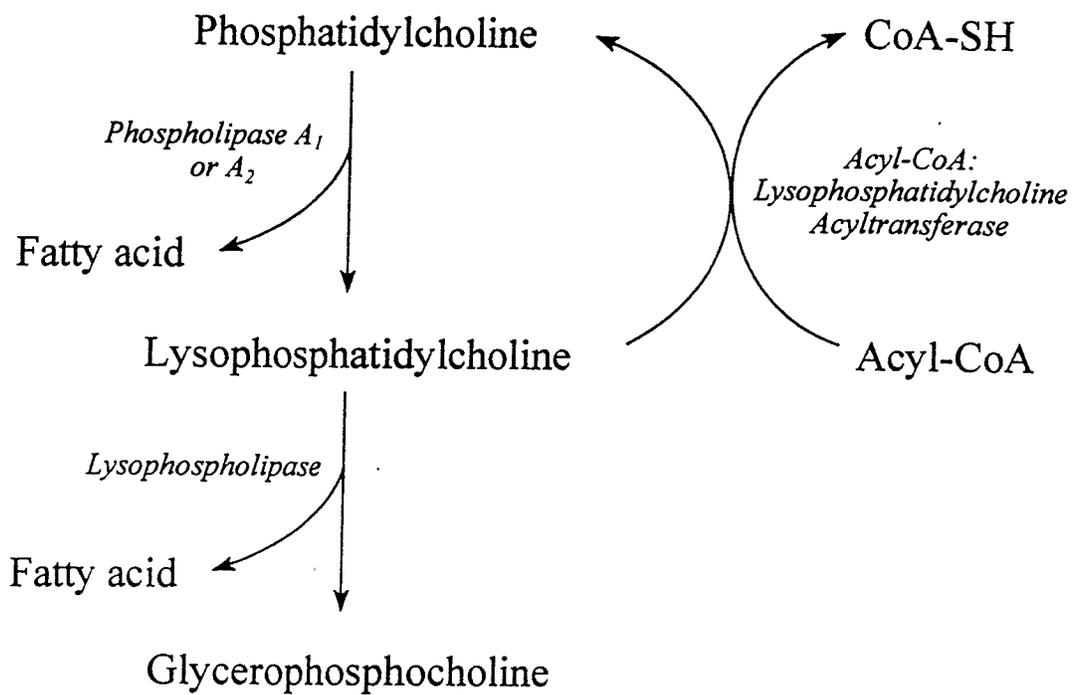


Figure 11. Deacylation / reacylation cycle

## 1.6 Research Aims and Hypothesis

Lipids form the building blocks of the biological membrane, and phosphatidylcholine is the principle lipid in mammalian tissues. The purpose of this research was to study the control of lipid metabolism in mammalian tissues. More specifically, the regulation of phosphatidylcholine metabolism, the deacylation / reacylation cycle and the direct acylation of glycerol were examined.

In the first part of the study, the control of phosphatidylcholine metabolism was examined in human umbilical vein endothelial cells. We hypothesized that phosphocholine, a phosphatidylcholine precursor could modulate phosphatidylcholine metabolism. In order to test this hypothesis, cells were treated with and without phosphocholine and incubated with radioactive choline. After cellular disruption and isolation of the choline-containing compounds, the radioactivities of the metabolites were determined. Activities of the enzymes in the CDP-choline pathway were also determined. The effect of phosphocholine on phosphatidylcholine catabolism was studied by measuring arachidonate release and determining cPLA<sub>2</sub> activity.

In the second part of the study, the deacylation / reacylation cycle of phosphatidylcholine was examined. Although purification of lysophosphatidylcholine acyltransferase was reported over a decade ago, further work on the purified enzyme has not progressed due to difficulties in reproducing the purification of the enzyme. We hypothesized that multiple acyltransferase isozymes exist, and that each isozyme is specific for a defined lysolipid and acyl group. Since direct evidence for the existence of acyltransferase isozymes

must come from studies with the purified enzyme, our initial attempt was to solubilize the enzyme from pig heart microsomes. Subsequently, column chromatography and gel activity assays were employed to purify the enzyme.

In the final part of the study, the direct acylation of glycerol was examined. Preliminary studies indicated that the direct acylation of glycerol was catalyzed by microsomal acyl-CoA: glycerol acyltransferase. We hypothesized that glycerol could be directly acylated for lipid biosynthesis. To test this hypothesis, pulse-chase studies were conducted with hepatocyte or myoblast cells using radiolabeled glycerol. The direct acylation of glycerol was further examined in pulse-chase experiments by varying the extracellular glycerol concentration or attenuating the glycerol-3-phosphate pathway. Studies were conducted to determine substrate specificity, pH and temperature optima of acyl-CoA: glycerol acyltransferase. Kinetic studies were conducted on the glycerol acyltransferase to determine the  $K_m$  and  $V_{max}$  values. FPLC gel filtration photoaffinity labeling and gel electrophoresis were employed to purify the enzyme. Immunological studies were performed to verify the purification, and amino acid sequencing was conducted to identify the enzyme.

The results of these studies enable us to better understand the control of glycerolipid metabolism in mammalian tissues.

## 2 MATERIALS AND METHODS

### 2.1 Materials

All lipid standards were obtained from Serdary Research Laboratories (London, Ontario, Canada). Thin-layer chromatography plates, (K6 Silica gel 60A) and DE81 (2.5cm) filter discs were purchased from Whatman Inc. (Clifton, New Jersey, USA). [*Methyl*-<sup>3</sup>H] choline (80.0 mCi/mmol) and CDP-[*Methyl*-<sup>14</sup>C] choline (42.2 mCi/mmol) were obtained from Dupont Canada Limited (Mississauga, ON, Canada). Phospho-[*Methyl*-<sup>3</sup>H] choline was synthesized from [*Methyl*-<sup>3</sup>H] choline as previously described (70). The radiolabeled compounds [5,6,8,11,12,14,15-<sup>3</sup>H(N)] arachidonate (230.5 Ci/mmol), [1-<sup>14</sup>C] arachidonoyl-CoA (51.6mCi/mmole), [Na<sup>125</sup>I] (16.5Ci/mg) and [1,3-<sup>3</sup>H] glycerol (3.5Ci/mmol) were obtained from PerkinElmer Life Sciences Canada (Woodbridge, ON, Canada). Radiolabeled 1-stearoyl-2-[1-<sup>14</sup>C] arachidonoyl-L-3-phosphatidylcholine (55 mCi/mmol) was purchased from Amersham International (Amersham, UK). Type I collagenase was obtained from Worthington Biochemical Corp. (Freehold, NJ, USA). Endothelial cell growth supplement was obtained from Collaborative Biomedical Products (Bedford, MA, USA). Protease inhibitor cocktail tablets were purchased from Roche Diagnostics Corporation (Indianapolis, IN, USA). The BCl<sub>3</sub>-methanol kit was purchased from Supelco Inc. (Bellefonte, PA, USA). All other chemicals were of analytical grade and obtained from Sigma Chemical Company (St. Louis, MO., USA). The HiTrap affinity column was obtained from Pharmacia Biotech (NJ, USA). PrepSep C<sub>18</sub> disposable extraction columns were purchased from Fisher

Scientific (Fair Lawn, NJ, USA). BCA protein assay reagents and ImmunoPure® Immobilized Protein A were purchased from Pierce (Rockford, IL, USA). Millex®-GP syringe driven filter units were purchased from Millipore Corporation (Bedford, MA, USA). The electrophoresis system and polyvinylidene difluoride membrane were obtained from Bio-Rad Laboratories Ltd. (Mississauga, ON, Canada). The FPLC system was obtained from Amersham Pharmacia Biotec, Inc. (Baie d'Urfe, QC, Canada).

## **2.2 Methods**

### **2.2.1 Cell Culture**

Endothelial cells were harvested from human umbilical vein using Type I collagenase as previously described (188). The cells were grown in flasks or culture dishes pre-treated with 0.2% gelatin, in Medium 199 (pH 7.4) supplemented with 25 mM HEPES, 30 µg/mL endothelial cell growth supplement, 90 µg/mL heparin, 10% fetal calf serum, 100 units/mL penicillin, 100 µg/mL streptomycin and 1.25 µg/mL Fungizone. The cells were incubated at 37°C in an atmosphere of 95% humidified air, 5% carbon dioxide until 70-80% confluency was achieved. Cells from the third passage grown in 60 mm culture dishes (approximately  $2.8 \times 10^6$  cells) were used for experiments.

H9c2 cells, a rat myoblast cell line and Chang liver cells were obtained from the American Type Culture Collection. They were cultured in petri dishes in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum, 100 units/mL of penicillin G, 10 µg/mL of streptomycin and 0.25 µg/mL amphotericin B. The cells were incubated at 37°C in an atmosphere of 95% humidified air, 5% carbon dioxide until 90% confluency was achieved.

### **2.2.2 Determination of choline uptake and phosphatidylcholine biosynthesis**

Endothelial cells were incubated with the indicated concentration of phosphocholine in the culture medium (2 mL) for 2 h at 37°C. [*Methyl*-<sup>3</sup>H] choline was added to a final concentration of 30 µM (0.17 µCi/nmol), and cells were further incubated for 1 h. The

culture medium was removed, and the cells were washed and harvested. Aqueous and organic metabolites were extracted by adding chloroform, methanol and water to final proportions of 4:2:3 by vol. Choline-containing metabolites in the aqueous phase were analyzed by thin-layer chromatography using a solvent system consisting of methanol / 0.6% NaCl / NH<sub>4</sub>OH (50:50:5, by vol). Phosphatidylcholine in the organic phase of the cell extracts was resolved by thin-layer chromatography using a solvent system consisting of chloroform / methanol / water / acetic acid (70:30:4:2, by vol). The radioactivity associated with the various metabolites was determined by liquid scintillation counting.

### 2.2.3 Choline kinase assay

Choline kinase activity was determined by measuring the production of radioactive phosphocholine. The reaction mixture (100 µL) contained 10 mM Tris-HCl (pH 8.0), 20 µM dithiothreitol, 1 mM magnesium chloride, 1 mM ATP and 1 mM [*methyl*-<sup>3</sup>H] choline (20,000 dpm/assay). The enzyme sample (10 µL) was added to the reaction mixture and incubated at 37°C for 15 min. Non-radiolabeled choline (final concentration 4 mM) was added, and the mixture was subsequently incubated at 100°C for 5 min. The sample was centrifuged at 10,000 x g for 5 min and the supernatant was applied to a thin-layer chromatography plate. The plate was developed in a solvent containing methanol / 0.6% NaCl / NH<sub>4</sub>OH (50:50:5, by vol), and exposed to iodine vapor to visualize the choline and phosphocholine bands. The silica gel corresponding to the authentic phosphocholine standard was scraped, and the radioactivity of phosphocholine was determined by scintillation counting.

#### **2.2.4 CTP: phosphocholine cytidyltransferase assay**

Phosphocholine cytidyltransferase activity was determined by measuring the production of radioactive CDP-choline. The reaction mixture (100  $\mu$ L) contained 100 mM Tris-succinate (pH 7.0), 12 mM magnesium acetate, 2.5 mM CTP and 1.0 mM phospho-[*methyl*- $^3$ H] choline (1,000 dpm/nmol). The enzyme sample ( $\sim$ 50  $\mu$ g protein) was added to the mixture and incubated at 37°C for 15 min. The reaction was stopped by incubating the mixture in a boiling water bath for 5 min. The reaction mixture was centrifuged at 5,000 g for 10 min, and the supernatant was applied to a thin-layer chromatography plate. The plate was developed in a solvent containing methanol / 0.6% NaCl / NH<sub>4</sub>OH (50:50:5, by vol). The location of CDP-choline on the plate was determined with a Bioscan System 200 Imaging Scanner (Bioscan Inc., Washington DC, USA). The silica gel corresponding to the CDP-choline fraction was scraped, and its radioactivity was determined by scintillation counting.

#### **2.2.5 CDP-choline: 1,2 diacylglycerol cholinephosphotransferase assay**

The cholinphosphotransferase activity was determined by measuring the production of radioactive phosphatidylcholine. The reaction mixture (1.0 mL) contained 100 Tris-HCl (pH 8.5), 10 mM magnesium chloride, 1 mM ethylenediaminetetraacetic acid, 0.4 mM CDP-[*methyl*- $^{14}$ C] choline (1.0  $\mu$ Ci/ $\mu$ mol) and 1.0 mM diacylglycerol (prepared in 0.015% Tween 20 by sonication). The enzyme sample ( $\sim$ 100  $\mu$ g protein) was added, and the reaction was incubated at 37°C for 15 min. The reaction was terminated by the addition of 3 mL chloroform / methanol (2:1 v/v) to the mixture. Water (0.5 mL) was added to the mixture

to cause phase separation. The organic phase was washed twice with 2 mL of 40% methanol, and the solvent in the lower phase was subsequently evaporated. The radioactivity associated with phosphatidylcholine was determined by scintillation counting. Analysis by thin-layer chromatography revealed that over 98% of the radioactivity in the lower phase was from phosphatidylcholine.

### **2.2.6 Measurement of arachidonate release**

The arachidonate released from cells was determined as described by Wong *et al.* (188). Endothelial cells were incubated for 20 h in the presence of 1  $\mu\text{Ci/mL}$  [ $^3\text{H}$ ] arachidonate in the culture medium. The cells were washed with HEPES-buffered saline (140 mM NaCl, 4 mM KCl, 5.5 mM glucose, 10 mM HEPES (pH 7.4), 1.5 mM  $\text{CaCl}_2$  and 1.0 mM  $\text{MgCl}_2$ ) containing 0.025% (w/v) essentially fatty acid-free bovine serum albumin. The cells were then incubated with HEPES-buffered saline containing phosphocholine and/or ATP as outlined in the text. The buffer was removed, and 50  $\mu\text{L}$  glacial acetic acid was added to halt any further reactions. The mixture was centrifuged for 5 min at 800 x g, and a 0.8 mL aliquot was used for lipid extraction in a solvent mixture consisting of chloroform / methanol / water (4:3:2, by vol). Oleic acid was added, and the fatty acids in the organic phase were resolved by thin-layer chromatography in a solvent system consisting of hexane / diethyl ether / acetic acid (70:30:1, by vol). The fatty acid band was visualized by iodine vapor, and the associated radioactivity was determined by liquid scintillation counting.

### 2.2.7 Determination of phospholipase A<sub>2</sub> activity

Phospholipase A<sub>2</sub> activity was assayed as described by Tran *et al.* (189). Cells were lysed by sonication in a buffer containing 50 mM Tris-HCl (pH 8.0), 1 mM EDTA, 10 μM leupatin, 10 μM aprotinin, 20 mM NaF and 10 mM Na<sub>2</sub>HPO<sub>4</sub>. The cell lysate was centrifuged at 100,000 g for 60 min. The supernatant was designated as the soluble fraction while the pellet was resuspended in the buffer described above and designated as the membrane fraction. Phospholipase A<sub>2</sub> activities in the subcellular fractions were determined by the hydrolysis of 1-stearoyl-2-[1-<sup>14</sup>C] arachidonoyl-*sn*-glycero-phosphocholine to yield free radiolabelled arachidonate. The substrate was resuspended in dimethylsulfoxide (less than 0.5% of the final volume) by vortexing and sonication in a water bath sonicator. The assay mixture contained 50 mM Tris-HCl (pH 8.0), 1.5 mM CaCl<sub>2</sub>, 0.9 nmole of stearoyl-2-[1-<sup>14</sup>C] arachidonoyl-*sn*-glycero-3-phosphocholine (100,000 dpm/assay) and approximately 10 μg protein in a final volume of 100 μL. The reaction mixture was incubated at 37°C for 30 min and then terminated by the addition of 1.5mL of chloroform / methanol (2:1 v/v). Total lipid was extracted, and the radioactivity of the free arachidonate was determined as described above.

### 2.2.8 Preparation of subcellular fractions

Pig heart was obtained fresh from a local abattoir. A 10% homogenate (w/v) was prepared in a buffer containing 10 mM Tris-HCl (pH 7.4), 0.25 M sucrose, 1 mM ethylenediaminetetraacetic acid and 0.1 mM phenylmethanesulfonyl fluoride. Subcellular fractions were obtained by differential centrifugation as previously described (7). The cross-

contamination of each subcellular fraction was assessed by enzyme markers. Fumarase (190) and succinate dehydrogenase (191) activities were used as mitochondrial markers. Glucose-6-phosphatase (192), 5'-nucleotidase (193) and  $K^+$ -stimulated *p*-nitrophenylphosphatase activities (193) were employed as microsomal markers. From the determination of these enzyme activities, the microsomal fraction was contaminated by 5% of the mitochondrial material, whereas the mitochondrial fraction was contaminated by 9% of microsomal material. The cytosolic fraction was contaminated by less than 1% of mitochondrial or microsomal materials. The protein content in each fraction was determined by the bicinchoninic acid method (194).

### **2.2.9 Acyl-CoA: lysophosphatidylcholine acyltransferase assay**

Lysophosphatidylcholine acyltransferase activity was determined by measuring the production of radioactive phosphatidylcholine. The reaction mixture (0.7 mL) contained 80 mM Tris-HCl (pH 7.4), 100  $\mu$ M [ $1-^{14}C$ ] arachidonyl-CoA (51.6 mCi/mmol) and 150  $\mu$ M lysophosphatidylcholine. The enzyme sample (~50  $\mu$ g protein) was added and incubated at 37°C for 10 min. The reaction was terminated by the addition of 3 mL chloroform / methanol (2:1 v/v). Non-radioactive phosphatidylcholine (10  $\mu$ g) was added as a carrier. Phase separation was caused by the addition of 0.8 mL water to the mixture. The lower phase was analyzed by thin-layer chromatography. The plate was developed in chloroform / methanol / water / acetic acid (70:30:4:2, by vol). The phosphatidylcholine band was visualized by exposure of the plate to iodine vapor. The silica gel containing the phosphatidylcholine was removed from the plate, and its radioactivity was determined by

scintillation counting.

### **2.2.10 Glycerol: acyl-CoA acyltransferase assay**

The glycerol acyltransferase activity was determined by measuring the production of radioactive monoacylglycerol. The enzyme sample (~10 µg protein) was incubated in a buffer containing 50 mM Tris-succinate (pH 6.0), 32.2 µM [ $1-^{14}\text{C}$ ] arachidonyl-CoA (51.6 mCi/mmole), 0.1 mM ethylenediaminetetraacetic acid and 5% (v/v) glycerol in a total volume of 60 µL. The reaction was terminated by addition of 0.75 mL chloroform / methanol (2:1 v/v). Water (0.25 mL) was added to mixture, and centrifugation was used to speed up phase separation. The organic phase was recovered and analyzed by thin-layer chromatography. The radioactivity associated with monoacylglycerol was determined by scintillation counting. Counts recovered from a control assay mixture lacking glycerol were subtracted from experimental values.

### **2.2.11 Glycerokinase assay**

Glycerokinase activity was determined using a modified method described by Westergaard *et al.* (195). The enzyme activity was determined by measuring the rate of [ $1,3-^3\text{H}$ ] glycerol-3-phosphate production. H9c2 or Chang liver cells were scraped from the petri dish and suspended in a phosphate buffered saline solution (pH 7.4). A cocktail of protease inhibitors was added to the cell suspension, and a homogenate (~0.1 mg protein/mL) was produced by sonication. A 10 µL aliquot of cell homogenate was incubated with a reaction mixture containing 25 mM Hepes buffer (pH 7.4), 3 mM ATP, 2.5 mM  $\text{MgCl}_2$  and 32.3 µM

[1,3-<sup>3</sup>H] glycerol (3.5 Ci/mmol) in a total volume of 100 µL for 10 min at 37°C. The reaction was terminated by incubating the mixture at 100°C for 5 min. The mixture was centrifuged for 5 min at 10,000 g, and the supernatant was applied onto a DE3-81 Whatman filter disc. The disc was placed onto a scintered glass filter under vacuum and slowly washed with 25 mL 80% ethanol. The radioactive glycerol-3-phosphate associated with the filter was determined by scintillation counting. Counts from a control assay mixture lacking ATP were subtracted from experimental values.

#### **2.2.12 Pulse-chase analysis**

H9c2 cells or Chang liver cells were grown in 35 mm petri dishes or 24 well plates until they became 90% confluent. Culture medium containing [1,3-<sup>3</sup>H] glycerol (3.5 Ci/mmol) was added to each dish for the prescribed time. The medium containing the label was removed from the dish, and the cells were incubated in culture media containing non-radiolabeled glycerol for various times. Subsequently, the dishes were rinsed 3 times with ice cold phosphate buffered saline solution (pH 7.4). Cells were scraped into a test tube with 1 mL methanol / HCl (100:1 v/v), and chloroform (1.3 mL) and water (0.7 mL) were added to the tube. The lipid fraction was recovered in the organic phase and analyzed by thin-layer chromatography.

#### **2.2.13 Separation of lipids**

The simultaneous separation of radiolabeled neutral lipid and phospholipid was performed by one-dimensional thin-layer chromatography using multiple development

systems. The thin-layer chromatography plate (20 cm x 20 cm) was activated by incubating at 135°C for at least 1 h and allowed to cool to room temperature before sample application. The plate was developed in a solvent containing chloroform / methanol / water / acetic acid (70:30:4:2, by vol) until the solvent front reached 11 cm from the origin. After drying, the plate was fully developed in a second solvent containing benzene / diethyl ether / ethanol / acetic acid (50:40:2:0.2, by vol). Again the plate was dried and then developed in the first solvent to 12.5 cm for further separation of phospholipids. Lipid fractions were visualized by exposure to iodine vapor. The bands on the thin-layer chromatography plate corresponding to authentic lipid standards were scraped into scintillation vials, and their radioactivities were determined.

#### **2.2.14 Lipid determination**

For the determination of monoacylglycerol, diacylglycerol, triacylglycerol, lysophosphatidate, phosphatidate and phosphatidylcholine, the acyl groups were converted to the respective methyl esters by reaction with BCl<sub>3</sub>-methanol (196). The total fatty acid methyl esters were quantified using a Hewlett Packard HP 5890A gas chromatograph equipped with a Supelcowax 10 30 m x 0.25 mm, 0.25 μm fused silica capillary column. Heptadecanoic acid methyl ester was used as a standard for quantification.

#### **2.2.15 Solubilization and purification of glycerol: acyl-CoA acyltransferase**

Glycerol acyltransferase was solubilized from the microsomal membrane by detergent treatment. The appropriate amount of detergent was added to the microsomal sample, and

the mixture was incubated at 4°C for 1 h with gentle stirring. The enzyme activity in the supernatant after centrifugation at 100,000 g for 60 min was regarded as the solubilized form of the enzyme.

Microsomal protein from the pig heart was solubilized with Nonidet P-40 (0.2%), and passed through a 0.22 µm pore filter. Purification of glycerol acyltransferase was performed by FPLC using a Sepharose 6B 16/50 HR column. The column was equilibrated with 10 mM Tris-HCl (pH 7.0), 0.1 mM ethylenediaminetetraacetic acid, 0.1 M KCl, and 10% (v/v) glycerol. After application of the sample to the column, the same buffer was used to elute the sample at a flow rate of 0.75 mL/min. Fractions of 1.8 mL were collected, and the elution profile was monitored by absorbance at 280 nm.

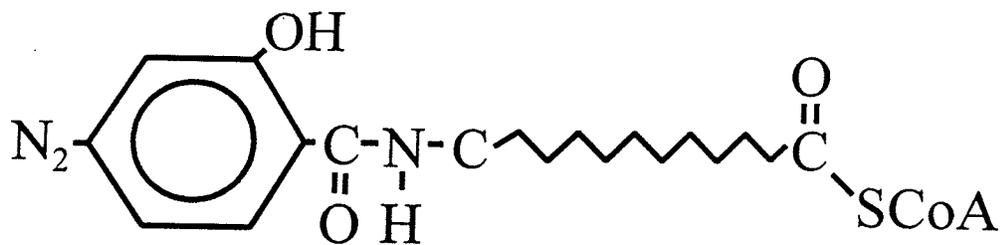
#### **2.2.16      Synthesis of [<sup>125</sup>I] labeled 12-[(4-azidosalicyl)amino]-dodecanoyl-CoA**

Azidosalicylamino-dodecanoic acid (ASD) was synthesized in the dark as previously described (197). A mixture of 12-aminododecanoic acid (12 mg), the N-hydroxysuccinimide ester of 4-azidosalicylic acid (27 mg), anhydrous 1,2,3,4-tetrahydro-9-fluorenone (2.0 mL) and pyridine (0.2 mL) was stirred for 72 h in the dark at room temperature. The entire mixture was evaporated to dryness under a nitrogen stream after the addition of 11.6 M HCl (0.2 mL). The residue was dissolved in 5 mL ethyl acetate and extracted twice with 5 mL of 30 mM HCl. The organic phase was dried over sodium sulfate. The product was evaporated under a nitrogen stream and resuspended at 10 mg/mL in ethyl acetate. The correct product formation was confirmed by thin-layer chromatography analysis using a solvent consisting of chloroform: methanol (5:1 v/v). The R<sub>f</sub> values for 12-aminododecanoic

and ASD were 0.00 and 0.63 respectively as previously reported (197).

12-[(4-Azidosalicyl)amino]-dodecanoyl-CoA (ASD-CoA) was synthesized enzymatically as previously described (197). The structure of ASD-CoA is depicted in Figure 12. The reaction mixture contained 1.5 mg azidosalicylaminododecanoic acid, 50 mM Tris-HCl (pH 8.0), 7.5 mM MgCl<sub>2</sub>, 0.3% Triton X-100, 4.3 mM CoA and 5 mM ATP in a volume of 1.8 mL. The mixture was sonicated for 10 min to emulsify the substrate, and 0.4 units of acyl-CoA synthetase were added to give a final volume of 2.2 mL. The reaction was carried out for 2 h at 28°C and stopped by boiling for 2 min. ASD-CoA was purified using the PrepSep C<sub>18</sub> column previously washed with 2.0 mL methanol, 2.0 mL water and 2.0 mL 10 mM KH<sub>2</sub>PO<sub>4</sub> (pH 5.3). After sample loading, the column was washed with 3.0 mL 10 mM KH<sub>2</sub>PO<sub>4</sub> (pH 5.3). ASD-CoA was eluted with 4 mM methanol into a pre-weighed test tube. The solvent was evaporated to dryness, and the residue was dissolved in ethyl acetate / methanol (2:1 v/v).

ASD-CoA was iodinated as previously described (198). The reaction mixture contained 60 µL chloramine T, 0.5 µg ASD-CoA, 200 µL ethyl acetate / methanol (2:1 v/v) and 0.25 mCi [Na<sup>125</sup>I]. The mixture was incubated for 30 min at room temperature in the dark, and 100 µL ethyl acetate and 100 µL 10% NaCl (w/v) were added to the reaction mixture. The upper phase was collected, and the aqueous phase was extracted twice more with ethyl acetate. The [<sup>125</sup>I] labeled ASD-CoA was stored in ethyl acetate at -70°C.



**Figure 12. Structure of ASD-CoA**

### **2.2.17 Photoaffinity labeling**

Photoaffinity labeling was performed by incubating fractions from gel filtration chromatography with the photoaffinity probe at room temperature in the dark for 15 min. The reaction mixture (215  $\mu$ L) contained 1.9  $\mu$ Ci [ $^{125}$ I] ASD-CoA and a fraction aliquot (0.1 mL) in 20 mM Tris-Succinate (pH 6.0), 40  $\mu$ M ethylenediaminetetraacetic acid and 4.2% (v/v) glycerol. Cross-linkage of the photoaffinity probe to the enzyme was induced by exposing the reaction mixture to ultraviolet light. A hand-held ultraviolet lamp (Model UVS-54, from Ultraviolet Productions Inc. San Gabriel, CA) was held at a distance of 5 cm from the sample for 15 min. Trichloroacetate (10% w/v) was added to stop the reaction and to cause protein precipitation. The mixture was incubated at  $-20^{\circ}\text{C}$  for 15 min to allow full precipitation of the labeled protein. The precipitated protein was sedimented at 10,000 g for 5 min, and the pellet was resuspended in 15  $\mu$ L 0.1 N NaOH. Sample buffer (15  $\mu$ L) was added to each sample and analyzed directly by tricine-sodium dodecyl sulfate-polyacrylamide gel electrophoresis (Tricine-SDS PAGE) (43). The gel was stained for protein using Serva Blue G, dried, and the labeled protein bands were identified by autoradiography. Band density was analyzed by Scion Image software (Scion Corp., Frederick, Maryland, USA).

### **2.2.18 Gel electrophoresis**

Tricine-SDS PAGE was used to separate low molecular weight proteins with exceptionally high resolution. Tricine, used as the trailing ion, allows resolution of small proteins at lower acrylamide concentrations than in glycine-SDS-PAGE systems (199). The stacking and resolving gels containing 4% and 10% polyacrylamide respectively were cast

in a mini gel apparatus. The anode buffer was composed of 100 mM Tris-HCl (pH 8.9), and the cathode buffer consisted of 100 mM Tris-HCl (pH 8.25), 100 mM Tricine and 1.0% SDS. The protein sample was prepared by incubating with sample buffer containing 150 mM Tris-HCl (pH 7.0), 30% glycerol, 12% SDS, 6%  $\beta$ -mercaptoethanol, and 0.05% Serva Blue G for 30 min at 40°C. After electrophoresis, the protein sample was electrophoretically transferred from the Tricine-SDS gel onto a polyvinylidene difluoride membrane using a Bio-Rad semi-dry transfer cell. The protein band in the membrane was carefully excised and used for amino acid sequencing. Sequencing was conducted by the Protein Microsequencing Laboratory (Victoria, BC, Canada).

#### **2.2.19 Production of antibody**

Rabbit polyclonal antibody was produced by National Biological Laboratory Ltd. (Winnipeg, MB, Canada). Antiserum was produced against each protein band identified by [<sup>125</sup>I] ASD-CoA photoaffinity labeling. Each protein band was electrophoretically eluted from the polyacrylamide gel and recovered in an enclosed dialysis bag. The protein sample (100  $\mu$ g) was injected subcutaneously into the rabbit with Freud's incomplete adjuvant followed by two additional booster shots (300-320  $\mu$ g protein) over four weeks. Control serum was obtained from the the pre-immunized animal. The IgG fraction was purified from the rabbit serum using a HiTrap affinity column. The purity of the antibody was assessed by polyacrylamide gel electrophoresis.

### **2.2.20 Immunoprecipitation of glycerol acyltransferase**

The immunoprecipitation reaction was conducted by incubating various amounts of IgG with solubilized microsomal protein for 30 min at 4°C. The incubation mixture contained 50 mM Tris-HCl (pH 7.4), 150 mM NaCl, and 5 mM ethylenediaminetetraacetic acid in a final volume of 200 µL. Protein A immobilized onto agarose beads (15 µL) was added to the mixture and incubation continued for 30 min with gentle shaking. The mixture was then centrifuged at 1,000 g for 2 min, and the enzyme activity remaining in the supernatant was determined.

### **2.2.21 Statistical analysis**

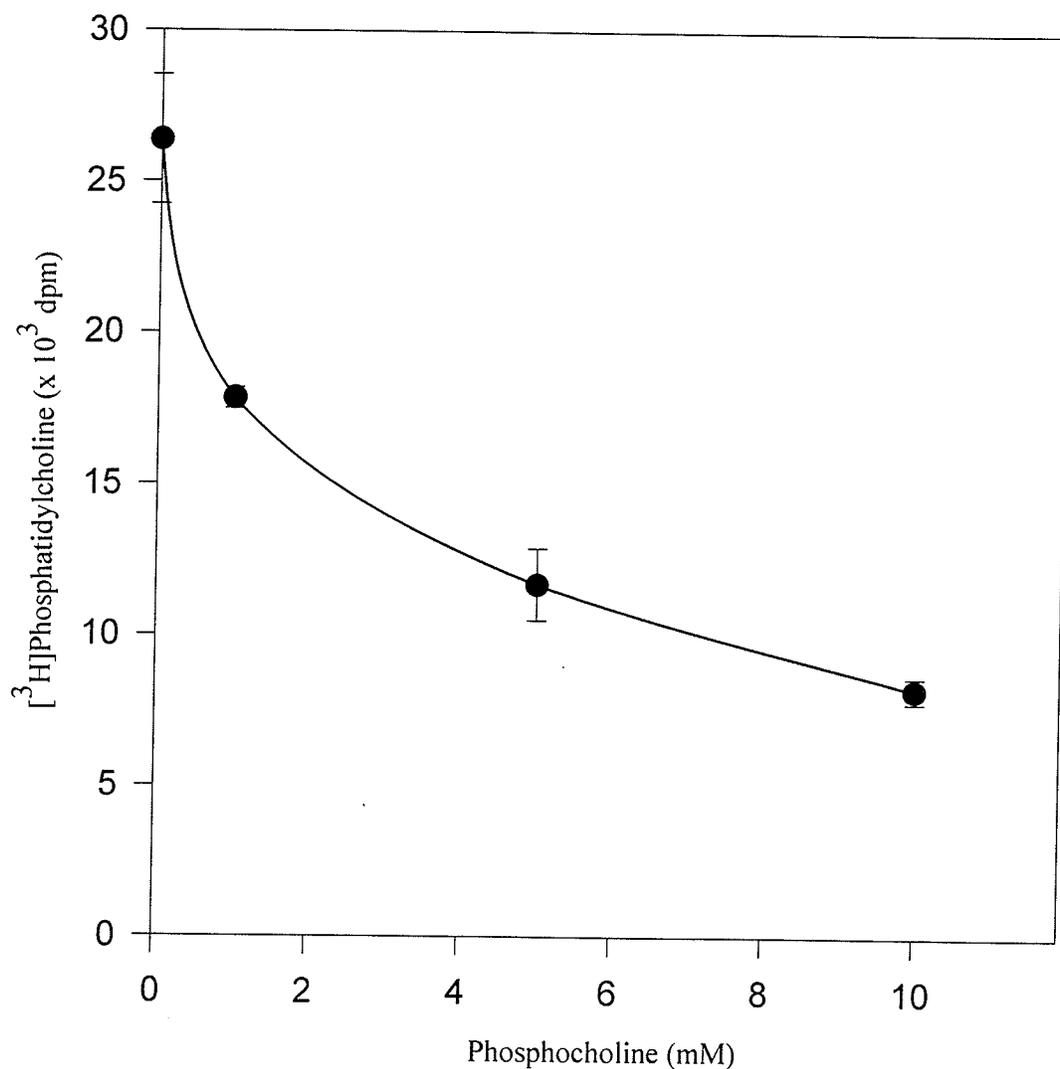
Data were analyzed using the paired Students' *t* test unless otherwise indicated. The level of statistical significance was defined as  $p < 0.05$ . The data presented without statistical analysis were performed in duplicate. The values from duplicate experiments were always within 10% of each other and were reproducible in at least three separate experiments.

### 3. EXPERIMENTAL RESULTS

#### 3.1 Studies on Phosphatidylcholine Metabolism in Human Endothelial Cells

##### 3.1.1 Effect of phosphocholine on phosphatidylcholine biosynthesis in human umbilical vein endothelial cells

The effect of exogenous phosphocholine on phosphatidylcholine biosynthesis in human umbilical vein endothelial cells was examined. In a preliminary study, the cells were incubated with phospho-[*methyl*-<sup>3</sup>H] choline for 2 h, washed and the radioactivity in the cells was determined. Less than 0.01% of the labeled material was found in these cells indicating that phosphocholine was not taken up by the cells. To study the effect of phosphocholine on phosphatidylcholine biosynthesis, the endothelial cells were preincubated with 0-10 mM phosphocholine for 2 h, and [*methyl*-<sup>3</sup>H] choline was added (final concentration 30 μM) to the incubating mixture. The cells were incubated for another 60 min, and the radioactivity incorporated into phosphatidylcholine was determined. Pre-incubation of the cells with phosphocholine caused the reduced incorporation of [*methyl*-<sup>3</sup>H] choline into phosphatidylcholine (Figure 13). In the presence of 1.0 mM phosphocholine phosphatidylcholine labeling was decreased by 32%, and at 5.0 mM phosphocholine, the labeling of phosphatidylcholine was reduced by more than half of the control value (56%). Increase in phosphocholine concentration to 10.0 mM caused a further inhibition in the labeling of phosphatidylcholine.



**Figure 13. Effect of phosphocholine on phosphatidylcholine biosynthesis in human umbilical vein endothelial cells.** Confluent monolayers of human umbilical vein endothelial cells were incubated with the indicated concentration of phosphocholine for 2 h. [*Methyl*-<sup>3</sup>H] choline (0.17  $\mu$ Ci/nmol) was added to a final concentration of 30  $\mu$ M, and the cells were incubated further for 1 h. The cells were harvested, and the radioactivity in the phosphatidylcholine fraction was determined as described in Materials and Methods. These data represent the mean  $\pm$  standard deviation of three experiments.

### 3.1.2 Effect of phosphocholine on choline containing metabolites

The observed decrease in phosphatidylcholine labeling may be reflected in changes in the labeling of intermediates in the CDP-choline pathway. Thus, the effect of phosphocholine on the incorporation of [*methyl*-<sup>3</sup>H] choline into the choline-containing metabolites which are found in the aqueous phase of the cell extracts was investigated. When cells were pre-incubated with 5 mM phosphocholine, the amount of radioactivity in the aqueous fraction was decreased by 15 % (Table 3). Analysis of the choline-containing metabolites revealed that the *in vivo* labeling of phosphocholine was significantly reduced. Since changes in the labeling of phosphocholine could result from changes in the enzyme activities in the CDP-choline pathway, the activities of choline kinase, phosphocholine cytidyltransferase and cholinephosphotransferase were determined in cells with and without pre-incubation with phosphocholine. No significant changes in enzyme activities were detected.

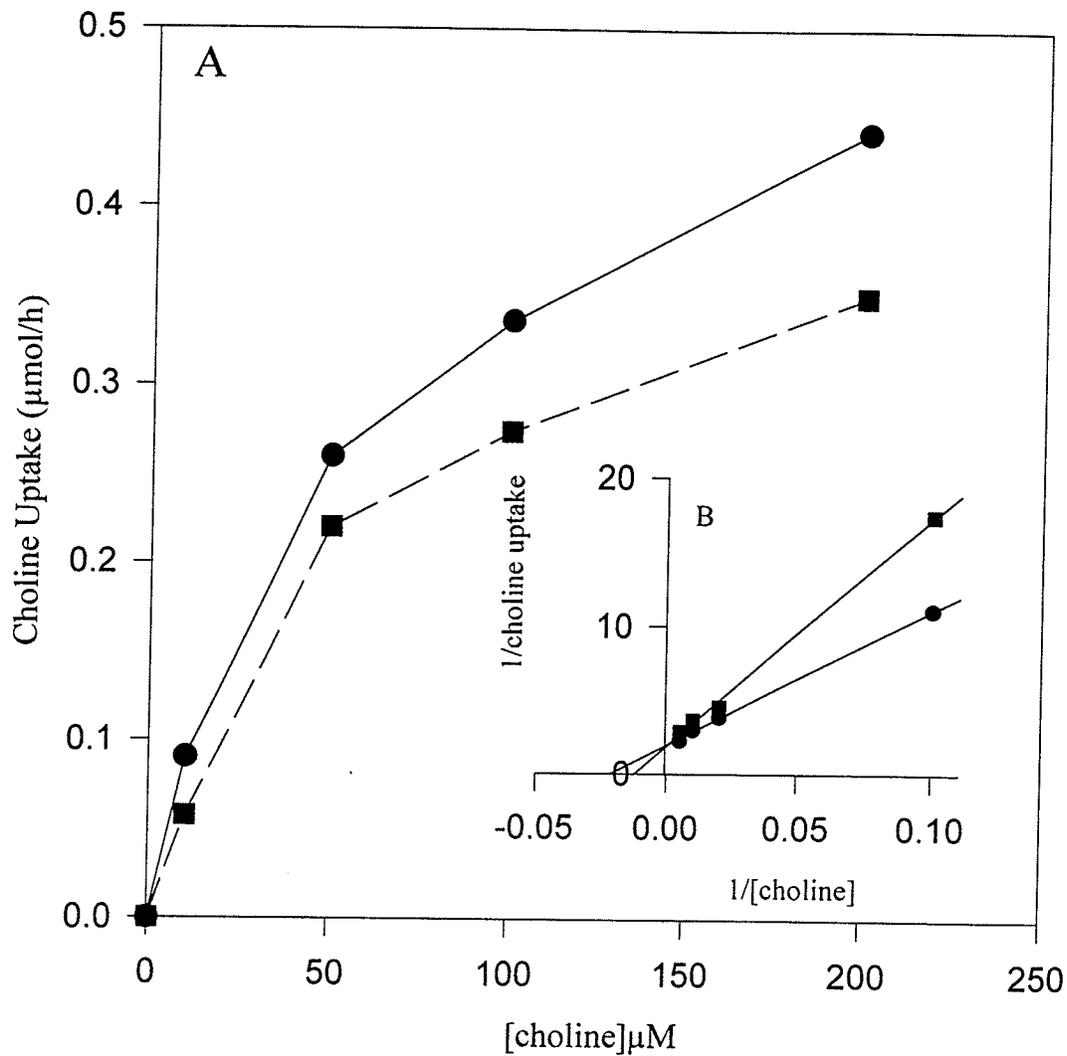
**Table 3. Radioactivity of aqueous choline-containing metabolites.**

Confluent monolayers of human umbilical vein endothelial cells were incubated with 0 or 5.0 mM phosphocholine for 2 h. [*Methyl*-<sup>3</sup>H] choline (0.17  $\mu$ C/nmol) was added to a final concentration of 30  $\mu$ M, and the cells were incubated further for 1 h. The radioactivity associated with the choline-containing metabolites was determined as described in Materials and Methods. These data represent the mean  $\pm$  standard deviation of three experiments. \**p* < 0.05 compared with the control.

	<b>[<sup>3</sup>H] Radioactivity (dpm x 10<sup>3</sup>/dish cells)</b>	
	<b>control</b>	<b>5 mM phosphocholine</b>
Aqueous fraction	56.2 $\pm$ 5.9	47.9 $\pm$ 2.9 *
Choline	0.7 $\pm$ 0.2	0.7 $\pm$ 0.1
Phosphocholine	43.9 $\pm$ 4.7	35.6 $\pm$ 2.7 *
CDP-choline	10.1 $\pm$ 3.6	10.9 $\pm$ 1.2

### 3.1.3 Effect of phosphocholine on choline uptake

Studies were conducted to determine the mechanism for the reduced uptake of labeled choline by phosphocholine. The human umbilical vein endothelial cells were incubated with different concentrations of [*methyl*-<sup>3</sup>H] choline in the absence and presence of 5.0 mM phosphocholine. Total choline uptake was inhibited in the presence of 5.0 mM phosphocholine (Figure 14). Analysis of the data in a double-reciprocal plot showed that choline uptake has a  $V_{\max}$  of 0.5  $\mu\text{mol/h}$ , with a  $K_m$  of 47  $\mu\text{M}$  (Figure 14, inset). In the presence of 5.0 mM phosphocholine, the  $V_{\max}$  of choline was unchanged, while a  $K_m$  of 83  $\mu\text{M}$  was obtained. These data suggest that the inhibition of choline uptake by phosphocholine was competitive.

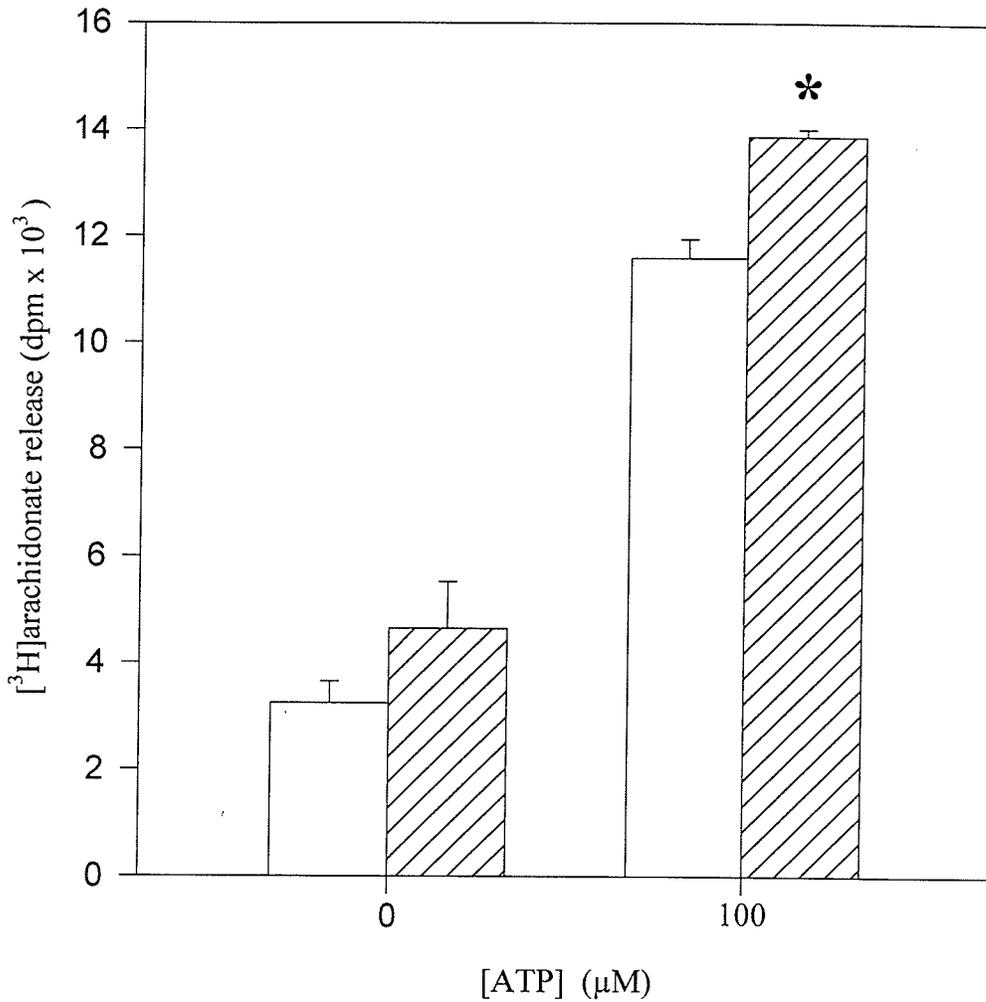


**Figure 14. Effect of phosphocholine on choline uptake** by endothelial cells. In (A), confluent monolayers of human umbilical vein endothelial cells were incubated with 0 mM (●) or 5.0 mM (■) phosphocholine for 2 h. [*Methyl-<sup>3</sup>H*]choline (10 μCi total) was added at the indicated concentrations, and the cells were incubated further for 1 h. The uptake of choline into the cells was determined. In (B), the data in (A) were re-plotted as a double-reciprocal plot.

### 3.1.4 Effect of phosphocholine on ATP-induced arachidonate release

The effect of phosphocholine on arachidonate release in endothelial cells was investigated. After pre-labeling of cellular lipids with [<sup>3</sup>H] arachidonate as described in Materials and Methods, the cells were incubated with 0 or 1.0 mM phosphocholine for 2 h. The cells were then exposed to 0 or 100 μM ATP, a known stimulator of arachidonate release (200). The release of arachidonate into the medium was determined. A small enhancement of arachidonate release was observed in the cells incubated with 1.0 mM phosphocholine, but the difference was not statistically significant (Figure 15). When cells were incubated with 1.0 mM phosphocholine in the presence of 100 μM ATP, a significant (20%) enhancement of arachidonate release was observed when compared to cells incubated without phosphocholine.

The importance of cytosolic phospholipase(s) A<sub>2</sub> in arachidonate release from endothelial cells has been previously demonstrated (188, 189). Thus the phospholipase A<sub>2</sub> activity in the endothelial cells after phosphocholine treatment was examined. Cells were incubated for 2 h with 0 or 5.0 mM phosphocholine. The cells were lysed and the phospholipase A<sub>2</sub> activity was determined in the total cell lysate and soluble and membrane fractions. Pre-incubation of the cells with phosphocholine did not result in detectable changes in phospholipase A<sub>2</sub> activities in these two sub-cellular fractions (Table 4).



**Figure. 15. Effect of phosphocholine on ATP-induced arachidonate release in endothelial cells.** Near-confluent monolayers of human umbilical vein endothelial cells were incubated for 20 h with 1  $\mu\text{Ci}/\text{mL}$  [ $^3\text{H}$ ] arachidonate (200  $\mu\text{Ci}/\text{nmol}$ ). After incubation with [ $^3\text{H}$ ] arachidonate, the cells were incubated with 0 mM (open bars) or 1 mM phosphocholine (hatched bars) for 2 h. The cells were then incubated with 0 or 100  $\mu\text{M}$  ATP for 10 min, and the [ $^3\text{H}$ ] arachidonic acid released into the medium was determined as described in Materials and Methods. These data represent the mean  $\pm$  standard deviation of three experiments. \* $p < 0.05$  compared with cells incubated with 0 mM.

**Table 5. Effect of phosphocholine on phospholipase A<sub>2</sub> activity**

Confluent monolayers of human umbilical vein endothelial cells were incubated for 2 h with 0 mM (control) or 5.0 mM phosphocholine. After incubation, phospholipase A<sub>2</sub> activity was determined in total cell lysate and membrane and soluble fractions as described in Materials and Methods. These data represent the mean  $\pm$  standard deviation of three experiments.

	<b>Phospholipase A<sub>2</sub> Activity (pmol/min/mg protein)</b>	
	<b>control</b>	<b>5 mM phosphocholine</b>
Total activity	9.01 $\pm$ 1.28	9.10 $\pm$ 0.29
Membrane fraction	1.85 $\pm$ 0.29	2.45 $\pm$ 0.55
Soluble fraction	6.91 $\pm$ 0.88	6.67 $\pm$ 0.21

## **3.2 Studies on the Deacylation / Reacylation Cycle**

The deacylation / reacylation cycle is an important process in establishing the proper fatty acid composition in the phospholipid. Phospholipases have been extensively studied (169, 170), but studies on lysophospholipid acyltransferases have been limited. Although MacQuarrie and colleagues have reported the purification of lysophosphatidylcholine acyltransferase, the purified enzyme has not been employed in any subsequent studies (185, 186). In this study, FPLC chromatography and gel activity assays were employed to obtain the purified enzyme. Unfortunately, the various approaches have not been successful, and further work is necessary for identification of the enzyme. Because these studies ultimately led to the study of glycerol acylation and the discovery of a novel lipid biosynthetic pathway (Section 3.3), a brief description on the approaches used to purify lysophosphatidylcholine acyltransferase is given in the following sections.

### **3.2.1 Gel filtration chromatography**

The solubilization of lysophosphatidylcholine acyltransferase from the microsomal membrane was completely re-evaluated by Mukherjee (182). A significant portion (35%) of the enzyme activity was solubilized by a combination of 1% Chaps and 1 M KCl. Solubilized pig heart microsomes were applied onto an FPLC gel filtration column containing Sepharose 6B. The severe loss of enzyme activity during chromatography was partially overcome by including 20% glycerol in the elution buffer. Acyltransferase activity was determined in each fraction by measuring the production of radioactive

phosphatidylcholine as described in Materials and Methods. A major activity peak eluted near the void volume, and a second activity peak eluted slightly ahead of bovine serum albumin (data not shown). Unfortunately, attempts to further purify the enzyme by other chromatographic methods resulted in the complete loss of enzyme activity.

Unexpectedly, several fractions catalyzed the production of a radioactive compound which did not correlate to lysophosphatidylcholine, arachidonyl-CoA, arachidonate or phosphatidylcholine. The radioactive compound migrated near the solvent front when analyzed by thin-layer chromatography using a solvent system containing chloroform / methanol / water / acetic acid (70:30:4:2, by vol). The radioactive compound was re-analyzed by thin-layer chromatography using a solvent system containing benzene / diethylether / ethanol / water (50:40:2:0.2, by vol). The radioactive compound co-migrated identically with the monoacylglycerol standard. The detection of radioactive monoacylglycerol suggested that a microsomal enzyme catalyzed the direct acylation of glycerol. The direct acylation of glycerol catalyzed by pig heart microsomes was further investigated as described in Section 3.3.

### **3.2.2 Gel activity assay**

The activity gel assay used to detect histone acetyltransferase from *Tetrahymena* macronuclei represented an alternative approach for the purification and identification of lysophosphatidylcholine acyltransferase (201). Brownell and Allis reported the detection of histone acetyltransferase after gel electrophoresis by the incorporation of radioactive acetate into histone substrates polymerized directly into the gel.

In a typical experiment, pig heart microsomal protein was separated by SDS-PAGE. After electrophoresis, the gel was incubated in a buffer containing 15% isopropanol to remove the SDS from the gel. Subsequently, the gel was washed in buffer containing either 7 M guanidine or 8 M urea to completely denature the acyltransferase. The gel was then incubated in a buffer containing detergent (e.g. sodium cholate, Nonidet P-40, tween 20 or tween 40) to allow the protein to refold into the native enzyme. To detect lysophosphatidylcholine acyltransferase activity, the gel was incubated with [<sup>14</sup>C] arachidonyl-CoA in the presence and absence of lysophosphatidylcholine. In theory, the newly formed phosphatidylcholine would become insoluble and localized to the enzyme in the gel. The location of the radioactivity was initially determined by autoradiography. The production of phosphatidylcholine was determined by performing lipid extraction from the gel, and the extract was analyzed by thin-layer chromatography. Although several protein bands were labeled, the production of phosphatidylcholine was not detected. These results indicate that under the experimental conditions tested, the gel activity assay was not successful for the purification of lysophosphatidylcholine acyltransferase.

### **3.3 Studies on the Direct Acylation of Glycerol.**

#### **3.3.1 Confirmation of monoacylglycerol production**

Rigorous analysis was conducted to confirm the identity of monoacylglycerol formed by the glycerol acyltransferase reaction. The glycerol acyltransferase reaction was conducted using pig heart microsomes as described in Materials and Methods. The radioactive product was analyzed by thin-layer chromatography by employing over a dozen solvent systems for the analysis. In every case, the radiolabeled product migrated identically with the monoacylglycerol standard (Table 5). The monoacylglycerol isomers were analyzed by using a boric acid impregnated thin-layer chromatography plate (202). After sample application, the plate was developed in a solvent containing chloroform / acetone (96:4 v/v). The radioactivity associated with each isomer was determined, and the analysis indicated that 10% of the monoacylglycerol produced was of the 2-isomer and 90% was of the 1(3)-isomer. Incubation of radioactive monoacylglycerol with pig heart microsomes resulted in the production of radioactive arachidonate and diacylglycerol which was determined by thin-layer chromatography (data not shown). The conversion of monoacylglycerol to diacylglycerol is consistent with the presence of monoacylglycerol acyltransferase in cardiomyocytes (54).

**Table 5. Identification of monoacylglycerol**

The glycerol acyltransferase reaction was performed as described in Materials and Methods. Multiple solvent systems were employed to verify the identification of monoacylglycerol. The solvent ratio is indicated by vol. The TLC plate was developed fully in the indicated solvent system unless otherwise noted. TLC, thin-layer chromatography.

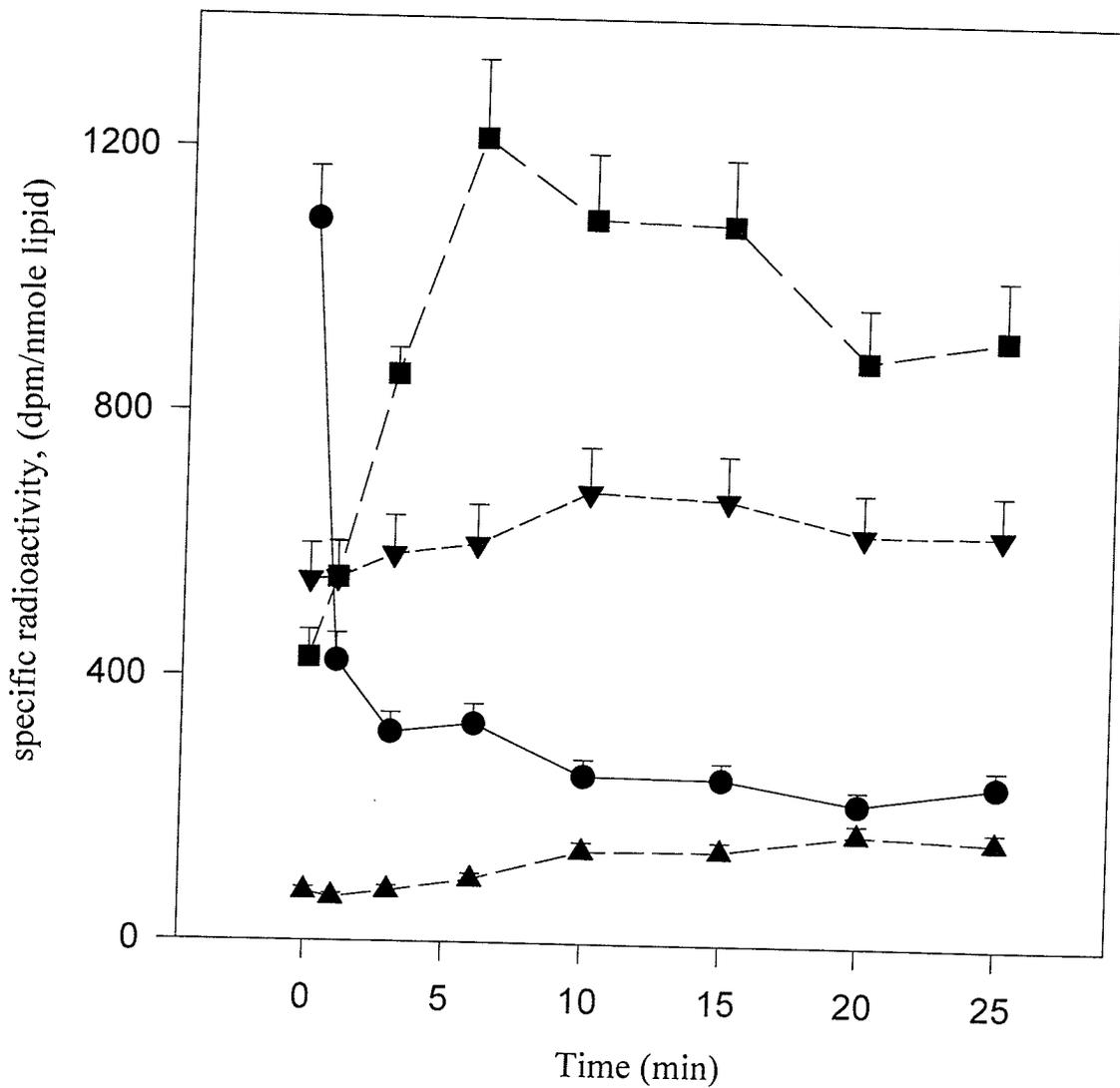
Chromatographic system	Solvent system	R <sub>f</sub> value of MG
One dimensional TLC	Diethyl ether: toluene: ethanol: acetic acid (40/50/1/0.5)	0.08
	Hexane: diethyl ether: acetic acid (70/30/1)	0.02
	Bezene: diethyl ether: ethanol: acetic acid (50/40/2/0.2)	0.23
	Hexane: diethyl ether: acetic acid (70/30/1)	0.02
	Petroleum ether: diethyl ether: acetic acid (50/50/1)	0.08
	Heptane: diethyl ether: methanol: acetic acid (90/20/2/3)	0.05
	Hexane: isopropyl ether: diethyl ether: acetone: acetic acid (85/12/1/4/1)	0.01
	Heptane: isopropyl ether: acetic acid (60/40/4)	0.04
	Chloroform : acetone (96/4) (Boric acid impregnated plated used)	0.05 (1(3)-MG) 0.1(2-MG)
Multi one dimensional TLC	Diethyl ether: hexane (60/40) 7cm; Diethyl ether: petroleum ether: formic acid (90/10/1) 18cm	0.03
	Isopropyl ether: acetic acid (90/10/1) 13cm; Petroleum ether: diethyl ether: acetic acid (85/12/1/4/1) 18cm	0.15
	Diethyl ether: toluene: ethanol: acetic acid (45/50/1/0.5) 12cm; Diethyl ether: hexane (8/92) 18cm	0.16
	Chloroform: methanol: acetic acid: water (70/30/4/2) 9cm; Diethyl ether: hexane: ethanol: acetic acid (40/50/2/0.2) 18cm; Chloroform: methanol: acetic acid: water (70/30/4/2) 13cm	0.68
Two Dimensional TLC	Benzene: diethyl ether: ethanol: acetic acid (50/40/2/0.2); Benzene: diethyl ether: ethanol: ammonium hydroxide (50/40/2/0.2)	
	Benzene: diethyl ether: ethanol: acetic acid (50/40/2/0.2); Diethyl ether: hexane: acetic acid (60/40/1)	

### 3.3.2 The direct acylation of glycerol

The acylation of glycerol-3-phosphate is regarded as the first committed step for glycerolipid biosynthesis. However, since the direct acylation of glycerol occurs *in vitro*, the pathway for glycerol acylation was reassessed in H9c2 cells by conducting pulse-labeling and chase studies. H9c2 cells were pre-incubated with culture medium containing 0.1 mM [1,3-<sup>3</sup>H] glycerol (3.5Ci/mmol) for 1 min and then incubated in culture medium containing 1 mM non-radiolabeled glycerol for various times. The specific radioactivities of monoacylglycerol, diacylglycerol, lysophosphatidate and phosphatidate were determined at each time point. As depicted in Figure 16, the monoacylglycerol pool was immediately radiolabeled at the beginning of the chase period, and its specific radioactivity rapidly diminished within 3 min. The specific radioactivity of diacylglycerol peaked after 5 min of incubation. The labeling of lysophosphatidate was almost linear, and its specific radioactivity remained low at all time points. The labeling of phosphatidate was not clearly defined but appeared to peak after 10-15 min of incubation. The labeling profile indicates that a considerable portion of the radiolabeled glycerol taken up by the cell was directly acylated to form monoacylglycerol. Some of the newly formed monoacylglycerol was converted to diacylglycerol and possibly phosphatidate. The importance of diacylglycerol kinase for the conversion of diacylglycerol to phosphatidate is well documented (203), and the presence of this enzyme in cardiac tissues has been reported (204).

Since the direct acylation of glycerol appeared to be rapid, the specific activity of glycerol acyltransferase was determined and compared to the specific activity of glycerokinase. Glycerol acyltransferase activity was  $310 \pm 18$  pmole/min/mg protein whereas

the glycerokinase activity was found to be  $8.3 \pm 1.8$  pmole/min/mg protein. The low glycerokinase activity may account for the low [1,3- $^3\text{H}$ ] glycerol incorporation into lysophosphatidate during the pulse-chase study.



**Figure 16. Pulse-labeling and chase analysis on glycerol metabolism in H9c2 cells.** H9c2 cells grown in 35mm dishes were pre-incubated with culture medium containing 0.1 mM [1,3-<sup>3</sup>H] glycerol (3.5 Ci/mmol) for 1 min. The pre-incubation medium was removed, and the cells were further incubated in culture medium containing 1 mM non-radiolabeled glycerol for different times. The specific radioactivities of monoacylglycerol (●), diacylglycerol (■), lysophosphatidate (▲) and phosphatidate (▼) were determined after each incubation.

### **3.3.3 Inhibition of glycerokinase**

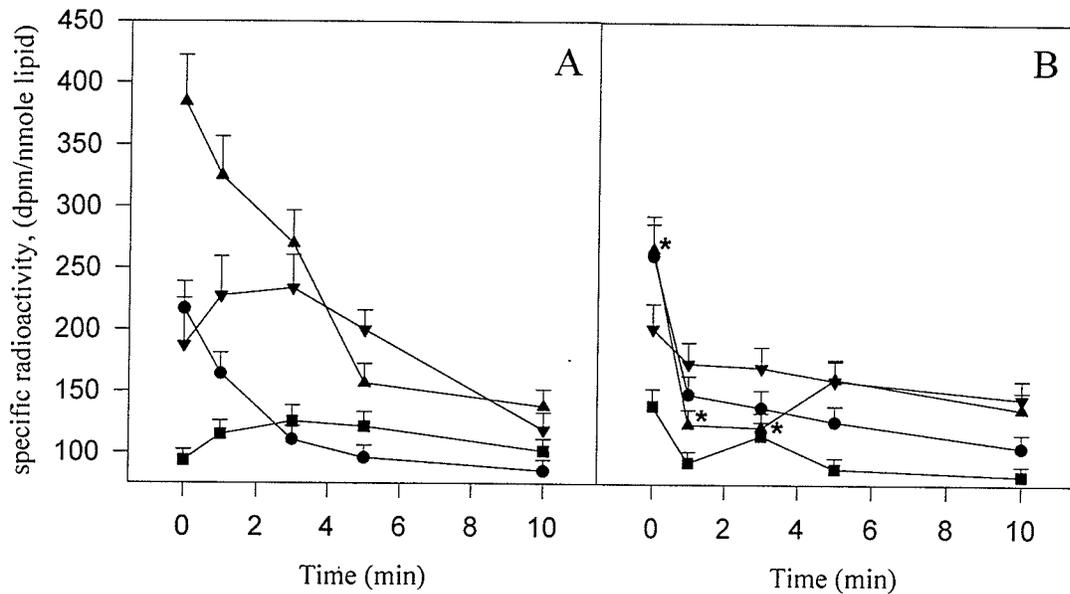
Monobutyryn is a known inhibitor of glycerokinase (205) and was chosen for the following studies since its ability to enter the cell has been demonstrated (195). The compound could be used to study the contribution of the direct acylation of glycerol by attenuating the glycerol-3-phosphate pathway. Our approach was to select a cell line with a relatively high degree of glycerokinase activity which could be attenuated by monobutyryn without inhibition of the glycerol acyltransferase activity. To determine whether monobutyryn could inhibit cellular glycerokinase *in vivo*, Chang liver cells were incubated for 1 h in the growth medium in the presence and absence of 10 mM monobutyryn. Subsequent to incubation, the cells were removed from the dish and suspended in a phosphate buffered saline solution (pH 7.4) containing a cocktail of protease inhibitors. The cells were disrupted by sonication, and the glycerol acyltransferase and glycerokinase activities were determined. Monobutyryn reduced the glycerokinase activity by 43% from  $119.5 \pm 10$  nmole/min/mg protein to  $68 \pm 10$  nmole/min/mg protein. In contrast, glycerol acyltransferase activity was stimulated by 38% from  $92.7 \pm 4$  pmole/min/mg protein to  $128 \pm 9$  pmole/min/mg protein. It is clear that monobutyryn has the ability to selectively attenuate the glycerol-3-phosphate pathway.

### **3.3.4 Attenuation of the glycerol-3-phosphate pathway and monoacylglycerol production**

The effect of monobutyryn on the acylation of glycerol in the Chang liver cells was examined by a pulse-chase experiment. Cells were pre-incubated with or without 10 mM

monobutyryn and then incubated with a medium containing 0.1 mM [1,3-<sup>3</sup>H] (3.5 Ci/mmol) glycerol for 2 min. The media containing the label was removed from the dish, and the cells were subsequently incubated in culture media containing 1 mM non-radiolabeled glycerol for different times. The specific radioactivities of monoacylglycerol, diacylglycerol, lysophosphatidate and phosphatidate were determined, and the results are depicted in Figure 17. In the absence of monobutyryn, lysophosphatidate was immediately labeled (Figure 17A). The specific radioactivity of lysophosphatidate decreased as the incubation time progressed, whereas the specific radioactivity of phosphatidate peaked after 3 min of incubation. The labeling profile of monoacylglycerol was parallel to that of lysophosphatidate but never exceeded the latter in specific radioactivity. The diacylglycerol profile closely resembled the phosphatidate profile but was never higher in specific radioactivity. In concurrence with the glycerol acyltransferase and glycerokinase activities in Chang liver cells, the majority of glycerol was metabolized through the glycerol-3-phosphate pathway. After the uptake of glycerol, lysophosphatidate was synthesized and subsequently converted to phosphatidate. Alternatively, a smaller percentage of glycerol was directly acylated to form monoacylglycerol and acylated again to form diacylglycerol.

When Chang cells were pre-incubated with 10 mM monobutyryn (Figure 17B), the specific radioactivity profile for lysophosphatidate was reduced during the first minute of incubation when compared to cells treated without monobutyryn. In contrast, the specific radioactivity in monoacylglycerol increased by 20% initially and as much as 32% midway during the chase period. These results indicate that the direct acylation pathway could function as a shunt when the glycerol-3-phosphate pathway is attenuated.

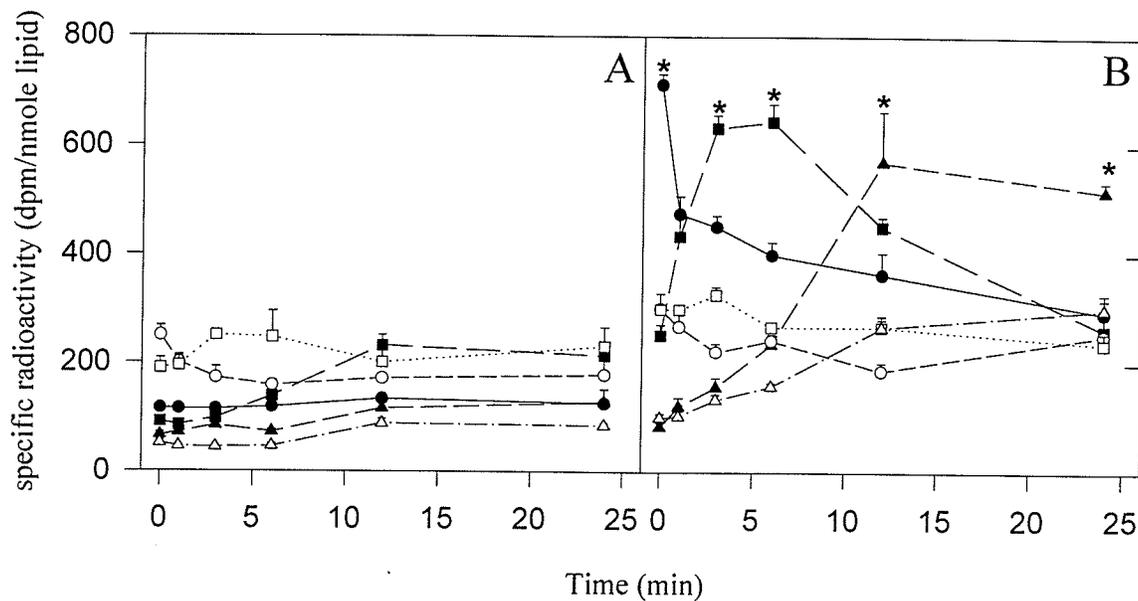


**Figure 17. Effect of attenuated glycerol-3-phosphate pathway on glycerol metabolism.** Chang cells in 35mm dishes were incubated with culture medium without (A) or with (B) 10 mM monobutyryn for 1 h. Subsequent to the monobutyryn exposure, the cells were incubated in a medium containing 0.1 mM [1,3-<sup>3</sup>H] glycerol (3.5 Ci/mmol) for 1 min. The medium was removed, and the cells were further incubated with medium containing 1 mM non-radiolabeled glycerol for different periods. The specific radioactivities of monoacylglycerol (●), diacylglycerol (■), lysophosphatidate (▲) and phosphatidate (▼) were determined and expressed as means  $\pm$  SEM, n=4; \* p<0.05 when compared to the corresponding value in (A).

### 3.3.5 Effect of glycerol concentration on the direct acylation pathway

To determine whether the direct acylation of glycerol could be affected by exogenous glycerol concentration, pulse-chase experiments using H9c2 cells at different glycerol concentrations were conducted. The cells were grown in a 24 well plate to reduce the surface area and the amount of radiolabel required. The cells were pulse labeled for 5 min with either 0.2 or 2.0 mM [1,3-<sup>3</sup>H] glycerol (3.5 Ci/mmole) in serum free culture medium. Serum, which contained glycerol, was excluded from the labeling media to avoid diluting the specific radioactivity of glycerol. The cells were subsequently incubated in culture medium containing 1 or 10 mM glycerol respectively for different times, and the specific radioactivities of the lipids were determined after each incubation. In comparison to the first pulse-chase experiment (Figure 16), the labeling profiles for monoacylglycerol and diacylglycerol were noticeably altered (Figure 18A). The differences may be due to the absence of serum in the labeling medium and the consequent reduced uptake of glycerol (195, 206). At 0.2 mM glycerol, the specific radioactivity curves for monoacylglycerol, triacylglycerol, and phosphatidylcholine were low and remained linear throughout the incubation. Lysophosphatidate was immediately labeled at the beginning of the chase period, and the specific radioactivity rapidly decreased within 3 min. The specific radioactivity of phosphatidate peaked after 3 min and decreased as the incubation progressed. The specific radioactivity of diacylglycerol peaked after 12 min of incubation. The labeling profiles indicate that under low glycerol concentrations, a small amount of the labeled glycerol is converted to lysophosphatidate subsequently to phosphatidate and diacylglycerol in H9c2 cells.

When H9c2 cells were pulse-labeled with high levels of glycerol, the labeling profiles of phosphatidylcholine, monoacylglycerol, diacylglycerol, and triacylglycerol were significantly altered (Figure 18B). Monoacylglycerol was immediately labeled, and its specific radioactivity rapidly decreased within 1 min. Diacylglycerol and triacylglycerol were also labeled and their specific radioactivity curves peaked after 6 and 12 min of incubation respectively. The specific radioactivity of phosphatidylcholine gradually increased as the incubation progressed. These results indicate that at physiological levels, glycerol is metabolized through the glycerol-3-phosphate pathway in H9c2 cells. In contrast, a considerable amount of glycerol is shunted toward the direct acylation pathway for lipid biosynthesis when glycerol levels become elevated.



**Figure 18. Effect of exogenous glycerol concentration on the direct acylation pathway.** H9c2 cells in 24 well plates were pulse labeled with 0.2 mM (A) or 2.0 mM (B) [1,3-<sup>3</sup>H] glycerol (3.5 Ci/mmol) in serum free medium for 5 min. The medium was removed and the cells were further incubated with medium containing 1 mM (A) or 10 mM (B) non-radiolabeled glycerol for different time periods. The specific radioactivities of monoacylglycerol (●), diacylglycerol (■), triacylglycerol (▲) lysophosphatidate (○), phosphatidate (□) and phosphatidylcholine (Δ) were determined. Values are expressed as means ± SEM of two separate experiments done in quadruplicate; \* p<0.05 when compared to the corresponding value in (A).

### 3.3.6 Characterization of glycerol: acyl-CoA acyltransferase

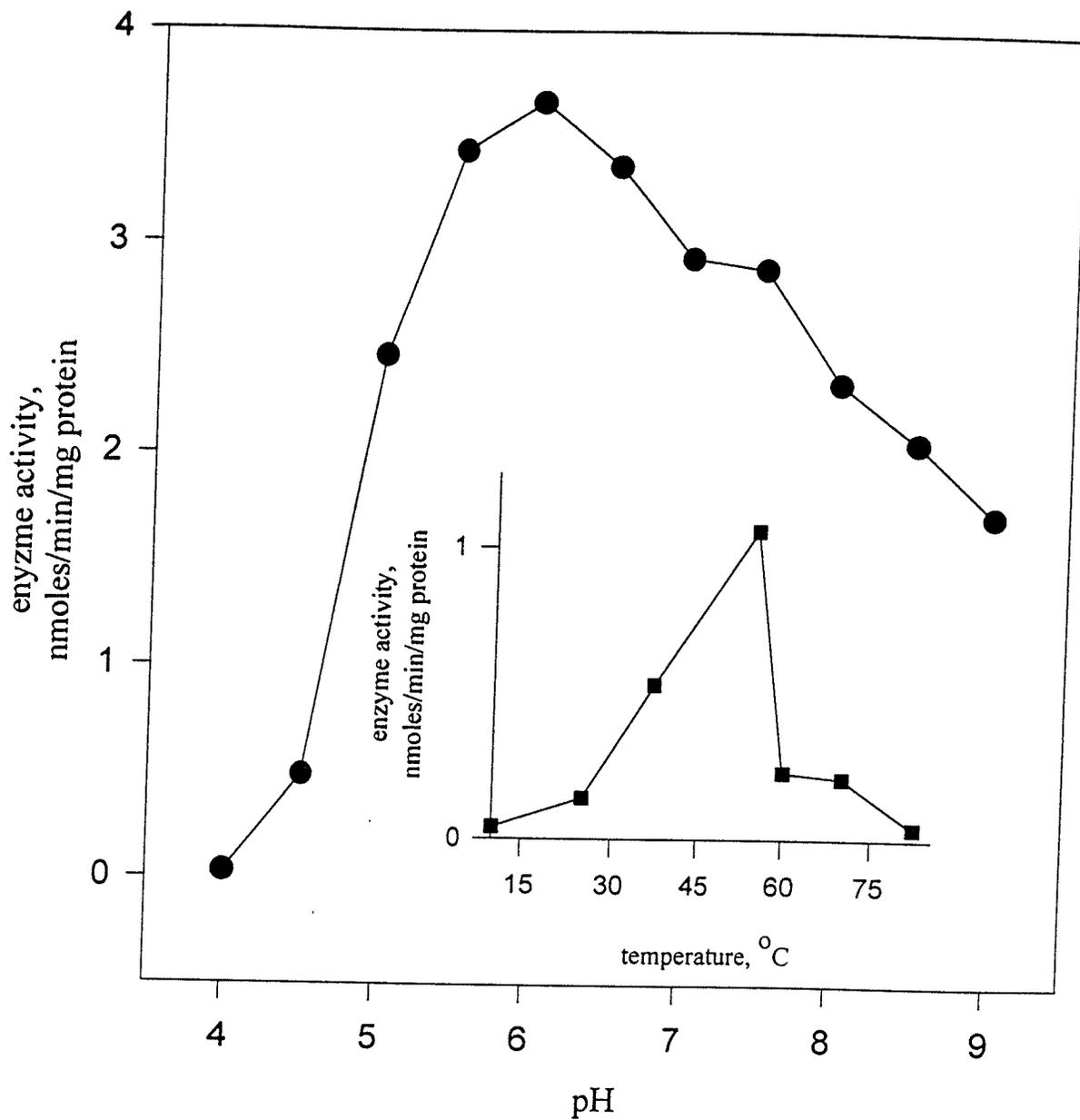
Since the identity of glycerol acyltransferase activity has not been previously demonstrated, the activity of this enzyme was determined in a variety of tissues. As shown in Table 6, the distribution of the enzyme appears to be tissue and species specific. Because of the high glycerol acyltransferase activity in pig heart, this tissue was chosen as the source for enzyme purification. In the heart, a substantial amount of glycerol is produced during the lipolysis of triacylglycerol (207). Glycerokinase activity is nearly undetectable in mammalian cardiac tissues (208), and consequently the glycerol is not recycled for the glycerol-3-phosphate pathway.

The majority of glycerol acyltransferase activity was localized in the microsomal fraction. The microsomal enzyme was active over a broad pH range, with the optimal activity at pH 6.0 (Figure 19). Enzyme activity was found to be optimal at 55°C (Figure 19, inset), but incubation at 60°C resulted in an irreversible inactivation of the enzyme. The enzyme displayed the highest activity with arachidonyl-CoA ( $C_{20:4}$ ) (100%) but lower activity with palmitoyl-CoA ( $C_{16:0}$ ) (25%), stearoyl-CoA ( $C_{18:1}$ ) (40%) and oleoyl-CoA ( $C_{18:1}$ ) (60%). No activity was observed when arachidonate was used. The apparent  $K_m$  values of the enzyme for glycerol and acyl-CoA were determined by varying one substrate concentration while maintaining the other substrate concentration constant (Figure 21). From the Lineweaver-Burke plots, the apparent  $K_m$  for glycerol was 1.1 mM whereas the apparent  $K_m$  for arachidonyl-CoA was 0.17 mM.

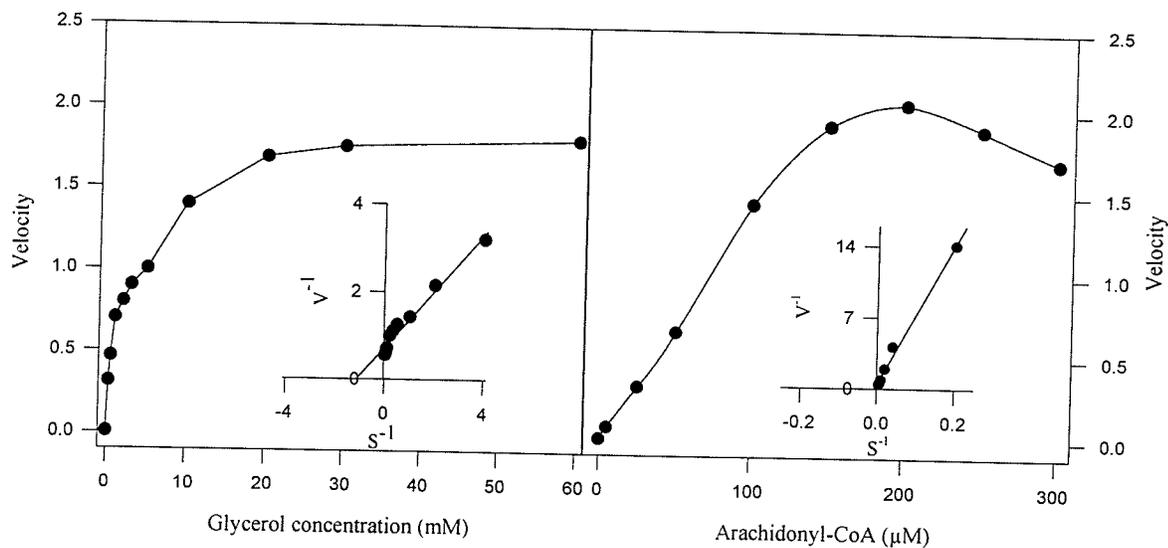
**Table 6. Glycerol acyltransferase activity in microsomal rat and porcine tissue.**

The reaction mixture contained 10  $\mu\text{g}$  of protein, 64.4  $\mu\text{M}$  [ $1\text{-}^{14}\text{C}$ ] arachidonyl-CoA (51.6 mCi/mmole) and 10% (v/v) glycerol in 50 mM Tris-HCl (pH 7.8). The reaction mixture was incubated at 37°C for 30 min. The enzyme activity was determined as described in Materials and Methods. ND, not determined.

Tissue	Specific activity (nmoles/min/mg protein)	
	Rat	Pig
heart	0.56	3.88
liver	1.06	1.04
skeletal muscle	0.86	3.78
kidney	0.35	2.16
brain	ND	0.96



**Figure 19. Effect of pH and temperature (inset) on glycerol acyltransferase activity.** Enzyme activity at the indicated pH or temperature was determined as described in Materials and Methods.



**Figure 20. Kinetic analysis of glycerol acyltransferase.** The apparent  $K_m$  values for glycerol (A) and arachidonyl-CoA (B) were determined by varying one substrate concentration while maintaining the other substrate concentration constant. Enzyme assays were conducted as described in Materials and Methods. The arachidonyl-CoA concentration was held at 200  $\mu\text{M}$  while the glycerol concentration was varied (A). The glycerol concentration was held at 5% (v/v) (0.68 M) while the arachidonyl-CoA concentration was varied (B). Inset: Lineweaver-Burk plots. Velocity is expressed as nmoles/min/mg protein.

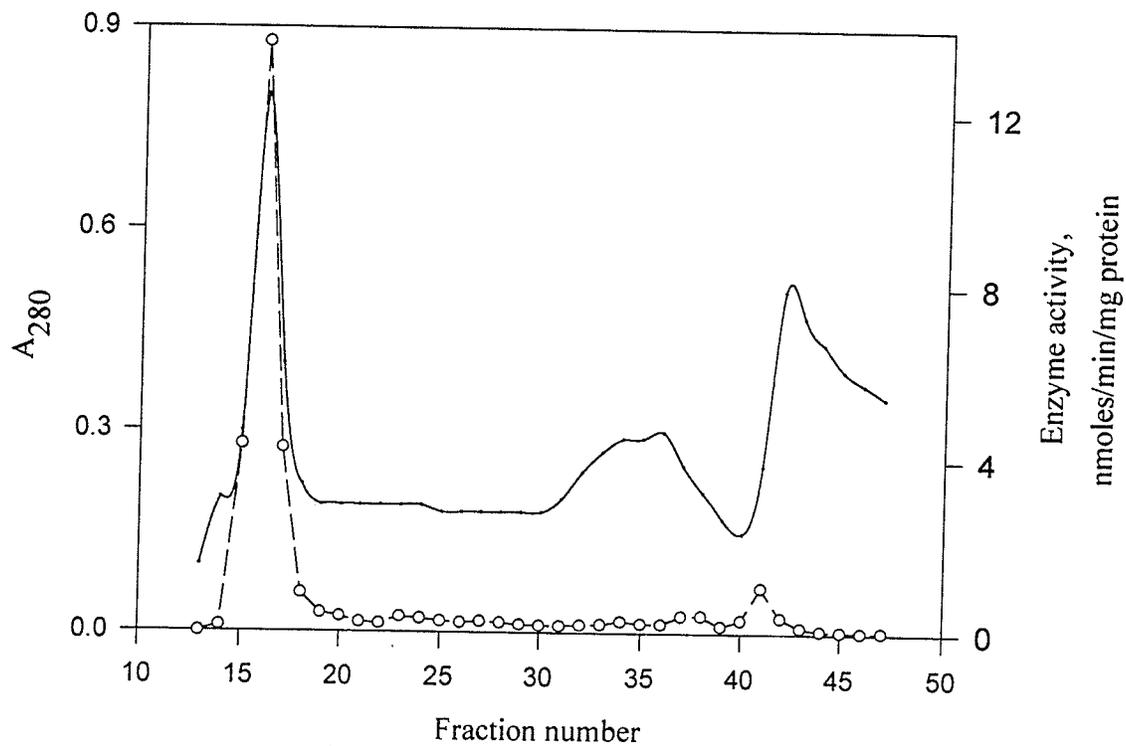
### 3.3.7 Purification of glycerol: acyl-CoA acyltransferase

The solubilization of membrane protein is commonly accomplished by the use of detergent (209). Hence, the microsomal fraction was incubated with Triton X-100, Chaps or Nonidet P-40 at various concentrations (Table 7). While all the detergents appeared to be effective, Nonidet P-40 (0.2%) was employed in subsequent experiments because the solubilized enzyme was most stable in this detergent. When the solubilized enzyme was applied to a gel filtration column and analyzed by FPLC, two enzyme activity peaks were obtained (Figure 21). A major activity peak was eluted from the column near the void volume that corresponded to the elution of mixed micelles. A minor activity peak was detected in fractions containing low molecular weight protein. The fractions from the minor activity region were analyzed by Tricine-SDS PAGE. Densitometric analysis of the protein bands in each fraction revealed the concomitant elution of an 18 kDa protein with glycerol acyltransferase activity (Figure 22A and C). Further purification of glycerol acyltransferase could not be achieved by conventional techniques. The enzyme was completely inactivated when the active fraction from the gel filtration column was further purified by ion-exchange or affinity chromatography.

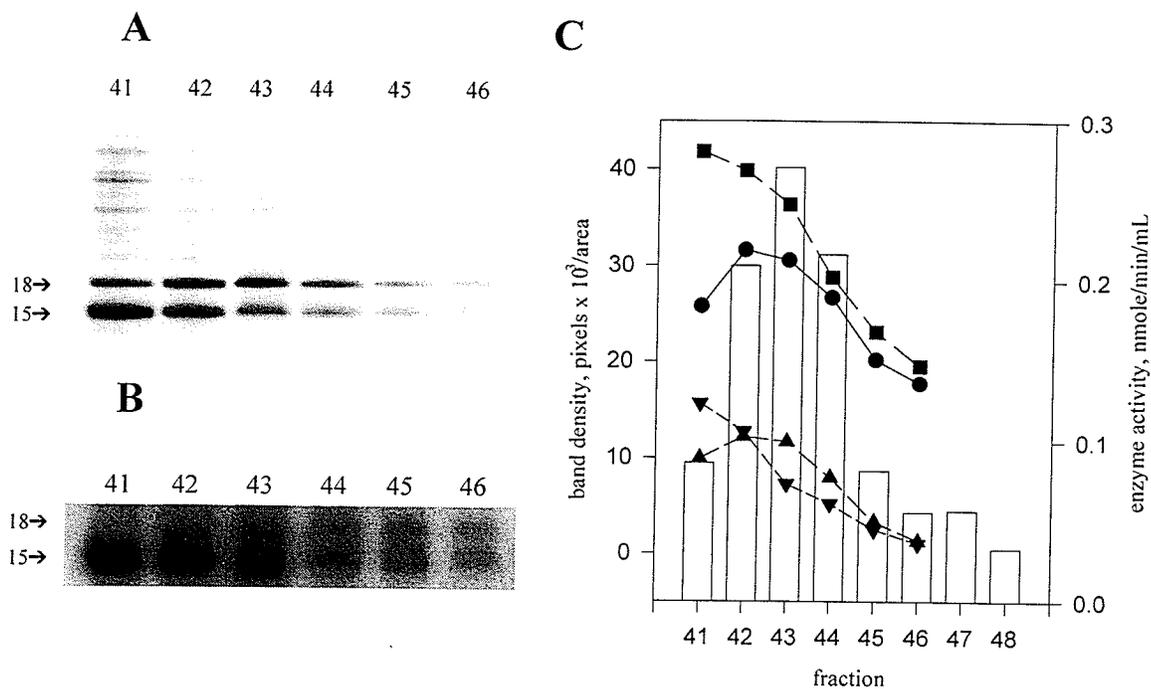
**Table 7. Detergent solubilization of glycerol acyltransferase.**

Pig heart microsomes were incubated with various detergents at 4°C for 1 h as described in Materials and Methods. The mixture was centrifuged at 100,000 g for 1 h, and glycerol acyltransferase activity in the supernatant was determined. Enzyme activity of control was 2.63 nmoles/min/mg protein.

<b>Detergent</b>	<b>% total activity</b>
control (no detergent)	100
Nonidet P-40	
0.01%	21
0.02%	22.1
0.05%	24.4
0.1%	30.4
0.2%	39
0.3%	31.9
Triton X-100	
0.01%	35.3
0.02%	39.7
0.05%	35.9
Chaps	
2mM	25
5mM	26.6
10mM	31.6



**Figure 21. Gel filtration analysis of solubilized microsomes.** Nonidet P-40 (0.2%) solubilized pig heart microsomes were applied onto an FPLC Superose 6B 16/50 HR gel filtration column. The flow rate was set at 0.75 mL/min and 1.8 mL fractions were collected. The eluent was monitored with a single path UV-1 monitor (Pharmacia Biotech, NJ, USA). Each fraction was assayed for glycerol acyltransferase activity (O) and protein concentration (•).



**Figure 22. Identification of glycerol acyltransferase by photoaffinity labeling and gel electrophoresis.** A. Proteins eluted from the gel filtration column were analyzed by 10%Tricine-SDS PAGE after photoaffinity labeling with [<sup>125</sup>I] ASD-CoA. Bands were visualized by Serva G staining. Fractions are indicated above each lane. B. Autoradiogram of [<sup>125</sup>I] photoaffinity labeled proteins separated by gel filtration and Tricine-SDS PAGE. The lanes correspond to the lanes shown in A. C. Elution profile of glycerol acyltransferase activity from the gel filtration column (open bars) and densitometric analysis of protein and autoradiographic bands. The 18 kDa protein band (●), 15 kDa protein band (■), [<sup>125</sup>I] labeled 18 kDa band (▲) and [<sup>125</sup>I] labeled 15 kDa band (▼) intensities were determined by using Scion Image software.

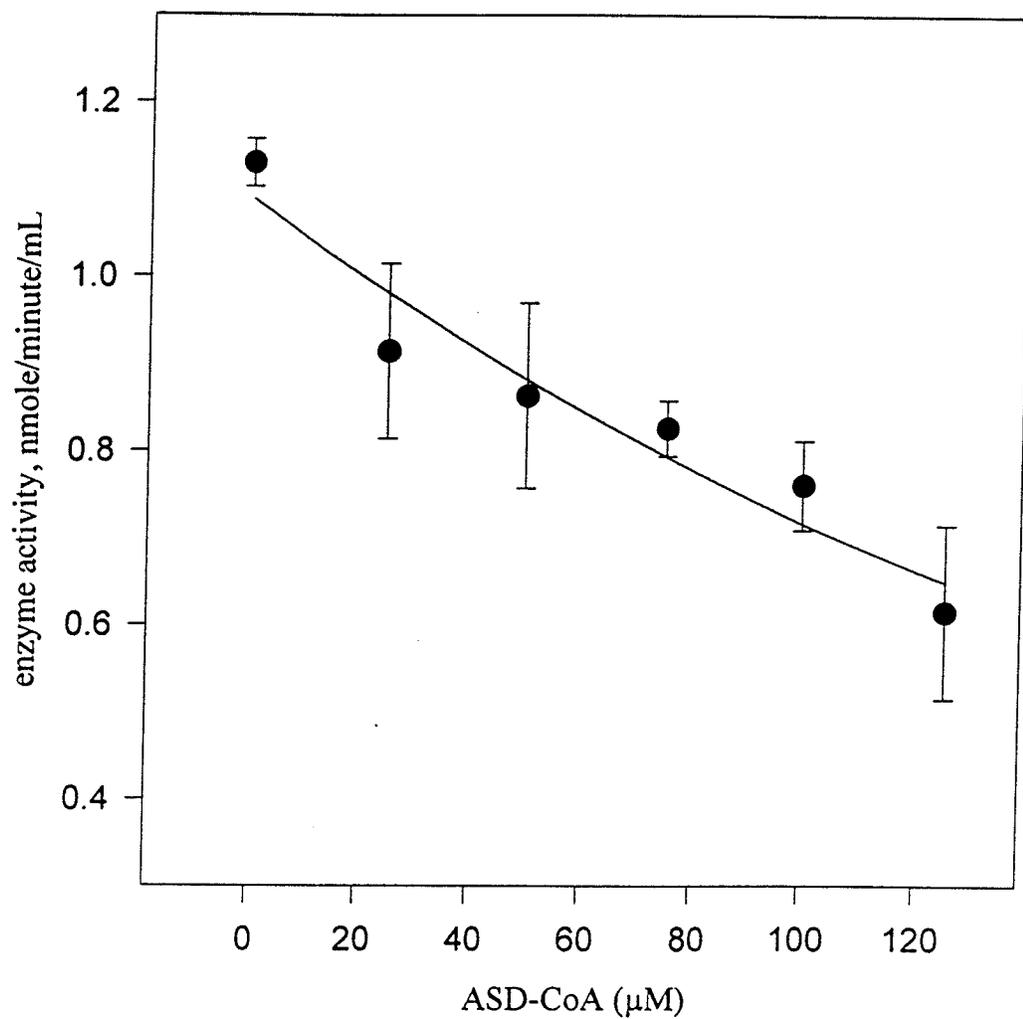
### 3.3.8 Photoaffinity labeling glycerol: acyl-CoA acyltransferase

Since further purification of the glycerol acyltransferase could not be achieved by column chromatography, the enzyme was identified in its inactive form by photoaffinity labeling. Photoaffinity labeling has been successfully used for the identification of several acyltransferases from plant sources (210, 211). Based on these studies, a photoaffinity probe for the detection of glycerol acyltransferase was developed. The approach was to synthesize a radiolabeled photoaffinity probe, which would be specific to the enzyme. Upon irradiation by ultraviolet light, the radiolabeled probe would be covalently linked to the enzyme in order to facilitate the identification of the enzyme band after gel electrophoresis.

To establish whether ASD-CoA could serve as a suitable photoaffinity probe for glycerol acyltransferase, inhibition and photoinactivation studies were performed. When the microsomal enzyme was assayed in the dark in the presence of ASD-CoA, the incorporation of [ $1-^{14}\text{C}$ ] arachidonyl-CoA into glycerol was decreased in a dose-dependent manner (Figure 23). The inhibition of the enzyme activity by ASD-CoA was competitive in nature (data not shown). In another set of experiment, microsomes were incubated with ASD-CoA and exposed to ultraviolet light in the presence and absence of arachidonyl-CoA. The inhibition of enzyme activity was more pronounced when the reaction mixture was exposed to ultraviolet irradiation (Table 8). Photoinactivation of the enzyme increased with higher amounts of ASD-CoA. The inhibition was not reversible by subsequent addition of arachidonyl-CoA. Alternatively, when arachidonyl-CoA was present in the assay mixture prior to ultraviolet irradiation, inhibition of the enzyme activity could be alleviated (Table 8). Taken together, it is plausible that both arachidonyl-CoA and ASD-CoA competed for

the same binding site on the enzyme, and hence ASD-CoA might be a suitable probe for the photoaffinity labeling of the enzyme.

Active fractions from both peaks eluted from the gel filtration chromatography were incubated with [<sup>125</sup>I] labeled ASD-CoA. The mixture was exposed to ultraviolet light, and the labeled proteins were analyzed by Tricine-SDS PAGE. An autoradiogram was produced from the gel, and several protein bands (15, 18, 43, 67, and 94 kDa) were found to be labeled (data not shown). In fractions containing the low molecular weight proteins, the 15 and 18 kDa bands were prominently labeled (Figure 22B). Densitometric analysis of the autoradiogram indicated that the 18 kDa band was labeled with [<sup>125</sup>I] ASD-CoA in a manner consistent with elution of glycerol acyltransferase activity from the gel filtration column (Figure 22C). The other photoaffinity labeled protein bands (15, 43, 67, 94 kDa ) were found to be unrelated to the elution of glycerol acyltransferase activity. In addition, none of the photoaffinity labeled protein bands (including the 18 kDa protein) from the major activity peak could be correlated to the elution of glycerol acyltransferase activity since the proteins likely eluted in the form of mixed micelles.



**Figure 23.** The effect of ASD-CoA on glycerol acyltransferase. Pig heart microsomes (~10μg protein) were incubated in the dark with various concentrations of ASD-CoA. Enzyme activity was determined as described in Materials and Methods.

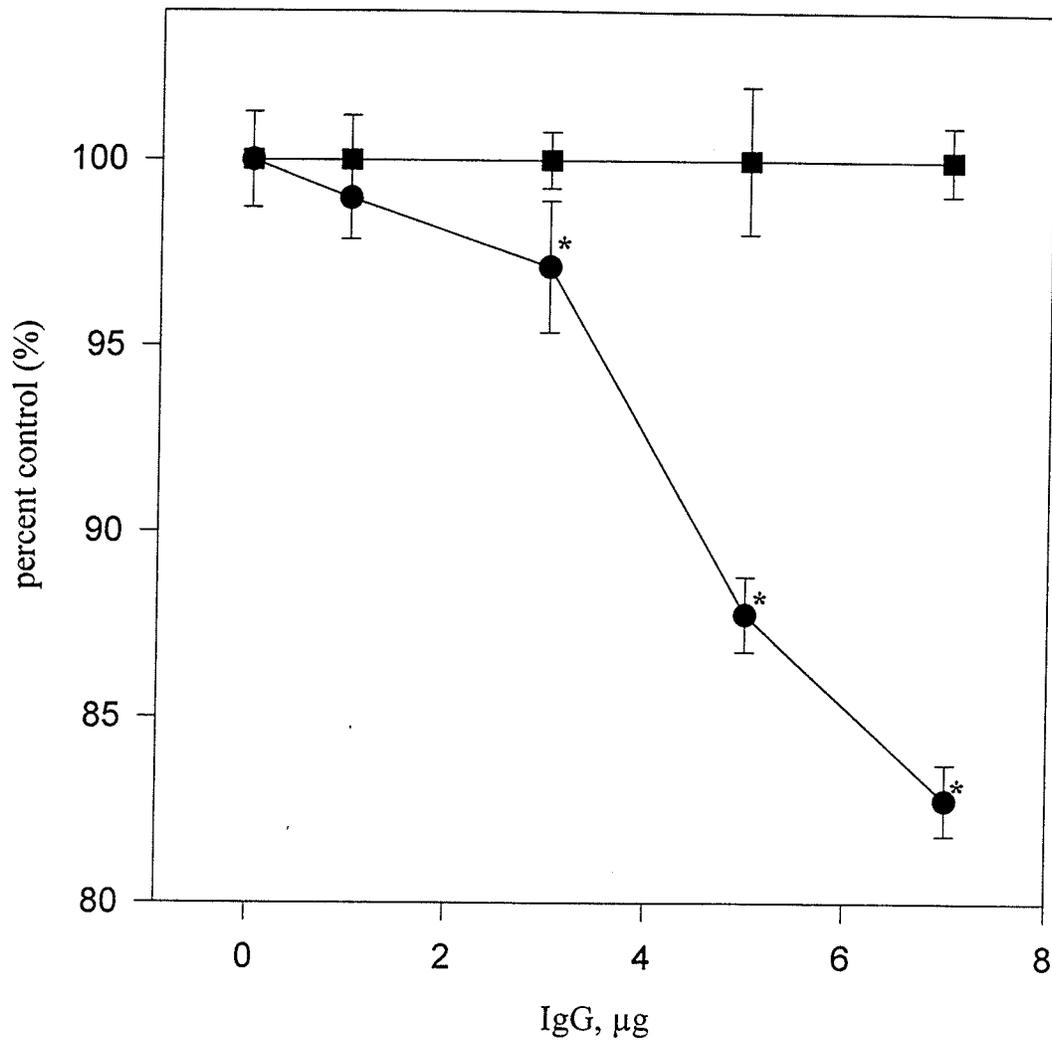
**Table 8. ASD-CoA photoinactivation of glycerol acyltransferase.**

Glycerol acyltransferase was solubilized from the microsomal membrane with 0.2% (v/v) Nonidet P-40. The enzyme was incubated in the presence and absence of ASD-CoA and arachidonyl-CoA. The mixture was exposed to ultraviolet light for 5 min and enzyme activity was determined as described in Materials and Methods. Enzyme activity of the control was 0.95 nmole/min/mg protein.

<b>Reaction mixture</b>	<b>Relative activity</b>
Enzyme + UV	100%
Enzyme + 0.05mM ASD-CoA + UV	62%
Enzyme + 0.05mM ASD-CoA + UV + 0.05mM arachidonyl-CoA	62%
Enzyme + 0.1mM ASD-CoA + UV	42%
Enzyme + 0.05mM ASD-CoA + 0.05mM arachidonyl-CoA + UV	70.2%

### **3.3.9 Immunoprecipitation of glycerol acyltransferase**

Immunological studies were conducted to verify that glycerol acyltransferase was correctly identified as the 18 kDa band. Polyclonal antibodies were produced against each of the photoaffinity labeled protein bands. Of the antibodies developed, only the anti-18 kDa antibody was found to inhibit the glycerol acyltransferase activity. Incubation of the anti-18 kDa antibody with solubilized enzyme sample caused the immunoprecipitation of the enzyme in a dose dependent manner (Figure 24). In contrast, the pre-immune IgG did not cause any immunoprecipitation but produced a slight stimulation to the glycerol acyltransferase activity. The ability of the anti-18 kDa antibodies to inhibit the glycerol acyltransferase activity in the solubilized enzyme sample provided us with an unambiguous identity of the 18 kDa band in the Tricine-SDS-PAGE.



**Figure 24. Immunoprecipitation of glycerol acyltransferase by anti-18 kDa IgG.** The anti-18 kDa IgG (●) and pre-immune IgG (■) were incubated with Nonidet P-40 (0.2%) solubilized microsomes. Immunoprecipitation was assessed by determining the residual enzyme activity remaining in the supernatant and expressed as percent of control (0.0045 nmole/min/mL). Values are expressed as means  $\pm$  SEM of three separate experiments done in quadruplicate; \*  $p < 0.05$  when compared to the control.

### 3.3.10 Sequence Analysis

The 18 kDa band obtained from the most active fraction (fraction 42) was isolated from the Tricine-SDS PAGE, transferred to a polyvinylidene difluoride membrane and used for N-terminal amino acid sequencing. N-terminal analysis of the 18 kDa blotted protein resulted in a sequence which matched pig myoglobin (Figure 25).

The addition of pig myoglobin to the enzyme assay mixture did not cause any acylation of glycerol. When myoglobin (1-5  $\mu\text{g}$ ) was preincubated with the microsomal fraction containing 50  $\mu\text{g}$  of protein for 1 h prior to assay, a linear increase of enzyme activity (up to 30%) was observed. In contrast, increased glycerol acyltransferase activity was not observed with other proteins such as albumin or globulin. Polyclonal antibodies produced from pig myoglobin produced similar effects as the anti-18 kDa IgG.

MYPG            **GLSDGEWQVVLNVWCKVEAD**VAGHGQEVLI R L R L F K G H P E T L E K F D K F K H L K S E D E M K A S E D  
 18kDa\_band **GLSDGEWQVVLNVWCKVEAD**-----  
                   1.....10.....20.....30.....40.....50.....

MYPG            L K K H G N T V L T A L G G I L K K K G H H E A E L T P L A Q S H A T K H K I P V K Y L E F I S E A I I Q V L Q S K H P  
 18kDa\_band -----  
                   61.....70.....80.....90.....100.....110.....

MYPG            G D F G A D A Q G A M S K A L E L F R N D M A A K Y K E L G F Q G  
 18kDa\_band -----  
                   121.....130.....140.....150.

**Figure 26. Alignment of amino acid sequences.** Pig myoglobin (MYPG) amino acid sequences are compared to the first 20 amino acid residues of the purified 18 kDa protein band.

## 4 DISCUSSION

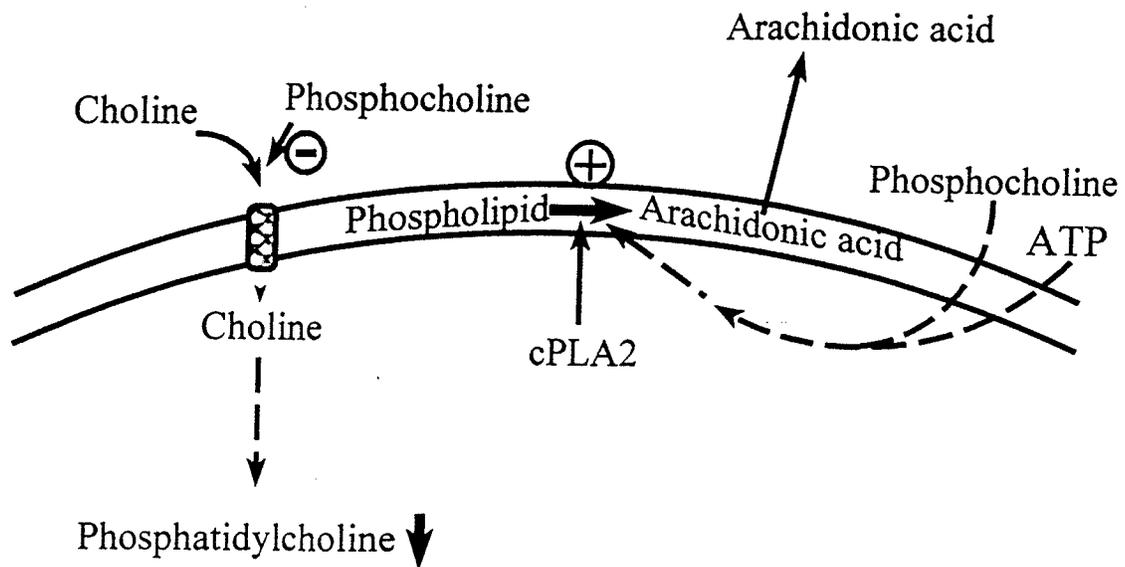
### 4.1 Studies on the Modulation of Phosphatidylcholine by Phosphocholine

The mitogenic effect of exogenous phosphocholine (1-25 mM) on NIH 3T3 cells has been clearly demonstrated (81, 212). In the NIH 3T3 cells, the normal intracellular phosphocholine concentration is about 0.5 mM, but the level may be elevated two- to four-fold in the ras-transformed cells. In the rat, phosphocholine concentrations in plasma is 0.1  $\mu$ M whereas it is much higher in the cerebrospinal fluid (1.7  $\mu$ M) (213). In the rat liver, phosphocholine level is 2-3  $\mu$ mol/g, and the level may be altered by fasting and re-feeding (214, 215). In rat lens, phosphocholine concentration is greater than 10 mM (216). Hence, the concentration of phosphocholine used in the present study to produce alterations in phosphatidylcholine metabolism are within the physiological range under certain pathological conditions.

In this study, the ability of phosphocholine to modulate phosphatidylcholine biosynthesis is clearly demonstrated. The study confirmed that exogenous phosphocholine was not taken up by the endothelial cells but competitively inhibited choline uptake (Figure 26). Consequently, although enzymes of the CDP-choline pathway were not affected, the extracellular phosphocholine caused the intracellular phosphocholine pool to decrease. Previous studies have shown that modulation of choline uptake and the inhibition of choline kinase activity are mechanisms for the regulation of phosphatidylcholine biosynthesis in mammalian tissues (70). Choline for phosphatidylcholine biosynthesis is transported across

the cellular membrane by a saturable mechanism, and the transport can be inhibited by choline analogs (70). For example, ethanolamine is a competitive inhibitor of choline uptake in the hamster heart and in baby hamster kidney-21 cells (217, 218). In addition, choline uptake is inhibited by hemicholinium-3 in a non-competitive manner (217). It is clear from this study that phosphocholine regulates phosphatidylcholine biosynthesis via its inhibition of choline uptake.

The release of arachidonate in mammalian tissues can be induced by ATP (219). In this study, the ATP induced arachidonate release was enhanced by phosphocholine in human umbilical vein endothelial cells. The cytosolic phospholipase A<sub>2</sub> has been shown to be the key enzyme for arachidonate release in endothelial cells (220). Cytosolic phospholipase A<sub>2</sub> can also be induced by another bioactive lipid, lysophosphatidylcholine, in human umbilical vein endothelial cells (188). The treatment of the cells with lysophosphatidylcholine causes activation of cPLA<sub>2</sub> by phosphorylation of the enzyme. In the present study, the cytosolic phospholipase A<sub>2</sub> activity was not activated by phosphorylation since incubation of the cells with phosphocholine did not affect enzyme activity. However, this study does not rule out that the intracellular phospholipases A<sub>2</sub> (173) was modulated by factors produced during phosphocholine incubation. Since ATP is known to increase intracellular calcium, by interaction with nucleotide receptors (221, 222), the level of intracellular calcium may be potentiated by phosphocholine incubation. This would cause the activation of the phospholipase and produce an enhancement of arachidonate release. It remains an open question whether the observed enhancement of ATP-induced arachidonate release by phosphocholine was due to perturbation in calcium levels.



**Figure 26. Effect of phosphocholine on phosphatidylcholine metabolism**

## 4.2 Studies on the Deacylation / Reacylation Cycle

Limited progress was made in the purification of lysophosphatidylcholine acyltransferase. However, the attempt was not fruitless since the direct acylation pathway was discovered during the effort. The existence of multiple acyltransferases, each of which is specific for a defined lysolipid and acyl group, has been postulated. Studies on the aging of microsomal enzyme at 4°C resulted in a differential loss of acyltransferase activity for different acyl donors (182). Additional support for multiple forms of acyltransferase comes from the observation that an increase in specificity for a particular acyl group occurs during enzyme purification (185, 186). For example, the enhancement of specificity for the arachidonyl-CoA with a corresponding loss in specificity of other acyl-CoA during purification was observed. Direct evidence for the existence of acyltransferase isozymes must come from studies with the purified enzyme.

### 4.3 Studies on the Direct Acylation of Glycerol

It has been well established that glycerol is first phosphorylated to glycerol-3-phosphate before acylation occurs. Consequently, results from previous studies on glycerol metabolism might have been misinterpreted especially when other metabolic routes for glycerol are present (Figure 27). Glycerol metabolism was re-examined in the current study, and there are several lines of evidence supporting a novel pathway for lipid biosynthesis. Firstly, pulse-chase studies with both H9c2 and Chang liver cells demonstrated that glycerol could be directly converted to monoacylglycerol and subsequently to other lipids. Secondly, we have identified, purified and characterized a novel enzyme for the direct acylation of glycerol. The glycerol acyltransferase has the ability to transfer various long chain acyl-CoA species, but displays a high degree of specificity for arachidonyl-CoA. Surprisingly, the newly identified glycerol acyltransferase shares the same identity as myoglobin.

Glycerol acyltransferase was readily solubilized from the microsomal fraction, but its purification by chromatographic methods remains to be a challenge. Some degree of purification was achieved by the use of gel filtration chromatography, ion exchange chromatography, membrane filtration and selective heat denaturation. Unfortunately, attempts to obtain a higher degree of purification by coupling these methods in tandem inevitably resulted in the complete loss of enzyme activity. Due to the instability of the enzyme, an alternative approach was developed to identify the enzyme in its inactive form. Photoaffinity labeling of the enzyme enabled identification of the inactive glycerol acyltransferase at every stage of purification. The ability to identify the inactive enzyme provided the opportunity to utilize other techniques, including the high-resolution power of

Tricine SDS-PAGE for protein isolation. The limitation of photoaffinity labeling is that the probe may not be entirely specific and more than one protein band may be labeled. As demonstrated in this study, ASD-CoA caused the labeling of five distinct protein bands. Fortunately, only the labeling associated with the 18 kDa protein band corresponded with enzyme activity in fractions eluted from the gel filtration chromatography. The positive identification of this protein band as the enzyme itself, however, would require additional confirmation. Hence, antibodies to each of the five protein bands were produced, and immunological studies confirmed the identity of the 18 kDa band as the glycerol acyltransferase protein.

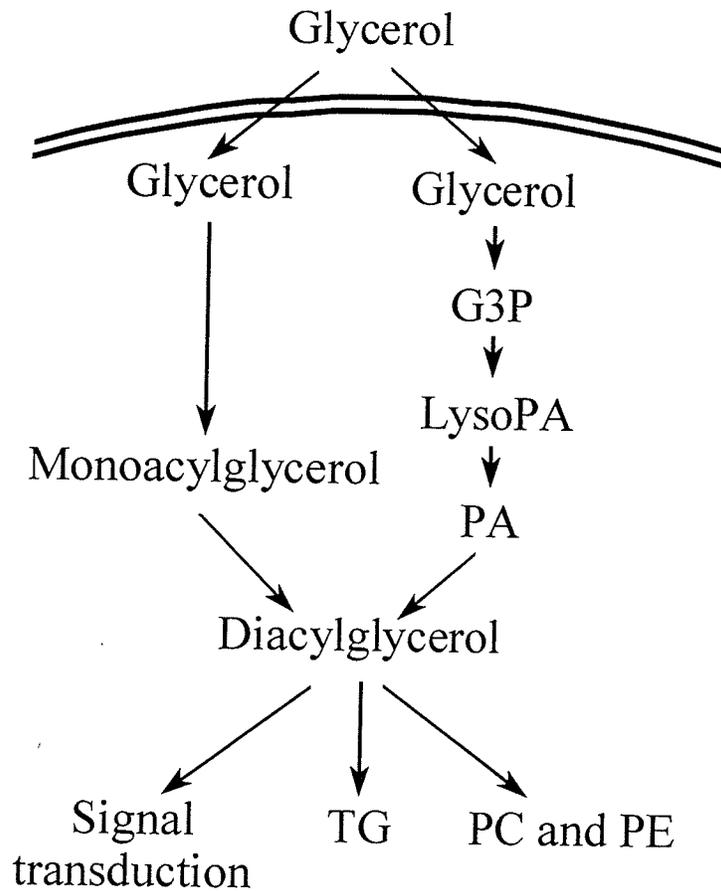
Although the physiological importance of the direct acylation pathway has not been completely established, this study has produced strong evidence that this novel pathway may serve as a shunt for glycerol metabolism. Glycerol is present in the mammalian body within and between all cells at a concentration of about 0.1 mM (223). In human serum, the glycerol level fluctuates from 0.04 to 0.4 mM (224). Kinetic studies of the enzyme revealed that it has an apparent  $K_m$  value of 1.1 mM for glycerol, indicating the rate of acylation is directly proportional to the intracellular glycerol concentration. As such, the direct acylation of glycerol may only occur in limited capacity during normal physiological conditions. Alternatively, the intracellular glycerol concentration is dramatically increased in certain forms of muscular dystrophy, diabetes, during fasting, extreme cold and ischemia of the heart (224, 225). Since the acylation of glycerol is regulated by glycerol availability, the direct acylation pathway may be of central importance during hyperglycerolemia. This notion is supported by the study on the metabolism of glycerol in Chang liver cells in the presence of

monobutyryn and in H9c2 cells. When the glycerol-3-phosphate pathway was inhibited in Chang liver cells, the acylation of glycerol was increased immediately as an alternative mechanism for the production of monoacylglycerol. In H9c2 cells, lipid biosynthesis increased dramatically when the direct acylation pathway was activated by elevated glycerol levels.

Unexpectedly, glycerol acyltransferase was found to share the same identity as myoglobin. It can be argued that glycerol acyltransferase may be a minor protein with similar molecular mass as myoglobin and would make it indistinguishable from myoglobin in the polyacrylamide gel. There are several lines of evidence against this argument. Firstly and the foremost, antibodies against myoglobin are effective in inhibiting the enzyme activity. Secondly, if the enzyme has a similar molecular mass as myoglobin, the enzyme that is covalently bound to the photoaffinity probe would have a different molecular mass, which would make it apparent in the autoradiogram. Indeed, no other protein band was observed near the 18 kDa region, with or without photoaffinity labeling. Thirdly, proteins other than myoglobin were not apparent in the 18 kDa protein band during amino acid sequence analysis. One intriguing aspect of the study is that myoglobin is a soluble protein, and its association with the pig heart microsomes to express acyltransferase activity is not clear. Its association with the microsomes indicates that the protein may have undergone some modification, perhaps at the post-translational stage. The fact that the purified myoglobin from pig heart has no ability to catalyze the acyltransferase reaction lends support to this notion.

Myoglobin belongs to the globin family, which is wide spread and has been

characterized in an expanding list of organisms including invertebrates, such as plants, fungi, protozoa, bacteria and archaeobacteria. Comparison of these proteins has unexpectedly revealed a great diversity in function and structure (226). For example, the myoglobin in *Halobacterium salinarum* functions to bind oxygen, and to transmit a signal through its C-terminal domain mediating chemotaxis (227). In *Rhizobium*, FixL is a chimeric protein which contains both globin and kinase domains (228). In *Alcaligenes eutrophus*, the globin contains a diaphorase activity (229), and in *Escherichia coli*, the globin shows a dihydropterine reductase activity (230). Since globin proteins with alternate functions are not novel, we speculate that glycerol acyltransferase is a specialized myoglobin molecule that has undergone some minor modification. Under normal conditions, the myoglobin facilitates the transport of oxygen to the mitochondria. Physiological stress causing hyperglycerolemia would result in modification of the myoglobin molecule and cause its translocation to the lipid membrane and the activation of its glycerol acyltransferase activity. Hence, myoglobin itself would not catalyze the acyltransferase, but if incubated with microsomes, the protein might undergo modification to acquire its catalytic activity. It is possible that myoglobin interacts with a fatty acid (231-233) and translocates to membrane structures (234-236). The bi-functional myoglobin would provide the cell a facile mechanism to cope with excess glycerol levels without the delay and energy requirements necessary for new protein synthesis.



**Figure 27. Pathways for glycerol metabolism.** The glycerol-3-phosphate pathway (right) and the direct acylation pathway (left). DHAP, dihydroxyacetone-3-phosphate; G3P, glycerol-3-phosphate; LysoPA, lysophosphatidate; PA, phosphatidate; TG, triacylglycerol; PC, phosphatidylcholine; PE, phosphatidylethanolamine.

## 5 SUMMARY

The research described in this thesis was designed to investigate the control of glycerolipid metabolism in mammalian tissues. In the first part of the study, the effect of exogenous phosphocholine on phosphatidylcholine biosynthesis and arachidonate release from human umbilical vein endothelial cells was examined. In the second part, several attempts to purify lysophosphatidylcholine acyltransferase were made. In the third part of this study, the direct acylation of glycerol was demonstrated and characterized.

In the first part of the study, phosphocholine (0-10 mM) was found to inhibit phosphatidylcholine biosynthesis in human umbilical vein endothelial cells. The extracellular phosphocholine reduced the intracellular phosphocholine pool while the choline and CDP-diacylglycerol were unaffected. Reduction of the phosphocholine pool was not caused by alterations in the enzyme activities of the CDP-choline pathway. Rather, the inhibition of phosphatidylcholine biosynthesis was caused by competitive inhibition of choline uptake. Extracellular ATP induced arachidonate release from the endothelial cells, and the release was significantly enhanced in the presence of phosphocholine. These studies demonstrate that phosphocholine may modulate phosphatidylcholine metabolism by affecting choline uptake and arachidonate release.

In the second part of the study, the deacylation / reacylation cycle of phosphatidylcholine metabolism was examined. Several attempts were made to purify the lysophosphatidylcholine acyltransferase but met with limited success. The enzyme was

rapidly inactivated after isolation by a second chromatographic column. In addition, enzyme activity was not recoverable in an activity gel assay. Further work is required for developing a procedure for the purification of the lysophosphatidate acyltransferase. Interestingly, studies on the deacylation / reacylation process led to the discovery of the novel direct acylation pathway.

In the final part of this study, the direct acylation of glycerol was examined in myoblast and hepatocyte cells. Glycerol was directly acylated to form monoacylglycerol, diacylglycerol and triacylglycerol. This pathway became prominent when the glycerol-3-phosphate pathway was attenuated and when glycerol levels became elevated. These studies clearly demonstrate the existence of a novel lipid biosynthetic pathway that may be important during hyperglycerolemia.

Glycerol acyltransferase activity was detected in the microsomal fraction of mammalian tissues. The enzyme was identified as an 18 kDa protein after gel filtration chromatography and photoaffinity labeling. Antibodies raised against the 18 kDa protein immunoprecipitated solubilized glycerol acyltransferase, thus confirming its identity. Sequence analysis of the 18 kDa protein revealed that it shares the same identity as myoglobin. These studies suggest that a specialized myoglobin is modified and translocated to the membrane where enzyme activity is then conferred.

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