THE METABOLISM OF PHOSPHOGLYCERIDES IN MAMMALIAN HEARTS

by

Monika Wientzek

a thesis presented to the

Faculty of Graduate Studies

University of Manitoba

In partial fulfillment of the

requirements for the degree

Master of Science

Department of Biochemistry

October 1986

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To my parents

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ABSTRACT

Plasmenylcholine (1-alkenyl-2-acyl-sn-glycero-3-phosphocholine) is an ether lipid which comprises 34% of the total choline glycerophospholipids in the guinea pig heart. The formation of plasmenylcholine from choline in mammalian tissues was not known and the biosynthesis of phosphatidylcholine in the guinea pig heart had not been studied. In this study, the biosynthesis of choline glycerophospholipids was investigated by perfusion of the guinea pig heart in the Langendorff mode with [Methyl-^{3}H]choline. An initial lag phase was observed for the first 40 min for the incorporation of labeled choline into the total choline glycerophospholipid fraction which was composed mainly of phosphatidylcholine and plasmenylcholine. After this lag period, the incorporation of labeled choline into these fractions became linear. This initial lag period in the incorporation of labeled choline into these choline glycerophospholipids suggests that the majority of these phospholipids were formed via the CDP-choline pathway. A minor contribution to their formation by the base-exchange reaction was also suggested.

The formation of plasmenylcholine from CDP-choline and 1-alkenyl-2-acyl-glycerol was demonstrated *in vitro* by the determination of CDP-choline : 1-alkenyl-2-acyl-glycerol phosphocholinetransferase activity in guinea pig heart microsomes. In addition, 1-alkenyl-2-acyl-glycerol was shown to be present in the guinea pig heart (17 nmol/g heart).

The formation of choline glycerophospholipids by the methylation of phosphatidylethanolamine or plasmenylethanolamine was investigated by

perfusion of the isolated guinea pig heart with [1-^{3}H]ethanolamine for 60 and 120 min. Significant amounts of labeled ethanolamine were incorporated into phosphatidylethanolamine and plasmenylethanolamine. Only a small amount of labeling of plasmenylcholine and phosphatidylcholine from labeled ethanolamine phosphoglycerides was observed suggesting that progressive methylation plays a minor role in the formation of plasmenylcholine.

Results for the incorporation of choline into choline glycerophospholipids indicate that the major pathway involved is the CDP-choline pathway. The rate-limiting steps of the CDP-choline pathway for the biosynthesis of choline glycerophospholipids were determined by pulse-chase experiments. Isolated guinea pig hearts were pulse-labeled for 15 min with [Methyl-^{3}H]choline and then chased with unlabeled choline for 0 to 120 min. The phosphorylation of choline (by choline kinase) and the conversion of phosphocholine to CDP-choline (by cytidylyltransferase) were found to be the rate-limiting steps in the biosynthesis of phosphatidylcholine and plasmenylcholine in the guinea pig heart. The rates of phosphatidylcholine and plasmenylcholine biosynthesis via the CDP-choline pathway were 2.69 nmol/min/g heart and 0.18 nmol/min/g heart, respectively.

The hydrolysis of plasmenylcholine by the enzyme plasmalogenase may be a source for lysophosphatidylcholine, which is cytolytic at high concentrations. Plasmalogenase activity towards plasmenylcholine had not been reported in the mammalian heart. A new spectrophotometric assay was developed in which the aldehyde released from the hydrolysis

by plasmalogenase was oxidized to carboxylic acid by the action of aldehyde dehydrogenase, with the production of the molar equivalent of NADH. The sensitivity of this spectrophotometric assay for plasmalogenase was shown to be 25-fold higher than with the methods described previously and enzyme activity could be detected with 1 µg of microsomal protein. Plasmalogenase activity was located exclusively in the cardiac microsomal fraction and the enzyme displayed a pH optimum at 8.5. The enzyme was active towards plasmenylethanolamine but not plasmenylcholine. It appears that in the heart, hydrolysis of the vinyl ether bond is not the initial step for the degradation of plasmenylcholine.

The role of elevated lysophosphatidylcholine levels has been implicated in the genesis of cardiac arrhythmias. Hence, the tissue level of this lipid must be accurately assessed. A new procedure for the determination of small amounts of lysophosphatidylcholine in cardiac tissue was developed. Lysophosphatidylcholine from canine heart was separated from the major phospholipids by column chromatography and then acetylated with labeled acetic anhydride. The acetylated lysophosphatidylcholine was isolated by thin layer chromatography and the lysophosphatidylcholine content was calculated from the radioactivity associated with the acetylated product. Although the sensitivity of the assay depends on the specific radioactivity of the acetic anhydride used, as low as 0.5 nmol of lysophospholipid in tissue samples can be readily quantitated. The results obtained from the control and ischemic canine cardiac tissues by this assay compare favorably with

those obtained by lipid-phosphorus assay and reconfirm the observed increase in lysophosphatidylcholine content in the ischemic cardiac tissue during cardiac arrhythmias.

ABBREVIATIONS

acyl-CoA	Acyl-Coenzyme A
ANSA	1-Amino-2-naphthol-4-sulfonic acid
ATP	Adenosine 5'-triphosphate
C	Carbon
CDP	Cytidine 5'-diphosphate
cm	Centimeter
CMP	Cytidine 5'-monophosphate
CTP	Cytidine 5'-triphosphate
dpm	Disintegrations per minute
EDTA	Ethylenediaminetetraacetic acid
g	Gram
g	Gravitational force
GPC	Glycero-3-phosphocholine
GPE	Glycero-3-phosphoethanolamine
h	Hour
H	Hydrogen
Kg	Kilogram
LPC	Lysophosphatidylcholine or 1 or 2-acyl-GPC
LPE	Lysophosphatidylethanolamine or 1 or 2-acyl-GPE
M	Molar
mg	Milligram
min	Minute
ml	Millilitre
mM	Millimolar
N	Normal
NADH	Nicotinamide adenine dinucleotide (reduced)

nm	Nanometer
nmol	Nanomole
PC	Phosphatidylcholine or 1,2-diacylglycerophosphocholine
PE	Phosphatidylethanolamine or 1,2-diacylglycerophosphoethanolamine
PI	Phosphatidylinositol or 1,2-diacylglycerophosphoinositol
pmol	Picomole
PS	Phosphatidylserine or 1,2-diacylglycerophosphoserine
Pte-H_4	Tetrahydropteridine
rpm	Revolutions per minute
SAM	S-adenosylmethionine
SEM	Standard error of the mean
TLC	Thin layer chromatography
µCi	MicroCurie
µg	Microgram
µl	Microlitre
µM	Micromolar
µmol	Micromole
U.V.	Ultraviolet
v	Volume
w	Weight

PREAMBLE

This treatise describes studies on phospholipid metabolism in the mammalian heart. The main thrust of the study is devoted to the control of plasmenylcholine biosynthesis in the guinea pig heart. The catabolism of the cardiac phospholipids is also studied.

Phosphatidylcholine (PC) is the major phospholipid in the mammalian heart. A large proportion of the PC (up to 40% in some mammalian hearts) exists as plasmenylcholine, which is a choline-containing ether glycerophospholipid. PC forms an integral part of the cardiac membrane and participates in the regulation of certain membrane bound enzymes. It may also be involved in the transmission of biological signals across the membrane.

The biosynthetic pathways for the diacylglycerophospholipid, phosphatidylcholine, in the heart have been reported. However, no information is available on the biosynthesis of plasmenylcholine in the heart or in any other mammalian tissue. In general, there is very little information available on the metabolism and physiological function of plasmenylcholine. As plasmenylcholine constitutes a significant portion of the PC in some mammalian hearts, studies on the biosynthesis of this lipid may provide insights into its functional role in the cardiac membrane.

The second area of this report is focused on the metabolism of lysophospholipids. Lysophosphatidylcholine (LPC) has been implicated as a major biochemical factor in the production of cardiac arrhythmias

after the development of cardiac ischemia. Addition of exogenous LPC has been shown to cause depression of transmembrane potential in cardiac fibres and decreased membrane excitability in Purkinje fibres. Although the events leading to the generation of cardiac dysfunction by the lysophospholipids remain obscure, it has been postulated that the incorporation of lysophospholipids into the membrane is critical for their action and the subsequent production of membrane alterations, electrophysiological abnormalities and cardiac arrhythmias.

Lysophosphatidylcholine is a product formed by the enzymic hydrolysis of PC and plasmenylcholine. In view of the relatively high concentration of plasmenylcholine in cardiac tissue, the study of the metabolism of LPC may aid our understanding of the biochemical events which lead to the production of cardiac arrhythmias.

INTRODUCTION

I. Lipids and Membranes

The organization of all higher cells depends mainly on the compartmentalization afforded by biological membranes. Biological membranes participate in a wide variety of cellular functions including the structural organization of the cell, secretion, transport, endocytosis, signal transmission and in many other regulatory processes (1).

The plasma membrane is the essential barrier at the cell surface. It defines the cell's extent and separates the interior of the cell from the environment surrounding it. This membrane is also a highly selective filter that maintains unequal concentrations of solutes and ions on either side, and allows nutrients and waste products to enter and leave the cell (2). Internal membranes of eukaryotic cells form the boundaries of organelles such as the endoplasmic reticulum, mitochondria, lysosome, nucleus, peroxisome, chloroplast, secretory granule and other types of vesicles (1). It seems that there is a degree of functional continuity between internal membranes and the plasma membrane (1).

Membrane structure is interpreted by the well known 'fluid mosaic' model (3). Essentially this model states that the plasma membrane and other internal membranes of eukaryotic cells are assemblies of protein and lipid molecules, held together by cooperative, noncovalent interactions. Membranes also contain carbohydrates that are linked to lipids and proteins (Fig.1). Membrane research has modified and ex-

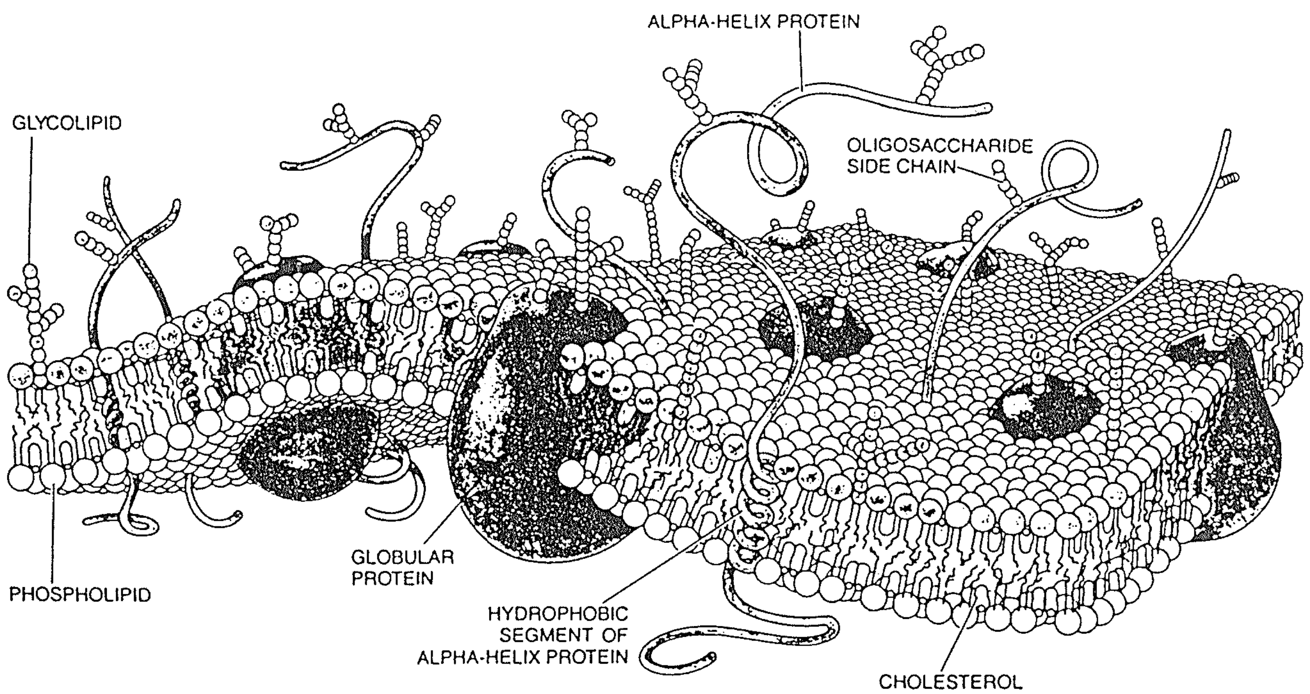


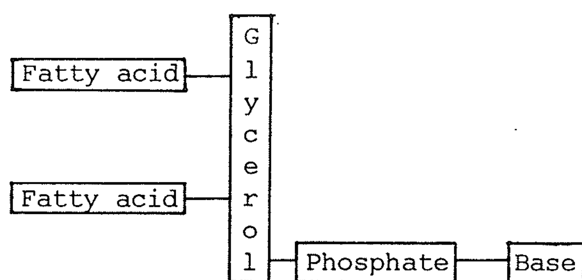
Fig. 1. A model of plasma membrane structure. (205)

tended the original model to provide specific details on membrane structure and function (4).

Membrane lipids constitute 50% of the mass of most animal cell plasma membranes (2). They are organized into an asymmetric bilayer, a continuous double layer, that is fluid, allowing lateral diffusion of lipid and protein in the plane of the membrane. Lipids establish the permeability barrier and provide a matrix with which transmembrane and peripheral proteins are associated.

When in an aqueous environment, lipid molecules have the ability to self-assemble into bilayers due to their amphipathic character (2). The head group of the molecule is polar or hydrophilic and the tail is nonpolar or hydrophobic. The lipid molecules in both leaflets of the bilayer align themselves so that the hydrophilic head groups face water on both sides of the bilayer. The oily, hydrophobic tails sequester themselves in the middle of the bilayer, thereby excluding water from it. Due to the hydrophobic interior, the bilayers are essentially impermeable to most water-soluble molecules such as amino acids, sugars, proteins, ions and nucleic acids.

The major membrane lipids belong to the group of glycerol-based phospholipids (Fig.2) and (5). They have a hydrophilic head group, on the C-3 position of the glycerol backbone, consisting of a phosphate esterified to a residue that can be either choline, serine, ethanolamine, inositol or a hydroxyl group. The most abundant and widely studied glycerophospholipids are those containing choline or ethanolamine residues, phosphatidylcholine (PC) (1,2-diacyl-sn-glycero-3-



Bases:

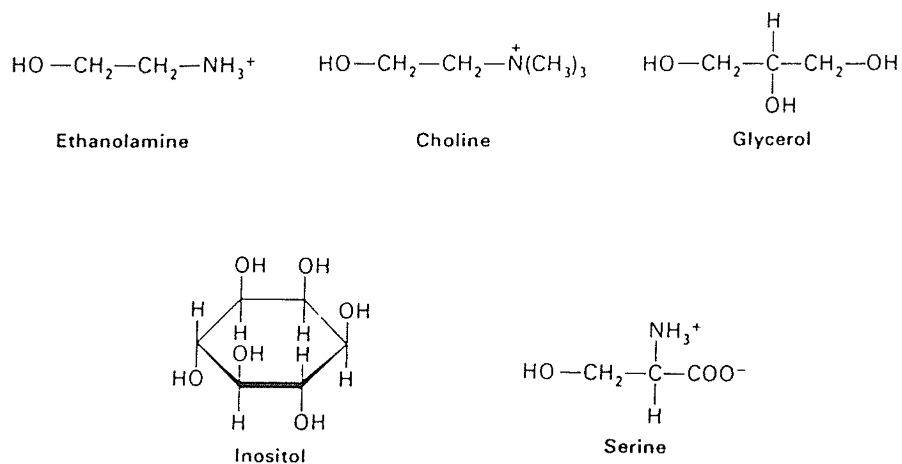


Fig. 2. Components of glycerophospholipids. (206)

phosphocholine) and phosphatidylethanolamine (PE) (1,2-diacyl-sn-glycero-3-phosphoethanolamine), respectively. Although the phospholipid content of mammalian tissues varies depending on tissue type and animal species, PC and PE are found to be the major phospholipids in most tissues (Table 1) (7). Attached to the two other positions of the glycerol backbone are two hydrophobic, hydrocarbon tails, each a fatty acid chain. Animal phospholipids usually contain fatty acids of chain lengths between 16 to 20 carbon (C) units. The major molecular species of PC and PE (Table 2 & 3) reveal that the 1-position of the glycerol backbone is usually esterified to a saturated fatty acid and the 2-position to an unsaturated fatty acid. The major diacylglycerophospholipids have their fatty acid chains attached to the glycerol moiety by ester linkages. There are also glycerophospholipids containing ether linkages (Fig.9). Alkenylacylglycerophospholipids (plasmalogens) have at least one fatty acid chain attached via a dehydrated hemiacetal (vinyl ether) linkage. The major lipids of this class are referred to as plasmenylcholine and plasmenylethanolamine. Alkylacylglycerophospholipids have at least one fatty acid chain attached through an ether linkage. Plasmanylcholine and plasmanylethanolamine are the major lipids of this type. Lysophospholipids are compounds that contain only one fatty acid chain, which can be attached via the ester, vinyl ether or the ether linkage. The lysophospholipids are formed by the action of phospholipases or plasmalogenases on the parent phospholipids (Fig.3) (6, 7).

Investigations into the chemistry of phospholipids in mammalian

Tissue		Total phospholipid (μmol/g)	Phosphatidyl-choline (% TPL)	Phosphatidyl-ethanolamine (% TPL)	Phosphatidyl-serine (% TPL)	Lysophos-phatidyl-choline (% TPL)
Brain,	rat	60.2	25	12	8	-
grey matter,	man	50.9	39	40 (incl. plasmal)	13	-
white matter,	man	82.8	31	34	16	-
myelin	man	-	24	7	21	-
Kidney	rat	36.6	34	27	7	1
	man	22.2	33	24	1	3
Lung	rat	-	54	20	6 (with PI)	-
	man	-	53	19	8	3
Spleen	rat	17.5	42	24	8	1
	man	24.7	41	25	8	2
Skeletal muscle	rat	11.3	51	22	4	3
	man	16.9	48	26	3	trace
Pancreas	ox	28.1	53	21	4	-
	guinea pig	30.6	50	18	4	-
Heart	rat	15.2	36	30	3	1
	man	21.5	40	26	3	4
Plasma	rat	1.5	64	1	-	23
	man	2.9	70	3	trace	7
Erythrocytes	rat	4.2	42	23	11	4
	man	3.9	29	28	14	2
Platelets (nmol/10^9)	man	436	40	28	9	1
Liver	rat	37.9	48	24	3	1
	man	41.3	44	28	3	1
Bile	rat	4.3	90	4	1	< 1
Amniotic fluid (μmol/100 ml)	man	14.4	68	12	8	< 1

Table 1. Phospholipid composition of various tissues. (7)

Fatty acids	Brain	Liver	Lung	Kidney	R.b.c.	Plasma	Gastric mucosa	Intestine	Bile
16:0/16:0	16.0	4.0	25.0, 41.4	8.0	20.3	–	31.0	–	–
18:0/16:0	4.0	–	4	–	–	–	7.0	–	–
16:0/16:1	–	–	12.0, 8.0	–	–	–	–	–	–
16:0/18:1	30.0	4.0	10.0, 16.6	–	14.5	5.5	–	6.5	–
18:0/16:1	–	–	–	–	–	–	–	–	–
18:0/18:1	12.0	–	–	–	–	–	14.0	–	–
16:0/18:2	–	15.7, 20.0	10.0, 6.4, 8.0	8.0	12.4	19.4	–	24.6	53.7
18:0/18:2	–	10.0, 9.0, 9.0	5.6	10.0	5.6	16.0	–	15.8	9.9
18:1/16:0	10.0	–	12.0	7.0	–	–	–	–	–
16:0/18:3	–	–	–	–	–	–	6.0	–	–
16:0/20:4	8.0	18.9, 22.0	15.0	22.0	5.4	5.8	–	14.0	14.7
18:0/20:4	10.0	25.0, 20.9	–	25.0	7.5	13.3	–	8.6	–
16:0/22:6	–	4.8	–	–	–	6.9	–	–	–
18:0/22:6	–	5.6	–	–	–	4.8	–	–	–
18:2/20:4	–	–	–	–	–	5.5	–	–	–
18:2/16:0	–	–	–	6.0	–	–	–	–	–

Table 2. Major molecular species of phosphatidylcholine in the rat. (as % of the total) (7)

	Skeletal muscle		Kidney		Sarcoplasmic reticulum of skeletal muscle
	Rat	Human	Dog	Pig	Rabbit
16:0/18:1	-	-	12.1	6.7	14.9
18:0/18:1	-	-	-	-	17.9
16:0/18:2	13.1	14.0	10.8	-	11.9
16:0/20:4	11.6	21.0	27.3	10.3	14.9
18:0/20:4	13.9	28.0	25.9	35.6	23.9
18:0/22:6	37.3	9.3	-	-	-
18:1/18:1				6.9	
18:1/20:4			11.2	18.1	

Table 3. Major molecular species of phosphatidylethanolamine in various tissues of different animals. (7)

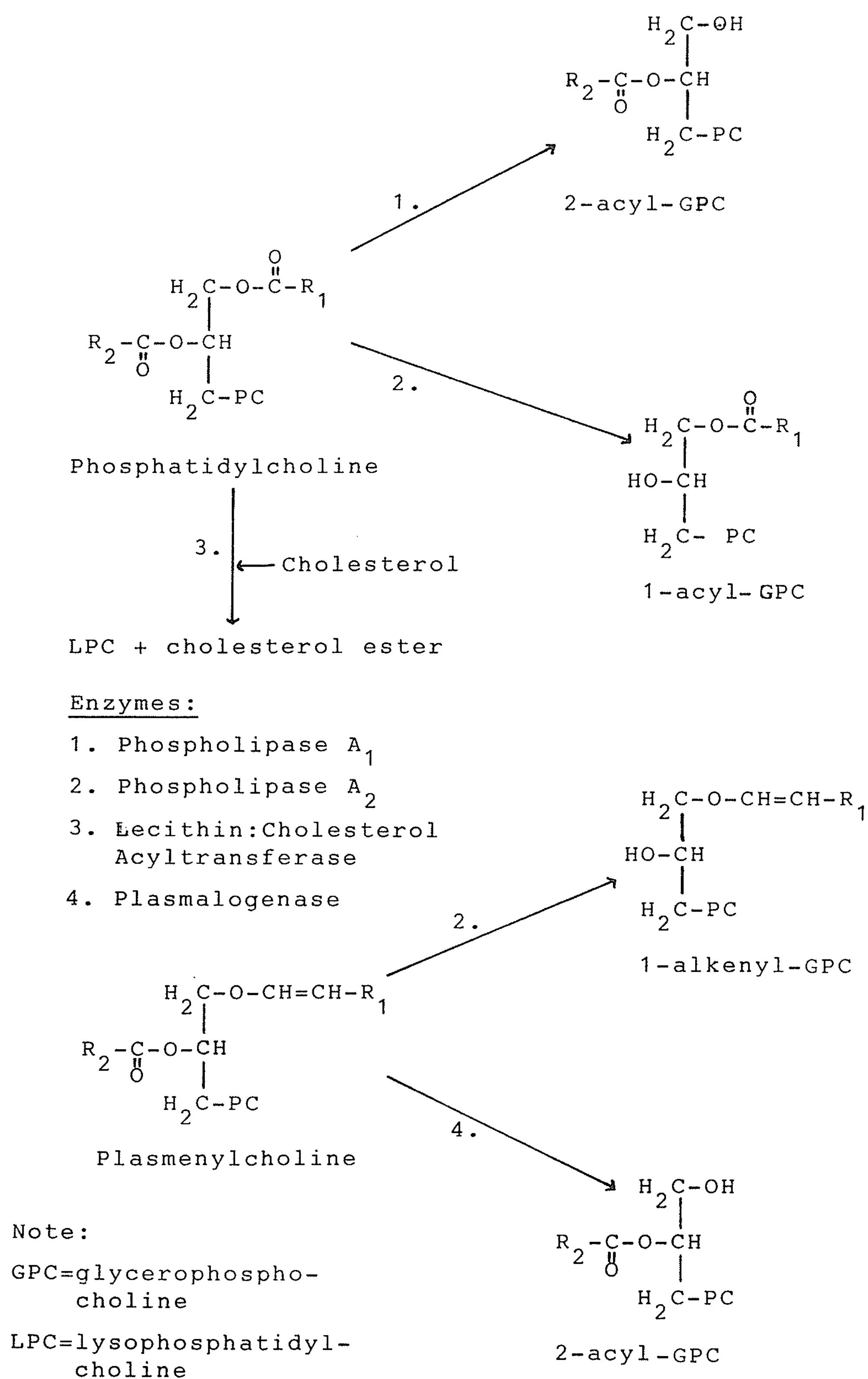


Fig. 3. Formation of lysophospholipids.

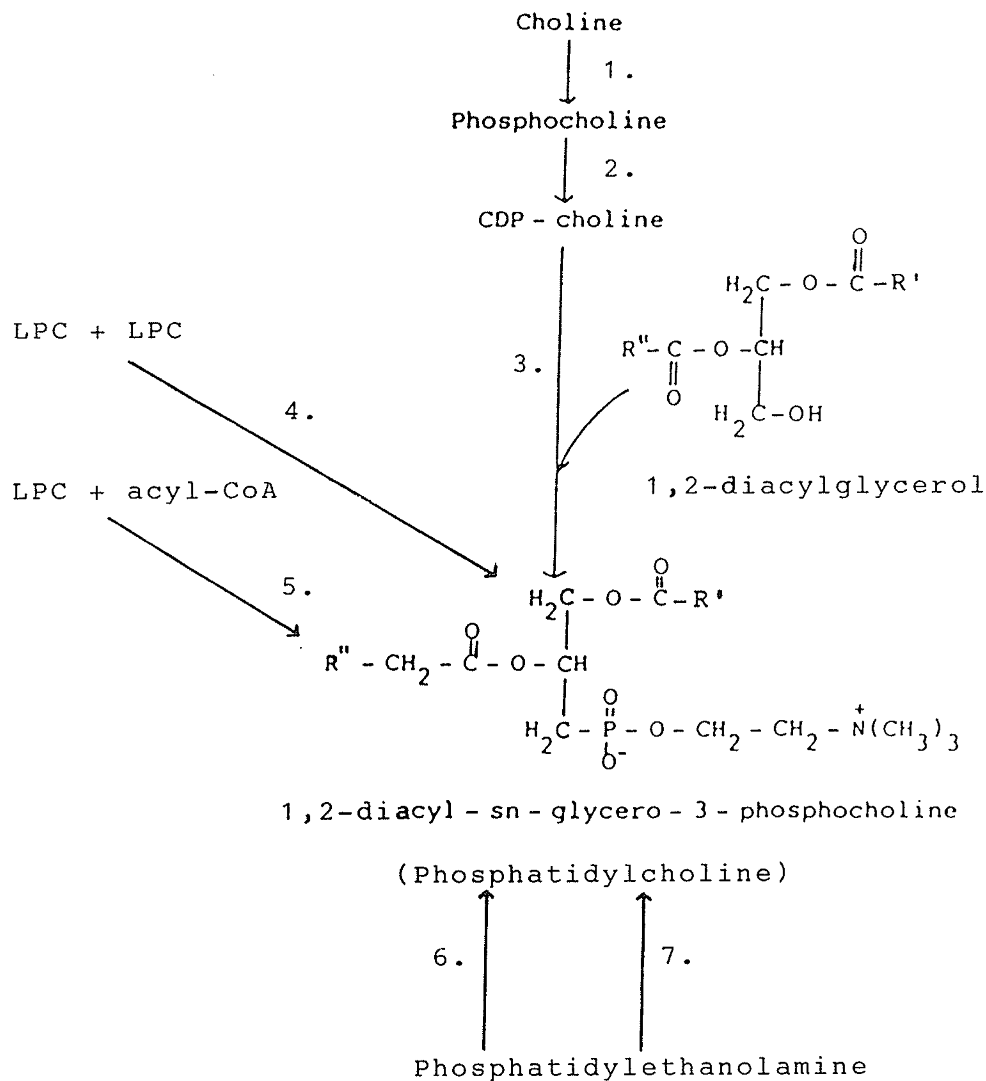
cells has illuminated some of the basic mechanisms responsible for their synthesis and catabolism. Some knowledge has also been obtained on their possible function in the membrane (76,77). A thorough understanding of the metabolism and function of phospholipids may provide insights into membrane function and assembly.

II. Biosynthesis of Phosphatidylcholine and Phosphatidylethanolamine

a) Biosynthesis of Phosphatidylcholine

In 1850, Gobley isolated "lecithin" from egg-yolk and brain (27). From this lipid he could obtain glycerophosphoric acid and fatty acids. Strecker (28) originally isolated lecithin from hog bile and in 1868, Diakanow (29) found that this lipid contained choline. They deduced a provisional structure for lecithin and in 1950, Baer and Kates (30) demonstrated via chemical synthesis that lecithin was based on L-α-glycerophosphate, like all other naturally occurring glycerophospholipids.

There are five different pathways for the biosynthesis of PC (Fig.4). The major 'de novo' pathway, in most tissues, is the Cytidine pathway (Fig.5), elucidated by Kennedy and Weiss (8) in the mid 1950's. These workers demonstrated that cytidine 5'-triphosphate (CTP) was a requirement for PC biosynthesis and described the reaction catalyzed by CTP : phosphocholine cytidylyltransferase whereby phosphocholine and CTP react to form CDP-choline. In many systems, the rate of this cytidylyltransferase reaction is the rate limiting determinant of PC biosynthesis (13). As well, Kennedy demonstrated that the

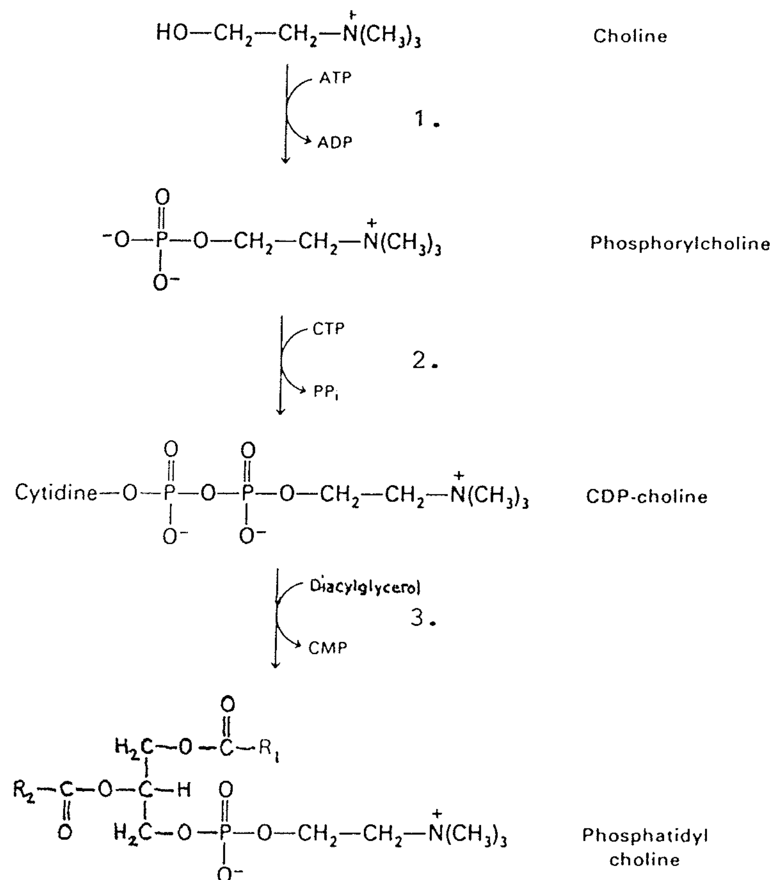


Enzymes:

1. Choline kinase
2. CTP:phosphocholine cytidylyltransferase
3. CDP-choline:1,2-diacylglycerol phosphocholine transferase
4. LPC:LPC transacylase
5. Acyl-CoA:LPC acyltransferase
6. Base-exchange enzyme
7. Phosphatidylethanolamine methyltransferase

(R' & R" = fatty acyl chains)

Fig. 4. Pathways for the biosynthesis of phosphatidylcholine



Enzymes:

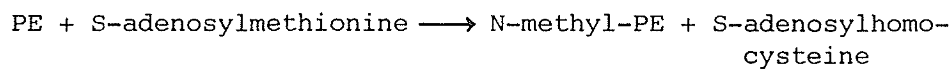
1. Choline kinase
2. CTP:phosphocholine cytidylyltransferase
3. CDP-choline:1,2-diacylglycerol phosphocholinetransferase

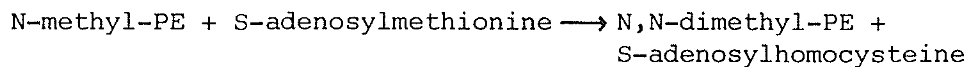
Fig. 5. The Cytidine pathway for the biosynthesis of phosphatidylcholine. (206)

final reaction, the condensation of CDP-choline with 1,2-diacylglycerol, was catalyzed by CDP-choline : 1,2-diacylglycerol phosphocholinetransferase. The 1,2-diacylglycerol backbone for all glycerolipid biosynthesis stems from glycerol-3-phosphate (G-3-P) or dihydroxyacetone phosphate (DHAP) (40). Previously, Wittenberg and Kornberg (9) had discovered choline kinase. This enzyme catalyzes the first step in the pathway, the phosphorylation of choline. Extensive reviews of this pathway and detailed descriptions of the enzymes involved may be found in (7) and (10).

The conversion of PE to PC can occur via two distinct routes. One path is via the stepwise methylation of PE, where methyl groups are successively introduced onto the terminal amino group of PE (Fig.6a). In 1960, Bremer and Greenberg (11) found that the N-methylation of PE proceded via the enzyme phosphatidylethanolamine methyltransferase, using S-adenosylmethionine (SAM) as the methyl donor. This pathway is of quantitative significance only in the liver (10). Dils and Hubscher (12) demonstrated the energy-independent, calcium dependent incorporation of choline into its corresponding phospholipid. This base-exchange activity (Fig.6b) results in the exchange of the bases choline, serine and ethanolamine with the bases of preexisting phospholipids. The significance for the base exchange of choline is unknown and it was found that this path makes a minor contribution to the biosynthesis of PC in the hamster heart (13).

Two types of PC resynthesis occur whereby the fatty acid chains of PC are modified as to the desired chain length and degree of unsatura-





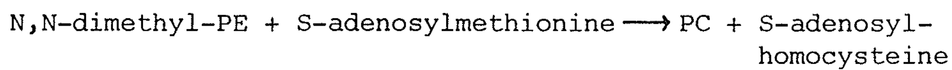


Fig. 6 a) Stepwise methylation of phosphatidylethanolamine.(PE)

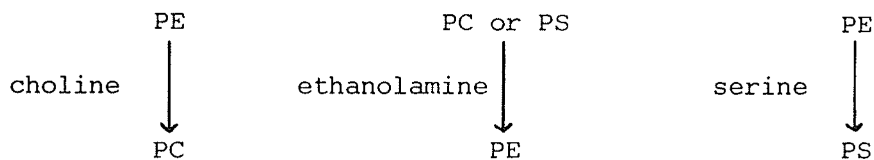
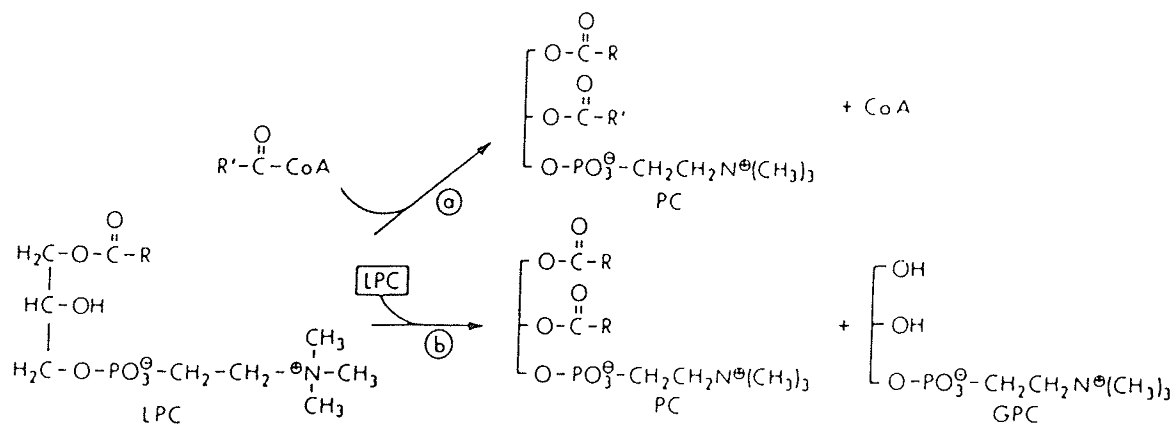


Fig. 6 b) Base-exchange reactions.

tion. The reacylation of 1- or 2-lysophosphatidylcholine (LPC) (1- or 2- acyl-sn-glycero-3-phosphocholine), first described by Lands (14) and LPC transacylation demonstrated in 1965 by Marinetti (15) lead to the synthesis of specific molecular species of PC (Fig.7). LPC is formed upon the deacylation of PC by phospholipases A1 and A2, which release the fatty acyl chains of PC at positions 1 and 2, respectively. For reacylation, a fatty acid is transferred from it's acyl-coenzyme A (acyl-CoA) to the free hydroxyl group at the 2-position of 1-acyl-sn-glycero-3-phosphocholine (1-acyl-GPC) by the enzyme acyl-CoA : 1-acyl-GPC-O-acyltransferase. At the 1-position of 2-acyl-GPC re-acylation with acyl-CoA occurs via acyl-CoA : 2-acyl-GPC -O-acyltransferase. The type of fatty acid transferred in the acylation of 1-acyl or 2-acyl-GPC is mainly determined by the position of the free hydroxyl group. When the free hydroxyl group is at the 1-position, saturated fatty acids are transferred and when the hydroxyl group in the 2-position is available unsaturated fatty acids are preferentially transferred in the acylation. The definite preference for arachidonic acid (20:4) in the acylation of the 2-position of 1-acyl-sn-glycero-3-phosphocholine reveals that acylation may be the major method for the incorporation of this fatty acid into PC in mammalian tissues (16).

The transacylation of LPC (Fig.7), discovered by Marinetti and Erbland (15), is catalyzed by LPC-LPC acyltransferase. This reaction produces PC and glycero-3-phosphocholine (GPC) from two molecules of 1-acyl-GPC. This route for the biosynthesis of PC is not of major import in the liver. Suggestions have been made that it may be significant in the lung (7) for the synthesis of the dipalmitoyl species of



Enzymes:

a) Acyl CoA : LPC acyltransferase

b) LPC : LPC transacylase

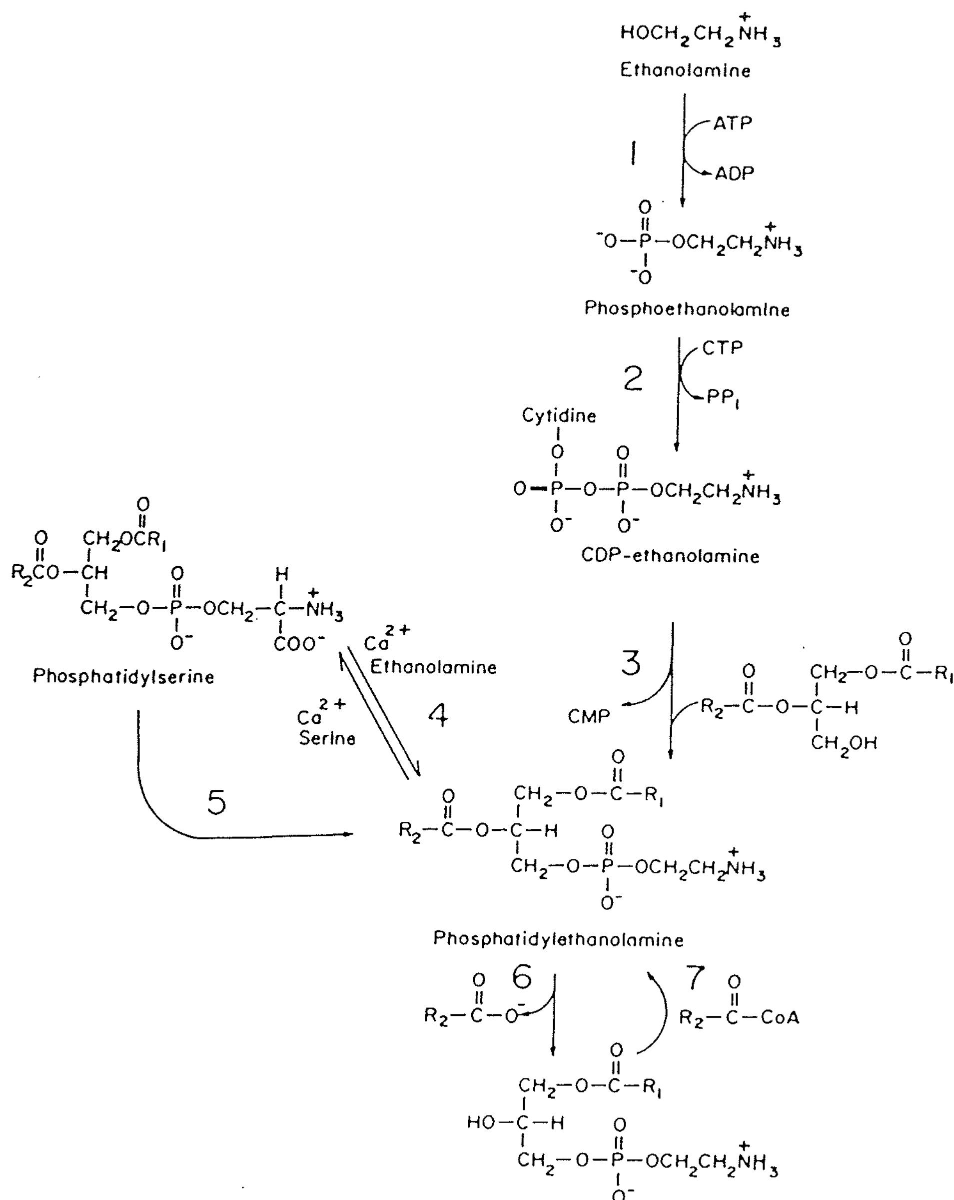
Fig. 7. Resynthesis of phosphatidylcholine from lysophosphatidylcholine.

PC, a major component of pulmonary surfactant. However, recently it was found that in rabbit alveolar macrophages (75) the dipalmitoyl species of PC is synthesized predominantly by a 'de novo' pathway and not the deacylation - reacylation pathway.

b) Biosynthesis of Phosphatidylethanolamine

A nitrogen and phosphorus containing lipid fraction, that was relatively insoluble in warm ethanol, was isolated from brain tissue by Thudichum (17) in 1884. He found that he could obtain ethanolamine from it as a hydrolysis product. In 1930, Rudy and Page (18) isolated ethanolamine glycerophospholipid from this same fraction of brain tissue. However, it is likely that this preparation contained plasmenylethanolamine and the first pure preparations of PE are attributed to Lea et al. (19) from egg-yolk and Klenk and Dohmen (20), from liver.

The biosynthesis of PE proceeds via four established pathways (Fig.8). PE biosynthesis has pathways analogous to those found for the biosynthesis of PC. The cytidine (CDP-ethanolamine) pathway, the major 'de novo' route, was first described by Kennedy and Weiss (8). This pathway contains enzymes whose functions are analogous to those participating in the cytidine (CDP-choline) pathway. Ethanolamine kinase, CTP : phosphoethanolamine cytidylyltransferase and CDP-ethanolamine : 1,2-diacylglycerol phosphoethanolaminetransferase are the enzymes involved in the biosynthesis of PE via this pathway. There is some controversy over whether choline and ethanolamine kinase activities



Enzymes:

1. Ethanolamine kinase
2. CTP:phosphoethanolamine cytidylyltransferase
3. CDP-ethanolamine:1,2-diacylglycerol phosphoethanolamine transferase
4. Base-exchange enzyme
5. Phosphatidylserine decarboxylase
6. Phospholipase A_2
7. Acyl-CoA:lysophosphatidylethanolamine acyltransferase

Fig. 8. Pathways for the biosynthesis of phosphatidylethanolamine. (35)

reside on the same enzyme (21) or on two distinct enzymes (10). It is known that CTP : phosphoethanolamine cytidylyltransferase, which catalyzes the rate-limiting step, is a separate and distinct enzyme from the cytidylyltransferase for PC biosynthesis (7) (10). Choline and ethanolamine phosphotransferase have been separated and characterized by their different specificities for the diacylglycerol moiety (10,34).

Borkenhagen et al. (22) first demonstrated the base exchange reaction for the incorporation of ethanolamine into PE. By displacing choline and serine from PC and phosphatidylserine (PS), respectively, free ethanolamine is predominantly incorporated into hexaenoic (6:0) PE in brain microsomes (23). This pathway contributes approximately 9% of PE synthesis in hepatocytes (35) and is analogous to the base exchange reaction for choline.

Merkl and Lands (24) described the acylation of 1- or 2-acyl-sn-glycero-3-phosphoethanolamine (GPE) in 1963. This route for the biosynthesis of PE is similar to the one for the deacylation-reacylation of PC. The acyltransferase for lysophosphatidylethanolamine (LPE) utilizes highly unsaturated fatty acids to account for 95% of the 18:2 incorporation and 100% of 20:4 incorporation into PE (7).

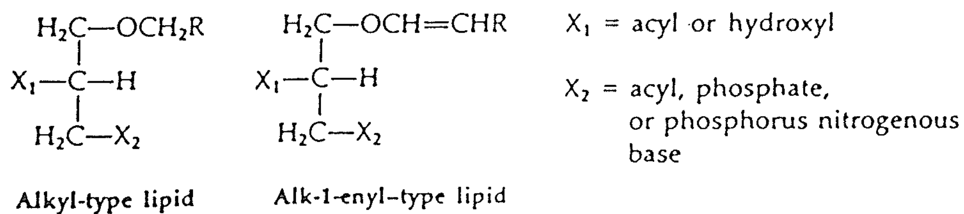
The decarboxylation of phosphatidylserine (PS) to produce PE in the liver was first described by Bremer et al. (25) in 1960. Only lipid-bound serine can be decarboxylated by the enzyme phosphatidylserine decarboxylase (26). This mechanism is a unique pathway for the biosynthesis of PE. However, this route for PE biosynthesis has not been

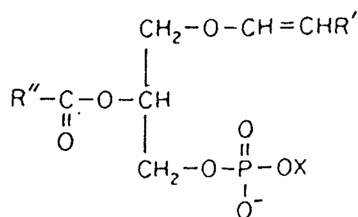
found to be of major importance in tissues except perhaps in hepatocytes where under certain conditions most of the PE arises from PS (35).

III. Plasmenylcholine and Plasmenylethanolamine

a) Structure and Nomenclature

Glycerolipids containing ether-linked aliphatic chains are found in neutral lipids and phospholipids. Phospholipids containing alkyl groups bound to glycerol are called glyceryl ethers and those with alkenyl groups are referred to as plasmalogens or vinyl ethers (Fig.9) (38). Alkenyl and alkyl refer to the presence or absence of unsaturation of the first and second carbons of the fatty acyl chain. Double bonds can occur at other sites along either acyl chain. The 1-position of the majority of ether glycerophospholipids is where the alkyl and alkenyl chains are located. As in diacylglycerophospholipids, at the 2-position of the glycerol backbone there is usually a fatty acyl chain attached through an ester linkage. Ether glycerophospholipids contain a phospho-head group on the third position of the glycerol moiety. The head group can be a phosphate, phosphocholine, phosphoethanolamine, phosphoserine or phosphoinositol. The chemical structure for plasmalogens reveals that the hydrocarbon chain is attached through a dehydrated hemiacetal or vinyl ether linkage, thus these chains are unsaturated at position-1. The double bond has a cis configuration in the alkenyl linkage of naturally occuring plasmalogens. For alkyl glycerophospholipids, there is an ether linkage at position-

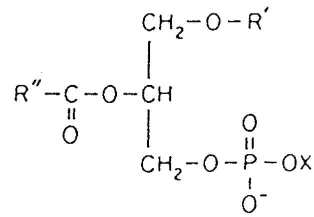




1 - Alkenyl - 2 - acyl - glycerophosphocholine

Plasmenylcholine

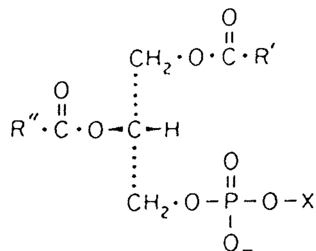
PC Plasmalogen



1 - Alkyl - 2 - acyl - glycerophosphocholine

Plasmanylcholine

Alkyl PC



1,2 - Diacyl - glycero - phosphocholine

Phosphatidylcholine (PC)

Fig. 9. Ether lipids

1 and therefore, it is saturated at carbons 1 and 2. The position on the glycerol backbone and the proportion of the alkyl and alkenyl chains, in a specific species of ether lipid, varies depending on the source of the lipid. For example, in bovine heart, 87% of the alkenyl moieties are found at position-1 and 13% at position-2. Whereas for pig heart, 75% was found at the first position and 25% at position-2 (71).

The names recommended by IUPAC-IUB (39) are plasmenyl (alkenyl) and plasmanyl (alkyl) for 1-alkenyl-2-acyl and 1-alkyl-2-acyl, respectively. Plasmenylcholine denotes 1-alkenyl-2-acyl-sn-glycero-3-phosphocholine (a choline plasmalogen) (Fig.9) (40). There are many molecular species for each type of ether lipid. They may contain more than one alkyl or alkenyl chain and there may be different numbers of carbon atoms and double bonds for each alkenyl, alkyl or acyl chain. Usually chain lengths of 16:0, 18:0 and 18:1 are found for the ether-linked acyl chains. There are also many unusual ether lipids in addition to the ether-lipids of neutral and phosphoglycerides (38, 40). Recently, evidence for a diplasmalogen of phosphatidylethanolamine was found (70). This ether lipid has two alkenyl moieties, one at the 1-position and one at the second position of the glycerol moiety. It does not contain an esterified acyl chain. This diplasmalogen occurs as 90% of the total phophatidylethanolamine of rabbit sperm (70).

b) History

More than 30 years were needed to resolve the unusual structure of plasmenylcholine and plasmenylethanolamine. To the present day, they

still pose the challenge of their function and there is still no method available for their separation from their diacyl counterparts, unless one or the other is derivatized. The following is a brief description encompassing the initial discovery of plasmalogens to the point wherein their final structure was resolved. The works that I have omitted, are described in detail in two excellent histories on plasmalogens (41,42).

The name plasmalogen was coined when glycerophospholipids containing a potential aldehyde were first discovered in the plasma of cells by Feulgen and Voit in 1924 (43), using the fuchsin-sulfurous acid stain that they had developed. Soon these workers, using these histochemical methods, found plasmalogen in the cells of every type of tissue from protozoa to human (44,45). In these early years, methods for the extraction, separation and analysis of phospholipids were virtually unknown. Thus, lipid biochemists of this time engaged in many painstaking and sometimes futile lines of investigation. Much progress in phospholipid knowledge was made during the 1940's and 50's when reliable techniques became established. Feulgen and Bersin in 1939 (46) were the first to isolate a phosphatide from bovine muscle that contained an aldehyde and ethanolamine. In 1951, using Folch's fractionation method (47), Klenk and Bohm (48) demonstrated that a serine plasmalogen was present in the human brain. Choline plasmalogens were also shown to exist in bovine heart muscle in 1953 when Klenk and Gehrmann (49) used alumina to isolate a lecithin fraction. Rapport (1954) (50) determined the nitrogen and phosphorus content of

plasmalogen-enriched phospholipid fractions. He concluded from these studies that native plasmalogens contained two fatty chains per phosphorus atom. That same year, Klenk and Debuch (51) demonstrated that in human brain , ethanolamine plasmalogens consisted of a fatty acid and an aldehyde moiety.

Independently, both Rapport's group and Klenk and his coworkers had also observed that the aldehyde present in choline and ethanolamine plasmalogen was sensitive to hydrogenation. After hydrogenation with platinum or mixed catalysts, it was found that the product could no longer be stained with the fuchsin-sulfurous stain nor easily hydrolyzed under acid or alkaline conditions. From this, Rapport suggested that the aldehydogenic linkage was in an α,β-unsaturated ether (53) and Klenk and Debuch (51) proposed structures that included an α,β-unsaturated ether. In the following year, Klenk and Debuch (52) showed that choline plasmalogens also contained a fatty acid chain and Debuch (54) illustrated conclusively that only unsaturated fatty acid was present in ethanolamine plasmalogen from brain. During 1955, Rapport and Alonzo (55) developed a method to determine the number of fatty acid esters in phospholipids. They found that bovine heart lecithin consists of 60% choline plasmalogen and that these plasmalogens were cleaved by snake venom phospholipase A to yield one ester chain per atom of phosphorus (56). This led to the conclusion that plasmalogens were very similar in structure to their diacyl counterparts. Erroneously, the ether linkage was placed at the 2-position of the glycerol, due to the misconception that snake venom phospholipase acted on position-1 of the glycerol backbone. Ethanolamine lysoplasmalogen was

prepared by Rapport et al. (57) in 1957 and chemical studies on this compound revealed that this molecule contained a single unsaturated bond. Independently, Debuch (58,59) also found that the aldehyde was in the enol form where there is a double bond between the α and β carbon atoms. Thus, the model where the ether linkage was of an α,β-unsaturated type was now greatly in favor. This ether structure was confirmed by Blietz (60) in 1958 for ethanolamine plasmalogens.

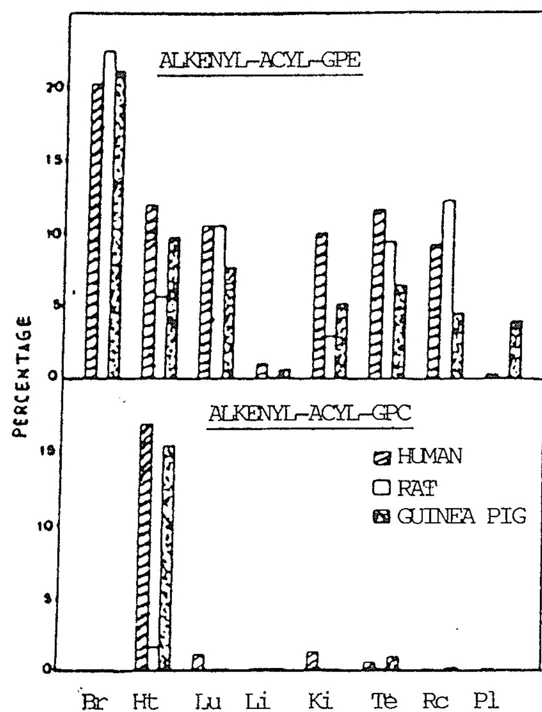
Marinetti and Erbland (61) had concluded from their work on pig heart plasmalogens that the aldehydes were at the 1-position on the glycerol backbone. However, many other investigators had evidence that the ether bond was actually at the 2-position (62, 63, 64, 65). In 1959, Debuch (66) conclusively proved the exclusive location of the ether (aldehyde group) at the 1-position of the glycerol moiety in ethanolamine plasmalogens from the brain.

c) Occurence

Ether glycerophospholipids are widely distributed in nature, being found in almost all bacterial (except maybe in strict aerobes) and animal cells. This section will focus mainly on the distribution of plasmenylcholine and plasmenylethanolamine in mammalian tissues since that is within the scope of this treatise. Alkenylacylglycerophospholipids account for 20% of the total phospholipids in adult man (Table 4) and (Fig.10). The highest levels are found in nervous tissue and striated muscle. There are very low levels present in plasma and liver (38), less than 2% in human (72). The tissue distribution of alkyl-

Tissue	Tissue weight (kg)	Plasmalogen content (mmol)	Phospholipid content (mmol)	Plasmalogen (% of PL)
Liver	2.3	1.6	201	0.8
Striated muscle	40.0	122	488	25
Kidneys	0.5	1.6	13.7	12
Skin	6.0	6.6	46.8	14
Alimentary tract	1.9	3.4	21.3	16
Heart	0.5	2.6	8.2	32
Nervous tissue	3.0	38.7	169	23
Skeleton	17.6	–	–	–
Adipose tissue	11.4	8.0	37.6	21
Total	83.2	184	986	19

Table 4. Plasmalogens in the tissues of adult man. (38)



Br (Brain) Ht (Heart) Lu (Lung) Li (liver)

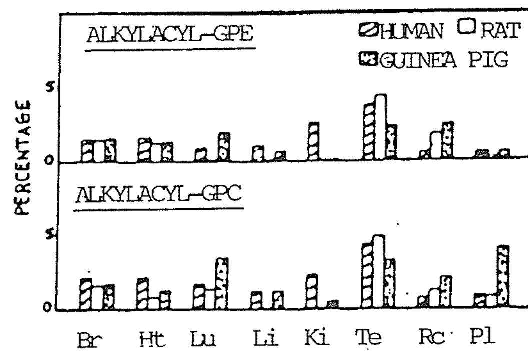
Ki (Kidney) Te (Testis) Rc (Redcell) Pl (Plasma)

Fig. 10. Tissue distribution of plasmenylcholine. (72)

acylglycerophospholipids is shown in Fig.11. In the central nervous system, as in many other tissues and cells, plasmenylethanolamine is the predominant ether lipid (Table 5), in the brains of humans, rats and guinea pigs, it represents approximately 55% of the total ethanolamine glycerophospholipids. In the brain, the alkylacyl subclass is found mainly in choline glycerophospholipids. The myelin sheath is enriched in plasmenylethanolamine, where plasmenylethanolamine makes up 32-43% of the total phospholipid in the central nervous system (Table 6). In peripheral nerve myelin, 27 to 30% of the total phospholipid is plasmenylethanolamine. Values for plasmenylethanolamine in the nervous system of various species depend on the degree of myelination and proportion of white matter. Increases occur during development in the content and proportion of plasmenylethanolamine in the human brain (67 & 68). The total plasmenylethanolamine percentage increases in myelin with age, in rats and humans (74).

Species differences are noted in the proportion of alkenylacylglycerophospholipids found in the heart. Mammalian heart tissue is unique as it contains a large proportion of plasmenylcholine. In humans, 39% of the total phosphatidylcholine in the heart exists as plasmenylcholine (Table 7). High levels of plasmenylcholine are also found in the guinea pig heart - 34% (154), 46% in bovine heart but only 4% in the rat heart (38). In young mammals, the ratio of plasmenylcholine to plasmenylethanolamine in heart muscle is different from that found in the adult but this was not thought to be a function of age (73).

Within a species, varying amounts of ether lipids are found in



Br (Brain) Ht (Heart) Lu (Lung) Li (Liver)

Ki (Kidney) Te (Testis) Rc (Redcell) Pl (Plasma)

Fig. 11. Tissue distribution of plasmanylcholine. (72)

Results represent percentages of total lipid phosphorus and are means ± S.D. of three duplicate determinations, except for human (two determinations).

Phospholipids	Human	Rat	Guinea pig
Choline glycerophospholipids			
diacyl	33.9 ± 1.5	38.5 ± 0.7	36.7 ± 1.8
alkylacyl	2.0 ± 0.2	1.4 ± 0.4	1.6 ± 0.4
alkenylacyl	traces	traces	traces
lysophosphatidylcholine	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
Ethanolamine glycerophospholipids			
diacyl	13.2 ± 0.1	15.3 ± 2.2	17.7 ± 2.3
alkylacyl	1.5 ± 0.1	1.4 ± 0.1	1.5 ± 0.2
alkenylacyl	20.2 ± 0.2	22.4 ± 1.2	21.1 ± 1.2
lysophosphatidylethanolamine	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
Serine glycerophospholipids	10.5 ± 1.2	10.2 ± 0.1	8.8 ± 2.6
Inositol glycerophospholipids	2.6 ± 0.5	1.6 ± 0.2	1.8 ± 0.5
Diphosphatidylglycerol	1.9 ± 0.3	2.5 ± 0.7	1.6 ± 0.1
Phosphatidic acid	0.0 ± 0.0	0.8 ± 0.2	traces
Sphingomyelin	13.8 ± 1.2	6.9 ± 0.3	8.8 ± 0.3
Total	99.6	101	99.7

Table 5. Phospholipid composition of the brain. (72)

Mole ratio, alk-1'-enyl groups: lipid P.

Myelin	Microsomes	Synaptosomes
0.32	0.19	–
0.43	0.19	–
0.42	0.27	–
0.34	0.16	–
0.42	–	0.27
0.30	–	–
0.27	–	–
0.38	0.16	–
0.39	0.27	–
0.32	0.28	–
–	0.17	0.18

Table 6. Plasmenylethanolamine content in subcellular fractions of the nervous system. (38)

Results represent percentages of total lipid phosphorus and are means ± S.D. of three duplicate determinations, except for human (two determinations).

Phospholipids	Human	Rat	Guinea pig
Choline glycerophospholipids			
diacyl	22.0 ± 2.8	39.4 ± 0.4	26.5 ± 2.3
alkylacyl	2.0 ± 0.6	0.6 ± 0.0	1.2 ± 0.5
alkenylacyl	16.9 ± 0.9 – 39 % of Total PC	1.6 ± 0.1	15.5 ± 0.8 – 36 % of Total PC
lysophosphatidylcholine	1.7 ± 1.7	0.0 ± 0.0	0.3 ± 0.2
Ethanolamine glycerophospholipids			
diacyl	14.0 ± 0.5	30.4 ± 3.4	20.7 ± 2.3
alkylacyl	1.6 ± 0.3	1.2 ± 0.1	1.3 ± 0.9
alkenylacyl	11.9 ± 0.6	5.6 ± 1.1	9.8 ± 1.5
lysophosphatidylethanolamine	1.6 ± 1.6	0.0 ± 0.0	0.5 ± 0.2
Serine glycerophospholipids	3.2 ± 0.2	1.7 ± 0.3	1.7 ± 0.2
Inositol glycerophospholipids	4.3 ± 0.6	2.0 ± 0.5	2.8 ± 0.9
Diphosphatidylglycerol	14.5 ± 0.2	15.0 ± 1.1	14.4 ± 0.3
Phosphatidic acid	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
Sphingomyelin	5.5 ± 0.5	3.1 ± 0.2	5.1 ± 0.7
Total	99.3	100.6	99.8

Table 7. Phospholipid composition of the heart. (72)

different types of skeletal muscle (Table 8). Other tissues and cells where plasmenylethanolamine and plasmenylcholine are abundant but found in different proportions, depending on species, are spleen, kidney (Table 9), testes (Table 10 & 11), bone marrow, erythrocytes (Table 12), macrophages, neutrophils, platelets, and in some tumor cell lines (Table 13) (38) (69).

d) Biosynthesis of Plasmenylethanolamine

i) The pathway

In 1962, Thompson and Hanahan (102) concluded from their studies on bone marrow that α-glycerophosphate or a similarly active compound was the precursor for the ether glycerophospholipids. That triose phosphates, such as glyceraldehyde-3-phosphate or dihydroxyacetone phosphate, rather than glycero-3-phosphate acted as the precursor of ether lipids was suggested by Friedberg and Greene in 1968 (98). Snyder et al. (99) had found that the glycerol backbone for the ether glycerophospholipids was a phosphorylated aldehydogenic gycerol derivative contained in the cytosolic fraction of mouse preputial tumor cells. This derivative could be substituted for by DL-glyceraldehyde-3-phosphate (GA-3-P) (100). In 1970, Hajra (94) demonstrated that dihydroxyacetone phosphate (DHAP) was the preferred substrate for ether bond formation in mouse brain microsomes and in guinea pig liver mitochondria even though D-GA-3-P, glycerol-3-phosphate (G-3-P) and dihydroxyacetone (DHA) (106) could also be utilized as precursors. To date, the biosynthetic pathways that have been elucidated for ether glycerophospholipids show that the precursor for the glycerol backbone is

	Alkenylacyl-GPC	Alkylacyl-GPC
	% choline glycerophospholipid	
Human rectus abdominis muscle	17	-
Human gastrocnemius muscle	27	-
Rat diaphragm	2.4	0.9
Rat soleus muscle	2.4	1.0
Rat rectus femoris muscle	3.0	0.9

Table 8. Ether-linked choline glycerophospholipids in skeletal muscle. (38)

Results represent percentages of total lipid phosphorus and are means ± S.D. of three duplicate determinations, except for human (two determinations).

Phospholipids	Human	Rat	Guinea pig
Choline glycerophospholipids			
diacyl	32.1 ± 1.0	38.9 ± 1.7	50.8 ± 4.3
alkylacyl	2.2 ± 0.0	traces	0.4 ± 0.7
alkenylacyl	1.3 ± 0.1	traces	0.0 ± 0.0
lysophosphatidylcholine	2.4 ± 0.9	0.0 ± 0.0	0.0 ± 0.0
Ethanolamine glycerophospholipids			
diacyl	17.0 ± 0.9	27.4 ± 0.9	18.8 ± 4.7
alkylacyl	2.6 ± 0.3	traces	0.0 ± 0.0
alkenylacyl	10.0 ± 0.8	2.9 ± 0.2	5.2 ± 1.1
lysophosphatidylethanolamine	1.9 ± 1.2	0.0 ± 0.0	0.0 ± 0.0
Serine glycerophospholipids	5.7 ± 1.0	4.2 ± 1.4	3.1 ± 1.3
Inositol glycerophospholipids	4.4 ± 0.3	3.4 ± 0.2	3.4 ± 1.1
Diphosphatidylglycerol	6.7 ± 0.2	6.8 ± 0.3	4.7 ± 0.3
Phosphatidic acid	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
Sphingomyelin	13.5 ± 0.2	16.3 ± 1.1	15.1 ± 2.3
Total	99.8	99.9	101.5

Table 9. Phospholipid composition of the kidney. (72)

Results represent percentages of total lipid phosphorus and are means ± S.D. of three duplicate determinations, except for human (two determinations).

Phospholipids	Human	Rat	Guinea pig
Choline glycerophospholipids			
diacyl	38.8 ± 1.8	47.7 ± 2.1	51.6 ± 3.6
alkylacyl	4.3 ± 0.3	4.8 ± 0.4	3.1 ± 0.8
alkenylacyl	0.6 ± 0.6	0.2 ± 0.4	1.0 ± 0.3
lysophosphatidylcholine	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
Ethanolamine glycerophospholipids			
diacyl	16.0 ± 2.7	13.3 ± 0.4	18.4 ± 3.5
alkylacyl	3.8 ± 0.7	4.4 ± 0.9	2.3 ± 0.5
alkenylacyl	11.6 ± 0.8	9.4 ± 1.9	7.1 ± 1.1
lysophosphatidylethanolamine	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
Serine glycerophospholipids	6.4 ± 0.1	3.9 ± 0.5	3.8 ± 0.3
Inositol glycerophospholipids	4.7 ± 0.0	3.2 ± 0.4	2.3 ± 0.4
Diphosphatidylglycerol	2.4 ± 0.1	3.7 ± 0.4	1.3 ± 0.4
Phosphatidic acid	traces	traces	traces
Sphingomyelin	11.1 ± 0.4	9.4 ± 0.8	10.1 ± 1.8
Total	99.7	100	101

Table 10. Phospholipid composition of testes. (72)

Animal	Alkenylacyl-GPC	Alkylacyl-GPC	Alkenylacyl-GPE	Alkylacyl-GPE
	% of total phospholipid			
Pig	11.6	27.4	11.3	13.8
Buffalo	19.4	-	3.4	-
Ox	36.8	-	9.0	-
Ox	34.8	9.5	9.4	4.7
Sheep	40.8	-	5.9	-
Man	2.3	-	9.3	-
Rhesus monkey	6.9	-	16.1	-
Dog	3.6	-	15.3	-
Chicken	7.8	-	5.2	-

Table 11. Choline and ethanolamine ether-linked glycerophospholipids in spermatozoa. (38)

Results represent percentages of total lipid phosphorus and are means ± S.D. of three duplicate determinations.

Phospholipids	Human	Rat	Guinea pig
Choline glycerophospholipids			
diacyl	31.0 ± 2.0	47.0 ± 5.1	48.6 ± 1.6
alkylacyl	0.6 ± 0.2	1.1 ± 0.9	2.0 ± 0.6
alkenylacyl	0.0 ± 0.0	0.0 ± 0.0	0.3 ± 0.4
lysophosphatidylcholine	0.6 ± 0.5	1.3 ± 0.9	0.0 ± 0.0
Ethanolamine glycerophospholipids			
diacyl	19.2 ± 2.5	9.0 ± 1.5	14.8 ± 3.5
alkylacyl	0.6 ± 0.3	1.8 ± 0.6	2.5 ± 0.7
alkenylacyl	9.2 ± 0.7	12.2 ± 2.2	4.5 ± 1.2
lysophosphatidylethanolamine	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
Serine glycerophospholipids	13.4 ± 1.3	9.4 ± 2.2	11.3 ± 0.8
Inositol glycerophospholipids	1.2 ± 0.2	4.5 ± 1.3	2.0 ± 0.3
Diphosphatidylglycerol	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
Phosphatidic acid	1.7 ± 0.7	0.0 ± 0.0	2.7 ± 0.2
Sphingomyelin	23.5 ± 2.4	13.3 ± 1.5	11.6 ± 1.3
Total	101	99.6	100.3

Table 12. Phospholipid composition of erythrocytes. (72)

Source	Alkenylacyl-GPC	Alkenylacyl-GPE	Alkylacyl-GPL
	% phospholipid		
Hamster astrocytes	2.7	11.5	2.2
NN astroblasts	3.1	10.0	2.5
C-6 astrocytoma	<0.3	11.7	2.1
C-6 astrocytoma	2.6	10.5	3.5
Glioma 12–18	1.9	13.8	–
Fetal neural CH	1.4	13.8	–
C1300 neuroblastoma	0	12.8	–
$HSDM_1C_1$ fibrosarcoma	6.9	12.0	–
Ehrlich ascites	2.8	6.1	11.0
L fibroblasts	8.2	4.1	–

Table 13. Plasmenylcholine and plasmenylethanolamine content of cultured cells. (38)

DHAP (Fig.12) (97, 103, 105).

DHAP can be directly acylated with acyl-CoA, at the 1-position, via acyl-CoA : DHAP acyltransferase, to form acyl-DHAP (86). Acyl-DHAP is then enzymatically reduced at the 2-position by NADPH : acyl-DHAP oxidoreductase and NADPH to form 1-acyl-sn-glycero-3-phosphate (lysophosphatidate). Alternatively, lysophosphatidate can be formed from glycerol-3-phosphate (G-3-P), which is acylated by acyl-CoA : G-3-P acyltransferase and acyl-CoA. G-3-P arises either from glycolysis, phosphorylation of glycerol or by the reduction of DHAP by NADH : DHAP oxidoreductase (NAD^+ linked G-3-P dehydrogenase) (88,89). There is some controversy over the importance of the acylation of DHAP as opposed to the acylation of G-3-P for the biosynthesis of ester-linked glycerophospholipids (87). Acyl-DHAP is the direct precursor required for the biosynthesis of the ether bond of glycerophospholipids in higher organisms (94). The contribution of the acyl-DHAP pathway for ester-linked glycerolipids has not been established directly (87, 90, 91). For ester-linked species, lysophosphatidate (either from G-3-P or acyl-DHAP) is acylated by acyl-CoA : 1-acyl-sn-glycero-3-phosphate acyltransferase to form 1,2-diacyl-sn-glycero-3-phosphate (phosphatidic acid). This lipid is the common precursor for PC, PE, phosphatidylinositol (PI), phosphatidylglycerol (PG) and di- and triglycerides (88,89).

The ether bond in alkyl glycerophospholipids originates from acyl-DHAP and long-chain fatty alcohols. Evidence that long-chain fatty alcohols were the direct precursors for the alkyl chain was first

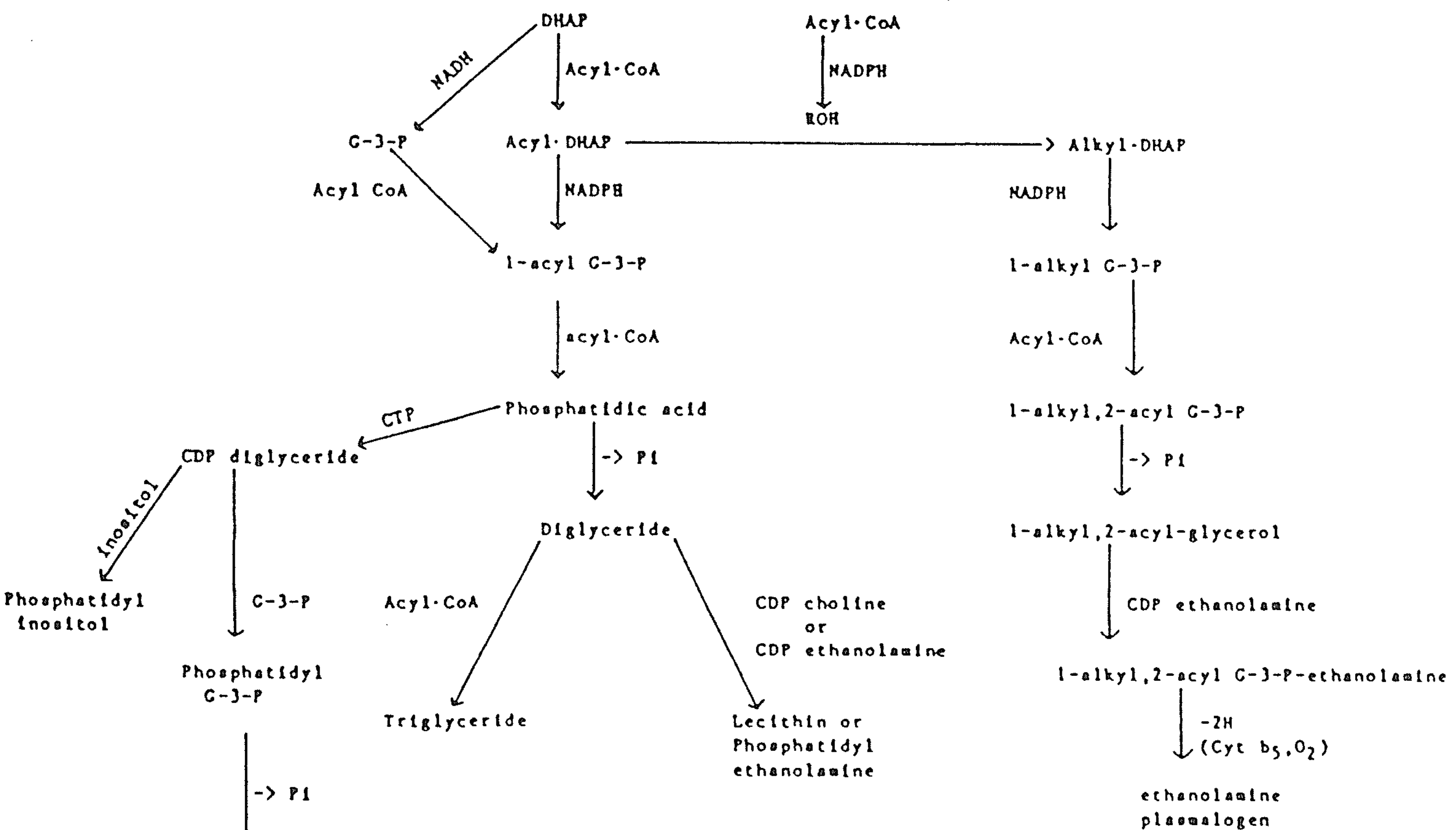


Fig. 12. Biosynthesis of glycerolipids. (97)

provided in 1967 by Friedberg and Greene (96). Later it was also found that long- chain aldehydes could not substitute for the alcohols (103). The fatty alcohols are formed from acyl-CoA, via an aldehyde intermediate, by a membrane associated acyl-CoA reductase (92,95). This enzyme requires NADPH in mammalian cells (93). The biosynthetic rate of formation of the ether lipids may be controlled by the levels of long-chain alcohols since they are the precursors for the alkyl bond (101).

Catalyzed by alkyl-DHAP synthase, acyl-DHAP reacts with a long-chain fatty alcohol to form alkyl-DHAP (1-alkyl dihydroxyacetone-3-phosphate) (94). This is a novel reaction where an acyl group is replaced by an alkyl group, releasing the fatty acid. This substitution can be inhibited by CoA (107), fatty acids (108) and NADPH (100). The enzyme reacts with acyl-DHAP and long-chain alcohols of length C_{10} to C_{22} (111, 112). The presence of fatty alcohols is required for the cleavage of the fatty acid by purified preparations of alkyl-DHAP synthase (132).

Alkyl-DHAP is then reduced at the 2-position by NADPH : alkyl-DHAP oxidoreductase and NADPH to form 1-alkyl-sn-glycero-3-phosphate (110). This oxidoreductase is capable of reducing both alkyl and acyl-DHAP and it has been concluded that this enzyme is the same one that catalyzes the reaction for acyl-DHAP (109). An alternate route for the synthesis of 1-alkyl-sn-glycero-3-phosphate is from alkylglycerols which are taken up from the diet. Alkylglycerols are phosphorylated by ATP : alkylglycerol phosphotransferase to form 1-alkyl-sn-glycero-3-

phosphate (118). With acyl-CoA, 1-alkyl-sn-glycero-3-phosphate may then be acylated by acyl-CoA : 1-alkyl-sn-glycero-3-phosphate acyltransferase to synthesize 1-alkyl-2-acyl-sn-glycero-3-phosphate, the alkyl analogue of phosphatidic acid (103,104). The acyltransferase regulates the acyl group composition of ether glycerophospholipids in brain (113) and in bovine heart (114), due to its specificity for polyunsaturated acyl-CoA's (113). The phosphate of 1-alkyl-2-acyl-sn-glycero-3-phosphate is removed from position-3 by 1-alkyl-2-acyl-sn-glycero-3-phosphate phosphohydrolase, to form 1-alkyl-2-acyl-glycerol, a structure analogous to 1,2-diacylglycerol (117). This ether glycerol is used as a substrate by the cytidine pathway, elucidated by Kennedy et al. in 1956 (8).

The enzyme CDP-choline or CDP-ethanolamine : 1-alkyl-2-acyl-glycerol phosphotransferase adds the choline or ethanolamine base to form 1-alkyl-2-acyl-sn-glycero-3-phosphocholine (1-alkyl-2-acyl-GPC) (plasmanylcholine) or 1-alkyl-2-acyl-GPE (plasmanylethanolamine) (115, 116). A study of these two phosphotransferase activities in rat liver and brain microsomes revealed that fatty acids inhibit the synthesis of plasmanylethanolamine in both tissues (152). Also, studies show that the same choline and ethanolamine phosphotransferases utilized for the synthesis of 1-alkyl-2-acyl-GPC or GPE are used to synthesize 1,2-diacyl-GPC or GPE (163). Plasmanylethanolamine may also arise from labeled 1-alkyl-2-acyl-glycerol by the reverse reaction of ethanolamine phosphotransferase on PE (161). The reverse phosphotransferase activity removes the phosphoethanolamine from endogenous PE and con-

tributes it to the labeled 1-alkyl-2-acyl-glycerol. This reverse reaction was enhanced by the addition of cytidine monophosphate (CMP) (161). Plasmanylcholine is now known to be the precursor storage form of platelet activating factor, 1-alkyl-2-acetyl-GPC (40). Recently, a metabolic link, such as methylation or base exchange, between 1-alkyl-2-acyl-GPE and 1-alkyl-2-acyl-GPC has been proposed on the basis of their tissue content (72). 1-alkyl-2-acyl-glycerol may also be acylated by acyl-CoA : 1-alkyl-2-acyl-glycerol acyltransferase forming 1-alkyl-2,3-diacylglycerol, a triradylglycerol (116).

Early in vivo studies on Ehrlich ascites cells, indicated that 1-alkyl-glycerols are converted to 1-alkenyl lipids by a substitution reaction (130). Alkylacyl and alkenylacyl glycerophospholipids are very similar structurally and it was postulated that there was a precursor-product relationship between 1-alkyl-2-acyl-GPE and 1-alkenyl-2-acyl-GPE on the basis of their specific radioactivities (120,128,129). Some studies demonstrated that 1-alkyl-GPE rather than 1-alkyl-2-acyl-GPE is utilized as the precursor (121). In 1973, Paltauf (122) demonstrated that adenosine triphosphate (ATP) and CoA were not required as cofactors for the reaction when 1-alkyl-2-acyl-GPE was the substrate but were required when the substrate was 1-alkyl-GPE. It has been confirmed in vitro that plasmanylethanolamine is the direct precursor of plasmenylethanolamine via a desaturation reaction of the alkyl bond (119,120,123).

Alkylacyl-GPE desaturase (Δ^1-alkyl desaturase) is the enzyme that catalyzes this terminal step during aerobic biosynthesis of plasmenyl-

ethanolamine in mammals (124). A microsomal mixed-function oxidase, the desaturase subtracts hydrogen atoms from positions 1 and 2 of the alkyl chain of 1-alkyl-2-acyl-GPE to form the double bond of 1-alkenyl-2-acyl-GPE (126). This enzyme is similar in function to fatty acyl CoA desaturase (122,125) and for optimum activity prefers that the 1-alkyl-2-acyl-GPE contains an unsaturated acyl chain at position-2 (124). The desaturase from hamster small intestine mucosa is specific with regard to the glycerol substrate and does not act on 3-alkyl-2-acyl-glycero-1-PE (a stereoisomer of 1-alkyl-2-acyl-GPC), 1-alkyl-GPE or 1-alkyl-2-acyl-glycero-3-phospho(N-dimethyl)ethanolamine (122). Desaturation requires cytochrome b_5 (127), oxygen (122,123,127), NADPH (119,122,123,127) and a cytosolic cyanide-sensitive factor, which stimulates the activity of the enzyme (119,122,123,124). This factor is identical to a desaturase protein and details of this protein are to be found in (124). Recent studies have shown that a high-affinity uptake system is needed to provide free ethanolamine for the biosynthesis of plasmenylethanolamine, as it does not all arise from the decarboxylation of serine (131).

ii) The enzymes

DHAP acyltransferase is a membrane-bound enzyme (133) that is located on the internal side of membrane vesicles from rabbit harderian glands and rat brain (40). In mammals, two types of DHAP acyltransferase are thought to occur. One is localized in peroxisomes and can not use G-3-P as a substrate and the other in microsomes, which can use DHAP or G-3-P as substrate (40). Originally, DHAP acyltrans-

ferase was localized in the microsomes of brain, lung, testes and adipose tissue and in the mitochondrial fraction of liver and kidney (110). Only recently in guinea pig liver and rat liver has this acyltransferase been found in peroxisomes (133,134). In the rat brain, acyltransferase activity was localized in microperoxisomes (97). However, Ehrlich ascites cells, which contain a high proportion of ether lipids, lack peroxisomes and high enzyme activity was found to be microsomal (104).

The DHAP acyltransferase from guinea pig liver peroxisomes has properties distinct from the microsomal DHAP acyltransferase (40) and G-3-P acyltransferase (133). Chronic treatment of rats with hypolipedemic drugs (which cause peroxisome proliferation in liver) resulted in increased activity of both DHAP acyltransferase and G-3-P acyltransferase (97,136). DHAP acyltransferase activity is also stimulated by detergents and the enzyme is resistant to heat and trypsin in the absence of detergents (133). Recently however, approximately 30% of liver DHAP acyltransferase activity has been found to be sensitive to trypsin (97). This may indicate that a portion of this enzyme is localized on the outer peroxisomal membrane. DHAP acyltransferase is generally associated with alkyl-DHAP synthase but the activities of these two enzymes vary in different tissues (135).

NADPH : acyl-DHAP oxidoreductase is present in liver microsomes (134) and with NADPH : alkyl-DHAP oxidoreductase is also found in liver peroxisomes (97). Acyl-DHAP oxidoreductase has also been localized in brain microsomes (97). NADPH : alkyl-DHAP oxidoreductase

specifically transfers hydrogen from the B side of NADPH to form 1-alkyl-sn-glycero-3-phosphate (110). This enzyme was partially purified from the microsomes of Ehrlich ascites cells and guinea pig liver mitochondria (147).

Alkyl-DHAP synthase is a membrane bound enzyme (137) which is not exposed on the cytoplasmic surface (that is, it is on the luminal side) of intact microsomes (138). This microsomal alkyl-DHAP synthase is stimulated by detergents and in the absence of detergents is not sensitive to trypsin (138). It has mainly been studied in microsomes but considerable activity is also found in peroxisomes (97). In guinea pig liver, activity is mainly localized in the light mitochondrial fraction (139) or peroxisomes (97) and in rat liver, a bimodal distribution between peroxisomes and microsomes is found (97). This enzyme is also found in brain microperoxisomes (97) and microsomes of Ehrlich ascites cells (40). Thus, it seems that in guinea pig liver, the enzymes of the acyl-DHAP pathway including: DHAP acyltransferase, acyl/alkyl-DHAP oxidoreductase and alkyl-DHAP synthase are localized in peroxisomes. In general, in organs other than liver and kidney these enzymes are localized in microperoxisomes (97).

The proposed mechanism by which alkyl-DHAP synthase substitutes a fatty alcohol for an acyl moiety is outlined in the following section. The substrate, acyl-DHAP must contain a ketone function (40). Alkyl-DHAP synthase is sensitive to sulfhydryl and amino functional group modifiers , an amino acid functional group at the active site binds the DHAP of acyl-DHAP (40). The acyl group of acyl-DHAP is then

cleaved to form an enzyme-DHAP intermediate before the fatty alcohol is added (40). Acyl-DHAP acylhydrolase activity is not associated with the purified alkyl-DHAP synthase (40). It is thought that this hydrolysis occurs in the absence of long-chain alcohols (145). However, recently it was demonstrated that the cleavage was dependent on the presence of the fatty alcohol and that the amount of fatty acid produced was equal to the alkyl-DHAP formed (132). After hydrolysis, the activated enzyme-DHAP intermediate can bind fatty acids or alcohols to produce acyl-DHAP or alkyl-DHAP, respectively (40). During this process, the pro-R hydrogen at C-1 of DHAP exchanges stereospecifically with water and there is a net retention of configuration in the product (140,141,142,143,144). A ping-pong mechanism has been suggested by the kinetic properties of a purified enzyme which supports the formation of an enzyme-DHAP intermediate (146). In the ether linkage of alkyl-DHAP, the oxygen is provided by the fatty alcohol and both oxygens in the acyl linkage of acyl-DHAP are found in the fatty acid released, this was recently confirmed by Brown et al. (132).

Alkylacyl-GPE desaturase, a membrane-bound enzyme, has over 70% of it's activity in the microsomes (124). This enzyme produces the alkenyl bond by the Z-elimination of erythro-1(S),2(S) hydrogens from the alkyl moiety (126). In microsomes from hamster small intestine, enzyme activity is 3.5 pmol/mg protein/h (149), 56.0 pmol/mg protein/h for adult rat brain microsomes (150) and 2.0 nmol/mg protein/h for pig spleen microsomes (127). In rat brain microsomes, the specific activity decreases with age until in adults the activity is 15% that of the 12-14 day old rats (150).

This desaturase appears to be a system composed of three proteins : NADH : ferricytochrome b_5 reductase (148), cytochrome b_5 (127) and an iron-containing protein that is sensitive to cyanide and identical to terminal desaturase (122,123,124). The reaction is inhibited by ethylenediamine tetraacetic acid (EDTA), sodium azide, N-ethylmaleimide, p-chloromercuribenzoate, vitamin K , Tween 80 and sodium deoxycholate but not by carbon monoxide (122). The mechanism of action for alkyl-acyl-GPE desaturase is similar to that for fatty acyl-CoA desaturase. Both enzyme systems produce a Z-double bond in an oxygen and NAD(P)H dependent reaction and in both processes the manner in which the oxygen is reduced is unknown (125). Rats maintained on a fat-free diet have increased fatty acyl-CoA desaturase activity but their alkylacyl-GPE desaturase activity does not increase (151). Thus, it has been suggested that the two desaturases are controlled at different sites of the electron transport system (124).

e) Biosynthesis of Plasmenylcholine

The entire pathway responsible for the biosynthesis of plasmenylcholine (1-alkenyl-2-acyl-GPC) in mammalian tissues is not known. The desaturation of 1-alkyl-2-acyl-GPC does not occur to form the alkenyl moiety (122,153,156). Also, alkylacyl-GPE desaturase can not utilize 1-alkyl-2-acyl-GPC as a substrate (40). Plasmenylcholine occurs in significant quantities in the mammalian heart (38,154) and the mechanism of its formation remains unsolved. Evidence indicates that long-chain fatty alcohols are incorporated into the alkenyl group (153)

however, it is not known how this occurs. Labeled phosphate can also be incorporated into plasmenylcholine (155), again the mechanisms involved have not been elucidated. Analysis of the fatty acyl chains at the 2-position of the glycerol moiety do not support a direct metabolic relationship between 1,2-diacyl-GPC and plasmenylcholine (154). Several possible pathways for the formation of plasmenylcholine include the methylation of plasmenylethanolamine, base exchange and a mechanism coupling the activities of phospholipases with CDP-choline or CDP-ethanolamine phosphotransferase. Possible pathways are outlined in Fig.13.

The methylation of plasmenylethanolamine to form plasmenylcholine has been demonstrated in rabbit myocardial membranes (157) and rat brain (158). These reports suggest that only small amounts of plasmenylcholine are formed via this mechanism. The base exchange reaction is known to participate in the formation of plasmenylethanolamine and plasmenylserine in the rat brain (170). As yet, there is no *in vivo* evidence of the exchange reaction for the formation of plasmenylcholine (124). *In vitro* studies show that there is a decrease in the rate of base exchange into plasmenylcholine in liver microsomes but not brain microsomes of aged rats (164).

Only trace amounts of plasmenylcholine are formed from 1-alkyl-2-acyl-glycerol and incorporation of labeled CDP-choline apparently results from the reversal of phosphotransferase activities (169). Plasmenylcholine may be formed *in vitro* from 1-alkenyl-2-acyl-glycerol and CDP-choline by a phosphotransferase activity (152,159,160). *In*

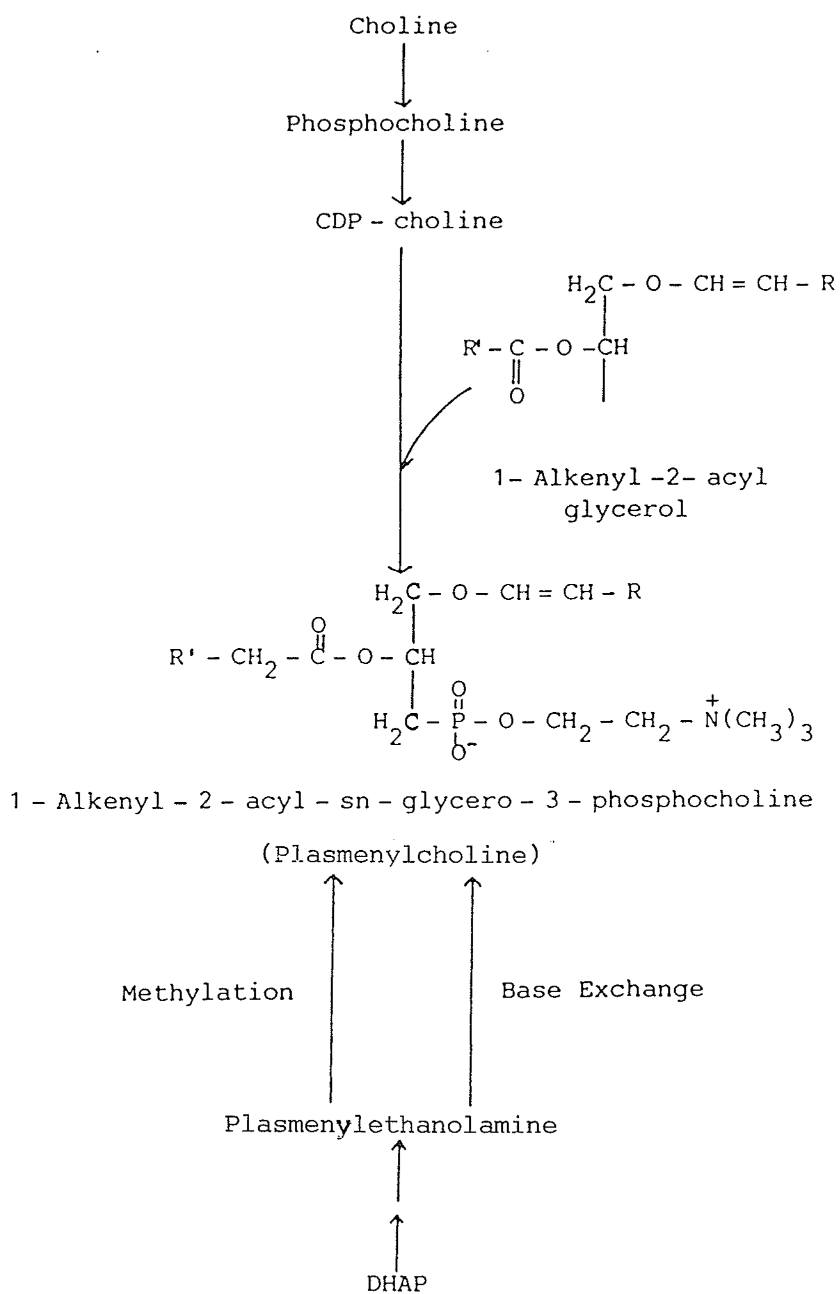


Fig. 13. Biosynthesis of plasmenylcholine.

vivo, the 1-alkenyl-2-acyl-glycerol required for this process may be produced by the removal of the polar head-group by reverse actions of CDP-choline or CDP-ethanolamine phosphotransferase with CMP (161,162). *In vitro*, the polar headgroup can be removed by phospholipase C (165,166,167). Recently, phospholipase C has been found to stimulate the incorporation of labeled choline into plasmenylcholine (165). After removal of the polar headgroup, plasmenylcholine is then formed by the transfer of phosphocholine to the 1-alkenyl-2-acyl-glycerol (152,159,160). The incorporation of CDP-choline into plasmenylcholine is increased significantly by the addition of alkylacylglycerols but not diacylglycerols (168). In liver microsomes, the rate of incorporation of phosphocholine into plasmenylcholine with alkylacylglycerols and CDP-choline is 15.0 nmol/mg protein/h (163).

Alkenyl species of glycerophospholipids are found in a variety of phospholipid classes such as choline, ethanolamine, serine and inositol. The only defined pathway known to date is for the formation of 1-alkenyl-2-acyl-sn-glycerophospho-3-ethanolamine. Thus, it is reasonable to speculate that these other alkenyl glycerophospholipids are probably derived from interconversions of plasmenylethanolamine by a remodelling process (Fig.14). This process possibly involves phospholipases and forward and reverse reactions of the phosphotransferases (124).

IV. Catabolism of Phospholipids

a) Phospholipases

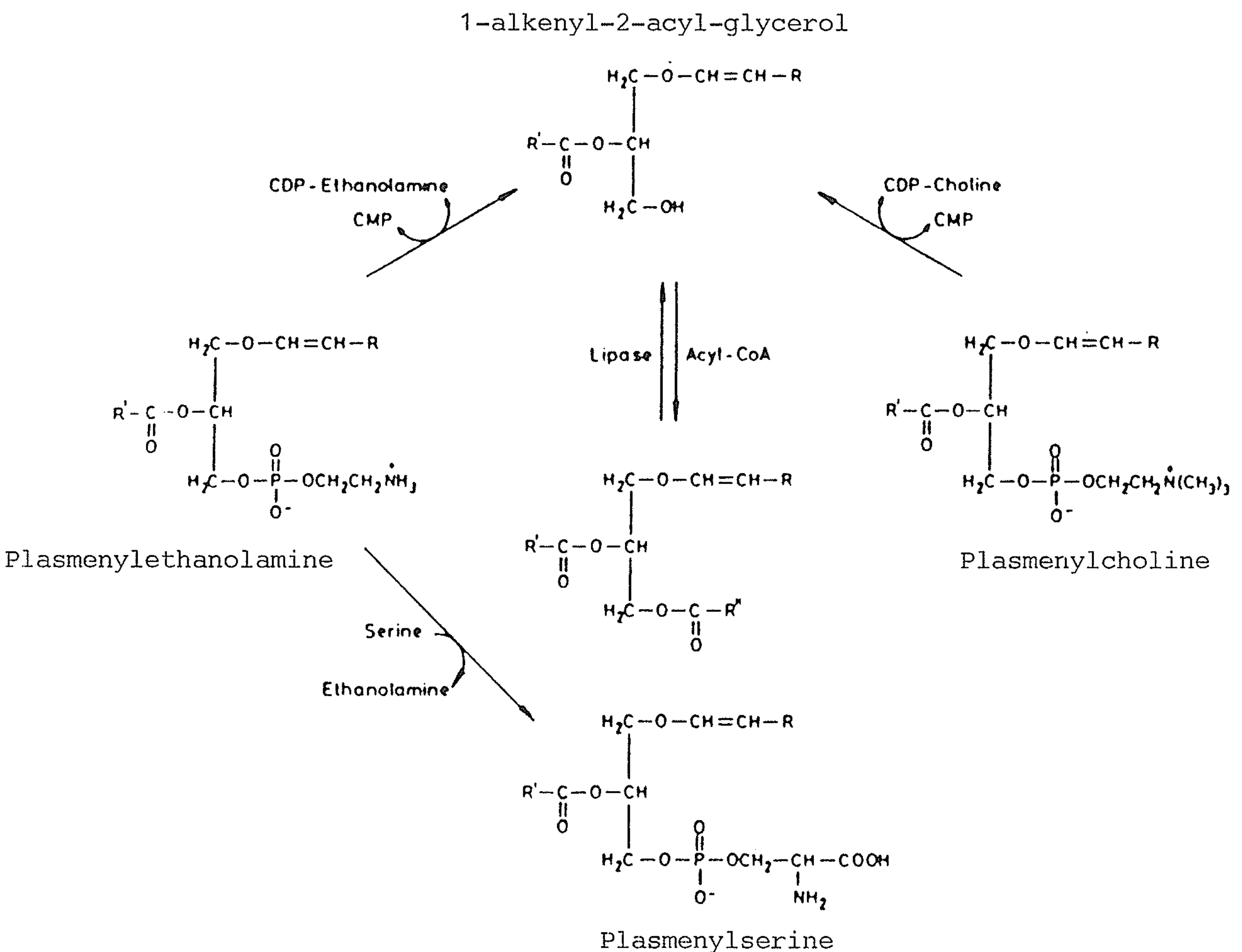


Fig. 14. Conversions of plasmenylethanolamine. (124)

Membrane phospholipids exist in a dynamic flux in which continuous biosynthesis is balanced by degradation. Phospholipases (Fig.15) are the enzymes responsible for the catabolism of the major diacylglycerophospholipids, PC and PE. Phospholipase A1 and A2 hydrolyze the acylester groups from the 1 and 2 positions, respectively, of the glycerol backbone to form the corresponding lysophospholipid (Fig.3). These phospholipases play an important role in the deacylation of phospholipids to lysophospholipids, thus allowing the remodelling process of reacylation to proceed. Phospholipase A1 and A2 are widely distributed in mammalian tissues and are the major phospholipases (36).

Plasmenylcholine and plasmenylethanolamine are resistant to the effects of phospholipase A1 on the alkenyl bond at the 1-position of the glycerol moiety (171). As well, the fatty acyl groups at the 2-position of these ether glycerophospholipids are less susceptible to phospholipase A2 action compared to the diacyl species (172,173). Phospholipase A2 activity from rat brain mitochondria and human cerebrum hydrolyze ether-linked choline glycerophospholipids but with a lower activity than with 1,2-diacyl-GPC and 1-alkyl-2-acyl-GPC (173,176). 1-alkyl-2-acyl-GPC and 1-alkyl-2-acyl-GPE are deacylated at the 2-position by soluble and membrane bound phospholipases A2 from liver lysosomes (174). Diacyl-GPC and 1-alkyl-2-acyl-GPC are cleaved at almost the same rate by phospholipase A2 from isolated rat epididymal fat cells, but the 1-alkenyl-2-acyl-GPC is cleaved at a lower rate (175). The specificity for long-chain acyl groups at the 2-position, reveals that linoleic acid is released faster than either linolenic or arachidonic acid (173) in these systems (175,176). The soluble phos-

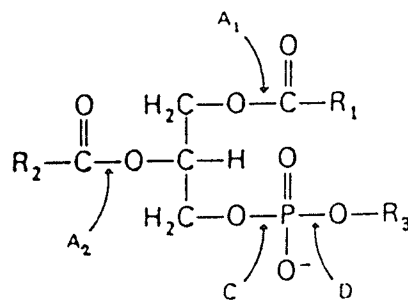


Fig. 15. Sites of phospholipase action. (206)

pholipases A1 and A2 isolated from bacteria, plants or snake venom show a higher affinity for diacyl than for alkylacyl or alkenylacyl glycerophospholipids (175). Phospholipase A1 and A2 have been used during the purification of some ether glycerophospholipids as they remove the majority of the diacyl contaminants efficiently (40), while leaving the majority of the ether glycerophospholipids intact.

Recently, a 'plasmalogen selective' phospholipase A2 activity was demonstrated in the cytosol of canine heart (177). This phospholipase is calcium-independent and specifically cleaves the fatty acid at the 2-position of plasmenylcholine five times faster than with diacyl-GPC (177). The activation of this enzyme could result in the selective release of arachidonic acid from phospholipids in the heart (202) and other tissues (203).

Phospholipase C cleaves the glycerophosphate ester bond of the diacylphospholipids to form 1,2-diacylglycerol and a phosphate mono-ester. Phospholipase C is commonly found in bacteria and is distri-buted in mammalian tissues (37). Most phospholipases C isolated from mammalian tissues are specific for phosphatidylinositol (PI) and ap-pear to be involved in the PI cycle (37).

Phospholipase C from bacteria (<u>B.cereus</u>, 166) and snake venom (<u>Naja naja</u>) demonstrate high activity with all three subclasses of GPC, 1,2-diacyl, 1-alkyl-2-acyl and 1-alkenyl-2-acyl (175). Interestingly, the phospholipase C from <u>B.cereus</u> cleaves alkylacyl-GPC faster than diacyl or alkenylacyl-GPC. A neutral active phospholipase C activity was

recently identified and partially purified from canine heart cytosol (177). This enzyme cleaves plasmenylcholine and diacyl-GPC with similar maximum velocities and does not hydrolyze PI or sphingomyelin (177). It has been proposed that the myocardial phospholipase C may function to shuttle 1-alkenyl-2-acyl-sn-glycerol from ethanolamine glycerophospholipids to choline glycerophospholipids (177). This may account for the high plasmenylcholine content of the heart (201).

Phospholipase D action is one of the mechanisms for transphosphatidylation . The enzyme acts by phosphatidate exchange with a covalent phosphatidyl-enzyme intermediate. When water is the phosphatidate acceptor the reaction is analogous to the hydrolysis of the base from the diacylglycerophosphate moiety. Alcohols substitute for water as the phosphatidate acceptor to form a variety of phospholipids by phosphatidate exchange. Phospholipase D is found predominantly in plants (37), however it has been purified from mammalian tissues (36).

Cabbage phospholipase D can convert 1,2-diacyl, 1-alkyl-2-acyl and 1-alkenyl-2-acyl-GPC from ox heart to phosphatidic acid, 1-alkyl-2-acyl-sn-glycero-3-phosphate and 1-alkenyl-2-acyl-sn-glycero-3-phosphate (179). However, this enzyme has a much slower rate of hydrolysis with the ether-linked choline glycerophospholipids than with diacyl-GPC (180).

Excellent descriptions of the detailed mechanisms and regulation of the four types of phospholipases are to be found in some recent reviews (36,37,175).

b) Plasmalogenases

In mammalian tissues, the enzyme plasmalogenase is thought to catalyze the hydrolysis of the alkenyl bond at the 1-position of plasmenylcholine and plasmenylethanolamine (38,40,186). The enzyme releases a long-chain aldehyde to produce 2-acyl-GPE (lysophosphatidylethanolamine) or 2-acyl-GPC (lysophosphatidylcholine) (185). These lysophospholipids can then be further catabolized by a lysophospholipase which causes deacylation at the 2-position (40). Plasmalogenase activity has been detected in mammalian brain, liver and in erythrocytes (178,181,182). In the brain, plasmalogenase activity is inhibited by diacylglycerophospholipids (38), increases during development (187), reflects the degree of myelination (188) and is higher in oligodendroglia than in neuronal perikarya or in astroglia (189). The presence of this enzyme in rat brain is controversial (183).

Arachidonic acid is a precursor for prostaglandins and thromboxanes (36) and is enriched at the 2-position of plasmenylethanolamine (38). Thus, mobilization of prostaglandin precursors from plasmenylethanolamine, may involve an initial cleavage of the alkenyl linkage by plasmalogenase, followed by the action of a lysophospholipase A2 on the resulting lysophospholipid, to release the arachidonic acid at the 2-position (40).

The catabolism of plasmenylcholine in the heart may occur by the same mechanism as that for the catabolism of plasmenylethanolamine: first, the cleavage by plasmalogenase and then the deacylation of the lysophospholipid by lysophospholipase A2. Alternatively, plasmenyl-

choline could first be deacylated by a phospholipase A2 and then the alkenyl bond of lysoplasmenylcholine (1-alkenyl-sn-glycero-3-phosphocholine) could be cleaved by a lysoplasmalogenase. The second alternative seems likely in light of the fact that plasmalogenase does not act on plasmenylcholine in the liver and a lysoplasmalogenase activity has been identified in this tissue (178). Recently, the absence of plasmalogenase activity for plasmenylcholine has been demonstrated in the canine heart (177).

c) Lysophospholipid Metabolism

Lysophospholipids are distributed throughout all mammalian tissues (31). Having no distinct biosynthetic pathway of their own, they are produced mainly by the hydrolytic action of intracellular phospholipases A on the parent phospholipids (Fig.3). Lysophosphatidylcholine (LPC) (1- or 2-acyl-GPC) is found to be the major lysophospholipid in mammalian cells. Due to its cytolytic property, the level of LPC in all tissues is rigidly controlled (32) and under normal circumstances is at low concentration in most biological membranes. LPC is regarded as an important intermediate in the catabolism of PC (33) and in the resynthesis of PC in most tissues by the deacylation-reacylation cycle (14) and transacylation (15) (Fig.7). These mechanisms are thought to play an important role in achieving the desired fatty acyl composition at the 2-position of phospholipids (33,204). Acylation of 1 or 2-acyl-lysophosphatidylcholine occurs via the enzyme acyl-CoA : 1- or 2-acyl-GPC acyltransferase (200). In serum, LPC is formed by the enzyme

lecithin-cholesterol acyl transferase (LCAT) whereby the fatty acyl chain at the 2-position of PC is transferred to cholesterol, producing cholesterol ester and LPC (7). Complete deacylation of LPC occurs by the action of lysophospholipases A. These enzymes hydrolyze the acyl-ester bonds in lysophospholipids to form glycerophosphocholine, upon the release of the fatty acyl chain (36).

Lysoplasmenylcholine (Fig.3) and lysoplasmenylethanolamine, like other lysophospholipids, are cytolytic and have to be actively metabolized to prevent their intracellular accumulation (32,197,198). Lysophospholipase D plays an important role in the degradation of cytotoxic lysophospholipids with ether bonds, as the acyl analogues are rapidly degraded by lysophospholipases A (175). The microsomal fraction of liver contains a lysophospholipase D that exclusively recognizes 1-alkyl-sn-glycero-3-phosphobases or 1-alkenyl-sn-glycero-3-phosphobases, as substrates (191). This phospholipase yields 1-alkyl or 1-alkenyl-sn-glycero-3-phosphates, upon the release of the amines and does not act on acyl lysophospholipids (192). Lysophospholipase D may also serve in the interconversions of plasmenylcholine and plasmenylethanolamine that involve acyltransferases as well as choline and ethanolamine phosphotransferases (193). This enzyme has also been found in rat kidneys, lung, intestine, testes and brain (193). An endogenous phosphohydrolase rapidly degrades 1-alkyl-sn-glycero-3-phosphate to 1-alkyl-sn-glycerol (38).

Oxidative cleavage of 1-alkyl-sn-glycerol, 1-alkyl-sn-GPC and 1-alkyl-sn-GPE at the 1-alkyl linkage occurs by a microsomal tetrahydro-

pteridine ($Pte-H_4$)-dependent alkyl monooxygenase (194). There is a transient formation of a hemiacetal intermediate (40) during this cleavage. The long-chain aldehydes released by this process can either be oxidized to the corresponding acid or reduced to the alcohol (40). This alkyl monooxygenase requires an alkyl group at the 1-position, a free hydroxyl group at the 2-position and a phosphobase group or a free hydroxyl at the 3-position (40). The enzyme also requires glutathione, ammonium ion and catalase as cofactors, in mammalian systems (194,195). This oxidative attack on the alkyl group is similar to that described for the hydroxylation of phenylalanine (40).

Lysoplasmalogenase (alkenyl hydrolase) from rat liver microsomes cleaves the alkenyl bond of 1-alkenyl-sn-GPC and 1-alkenyl-sn-GPE (178,196). The enzyme for lysoplasmenylcholine is solubilized by sodium deoxycholate (178) but the enzyme for lysoplasmenylethanolamine is inhibited by sodium deoxycholate and p-hydroxymercuribenzoate (196). Alternatively, 1-alkenyl-sn-GPC can be reacylated, back to the parent phospholipid. This was demonstrated in rabbit skeletal muscle but was not observed in rabbit brain or heart (199).

V. Reseach Aims and Synopsis

Plasmenylcholine (1-alkenyl-2-acyl-sn-glycero-3-phosphocholine) is a class of ether lipid which comprises 39% of the total choline phosphoglycerides in the human heart (38). No information was available on the biosynthesis of plasmenylcholine in the heart or in any other mammalian tissue.

Since the formation of plasmenylcholine inevitably requires choline, the initial approach used to elucidate the biosynthesis of this ether lipid was to label plasmenylcholine with radioactive choline. Specifically, perfusion of the guinea pig heart with radioactive choline was performed according to established procedure (13). The radioactively labeled plasmenylcholine was isolated and its specific radioactivity was examined as a function of perfusion time. The various radioactively labeled aqueous choline-containing metabolites were also examined.

Since the lipoidal portion of phosphatidylcholine originates from 1,2-diacylglycerol, it is possible that the lipoidal portion of plasmenylcholine may be derived from 1-alkenyl-2-acyl-glycerol. This possibility was examined and further studies were conducted to illuminate whether the CDP-choline pathway would be the major pathway for the incorporation of choline into plasmenylcholine.

In order to further elucidate the possible role of lysophosphatidylcholine (LPC) in cardiac arrhythmias, it would be helpful to be able to accurately determine the tissue level of LPC. In view of the

high plasmenylcholine content in cardiac cells,lysophosphatidylcholine may be formed from the hydrolysis of plasmenylcholine by the enzyme plasmalogenase. Plasmalogenase has been detected in a number of mammalian tissues, but this activity has not been reported in mammalian heart. A study was undertaken to identify and characterize plasmalogenase activity in the hamster and guinea pig hearts and to develop a rapid spectrophotometric assay for the determination of the plasmalogenase activity. With the availability of this new assay, the subcellular localization and other characteristics of the enzyme were studied. The possible attribution of plasmalogenase activity to the species differences noted in cardiac ether lipid content was also explored.

The possibility that LPC is a physiological factor in the genesis of cardiac arrhythmias (78,81,82,83) has been the subject of much debate. The major problem of studies involving LPC were due to the difficulties encountered in the extraction and quantitation of tissue LPC. High tissue concentrations of lysophospholipids in the ischemic heart had been initially reported (79). However, this might have been caused by the use of acidified butanol during extraction (84). With the usage of neutral organic solvents, lower concentrations of LPC in ischemic myocardium have since been reported (80,84,85). Nevertheless, the obvious lack of agreement between values obtained from different laboratories has not been explained. The discrepancies may result from the different methodologies employed for the extraction, isolation and quantitation of lysophospholipids. Hence, the development of a specific and reproducible method is essential for studies involving the

physiological significance of this lipid.

In this study, a procedure was developed for the determination of small amounts of LPC in cardiac tissue. LPC from canine heart was separated from the major phospholipids and then acetylated with labeled acetic anhydride. The LPC content was calculated from the radioactivity associated with the acetylated product. The results from control and ischemic canine cardiac tissues were compared.

This research may enable investigators to accurately assess the changes in lysophosphatidylcholine levels during cardiac ischemia and other cardiac disorders. The results obtained may provide additional information for the delineation of the physiological role of lysophosphatidylcholine in cardiac dysfunction.

Materials and Methods

I. Materials

a) Animals

Male, albino guinea pigs, weighing 200-250 g, were used for the study of plasmenylcholine biosynthesis. The animals were obtained from High Oak, Ontario, and were maintained on Purina Chow and tap water, *ad libitum*, in a light and temperature controlled room. Syrian golden hamsters, 100-150 g, were utilized for the study of plasmalogenase. Mongrel dogs of either sex, weighing 8-15 kg, were used for the quantitation of cardiac lysophosphatidylcholine.

b) Chemicals

Phosphatidylcholine (pig liver), sphingomyelin (beef brain), 1,2 and 1,3-diacylglycerides (pig liver) were the product of Serdary Research Laboratories (London, Ontario). L-α-Lysophosphatidylcholine (egg yolk) and lipid standard (containing: cholesterol, cholesterol oleate, methyl oleate, oleic acid and triolein) were purchased from Sigma Chemical Co. (St. Louis, MO). CDP-choline, phosphorylcholine chloride, choline chloride, choline iodide and aminoethanol (ethanolamine) were also obtained from Sigma. Palmitaldehyde sodium bisulfite was purchased from K & K Laboratories, Plainsville, NY. 3-heptanone, 4-heptanone and 2',7'-dichlorofluorescein were obtained from Eastman Kodak Co., NY. Thin layer chromatographic plates (SIL-G25) were produced by Macherey-Nagel (West Germany) and purchased through Brinkman Instruments, Rexdale, Ontario. Redi-Plate (Silica gel G), thin layer

chromatographic plates were obtained from Fisher Scientific Co. (Winnipeg, Manitoba). Silicic acid (Bio-Sil A) 100-200 mesh was obtained from Bio-Rad Laboratories, Ca. Celite 545 (AW) was obtained from Supelco Inc., PA. Adenosine 5'-triphosphate (equine muscle), glutathione (reduced form), decanaldehyde, β -nicotinamide adenine dinucleotide (grade III, yeast) and bovine serum albumin were purchased from Sigma. Butylated hydroxytoluene (BHT), potassium iodide, iodine, mercuric chloride and tetraphenylboron were also obtained from Sigma. Sucrose, Trizma base (reagent grade), deoxycholic acid (sodium salt), activated charcoal and ethylenediamine tetraacetic acid (EDTA) were purchased from Sigma. Absolute methanol was obtained from J.T. Baker Chemical Co., NJ. Acetic anhydride of analytical grade was obtained from BDH Chemicals (Poole, England) and kept desiccated over sodium sulfate at 4°C. Pyridine was obtained from Fisher Scientific Co. and kept desiccated over barium oxide at 4°C. Aqueous counting scintillant was purchased from Amersham Corporation (Oakville, Ontario). Anhydrous ether, petroleum ether, diethyl ether, chloroform, iso-butanol, ethanol, methanol and 69-72% perchloric acid were of certified A.C.S. grade from Fisher Scientific Co. All other chemicals were of reagent grade and were obtained from Fisher Scientific Co. All glassware was treated with dimethyldichlorosilane solution (2% in 1,1,1-trichloroethane, BDH Chemicals) before use. All solutions were prepared with glass-distilled water and adjusted to the desired pH.

c) Enzymes and Radiolabeled Compounds

Phospholipase C (*Clostridium welchii*), choline kinase (bakers

yeast) alkaline phosphatase (Escherichia coli), aldehyde dehydrogenase (potassium activated, bakers yeast) and phosphodiesterase I (type II) (Crotalus adamanteus) were purchased from Sigma Chemical Co. (St. Louis, MO).

1,2-Dipalmitoyl-sn-glycero-3-phospho[methyl-^{14}C]choline, [Methyl-^{3}H]choline chloride, [Methyl-^{14}C]cytidine diphosphocholine, ^{14}C-phosphocholine and [γ-^{32}P]adenosine 5'-triphosphate tetra(triethylammonium salt) were purchased from New England Nuclear Research Products (Boston, MA). 1-[1-^{14}C]palmitoyl,2-lyso-sn-glycero-3-phosphocholine, [1-^{14}C]acetic anhydride, [^{3}H]acetic anhydride and [1-^{3}H]ethan-1-ol-2-amine hydrochloride were obtained from Amersham Corporation (Oakville, Ontario).

II. Methods

a) Heart Tissue Preparations

i) Perfusion of the isolated guinea pig heart

Isolated guinea pig hearts were perfused with Krebs-Henseleit buffer (207) in the Langendorff mode (208). Immediately prior to perfusion, the buffer was prepared by combining 100 ml of solution A, 10 ml of solution B, 5 ml of solution C and distilled water to a volume of 1 litre (l). Solution A contains: 70.1 g/l sodium chloride, 21 g/l sodium bicarbonate and 9.91 g/l dextrose. Solution B consists of 3.55 g/100 ml potassium chloride, 2.94 g/100 ml magnesium sulfate and 1.63 g/100 ml sodium phosphate (monobasic). Solution C contained 3.73 g/100

ml of calcium chloride. All solutions were stored separately at 4°C.

Guinea pigs were sacrificed by decapitation, the hearts were excised and placed in Krebs-Henseleit buffer, pH 7.4, saturated with 95% oxygen and 5% carbon dioxide, at room temperature. The aorta was cannulated and the heart was perfused in the Langendorff mode. All perfusions were carried out at 37°C, with a coronary flow rate of 2.8 ml/min. Each heart was first perfused with Krebs-Henseleit buffer for 10 min to allow a period of stabilization for the isolated heart and to restore regular rhythm. Electrocardiac recordings were obtained by placing one electrode on the aortic cannula and the other on the apex of the heart. This placement of electrodes (lead 2) allowed the assessment of atrial and ventricular activities at the same time. Under our experimental conditions, normal EKG patterns were maintained for at least 4 h of perfusion. After stabilization, the hearts were perfused with Krebs-Henseleit buffer containing radioactive or nonradioactive compounds, for various time periods. Following perfusion, the hearts were perfused rapidly with 30 ml of Krebs-Henseleit buffer to remove the residual radioactive or nonradioactive compounds in the blood vessels. An additional 30 ml of air was forced through the cannula to remove the buffer. The heart was then cut open, blotted dry and the wet weight was determined.

ii) Preparation of ischemic heart tissue

Dogs were anesthetized by intravenous injection of sodium pentobarbitol (30 mg/kg body weight). After exposure of the heart, ischemia was produced by surgical occlusion of the left anterior descending

coronary artery by the Harris two-stage technique (209). The chest cavity was closed and the animal was allowed to recover. Appropriate dosages of morphine sulfate and diazepam were given as analgesic and sedative. The cardiac rhythm of these animals after surgery was monitored by electrocardiac recording (lead 2). The heart was excised 24 h after surgery and ischemic areas of tissue from the left ventricle were removed and placed on ice. Control cardiac tissue was obtained from the nonischemic areas of the left ventricle of the same heart.

iii) Subcellular fractionation

Guinea pigs and hamsters were sacrificed by decapitation and the hearts removed and placed on ice. The hearts were weighed, washed, cut into pieces and then homogenized in 0.25 M sucrose, 10 mM tris-HCl and 2 mM EDTA (pH 7.4) to yield a 10% (w/v) homogenate. Homogenization for 20 s was performed twice with a Polytron homogenizer (Brinkman PT10/35). The homogenate was centrifuged for 4 min at 100 x *g* with a bench centrifuge at 4°C. The supernatant was decanted and the pellet was rehomogenized. This procedure was repeated and the supernatants were combined with the last homogenate. This final homogenate was centrifuged at 2,000 x *g* for 10 min to pellet nuclei, cell debris and unbroken cells. The resulting supernatant was centrifuged at 20,000 x *g* for 10 min (12,500 rpm x 10 min in Sorvall RC-5 Superspeed Refrigerated Centrifuge with SS34 rotor). The supernatant was decanted and the centrifugation was repeated. The two resultant precipitates represented the mitochondrial fraction. The post mitochondrial supernatant was centrifuged at 100,000 x *g* for 60 min (37,000 rpm x 1 h in

Beckmann Ultracentrifuge with Ti 70 rotor). The pellet represented the microsomes and the supernatant the cytosol.

The microsomes were then washed as follows: The microsomal pellet was resuspended in 0.15 M tris-HCl (pH 8.0) using a Dounce homogenizer type B. The homogenate was centrifuged at 100,000 x g for 60 min. The microsomes (pellet) were then resuspended in 0.25 M sucrose and 10 mM tris-HCl (pH 7.4) and the centrifugation was repeated. The final, washed microsomes were resuspended in 0.25 M sucrose and 10 mM tris-HCl (pH 7.4) and stored at -20°C.

b) Preparation and Isolation of Lipids

i) Preparation of total lipid extracts

The wet weight of all cardiac tissue samples, taken either directly from the animal or after perfusion, were determined and the tissue samples were immediately cut into small pieces for homogenization. The tissue sample in chloroform/methanol (1:1; v/v) was homogenized twice with a Polytron homogenizer (Brinkman PT10/35) at a setting of 6, for 20 s each. The homogenizer was rinsed with 5 ml of chloroform/methanol (1:1; v/v) which was added to each tissue homogenate. Butylated hydroxytoluene (0.25%) was added to all samples prior to homogenization. Lipids were extracted from the homogenate by the method of Folch et al. (210). The tissue homogenate was centrifuged for 10 min at 2,000 rpm in a clinical centrifuge. The supernatant was decanted into a round bottom flask and chloroform was added to produce a chloroform/methanol ratio of 2:1; v/v. The tissue pellet was re-

extracted with chloroform/methanol (2:1; v/v) and the mixture allowed to stand for at least 10 min to facilitate extraction. The second extract was centrifuged as above and the resulting supernatant was decanted. Extraction of the pellet was then repeated. Supernatants (lipid extracts) from each extraction were combined into a round bottom flask and enough water was added to each to produce chloroform/methanol/water (4:2:2; v/v). The mixture was shaken vigorously and allowed to stand for 10 min, to improve phase separation. The upper phase (aqueous) was removed from each flask by pasteur pipette and placed in a round bottom flask and the volume of the solvent was reduced by evaporation *in vacuo*, at 45°C. The content was resuspended in 1 ml of water, transferred to a screw cap tube and stored at -20°C. The lower phase (organic) was filtered (Whatman 1PS filter paper) into a round bottom flask and the volume of the solvent was reduced by evaporation *in vacuo*, at 30°C. The content of each flask was resuspended in chloroform/methanol (2:1; v/v) and the solution was filtered (Whatman 4 qualitative filter paper) into a screw cap tube. The solvent was then totally removed by evaporation under nitrogen. A known volume of chloroform was added to each tube and the tubes were stored at -20°C. These tubes constitute the total lipid extract from each tissue sample.

ii) Separation of phospholipids

a) Silicic acid column chromatography

Phospholipid classes in the total lipid extract were separated by

silicic acid chromatography according to the protocol of Sheltawy and Dawson (211). Silicic acid, 100-200 mesh (Bio-Sil A, Bio-Rad) was washed several times with chloroform and then packed into columns. Total lipid extracts (in chloroform) were then loaded onto the top of each column. Fractions were collected and the volume of the solvent was reduced by evaporation at 30°C., *in vacuo*. After evaporation, lipid fractions were transferred to screw cap tubes and analyzed by thin layer chromatography to monitor the lipid composition of each fraction. Neutral lipids were eluted by passing sufficient volumes of 100% chloroform down the column. The total phospholipids were then eluted with 80% methanol in chloroform.

When phospholipids were eluted in a stepwise manner, the following series of eluants were used. Phosphatidic acid and phosphatidylglycerol were eluted from silicic acid columns with 5-10% methanol in chloroform. Phosphatidylethanolamine was obtained from the columns with 10-15% methanol in chloroform, phosphatidylserine with 15-20% methanol in chloroform and phosphatidylcholine was eluted with 40-50% methanol in chloroform. Sphingomyelin and lysophosphatidylcholine were eluted from the silicic acid columns with 80% methanol in chloroform.

b) Thin layer chromatography (TLC)

Neutral and phospholipids were regularly separated on thin layer chromatographic plates (Redi-Plate, Silica gel G, Fisher Scientific). Lipid samples were applied to the origin (1.5 cm from the bottom of the plates) via a Hamilton syringe. Blank lanes of silica gel (0.5-2.0 cm) were left on the sides of each plate. After application to the

plates, the samples were allowed to air dry. Chromatographic tanks were pre-saturated with the solvent mixture and all plates were run at room temperature. After chromatography, the plates were allowed to air dry, prior to visualization. Phosphatidylethanolamine, phosphatidylcholine and lysophosphatidylcholine were usually separated with a solvent mixture containing chloroform/methanol/water/acetic acid (70:30:4:2; v/v) or chloroform/methanol/water/ammonium hydroxide (65:50:4:11; v/v). When highly purified phospholipids were required, succesive chromatographies utilizing both solvent mixtures were employed.

Lipids on TLC plates were visualized by destructive (iodine, ninhydrin, phosphomolybdate-sulfuric acid, Dragendorff's reagent) and non-destructive methods (2',7'-dichlorofluorescein). Destructive methods were only employed when the lipids separated did not require further analysis after chromatography. All unsaturated lipids were easily visualized by exposure of the plates to iodine vapor, in a closed chamber for a few minutes. However, this is a poor method for the visualization of saturated lipids. Also, the iodination of double bonds causes the loss of unsaturated fatty acids and of the alkenyl bond of ether lipids. Iodine was removed from TLC plates by warming them for 30 min at 100°C.

Sprays were utilized to specifically visualize certain classes of lipids. Ninhydrin (0.25% in acetone) spray generated purple spots, after heating the plates, to visualize phosphatidylethanolamine, lysophosphatidylethanolamine and phosphatidylserine. A modified version of

Dragendorff's reagent (211) was used to visualize choline-containing lipids, as yellow spots on a grey background. Dragendorff's reagent was prepared by rapidly mixing a solution of 17% (w/v) bismuth nitrate in 20% (v/v) aqueous acetic acid in a ratio of 2:7 with 0.29% (w/v) potassium iodide. This mixture is highly unstable and was immediately sprayed onto TLC plates.

The method utilized for the detection of lipid-phosphorus on TLC plates was that developed by Dittmer and Lester (212), as described by Sheltawy and Dawson (211). All phosphorus-containing lipids appeared as blue spots, which gradually increased in intensity, after TLC plates were sprayed with the phosphomolybdate-sulfuric acid solution. This reagent was also used to char all types of lipids, by heating the sprayed plate for 30 min at 100°C. This spray was prepared by mixing two volumes of water with equal volumes of solutions A and B. Solution A was prepared by the addition of 40.11 g molybdenum trioxide to 1 litre of 25 N sulfuric acid (70%, v/v). The mixture was boiled until the molybdenum trioxide had dissolved. Solution B was prepared by the addition of 1.78 g powdered molybdenum to 500 ml of solution A. This mixture was boiled for 15 min, cooled and decanted from any residue present. Solutions A and B were stored separately until required.

When lipids were required for analysis subsequent to thin layer chromatography, the TLC plates were sprayed with 0.2% (w/v) 2',7'-dichlorofluorescein in ethanol. Lipids were visualized as yellow fluorescent spots against a green background, under ultraviolet light. To isolate the lipids, the appropriate fractions were removed from the

TLC plates and eluted from the silica gel by the method of Arvidson (213). To the silica gel in small test tubes was added 4 ml of chloroform/methanol/water/acetic acid (50:39:10:1; v/v) and the solution was mixed vigorously. The tubes were allowed to stand for 10 min, to facilitate the extraction of the lipids. Thereafter, the tubes were centrifuged for 10 min at 2,000 rpm in a clinical centrifuge. The supernatants were removed to large tubes (15 ml) and extraction of each silica gel pellet was repeated, twice. To the combined supernatants in the large tubes was added 4 ml of 4 M ammonium hydroxide and the tubes were mixed vigorously. After phase separation, the upper phases (containing the dye) were removed by suction. The lower phases were filtered (Whatman 1 PS filter paper) into screw cap tubes and evaporated to dryness under nitrogen. Each lipid sample was then resuspended in chloroform and stored at -20°C.

iii) Preparation of plasmenylcholine and plasmenylethanolamine

a) Care and storage

The vinyl ether (alkenyl) linkages of plasmenylcholine and plasmenylethanolamine are easily lost due to peroxidation, to form the ether (alkyl or hydroxy-alkyl) linkage. Hence, an antioxidant, butylated hydroxytoluene, was included in all lipid preparations containing alkenyl linkages. All test tubes and glassware used to store these lipids were covered with aluminum foil to exclude light. Lipid preparations were stored under nitrogen at -20°C., at all times. During all experiments, care was taken to avoid exposing the alkenyl linkages to

temperatures above 40°C., mercuric ions, iodine vapors or mildly acidic conditions. All of the above conditions will cause the cleavage of the alkenyl linkages by reducing the double bond and releasing the long-chain aldehyde, thereby producing the lysophospholipid.

b) Alkaline hydrolysis

The current methods available for thin layer chromatography and silicic acid column chromatography were not able to separate intact plasmenylcholine from the diacyl and alkylacylglycerophosphocholine classes. All three classes of lipids migrate as a single fraction with these methods. This difficulty also applies to the ethanolamine glycerophospholipid classes. It is therefore necessary to hydrolyze or derivatize one or more of these lipid classes, prior to their separation by chromatography. In our experiments, intact plasmenylcholine and plasmenylethanolamine were isolated from choline and ethanolamine glycerophospholipids (containing diacyl, alkenylacyl and alkylacyl classes) after alkaline hydrolysis. Alkaline conditions caused the hydrolysis of the ester linkages of glycerophospholipids releasing the fatty acyl chains, whilst leaving the ether (alkenyl and alkyl) linkages intact. Since alkylacylglycerophosphocholine constitutes less than 3% of the total choline glycerophospholipids of the guinea pig heart, it was allowed as a minor contaminant in all preparations of choline glycerophospholipids.

Large amounts of plasmenylcholine and plasmenylethanolamine were prepared by the method of mild alkaline hydrolysis by Renkonnen (215). Treatment for 15 min by mild alkaline hydrolysis caused the complete

cleavage of the ester linkages of the diacylglycerophospholipids but only 30-50% of the ester groups in plasmenylcholine. Specifically, the choline or ethanolamine glycerophospholipid sample was evaporated to dryness in a large round bottom flask, under a stream of nitrogen. The lipids were redissolved in 120 ml of chloroform/methanol (1:1; v/v). Thereafter, 20 ml of 0.35 N sodium hydroxide in 96% methanol was added. After vigorous mixing, the sample was incubated at room temperature for 15 min. Subsequent to incubation, 100 ml of chloroform and 0.8 ml of methanol were added to the flask. The flask was capped, shaken vigorously and 51 ml of water was added. After mixing and phase separation, the upper phase was removed and the solvent in the lower phase was reduced by evaporation *in vacuo* at 30°C. The lipids were redissolved in chloroform/methanol (2:1; v/v) and applied to the top of a silicic acid column. Intact plasmenylethanolamine was eluted from the column with 10-15% methanol in chloroform and plasmenylcholine was eluted with 30-40% methanol in chloroform, as described previously. Thin layer chromatography was used to establish the lipid composition of each fraction. When the incubation time was increased to 40 min, all of the ester linkages were destroyed. This resulted in the formation of lysoplasmenylcholine (from plasmenylcholine) and glycero-3-phosphocholine (from diacylglycerophosphocholine). Similar hydrolytic products were obtained for the ethanolamine phosphoglycerides. Under these conditions, lysoplasmenylethanolamine was eluted from the silicic acid columns with 20-25% methanol in chloroform and any residual diacylglycerophosphocholine was eluted with 50% methanol in chloroform. Lysoplasmenylcholine was eluted gradually between 55-80%

methanol in chloroform.

Mild alkaline hydrolysis by the method of Wells and Dittmer (214) was used to degrade all of the ester linkages of glycerophospholipid samples containing up to 20 µmol of lipid. This method was utilized to prepare lysoplasmenylcholine and lysoplasmenylethanolamine. Specifically, solvents were completely removed from the choline or ethanolamine glycerophospholipid samples by evaporation under nitrogen. The lipids were redissolved in 1 ml of chloroform/methanol (1:4; v/v) by vigorous mixing. To this solution was added 0.1 ml of 1.2 N sodium hydroxide in methanol/water (1:1; v/v). The solution was mixed thoroughly, covered with Parafilm and incubated at 37°C for 20 min in a shaking water bath. The sample was neutralized upon the addition of 0.15 ml of 1 N acetic acid and 2 ml of chloroform/methanol (9:1; v/v). After vigorous mixing, 1 ml of isobutanol and 2 ml of water were added. The mixture was shaken and then centrifuged for 10 min at 2,000 rpm in a clinical centrifuge to facilitate phase separation. The upper phase was removed by suction and the lower phase was re-extracted with methanol/water (1:2; v/v). After centrifugation, the solvent in the washed lower phase was evaporated with nitrogen. The lipids were redissolved in chloroform/methanol (2:1; v/v) and spotted onto a TLC plate, which was then developed in a solvent mixture of chloroform/methanol/water/acetic acid (70:30:4:2; v/v). After chromatography, the plate was allowed to air dry and was then sprayed with 0.2% 2',7'-dichlorofluorescein in ethanol to visualize the lipids. This procedure degrades diacylglycerophosphocholine to glycero-3-

phosphocholine, plasmanylcholine to lysoplasmanylcholine and plasmenylcholine to lysoplasmenylcholine. Similar hydrolytic products were formed from the ethanolamine glycerophospholipids. Therefore, the LPC or LPE fractions on the TLC plate contained only the ether classes of choline and ethanolamine glycerophospholipids, respectively. These lysophospholipid fractions were removed from the plate and the lipids were eluted from the silica gel by the method of Arvidson (213) as described previously.

c) Acidic hydrolysis

Phospholipid preparations, free of plasmenylcholine and plasmenylethanolamine, were obtained after acid hydrolysis by the method of Wells and Dittmer (214). This method hydrolyzes all of the alkenyl bonds of glycerophospholipids but leaves the ester and ether (alkyl) bonds intact. Specifically, the solvents of lipid samples (containing up to 1 µmol) were evaporated under nitrogen. The lipids were resuspended in 1.6 ml of chloroform/methanol (5:11; v/v) and mixed vigorously. The mixture was again vigorously mixed following the addition of 0.4 ml of 0.025 M mercuric chloride in 0.05 N hydrochloric acid. The tubes were capped with Parafilm and incubated for 20 min at 37°C in a shaking water bath. After incubation, 3.5 ml of chloroform and 0.9 ml of methanol were added and mixed thoroughly prior to the addition of 2.6 ml of water. After mixing, the phases were separated by centrifugation (10 min at 2,000 rpm in a clinical centrifuge). The upper phase was removed and the solvent in the lower phase was evaporated under nitrogen. The hydrolysis was then repeated. Following the

second hydrolysis, the lipids in the lower phase were redissolved in chloroform/methanol (2:1; v/v) and spotted onto a TLC plate. The plate was developed in a solvent mixture of chloroform/methanol/water/acetic acid (70:30:4:2; v/v). After development, the plate was allowed to air dry and the lipids were visualized by exposure to iodine vapor. By this procedure, long-chain fatty aldehydes were released from the C-1 position of plasmenylcholine and plasmenylethanolamine, resulting in the formation of lysophospholipids. Ester bonds and alkyl bonds of the phosphoglycerides were not attacked by this treatment and the migration of these lipids on the TLC plates remained unchanged.

iv) Isolation of lysophosphatidylcholine

Lysophosphatidylcholine (LPC) was isolated from the total lipid extract of canine heart, on silicic acid columns according to the protocol of Sheltawy and Dawson (211). The lipid extracts were suspended in a volume equivalent (ml) of chloroform/methanol (2:1; v/v) equal to the gram wet weight of the tissue homogenate. Silicic acid (0.5 g) was suspended in chloroform and packed into a 10 x 0.5 cm column. Lipid extract (20-100 µl) was applied to the column. The lipids were eluted from the column by the sequential application of 5 ml of chloroform, 5ml of chloroform/methanol (4:1; v/v), 25 ml of chloroform/methanol (1:1; v/v) and 40 ml of chloroform/methanol (1:9; v/v). LPC was eluted from the column with the last eluant and the volume of the LPC-containing fraction was reduced by evaporation *in vacuo*. The content was transferred to a test tube and the solvent was totally removed by evaporation under nitrogen.

v) Isolation of 1-alkenyl-2-acyl-glycerols

1-Alkenyl-2-acyl-glycerols were isolated and quantitated from the guinea pig heart. Total lipid extracts (in chloroform) were prepared from three samples (each containing 4 hearts). Neutral lipids present in the total lipid extracts were separated from the phospholipids by thin layer chromatography. The TLC plates were developed with a solvent mixture of chloroform/methanol/acetic acid (98:2:1; v/v). After chromatography, the neutral lipids were visualized under U.V. light with 0.2% 2',7'-dichlorofluorescein. Since 1-alkenyl-2-acyl-glycerol migrates with 1,2 and 1,3-diacylglycerols on TLC plates, the fractions corresponding to 1,2 and 1,3-diacylglycerols were removed from the chromatograms. The lipids were recovered from the silica gel by the method of Arvidson (213). The diradylglycerol preparation was further purified by thin layer chromatography with a solvent mixture of petroleum ether/diethyl ether/acetic acid (80:20:1; v/v). Following development, the 1,2 and 1,3-diacylglycerols were removed from the TLC plates and eluted as described above. These two diradylglycerol fractions were combined and the 1-alkenyl-2-acyl-glycerols were quantitated by their alkenyl content by a modification of the method by Gottfried and Rapport (216).

vi) Production of 1-alkenyl-2-acyl-glycerol

Plasmenylcholine-enriched fractions were obtained from bovine and canine hearts by silicic acid column chromatography after mild alkali hydrolysis (215). The alkenyl content of these fractions was estimated

by the method of Gottfried and Rapport (216). 1-Alkenyl-2-acyl-glycerols were prepared from plasmenylcholine by hydrolysis of the phosphocholine moiety with phospholipase C (C.welchii) according to the method of Renkonnen (167). The solvents of plasmenylcholine-enriched fractions (containing up to 30 µmol of plasmenylcholine) were evaporated under a stream of nitrogen. The lipids, in large covered tubes, were resuspended by sonication in 1 ml of 0.2 M tris-HCl (pH 7.35). Phospholipase C (100 units) was resuspended in 0.2 M tris-HCl (pH 7.35) containing 8 mM calcium chloride and added to the sonicated lipid suspension. Diethyl ether, up to 2 ml, was gently layered onto the top of the lipid-enzyme suspension. This preparation was incubated at 37°C in a shaking water bath, until the bottom layer had clarified. The ether was then evaporated under a stream of nitrogen and the lipids were extracted and phase separated as previously described. The lipid extract was applied to the top of a chloroform washed, silicic acid column and diradylglycerols were eluted from the column by passing sufficient volumes of 100% chloroform down the column. The diradylglycerols were further purified by thin layer chromatography, utilizing the two solvent system described in the previous section.

c) Analytical Methods and Assays

i) Determination of lipid-phosphorus

Total lipid phosphorus was determined by the method of Bartlett (217), using perchloric acid digestion as described by King (218). This assay was carried out on glycerophospholipid samples or spots from thin layer chromatographic plates. The sensitivity for this

method is 0.3 µg of lipid-phosphorus. Any organic solvents in the sample were removed by evaporation under a stream of nitrogen. Perchloric acid (1.2 ml) was added and the sample was digested at 160°C with an electric heater until clear (approximately 2 h). After cooling, 8 ml of water was added to the digested sample, to give a volume of 9 ml. After vigorous mixing, 0.8 ml of 5% ammonium molybdate solution was added followed by vigorous mixing. ANSA reagent was freshly prepared by dissolving 0.025 g of 1-amino-2 naphthol 4-sulfonic acid, 1.462 g of sodium bisulfite and 0.05 g of sodium sulfite in 10 ml of hot distilled water with constant stirring. The reagent was allowed to cool to room temperature and then filtered to remove any impurities. ANSA reagent (0.2 ml) was added to each sample and the tubes were mixed vigorously. The tubes were then covered with aluminum foil and heated in a boiling water bath for at least 10 min. After cooling, the absorbance of the solutions were measured at 830 nm. Standards of potassium phosphate, monobasic (10 µg/ml), for the standard curve and reagent blanks were assayed each time under the same conditions as the samples.

ii) Determination of alkenyl content

The alkenyl content of lipid samples was measured by a modification of the method of Gottfried and Rapport (216). Five test tubes for each lipid sample (about 0.1 µmol) were prepared and the solvent was evaporated with nitrogen. A series of five blank test tubes (with no sample) were treated exactly as the sample tubes hereafter. Absolute methanol (0.5 ml) was added to all tubes followed by brief sonication

in a bath sonicator, to disperse the lipid. The tubes were then warmed at 60 to 70°C for 2 to 3 min in a water bath. Tubes were mixed vigorously and 0.5 ml of iodine solution, containing 1 ml of 38 mg of iodine in 50 ml of 3% (w/w) aqueous potassium iodide and 9 ml of 3% aqueous potassium iodide, were added to three of the five tubes for each sample. To the other two tubes were added 0.5 ml of 3% aqueous potassium iodide. All tubes were mixed vigorously and allowed to stand at room temperature for 20 min. Ethanol (4 ml) was added to all tubes and the solution was mixed vigorously. The spectrophotometer was zeroed with ethanol and the absorbance of all tubes was measured at 355 nm. When the absorbance of the tubes containing the sample and the iodine solution was less than 0.1, then the assay was repeated with a smaller sample.

The content of alkenyl groups for each sample was calculated by subtracting the absorbance of the tubes that contain the sample and 3% potassium iodide from the absorbance of the tubes that contain the sample and the iodine solution. The final absorbance of the reagent blank was calculated by subtracting the absorbance of the tubes containing 3% potassium iodide from the absorbance of the tubes containing the iodine solution. Then, the final absorbance for the reagent blank was subtracted from the final absorbance of each sample and the concentration of alkenyl lipids in each sample were calculated using a molar extinction coefficient (ϵ) of 0.0275 (nmol/ml).

iii) Protein determination

The protein concentrations of the subcellular fractions were estimated by the method of Lowry et al. (219), with bovine serum albumin (BSA) as the standard. A series of test tubes with known amounts of BSA (0-100 µg/tube) were prepared in triplicate, for the standard curve. Triplicate tubes of samples were also prepared and the volume of all tubes was brought to 100 µl with distilled water. To each tube was add 0.1 ml of 5% (w/v) sodium deoxycholate and distilled water in order to bring the volume of each tube to 1 ml and the solution was mix vigorously. Equal volumes of 1% (w/v) copper sulphate and 2% (w/v) potassium sodium tartarate were mixed. The resultant solution was mixed with 2% (w/v) sodium carbonate in 0.1 M sodium hydroxide at a ratio of 1/50 (v/v). The mixed solution (4 ml)was added to each tube and allowed to stand at room temperature for 10 mins. The Phenol reagent (Folin-Ciocalteau) was prepared by diluting 1 ml of the commercial reagent (2 N) with 1.36 ml of distilled water. The diluted reagent (0.5 ml) was added to each tube and the tubes were mixed instantaneously. The tubes were heated at 60°C for 10 mins, in order to stabilize the color. After cooling, the absorbance of the tubes was measured at 730 nm.

iv) Assays for the determination of plasmalogenase activity

a) Disappearance of substrate

Plasmalogenase activity was measured by a modified procedure of Dorman et al.(189). Purified plasmenylethanolamine (2.6 µmol) was dispersed in 1 ml of water by sonication in a bath sonicator at 0°C, until clear. The reaction mixture contained 200 µl of dispersed plas-

menylethanolamine and 300 µl of 1 M tris-HCl buffer (pH 8.0). The reaction was started upon the addition of the subcellular fraction (containing 25-500 µg protein). The final reaction volume was 1.5 ml. After an incubation period of 0-60 min, the reaction was stopped by the addition of 6 ml of chloroform/methanol (2:1; v/v) and the sample was mixed vigorously. The upper and lower phases were separated by centrifugation and the upper phase was dicarded. Aliquots of the lower phase were removed for analysis of alkenyl content as described previously.

b) Spectrophotometric

Plasmalogenase activity was assayed by measuring the production of NADH at 340 nm on a double-beam spectrophotometer. The reaction mixture (1.5 ml) contained 200 mM tris-HCl (pH 8.5), 133 mM potassium chloride, 7 mM glutathione (reduced form), 8.3 mM NAD^+, 1 unit of aldehyde dehydrogenase and 347 µM plasmenylethanolamine (prepared as described above). The contents of the reaction mixture were added sequentially into a quartz cuvette, in the above order. Due to the presence of dispersed plasmenylethanolamine in the assay, the mixture was allowed to equilibrate in the spectrophotometer for 5 min, in order to obtain a stable baseline. The reaction was initiated by the addition of the enzyme preparation to the sample cuvette. When enzyme preparations containing mitochondrial particles were used, rotenone (2 µM) was present in the incubation mixture to inhibit NADH oxidation. The reference cuvette contained all the ingredients of the assay except the subcellular fraction, which was replaced by an equal volume

of 50% (w/w) glycerol in the homogenizing buffer. Enzyme activity was calculated from the change in absorbance at 340 nm.

v) Aldehyde dehydrogenase assay

Aldehyde dehydrogenase activity was measured spectrophotometrically by following the reduction of NAD^+ at 340 nm. The reaction mixture (1.5 ml) contained 333 mM tris-HCl (pH 8.0), 133 mM potassium chloride, 7 mM glutathione (reduced form), 8.3 mM NAD^+, 0.03 units aldehyde dehydrogenase and the aldehyde solution. The aldehyde solutions were prepared and utilized as follows: 1 mM solution of palmitaldehyde was prepared by sonication and 10 µl were used for assays. Decanaldehyde (0.1 M) was prepared by the method of Nakayasu et al. (220) and 10 µl were used for assays. When acetaldehyde was used as a substrate, 20 µl of 178 mM acetaldehyde solution were used in the assays.

vi) Quantitation of lysophosphatidylcholine

Lysophosphatidylcholine from canine heart was separated from the major glycerophospholipids by column chromatography as described previously and then acetylated with labeled acetic anhydride. Acetylation of LPC was conducted in the following manner. The reaction mixture contained 24 µl of pyridine, 36 µl (382 µmol) of [^{3}H]acetic anhydride (500-2000 dpm/nmol) and 0.6 µl of perchloric acid. These were added sequentially to each sample tube containing dry lipid sample. The tubes were sealed with Teflon-coated stoppers and the contents were mixed vigorously. Tubes were then incubated at 70°C for 30 min, mixed for 10 sec and reincubated for another 30 min. After incubation, the

solvents in the reaction mixture were evaporated with nitrogen. The residue was dissolved in 1 ml of chloroform followed by 1.25 ml of methanol/water (2:3; v/v) to cause phase separation. The upper phase was removed and the lower phase was re-extracted with 1.25 ml of methanol/water (2:3; v/v). The solvents in the lower phase were evaporated to dryness with nitrogen and the residue in each tube, including the reagent blanks, was redissolved in 25 µl of chloroform/methanol (2:1; v/v). The acetylated LPC was isolated by thin layer chromatography with a solvent system containing chloroform/methanol/water/acetic acid (70:30:4:2; v/v) and visualized by exposure of the TLC plate to iodine vapor. In some experiments, 10 nmol of non-labeled 1-acyl-2-acetyl-sn-glycerophosphocholine was used as a carrier during TLC separation. The amount of radioactivity in the acetylated LPC fraction was determined by scintillation counting. The theoretical yield on the acetylation of LPC was calculated from one-half of the specific radioactivity of [^{3}H]acetic anhydride used in the assay.

Hydrolysis of the acetylated LPC by phospholipase C (C.welchii) was conducted as described by Renkonnen (167). In order to detect any radioactivity associated with the long-chain acyl groups, both the acyl and the acetyl groups of the acetylated LPC (acetylated by [^{3}H]acetic anhydride) were converted into methyl esters, as described by Choy (221). The labeled methyl acetate formed from the reaction was removed from the long-chain methyl esters by evaporation of the solvents at 50°C under a stream of nitrogen. The acyl composition of LPC was determined by the methyl esters of the fatty acyl groups with gas-

liquid chromatography.

vii) Scintillation counting

Radioactive lipids in chloroform-based solvents were placed into 20 ml glass scintillation vials and the solvents were evaporated under a stream of nitrogen. Aqueous radioactively-labeled compounds were also placed into glass scintillation vials, but were not evaporated to dryness. Aqueous counting scintillant (10 ml) and 0.2 ml of acetic acid were added to each vial. The contents of the vial were mixed vigorously and the vials were allowed to stand in the dark overnight, to remove chemiluminescence. Radioactivity in each vial was determined with an LKB Minibeta liquid scintillation counter. Radioactivity was calculated from cpm by the Channels Ratio Calibration method.

Radioactive lipid fractions on thin layer chromatographic plates were visualized with iodine, outlined lightly with pencil and then, the iodine was removed from the TLC plates by warming them at 100°C for 30 min. The radioactive lipid fractions were removed from the TLC plates and placed into glass scintillation vials. Distilled water (2 ml) was added to each vial and the vials were allowed to stand at room temperature for 10 min. Aqueous counting scintillant and acetic acid were added as described above and the vials were then sonicated for 20 min. After sonication, the vials were allowed to stand in the dark overnight prior to radioactivity determination.

viii) The uptake of [Methyl-^{3}H]choline by the isolated guinea pig heart

a) Time-course studies

Isolated guinea pig hearts were perfused in the Langendorff mode (208), as described in section a) i). After the stabilization period, the hearts were perfused with 12 ml each of Krebs-Henseleit buffer containing 0.5 µM [Methyl-^{3}H]choline chloride (10 µCi/nmol). This pulse of radioactivity was recirculated through the hearts for 15 to 120 min. Aliquots of the perfusate were taken before and after perfusion of each heart for determination of radioactivity. After perfusion, the hearts were homogenized and total lipid extracts were prepared, as previously described. Aliquots of the total heart homogenates and of the aqueous and organic phases of the total lipid extracts were obtained for the determination of radioactivity.

b) Analysis of the choline glycerophospholipids

The choline glycerophospholipids were isolated from the other lipids present in the total lipid extracts by thin layer chromatography, as described previously. The TLC plates were developed in a solvent mixture of chloroform/methanol/water/acetic acid (70:30:4:2; v/v) and the lipid fractions on the TLC plates were visualized with 2',7'-dichlorofluorescein. The choline glycerophospholipid fractions were removed from the TLC plates and the lipids were eluted from the silica gel, as previously described. The radioactivity in lysophosphatidylcholine was determined by scintillation counting. The other choline glycerophospholipids were further purified by thin layer chromatography with a solvent mixture of chloroform/methanol/water/ammo-

nium hydroxide (65:50:4:11; v/v). After elution from the silica gel, the choline glycerophospholipids from each heart were redissolved in 1 ml of chloroform/methanol (2:1; v/v).

These choline glycerophospholipid fractions were analyzed for lipid-phosphorus (217), alkenyl content (216) and the total amount of radioactivity incorporated, as previously described. Aliquots of this choline glycerophospholipid fraction were also subject to acid hydrolysis (214). After the hydrolysis, the products were isolated by thin layer chromatography as described previously. The diacylglycerophosphocholine and lysophosphatidylcholine (from plasmenylcholine) fractions from the TLC plates, were analyzed for lipid-phosphorus content (217) and the amount of radioactivity incorporated into each phospholipid was determined by liquid scintillation counting.

ix) Pulse-chase studies on the metabolism of choline

Isolated guinea pig hearts were perfused in the Langendorff mode (208) as described in section a) i). After stabilization, the hearts were perfused with 12 ml each of Krebs-Henseleit buffer containing 0.5 µM [Methyl-^{3}H]choline chloride (10 µCi/nmol). This pulse of radioactivity was recirculated through the heart for only 15 min, after which time the hearts were perfused, in a non-recirculating manner, with Krebs-Henseleit buffer containing 5.0 µM choline chloride for 0 to 120 min. After perfusion, the hearts were homogenized and total lipid extracts were prepared, as previously described. The choline glycerophospholipids were analyzed as outlined in the previous section.

x) Isolation and quantitation of aqueous choline-containing metabolites

The aqueous choline-containing metabolites were found in the upper (aqueous) phase of the total lipid extracts of perfused guinea pig hearts. Prior to analysis, each sample was thawed and an aliquot was taken to determine the total radioactivity in the sample. The metabolites choline, phosphocholine and CDP-choline were separated from these samples by thin layer chromatography, as described by Yavin (222). Aliquots of each sample were applied to the origin (1.5 cm from the bottom of the plate) of Sil-G25 thin layer chromatographic plates (Brinkman Instruments) via an air displacement pipette. Blanks (0.5-2.0 cm) were left on the sides of each plate. Samples were dried by warm forced air during sample application. A carrier mixture containing 50 mM CDP-choline, 100 mM phosphocholine and 100 mM choline was applied on top of each dry sample in the same manner. The carrier mixture aided the separation and visualization of these metabolites. The TLC plates were developed at room temperature in methanol/0.6% sodium chloride/ammonium hydroxide (50:50:5; v/v). After development, the TLC plates were allowed to air dry overnight. Ultraviolet light was used to visualize the CDP-choline fractions and then the TLC plates were exposed to iodine vapors to visualize the choline and phosphocholine fractions. The iodine was removed from the plates by placing them in a 100°C oven for 30 min. The choline, phosphocholine and CDP-choline fractions were removed from the TLC plates and placed into glass scintillation vials. These metabolites were analyzed for radioactivity by scintillation counting, as described previously.

xi) Recovery of radioactive compounds after perfusion

Isolated guinea pig hearts were perfused with a continuous, recirculating pulse of 0.5 µM [Methyl-^{3}H]choline in Krebs-Henseleit buffer for 30, 60 and 120 min. Aliquots of the perfusate were taken before and after the perfusion of each heart for radioactivity determination. Subsequent to perfusion, the hearts were homogenized and an aliquot of each homogenate was taken to determine the total radioactivity incorporated. Total lipid extracts were prepared from the homogenates and an aliquot from each extract was obtained for the determination of radioactivity. The lipid extracts were phase separated and aliquots of the upper (aqueous) and lower (organic) phases were obtained for scintillation counting.

The labeled metabolites present in the upper (aqueous) phases were isolated by thin layer chromatography with methanol/0.6% sodium chloride/ammonium hydroxide (50:50:5; v/v). The radioactivity present in the choline, phosphocholine and CDP-choline fractions was determined.

The labeled lipids present in the lower (organic) phases were isolated by thin layer chromatography. Three aliquots of each sample were separated by thin layer chromatography with a solvent mixture of chloroform/methanol/water/acetic acid (70:30:4:2; v/v) and after development, the lipid fractions were visualized by exposure of the TLC plates to iodine vapor. The first aliquot was used for the determination of radioactivity in lysophosphatidylcholine, phosphatidylcholine and sphingomyelin fractions. The second aliquot was used to determine the total radioactivity in the sample after TLC. The entire length of

the TLC plate (from origin to solvent front) was removed and the total radioactivity determined. The third aliquot was used to determine the radioactivity profile of the sample following plate development. Fractions (0.5 cm) of silica gel were removed from the TLC plate beginning 0.5cm below the origin and continuing on up to the top of the solvent front. Each 0.5 cm fraction was placed into a glass scintillation vial and the radioactivity was determined.

Since there was a significant amount of radioactivity located near the origin of the TLC plate, the identity of the labeled material(s) was further investigated. Specifically, two aliquots from each sample were separated by thin layer chromatography with a solvent mixture of chloroform/methanol/water/acetic acid (70:30:4:2; v/v). After visualization of the lipids fractions, the iodine was removed from the TLC plates. The silica gel from the origin of the first aliquot was removed from the TLC plates and the radioactivity was determined by scintillation counting. The silica gel from the origin of the second aliquot was removed from the TLC plates and the radioactive material was eluted from the silica gel by successive washes with methanol/water (1:1; v/v). The silica gel pellet was dried and then washed once with chloroform/methanol/water/acetic acid (50:39:10:1; v/v) and twice with chloroform/methanol (2:1; v/v). These last three washes (organic washes) were combined and the solvent was removed under nitrogen. The radioactivity eluted from the organic washes and also the radioactivity remaining in the washed silica gel pellet were determined by scintillation counting. The methanol/water washes were combined and the

solvent was removed by evaporation *in vacuo* at 45°C. The residues were resuspended in 500 µl of water and an aliquot was taken to determine the total radioactivity present. These samples were then analyzed by thin layer chromatography with methanol/0.6% sodium chloride/ammonium hydroxide (50:50:5; v/v). Choline, phosphocholine and CDP-choline fractions were visualized after development and removed from the TLC plates for radioactivity determination. The areas of silica gel below the choline fraction (including the origin) and above the CDP-choline fraction (up to the solvent front) were also removed and any radioactivity present in these fractions was determined.

xii) The uptake of [^{3}H]ethanolamine by the isolated guinea pig heart

a) Time-course studies

Isolated guinea pig hearts were perfused in the Langendorff mode (208) as described in section a) i). After the stabilization period, the hearts were perfused with 12 ml each of Krebs-Henseleit buffer containing 0.5 µM [1-^{3}H]ethanolamine hydrochloride (10 µCi/nmol). The pulse of radioactivity was recirculated through the hearts for 60 and 120 min. Aliquots of the perfusate were obtained before and after the perfusion of each heart for the determination of radioactivity. After perfusion, the hearts were homogenized and total lipid extracts were prepared as outlined previously. Aliquots of the total heart homogenates and of the organic phases of the total lipid extracts were obtained for the determination of radioactivity.

b) Analysis of the ethanolamine and choline phosphoglycerides

The choline and ethanolamine glycerophospholipids were isolated and purified from the other lipids present in the total lipid extracts by thin layer chromatography as described earlier. The choline and ethanolamine glycerophospholipid fractions were analyzed for lipid-phosphorus (217), alkenyl content (216) and the amount of radioactivity incorporated into each phosphoglyceride, as outlined in section viii) b).

xiii) Phosphocholinetransferase assay

Phosphocholinetransferase activities were measured by a modification of the method described by Arthur and Choy (223). 1-Alkenyl-2-acyl-glycerols were synthesized from plasmenylcholine by hydrolysis of the phosphocholine moiety with phospholipase C (*C.welchii*) according to the method of Renkonnen (167) as previously outlined. Diacylglycerols (pig liver) were obtained from Serdary Research Laboratories. For the assay, a working solution of the 1-alkenyl-2-acyl-glycerols or the diacylglycerols (2 µmol/ml) were prepared in a solution of Tween-20 (0.015%). The mixtures were sonicated for 15 min at 0°C and the solutions were used immediately for the assay of phosphocholinetransferase activities. Washed microsomes were prepared from guinea pig hearts as described before.

The reaction mixture contained: 100 mM tris-HCl (pH 8.5), 10 mM magnesium chloride, 5 mM EDTA, 0.4 mM [Methyl-^{14}C]CDP-choline (0.1 µCi/µmol), 1,2-diacylglycerol solution (0.4 µmol) or 1-alkenyl-2-acyl-glycerol solution (0.4 µmol) and microsomal preparation (250 µg pro-

tein) to a final volume of 1 ml. The reaction was initiated by the addition of labeled CDP-choline. The reaction mixture was incubated at 37°C for 30 min, in a shaking water bath. The reaction was terminated by the addition of 2 ml of chloroform/methanol (1:1; v/v). Chloroform (1 ml) and distilled water (0.5 ml) were added to the reaction mixture to cause phase separation. The upper phase was discarded and the lower phase was washed three times with an equal volume of 40% methanol in water. When the diacylglycerols were utilized as the substrate, the lower phase was transferred to a scintillation vial and the solvent was removed by evaporation under nitrogen. Radioactivity in this lower phase was determined by liquid scintillation counting. When the 1-alkenyl-2-acyl-glycerols were used, the lower phase was evaporated to dryness under nitrogen in test tubes. The residue was then hydrolyzed with acid, as stated before and the radioactivity incorporated into the plasmenylcholine fraction was determined by scintillation counting of the LPC formed after acid hydrolysis.

xiv) Determination of choline, phosphocholine and CDP-choline contents in guinea pig hearts

a) Isolation of choline, phosphocholine and CDP-choline pools

Two groups of guinea pig hearts were used for this study: a) control, unperfused hearts and b) hearts perfused with 0.5 µM choline for 120 min (as outlined previously). The wet weights of the guinea pig hearts, taken either directly from the animal or after perfusion, were determined and the hearts were homogenized as described previously. To each total homogenate was added chromatographically pure

[Methyl-^{3}H]choline, [^{14}C]phosphocholine and [Methyl-^{14}C]CDP-choline, in order to estimate recoveries. Total lipid extracts were prepared and phase separated with water as outlined before. The lower (organic) phases were washed twice with methanol/water (2:1; v/v) and then discarded. The upper (aqueous) phases and the above washes were combined and the solvent was removed by evaporation _in vacuo_ at 45°C. The sample was resuspended in 1 ml of water and choline-containing metabolites in the entire sample were separated by thin layer chromatography, with methanol/0.6% sodium chloride/ammonium hydroxide (50:50:5; v/v). After development and visualization, the choline, phosphocholine and CDP-choline fractions on the TLC plate were removed and placed into test tubes. These compounds were extracted from the silica gel by the addition of 5 ml of methanol/water (1:1; v/v) (pH 9.2). The tubes were mixed vigorously and allowed to stand for at least 10 min, to facilitate the extraction process. Thereafter, the tubes were centrifuged for 10 min at 2,000 rpm in a clinical centrifuge. The supernatants were removed and placed in round bottom flasks and the extraction of each silica gel pellet was repeated three more times with the same mixture. The solvent in the combined extracts in the round bottom flasks was reduced by evaporation _in vacuo_ at 45°C. Each of the choline, phosphocholine and CDP-choline fractions were resuspended in 2 ml of water (pH 9.2) and aliquots were taken for the determination of radioactivity subsequent to further purification by thin layer chromatography.

The following enzymes were used for the hydrolysis of the phospho-

choline and CDP-choline fractions. E.coli alkaline phosphatase (100 units in 2.5 M ammonium sulphate) was dialyzed overnight against 5 mM tris-HCl buffer (pH 9.2). C.adamanteus phosphodiesterase I (type II) (5 units) was resuspended in 1 ml of water (pH 9.2). The phosphocholine fractions in 5 mM tris-HCl (pH 9.2) were digested with 10 units of the dialyzed alkaline phosphatase for 2 h at 37°C. The CDP-choline fractions in 5 mM tris-HCl (pH 9.2) were digested first with 0.6 units of phosphodiesterase I for 1 h at 37°C. Subsequent to this incubation, 10 units of dialyzed alkaline phosphatase were added and the mixture was further digested for 2 h at 37°C. The digested phosphocholine and CDP-choline samples, as well as the choline samples were then lyophilized. After lyophilization, the residues were extracted three times with 1 ml of 70% ethanol. The samples, in 70% ethanol, were placed into 1.6 x 12.5 cm screw cap tubes and the solvent was evaporated under nitrogen.

The choline, digested phosphocholine and CDP-choline fractions were separately dissolved in 3 ml of water with vigorous mixing. To these fractions was added 2 ml of tetraphenylboron (10 mg/ml) in 4-heptanone and the mixture was shaken vigorously. The tubes were centrifuged for 10 min at 2,000 rpm to cause phase separation and the top phases were removed and placed into clean 1.6 x 12.5 cm screw cap tubes. This process was repeated with another 2 ml of tetraphenylboron in 4-heptanone. The choline was back extracted from tetraphenylboron by 2 ml of 0.4 N HCl. After shaking vigorously, the tubes were centrifuged to separate the phases. The bottom phases (0.4 N HCl) were removed and placed into clean 1.6 x 12.5 cm screw cap tubes and the

back extraction was repeated with another 2 ml of 0.4 N HCl. Finally, the tetraphenylboron was washed with 2 ml of 1 N HCl. The combined HCl solutions for each fraction were lyophilized and each sample was redissolved in 1 ml of 50 mM sodium phosphate buffer (pH 8.0). An aliquot from each sample was taken for radioactivity determination. The yield was calculated from the total radioactivity recovered in each pool.

b) Quantitation of pools

The amount of extracted choline was determined by the quantitative conversion of choline to [^{32}P]phosphocholine. The conversion was catalyzed by choline kinase in the presence of [γ-^{32}P]ATP, by a modification of the method by McCamon and Stetzler (224). Choline kinase was prepared from stock by dilution with water (5 units/10 ml) at 0°C and the enzyme solution was then placed into an Amicon Minicon-B15 clinical sample concentrator at 4°C. The enzyme was allowed to concentrate to 10X of the initial volume before removal from the concentrator. This procedure allowed the removal of the ammonium salt from the enzyme stock before use. Usually 2.5 units of choline kinase in 500 µl were recovered.

The reaction mixture contained 55 µl of sample, 20 µl of assay cocktail and 25 µl of choline kinase (.125 units). The assay cocktail contained 0.1 M magnesium chloride, 0.5 M sodium phosphate buffer (pH 8.0) and 30 mM [γ-^{32}P]ATP (10 µCi/µmol). The components of the reaction mixture were added sequentially in the above order to 10 x 75 mm

test tubes on ice and gently mixed. The tubes were capped with Parafilm and incubated for 1 h at 37°C in a shaking water bath. The reaction was terminated by placing the tubes in ice. Excess labeled ATP was removed by the addition of 100 µl of 0.2 M 'ice cold' barium acetate. The tubes were gently mixed and allowed to stand on ice for 30 min. After this, the tubes were centrifuged at 4,000 rpm for 20 min at 0°C (Sorvall RC-5 Superspeed Refrigerated Centrifuge with SS34 rotor). Supernatants (150 µl) were removed to clean 10 x 75 mm test tubes on ice and the pellets were discarded. To the supernatants were added 6 µl of 30 mM ATP and 25 µl of 0.2 M 'ice cold' barium acetate. After gently mixing and standing on ice for 20 min, the tubes were centrifuged as above. Supernatants were removed to clean 10 x 75 mm test tubes and the pellets were discarded. An aliquot (100 µl) of each supernatant was applied to a 1.5 cm charcoal/celite column (1:1; w/w) and the [^{32}P]phosphocholine was eluted with 2 ml of water. The eluants were collected in glass scintillation vials and the radioactivity was determined by scintillation counting. A standard curve was established and the choline concentration from each original choline, phosphocholine and CDP-choline fraction was calculated from the curve. Samples containing an internal standard of 5 nmol of choline were used to check the complete conversion of choline to phosphocholine.

Experimental Results

I. Plasmenylcholine Biosynthesis

a) Incorporation of Choline into Plasmenylcholine

The formation of plasmenylcholine by both base-exchange and the CDP-choline pathway inevitably requires choline. Thus, the initial approach to elucidate the biosynthesis of this ether lipid was to determine if it could be labeled with radioactive choline. Perfusion of the isolated guinea pig heart with 0.5 µM [Methyl-^{3}H]choline (10 µCi/nmol) from 15 to 120 min was performed as described in "Materials and Methods". After perfusion, the labeled metabolites in the aqueous and organic phases of the heart homogenate were analyzed.

The incorporation of labeled choline into the total choline glycerophospholipid fraction (contains: 1,2-diacyl-sn-glycerophosphocholine and plasmenylcholine) of the guinea pig heart, is shown in Fig.16. There was a distinct lag phase in the incorporation of label during the first 45 min, after which time, the incorporation of labeled choline became linear. The incorporation of labeled choline into 1,2-diacylglycerophosphocholine was determined and also displayed an initial lag phase (Fig.17). Incorporation of labeled choline into this lipid became linear after 30 min. Labeled choline was also incorporated into plasmenylcholine (Fig.18). An initial lag phase was observed for the first 40 min, thereafter the incorporation of labeled choline into plasmenylcholine proceeded linearly.

The observation of a distinct lag in the incorporation of labeled

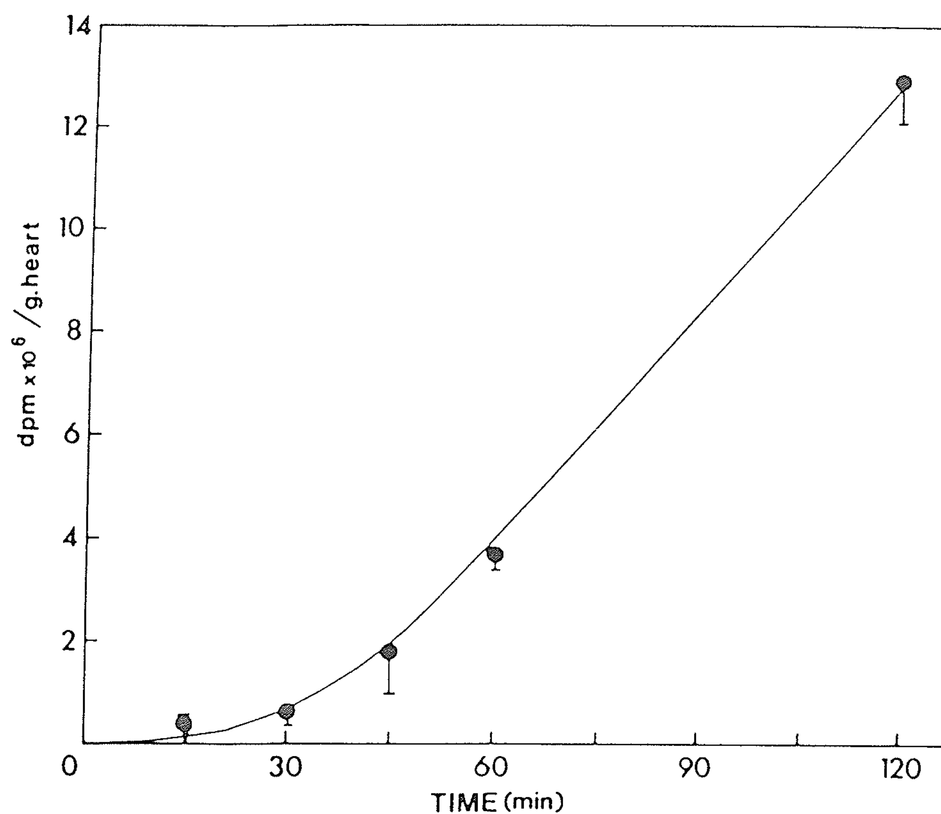


Fig. 16. Time course for the incorporation of [Methyl-^{3}H]choline into the total choline phosphoglyceride fraction.

Isolated guinea pig hearts were perfused with 0.5 μM [Methyl-^{3}H]choline in Krebs-Henseleit buffer from 15 to 120 min. After perfusion, hearts were homogenized in chloroform/methanol (1:1; v/v). Chloroform and water were added to obtain chloroform/methanol/water (4:2:2; v/v). The organic (chloroform) phase was analyzed by thin layer chromatography for radioactivity in the choline phosphoglyceride fraction. Each point represents the mean of at least three separate experiments. The vertical bars are standard errors of the mean (SEM).

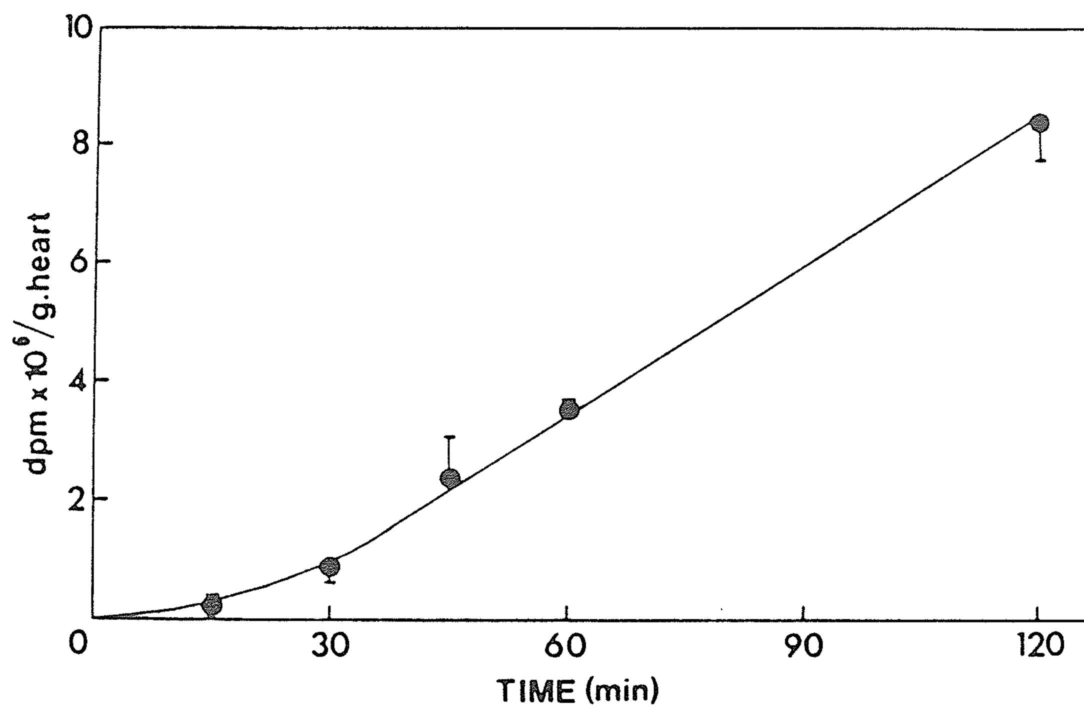


Fig. 17. Time course for the incorporation of [Methyl-^{3}H]choline into 1,2-diacylglycerophosphocholine.

The total choline phosphoglyceride fraction from Fig.16. was subject to acid hydrolysis and then analyzed by thin layer chromatography for the radioactivity in 1,2-diacylglycerophosphocholine. Each point represents the mean of at least three separate experiments. The vertical bars indicate SEM.

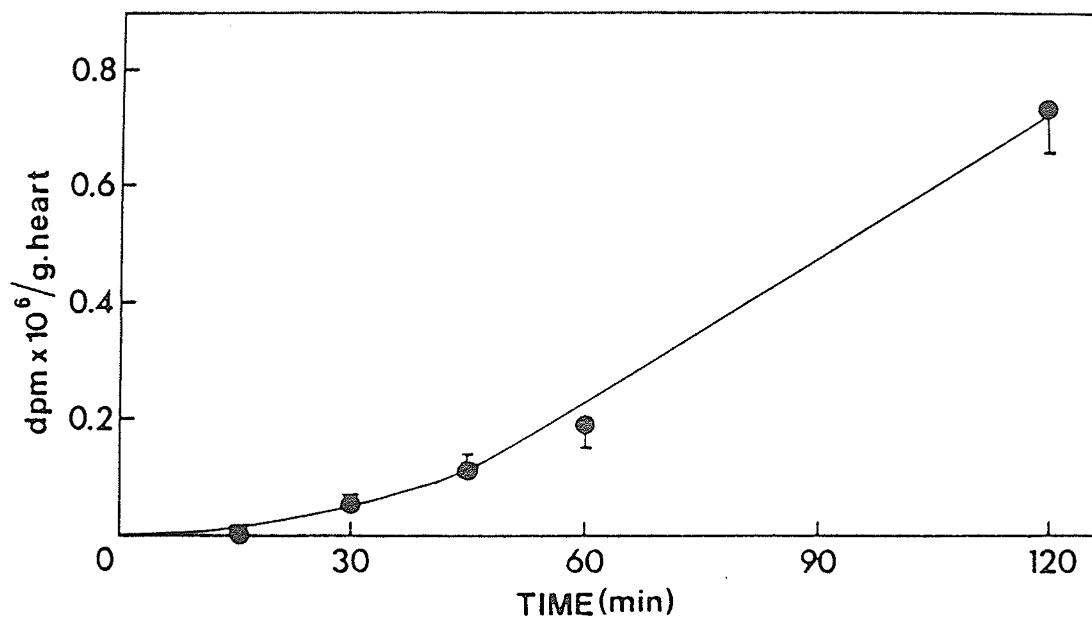


Fig. 18. Time course for the incorporation of [Methyl-^{3}H]choline into plasmenylcholine.

The radioactivity in plasmenylcholine was determined after isolation by thin layer chromatography from the choline phosphoglyceride acid hydrolysate (Fig.17). Each point represents the mean of at least three separate experiments. The vertical bars represent SEM.

choline into diacylglycerophosphocholine and plasmenylcholine indicates that the majority of the labeled choline glycerophospholipids in the guinea pig heart are not formed by the base-exchange reaction (13). Thus, the CDP-choline pathway appears to be the major pathway for the incorporation of choline into plasmenylcholine.

The time course for the incorporation of [Methyl-^{3}H]choline into the major aqueous choline-containing metabolites of the CDP-choline pathway (choline, phosphocholine and CDP-choline) is depicted in Fig.19. Radioactivity in choline is observed within 15 min of perfusion and declines slightly over the remaining perfusion period. The incorporation of labeled choline into phosphocholine and CDP-choline reach maxima at 60 and 90 min, respectively and then remain constant.

b) Recovery of radioactive compounds after perfusion

During these studies, attempts were made to ensure that all possible choline-containing compounds were accounted for. Isolated guinea pig hearts were perfused for 30, 60 and 120 min with 0.5 µM [Methyl-^{3}H]choline. Subsequent to perfusion, the hearts were homogenized and the aqueous and organic phases were separated as described in "Materials and Methods".

The radioactivity present in the organic phase was analyzed by thin layer chromatography (Fig.20). Radioactivity was determined by the analysis of 0.5 cm fractions of all chromatograms. The majority of the radioactivity, for all time points, was localized in the choline glycerophospholipid fraction. Small amounts of radioactivity were

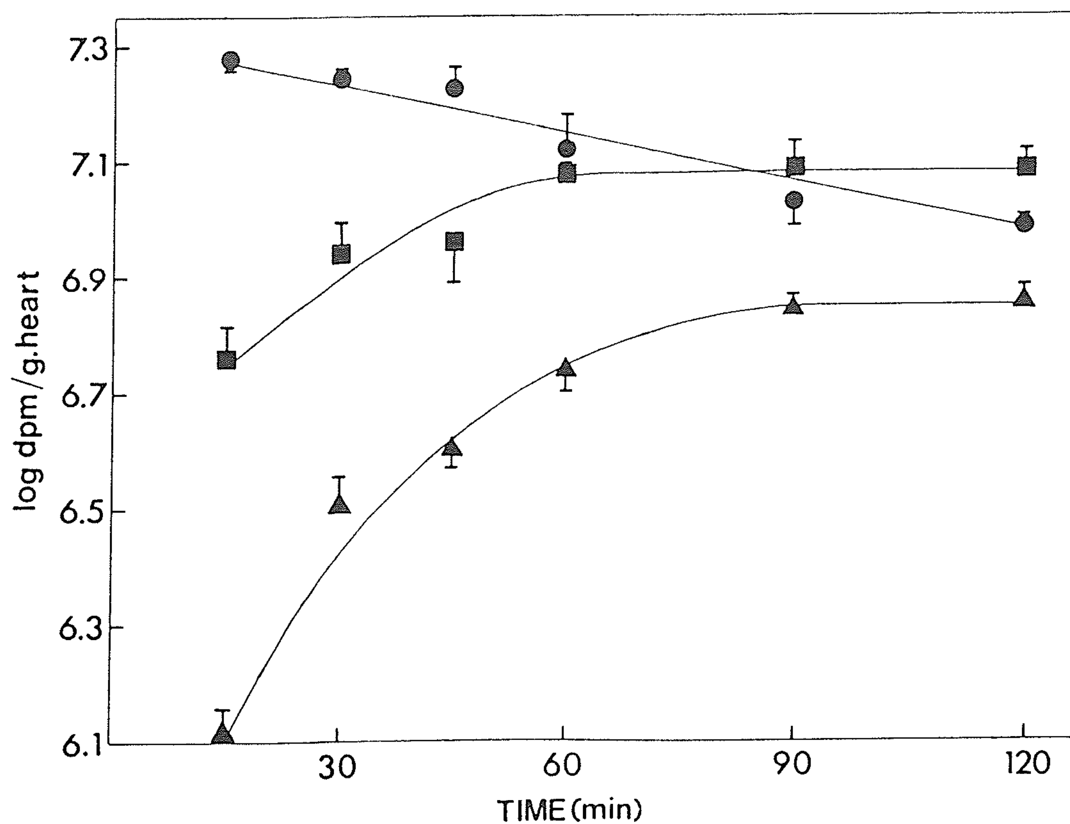


Fig. 19. Time course for the incorporation of [Methyl-^{3}H]choline into major aqueous choline-containing metabolites.

The aqueous (water and methanol) phase from Fig.16 was analyzed by thin layer chromatography for radioactivity in choline (●—●); phosphocholine (■—■) and CDP-choline (▲—▲). Each point represents the mean of at least three separate experiments. The vertical bars represent SEM.

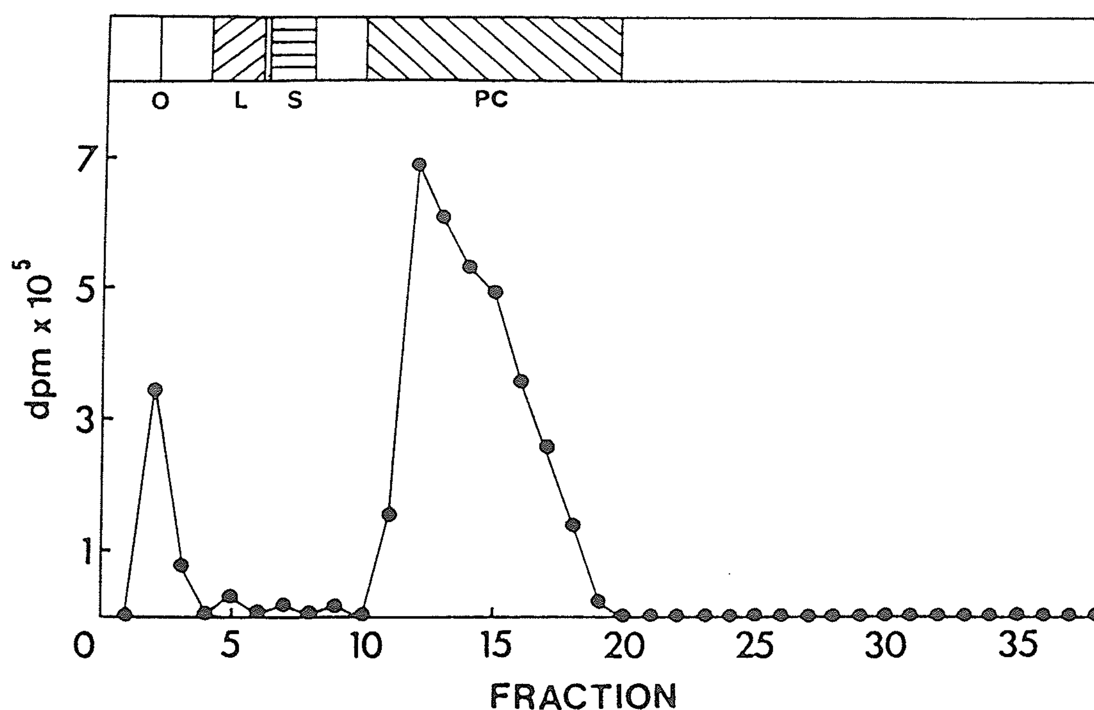


Fig. 20. Recovery of radioactivity in different lipid fractions in the organic phase after extraction.

Isolated guinea pig hearts were perfused with [Methyl-^{3}H] choline in Krebs-Henseleit buffer for 60 min. After perfusion, hearts were homogenized in chloroform/methanol (1:1; v/v). Aqueous and organic phases were separated upon the addition of chloroform and water to obtain chloroform/ methanol/water (4:2:2; v/v). The organic phase was analyzed by thin layer chromatography for labeled metabolites. Radioactivity on the thin layer chromatogram was localized at the origin (O), in the lysophosphatidylcholine fraction (L), the sphingomyelin fraction (S) and in the total choline phosphoglyceride fraction (PC). The radioactivity on the thin layer chromatogram was determined by analysis of 0.5 cm fractions of the chromatogram, beginning 0.5 cm below the origin and on up to the top of the chromatogram.

associated with lysophosphatidylcholine and sphingomyelin. The radioactivity localized at the origin of the chromatogram was re-analyzed by thin layer chromatography. This fraction was found to contain only choline, phosphocholine and CDP-choline. It was concluded that the radioactivity at the origin resulted from the contamination of the organic phase with the aqueous phase, during phase separation.

The radioactivity present in the aqueous phase was also analyzed by thin layer chromatography (Table 14). The radioactivity in the choline, phosphocholine and CDP-choline fractions were determined. Percentage recoveries indicate that these three compounds account for 99% of the radioactivity present in this phase. No significant amount of radioactivity was found in the glycerolphosphocholine fraction.

c) Phosphocholinetransferase Activities

The CDP-choline : 1,2-diacylglycerol phosphocholinetransferase activity of guinea pig heart microsomes was determined (Table 15) as described in "Materials and Methods". The existence of CDP-choline : 1-alkenyl-2-acyl-glycerol phosphocholinetransferase activity was also demonstrated (Table 15) and was found to be located in the microsomal fraction. In addition, 1-alkenyl-2-acyl-glycerol was also shown to be present in the guinea pig heart (17 nmol/g heart). It appears that guinea pig heart microsomes have the ability to condense 1-alkenyl-2-acyl-glycerol with CDP-choline.

d) Plasmenylcholine Biosynthesis from Ethanolamine

TABLE 14

Recovery of radioactivity in the aqueous phase after extraction.

Guinea pig hearts were perfused with [Methyl-^{3}H]choline in Krebs-Henseleit buffer for 30 min. After perfusion, hearts were homogenized in chloroform/methanol (1:1; v/v). Aqueous and organic phases were separated upon the addition of water, to obtain chloroform/methanol/water (4:2:2; v/v). The aqueous phase was analyzed by thin layer chromatography for labeled metabolites, as described in "Materials and Methods".

	dpm x 10 /g heart	%
Total radioactivity	12.52	100
Choline fraction	5.79	46
Phosphocholine fraction	4.61	37
CDP-choline fraction	1.94	16

TABLE 15

The activities of phosphocholinetransferase enzyme(s) in guinea pig heart microsomes.

Enzyme activities were assayed under optimal conditions as described in "Materials and Methods". Each value represents the mean of two separate experiments.

Enzyme	Activity
	nmol/h/mg protein
CDP-choline : 1,2-diacylglycerol phosphocholinetransferase	26.453
CDP-choline : 1-alkenyl-2-acylglycerol phosphocholinetransferase	5.215

Formation of plasmenylcholine by the methylation of phosphatidylethanolamine or plasmenylethanolamine was studied in the guinea pig heart. Isolated hearts were perfused with 0.5 µM [1-^{3}H]ethanolamine (10 µCi/nmol) for 60 and 120 min. The radioactivity incorporated into the total ethanolamine glycerophospholipid and the total choline glycerophospholipid fractions were determined (Table 16). Plasmenylethanolamine and plasmenylcholine were then isolated from their respective total fractions by acid hydrolysis as described in "Materials and Methods". Significant amounts of labeled ethanolamine were incorporated into plasmenylethanolamine. No significant labeling of plasmenylcholine from labeled ethanolamine was observed.

e) Rate-limiting Steps for Plasmenylcholine Biosynthesis

Results for the incorporation of choline into plasmenylcholine indicate that the major pathway involved is the CDP-choline pathway. This pathway was studied in detail by pulse-chase experiments to determine the rate-limiting steps for the biosynthesis of plasmenylcholine. Isolated guinea pig hearts were pulse labeled (via perfusion) for 15 min with 0.5 µM [Methyl-^{3}H]choline (10 µCi/nmol) and then chased with 5.0 µM nonradioactive choline for 0 to 120 min. After perfusion, the labeled metabolites in the aqueous and organic phases of the heart homogenate were analyzed.

Total uptake of radioactivity by the guinea pig heart was at the maximum immediately after the 15 min pulse period (0 min point of the chase period) (Fig.21). Total radioactivity in the heart declined over the chase period and remained constant after 40 min of chase. Linear

TABLE 16

The incorporation of [1-^{3}H]ethanolamine into choline glycerophospholipids.

Guinea pig hearts were perfused with [1-^{3}H]ethanolamine for 60 and 120 min. Radioactivity was determined for the total ethanolamine glycerophospholipid and choline glycerophospholipid fractions before acid hydrolysis to isolate plasmenylethanolamine and plasmenylcholine. Each value represents the mean of at least two separate experiments.

	Perfusion for 60 min	Perfusion for 120 min
	dpm/g heart	
Total ethanolamine phosphoglyceride	1,206,733.	3,695,350.
Plasmenylethanolamine	242,659.	654,594.
Total choline phosphoglyceride	24,094.	50,645.
Plasmenylcholine	776.	1,735.

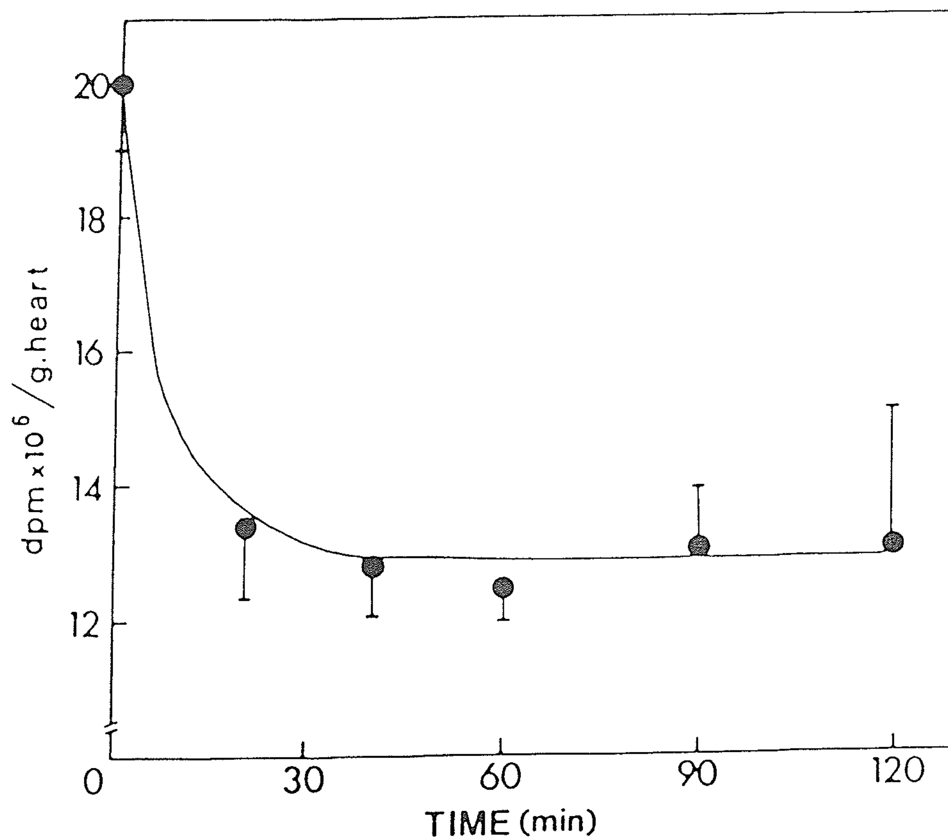


Fig. 21. Pulse-chase study on the total [Methyl-^{3}H]choline taken up by isolated guinea pig hearts.

Isolated guinea pig hearts were pulsed for 15 min with 0.5 µM [Methyl-^{3}H]choline and then chased with 5.0 µM choline for 0 to 120 min. The radioactivity taken up by the isolated hearts were calculated from the total radioactivities of the organic and aqueous phases, after homogenization. Each point represents the mean of at least three separate experiments. The vertical bars indicate SEM.

increases in the incorporation of radioactivity into choline glycerophospholipids (Fig.22), 1,2-diacylglycerophosphocholine (Fig.23) and plasmenylcholine (Fig.24) were observed over the entire chase period.

Maximum labeling of choline occured immediately after pulse labeling. The radioactivity in choline then decreased over the chase period and remained constant after 60 min of chase (Fig.25). The labeling of phosphocholine reached a maximum after 20 min of chase and then declined linearly over the remaining chase period. The radioactivity in CDP-choline attained maximum after 60 min of chase and then remained constant. These results indicate that there are at least two rate-limiting steps in the CDP-choline pathway for the guinea pig heart. The initial rate-limiting step is between choline and phosphocholine which can be seen from 0 to 20 min of the chase period. The other rate-determining step can be identified at the conversion of phosphocholine to CDP-choline, from 30 to 120 min of the chase period.

f) Pool Sizes of Aqueous Precursors for Plasmenylcholine

The specific radioactivity of the choline-containing metabolites may change if a change in the pool size of any of these precursors occurs during perfusion. This may in turn affect the rate of the incorporation of radioactivity into plasmenylcholine. Thus, the pool size of choline, phosphocholine and CDP-choline, in the guinea pig heart, were determined before and after perfusion with 0.5 μM choline. No significant ($p<0.05$) changes in the pool size of these three choline-containing metabolites were observed in the guinea pig heart

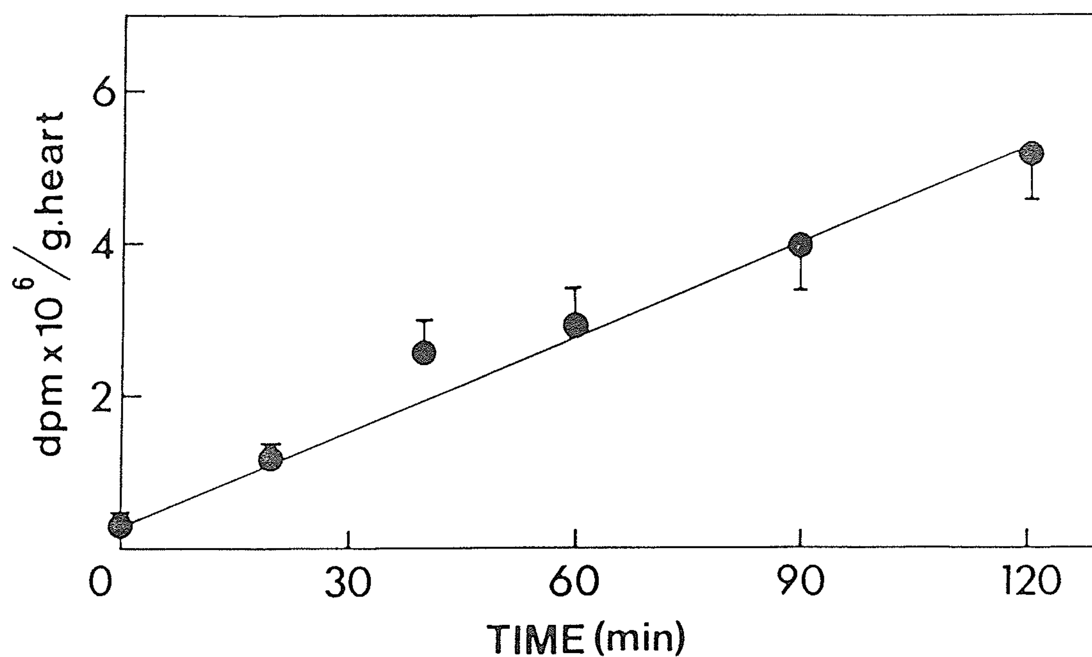


Fig. 22. Pulse-chase study on the incorporation of [Methyl-^{3}H] choline into the total choline phosphoglyceride fraction.

The organic phase from Fig.21 was analyzed by thin layer chromatography for radioactivity in the total choline phosphoglyceride fraction. Each point represents the mean of at least three separate experiments. The vertical bars represent SEM.

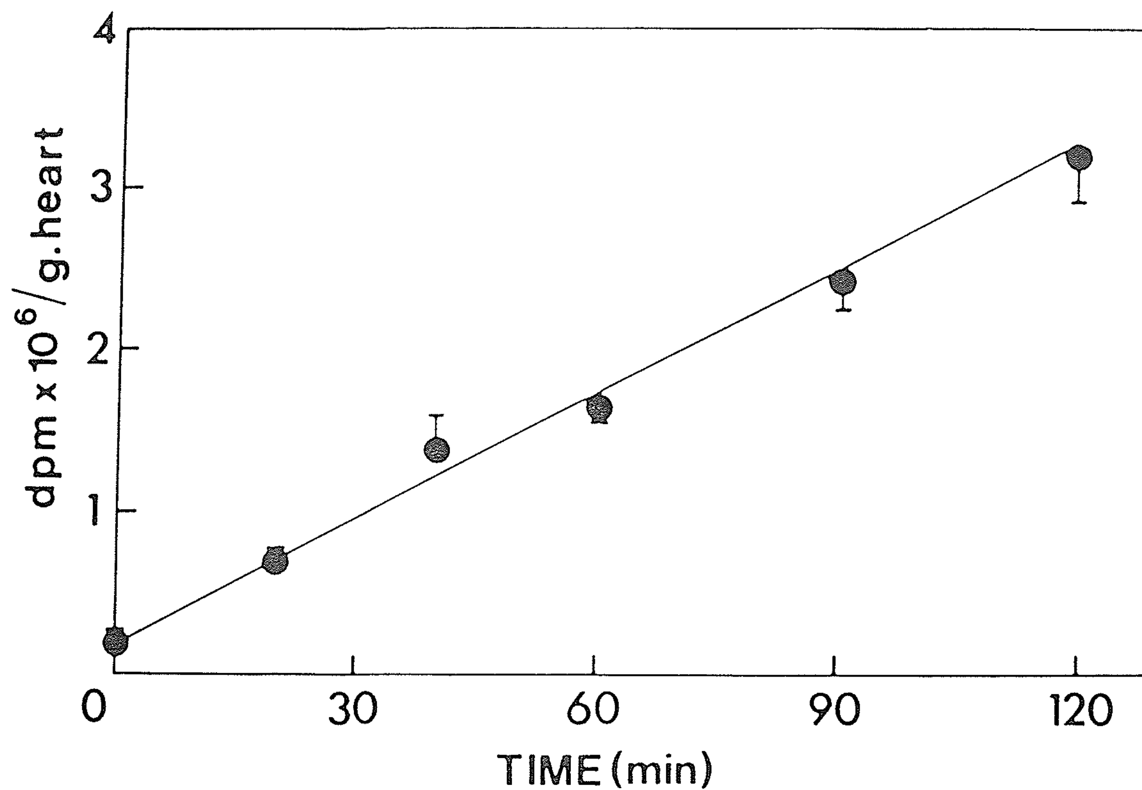


Fig. 23. Pulse-chase study on the incorporation of [Methyl-^{3}H]choline into 1,2-diacylglycerophosphocholine.

The total choline phosphoglyceride fraction from Fig.22 was subject to acid hydrolysis and then analyzed by thin layer chromatography for radioactivity in 1,2-diacylglycerophosphocholine. Each point represents the mean of at least three separate experiments. The vertical bars represent SEM.

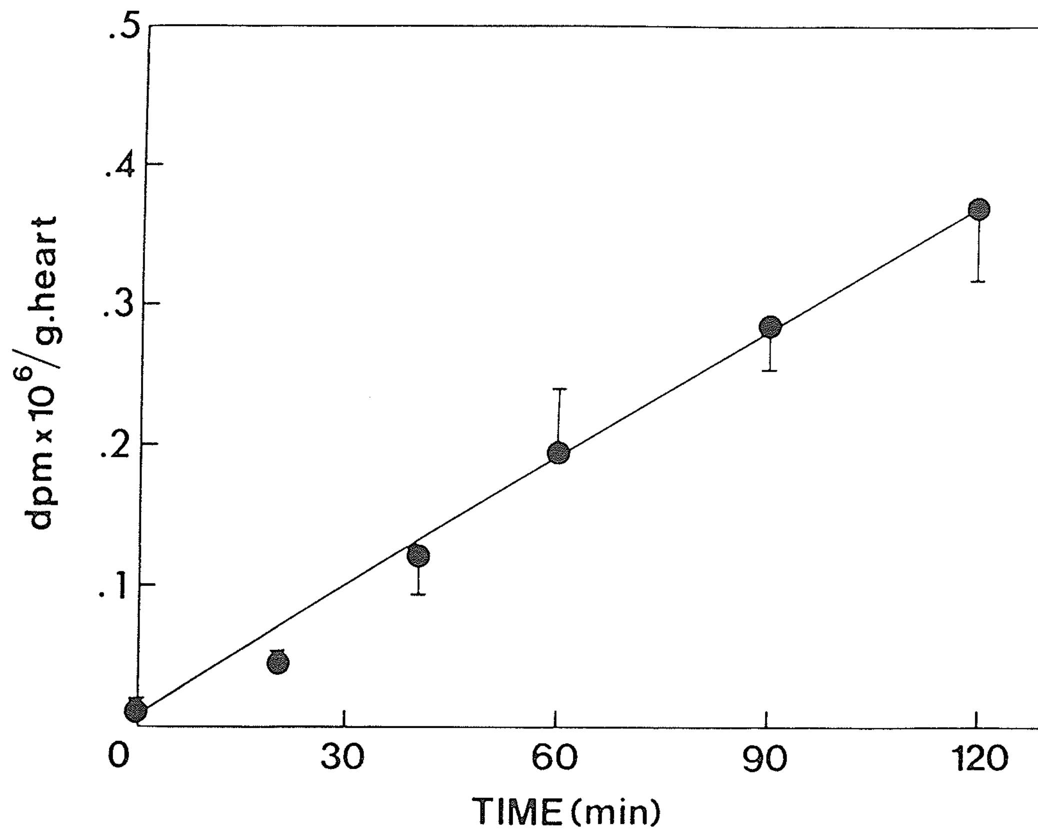


Fig. 24. Pulse-chase study on the incorporation of [Methyl-^{3}H] choline into plasmenylcholine.

The radioactivity in plasmenylcholine was determined after isolation by thinlayer chromatography from the acid hydrolysate of the total choline phosphoglyceride fraction (Fig.23). Each point represents the mean of at least three separate experiments. The vertical bars indicate SEM.

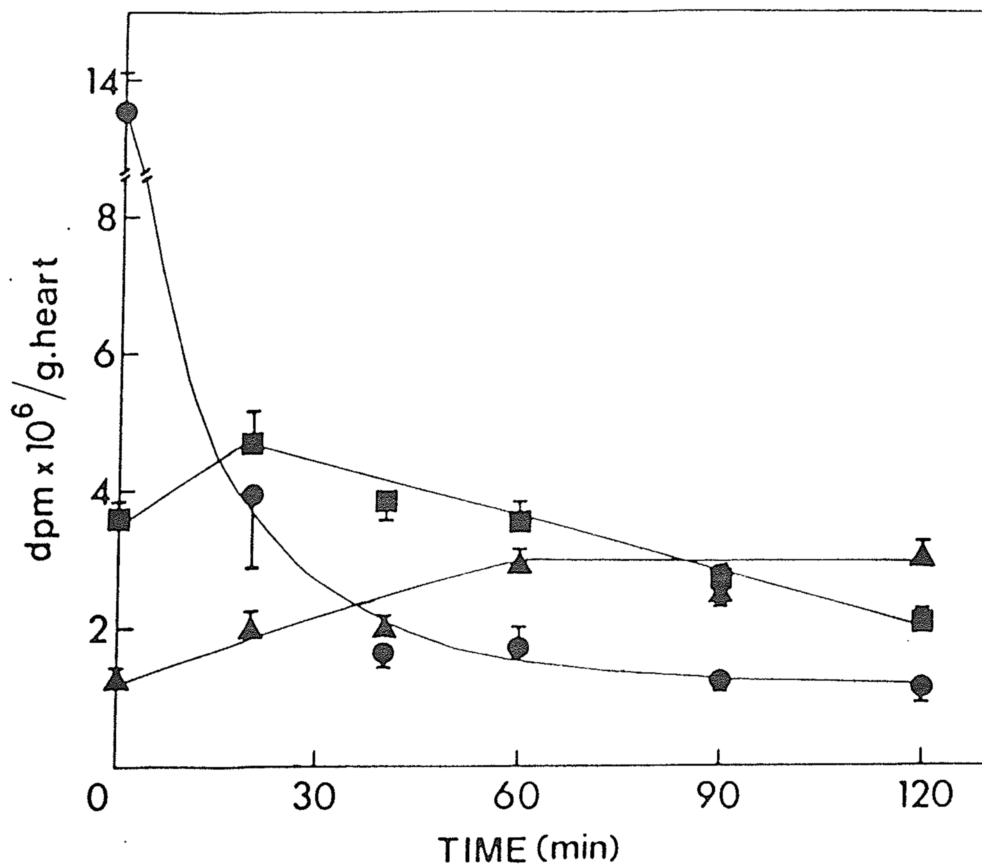


Fig. 25. Pulse-chase study on the incorporation of [Methyl-^{3}H] choline into the major aqueous choline-containing metabolites.

The aqueous phase from Fig.21 was analyzed by thin layer chromatography for radioactivity in choline (●—●); phosphocholine (■—■) and CDP-choline (▲—▲). Each point represents the mean of at least three separate experiments. The vertical bars represent SEM.

before and after perfusions (Table 17).

From the average pool size and specific radioactivity of CDP-choline at 90 to 120 min of perfusion, the rate of plasmenylcholine formation in the guinea pig heart via the CDP-choline pathway was estimated to be 0.183 nmol/min/g heart. The rate of formation of 1,2-diacylglycerophosphocholine was calculated to be 2.69 nmol/min/g heart.

II. Cardiac Plasmalogenase Activity

a) Alkenyl Phosphoglyceride Content of the Hamster Heart

Ethanolamine and choline glycerophospholipid fractions from hamster heart lipid extracts were analyzed for their alkenyl content and the results are given in Table 18. Plasmenylcholine accounted for 2% of the choline glycerophospholipids and plasmenylethanolamine for 8.5% of the ethanolamine glycerophospholipids. Since a higher alkenyl content was present in the ethanolamine glycerophospholipids, plasmenylethanolamine was used as the substrate for the investigation of plasmalogenase activity in the hamster heart.

b) Quantitation of Plasmalogenase Activity

Initially, plasmalogenase activity was determined by measuring the disappearance of substrate with time (0-60 min) using microsomal, mitochondrial and cytosolic fractions. Hamster heart plasmalogenase activity was located exclusively in the microsomal fraction and enzyme activity was found to be linear for the first 11 min. Enzyme activity

TABLE 17

Concentration of choline, phosphocholine and CDP-choline in guinea pig heart.

The pool sizes of the aqueous choline-containing metabolites in guinea pig hearts before and after 120 min of perfusion were measured as described in "Materials and Methods".

	Before perfusion	After perfusion for 120 min
	nmol/g heart	
Choline	341[a] ± 81[b] (3)[c]	363 ± 156 (3)
Phosphocholine	356 ± 49 (4)	415 ± 115 (8)
CDP-choline	87 ± 22 (4)	66 ± 5 (5)

[a] Mean
[b] Standard error of the mean
[c] Number of experiments

TABLE 18

Alkenyl content of choline and ethanolamine glycerophospholipid fractions from the hamster heart.

The choline and ethanolamine glycerophospholipids from hamster heart total lipid extract were separated by thin layer chromatgraphy. Total lipid-phosphorus and alkenyl content in each fraction was determined, as described in "Materials and Methods".

	Choline glycerophospholipids	Ethanolamine glycerophospholipids
	umol/g heart	
Total lipid-P	12.9[a] ± 0.40[b] (4)[c]	10.7 ± 0.25 (4)
Total alkenyl lipid	0.285 ± 0.08 (3)	0.935 ± 0.115 (3)
% alkenyl lipid	2.21 ± 0.54	8.74 ± 0.69

[a]Mean
[b]Standard error of the mean
[c]Number of experiments

was detected with a minimum of 25 µg of microsomal protein and a specific activity of 83.6 nmol plasmenylcholine hydrolyzed /min /mg protein was obtained with 40 µg of microsomal protein.

The determination of plasmalogenase activity by measuring the rate of disappearance of plasmenylethanolamine required a substantial amount of substrate and was also time consuming. To overcome these limitations, an assay was developed in which the long-chain aldehydes produced by the plasmalogenase activity were converted to fatty acids by the action of aldehyde dehydrogenase. In this assay, 1 mol of NADH would be produced per 1 mol of aldehyde formed, when the plasmenylethanolamine was oxidized. Hence, plasmalogenase activity could be monitored in this coupled reaction by the change in absorbance with time at 340 nm. To ensure that the reaction catalyzed by aldehyde dehydrogenase was not rate limiting, the dehydrogenase activity towards long-chain aldehydes was investigated. Palmitaldehyde was found to be a poor substrate for aldehyde dehydrogenase and the reaction proceeded at 8.5% of the rate when acetaldehyde was used. However, the slow rate of the reaction with palmitaldehyde as substrate could be enhanced in a linear fashion by the addition of more enzyme. In order to utilize this assay to study the pH profile of plasmalogenase in the heart, the effects of pH on aldehyde dehydrogenase were determined. Aldehyde dehydrogenase activity was greatly diminished between pH 6.5 and 7.5, but adequate enzyme activity at pH 6.5-7.5 for the coupled reaction could be obtained with the addition of more enzyme. Therefore, in the study of the pH profile of the cardiac plasmalogenase, the amount of aldehyde dehydrogenase used in each assay was adjusted

to at least 10-fold higher than the plasmalogenase activity and therefore, the reaction catalyzed by aldehyde dehydrogenase would not be rate limiting in the production of NADH. The effect of Ca^{2+} and Mg^{2+} on the dehydrogenase activity was also investigated. The two cations had very little effect on the activity of aldehyde dehydrogenase at the concentrations tested (0-16 mM).

Since aldehyde dehydrogenase requires glutathione and potassium chloride for maximum enzyme activity, the effects of these compounds on the cardiac plasmalogenase activity were also investigated. Plasmalogenase activity in the microsomal fraction was assayed by the disappearance of plasmenylethanolamine in the presence of potassium chloride and glutathione. The activities obtained in the presence and absence of these compounds were identical, which indicates that these compounds did not affect plasmalogenase activity in the assay.

To demonstrate the specificity of the coupled reaction for plasmalogenase activity, a number of controls were established (Table 19). The presence of microsomes and plasmenylethanolamine in the assay were essential for the expression of any activity. Substitution of plasmenylethanolamine with synthetic or natural 1,2-diacylglycerophosphoethanolamine, lysophosphatidylethanolamine and lysoplasmenylethanolamine did not elicit any activity. The omission of NAD^+ or aldehyde dehydrogenase from the assay resulted in an increase in absorbance with time at 340 nm. It was discovered that the reaction mixture was becoming turbid in the absence of NAD^+ or aldehyde dehydrogenase. Such an increase in absorbance with time was also observed when the reac-

TABLE 19

Specificity of the spectrophotometric procedure for the determination of plasmalogenase activity in the hamster heart microsomal fraction.

The specificity of the spectrophotometric assay for plasmalogenase was analyzed by the control experiments as indicated in the table. The concentration of each component was described in "Materials and Methods". The final concentration of each lipid component used in the reaction was 347 µM and a microsomal preparation containing 8 ug of protein was used in each assay. The final volume of the reaction mixture was 1.5 ml. The absorbance at 340 nm was calculated from the mean of two separate assays. The presence of a component in the assay is indicated by + , whereas the omission of a component in the assay is indicated by –. N.D. indicated that no change in absorbance was detected after 10 min of assay.

Assay component	Presence or absence								
Tris-HCl buffer + NAD^+	+	+	+	+	+	+	+	+	+
Microsomal preparation	+	–	+	+	+	+	+	–	+
Aldehyde dehydrogenase	+	+	+	+	+	+	+	+	–
Ethanolamine plasmalogens	+	+	–	–	–	–	–	–	–
Ethanolamine lysoplasmalogens	–	–	–	+	–	–	–	–	–
Dioleoylphosphatidylethanolamine	–	–	–	–	+	–	–	–	–
Phosphatidylethanolamine	–	–	–	–	–	+	–	–	–
Mixed fatty acids	–	–	–	–	–	–	+	–	–
Absorbance at 340 nm/min	0.035	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.

tion was monitored at 500-700 nm. Since the absorbance of NADH is minimal at 500-700 nm, we concluded that such absorbance was caused by the turbidity of the reaction. In the presence of NAD^+ or aldehyde dehydrogenase, no increase in absorbance was observed at 500-700 nm. These results suggest that when the long-chain aldehydes released from the plasmenylethanolamine were not converted to fatty acids, their continued accumulation would exceed their solubility in the reaction mixture, which would result in the formation of a turbid solution.

c) Characterization of Hamster Heart Plasmalogenase Activity

The hamster heart microsomal plasmalogenase was characterized using the spectrophotometric assay developed. The sensitivity of this method enabled the detection of enzyme activity when as little as 1 µg of microsomal protein was used in the assay at pH 8.5. When 1 unit of aldehyde dehydrogenase was used for the coupled reaction, the assay of microsomal plasmalogenase activity was linear to 8 µg microsomal protein. The imposition of this upper limit was due to the maximum ability of the aldehyde dehydrogenase employed in the assay to convert all the aldehyde produced to acid under the assay conditions. Any higher amount of microsomal proteins used without a corresponding increase in the amount of aldehyde dehydrogenase caused an accumulation of aldehydes leading to problems with turbidity. Under the conditions of the assay described, a reaction rate of 0.035 absorbance units/min was the limit beyond which turbidity interfered with A_{340} measurements.

The time-course and substrate dependence of the reaction were studied using the above spectrophotometric assay. The reaction was linear up to 12 min and maximum velocity was obtained with 300 µM of plasmenylethanolamine (Fig.26). The enzyme did not show an absolute requirement for divalent metal ions and enzyme activity was slightly enhanced by Mg^{2+}. The enzyme was activated by 3-10 mM Ca^{2+}, but higher concentrations of Ca^{2+} completely inhibited the enzyme activity (Fig.27). The enzyme was active a broad pH range, with maximum activity at pH 8.5 (Fig. 28).

The subcellular distribution of plasmenylethanolamine plasmalogenase activity was also re-examined using the spectrophotometric assay method. Activity was measured at pH 8.5 and 7.4. At pH 8.5, the majority of the enzyme activity was detected in the microsomal fraction (102 ± 6 nmol NADH formed/min/mg protein) and only a small amount of activity was found in the cytosolic fraction (0.78 nmol NADH formed/min/mg protein). At pH 7.4, enzyme activity was only detected in the microsomal fraction (95 ± 4.5 nmol NADH formed/min/mg protein). These results are essentially in agreement with the data obtained using the assay method in which the disappearance of substrate was monitored (84 ± 3.5 nmol/min/mg protein). The results confirm that plasmalogenase activity is essentially microsomal.

d) Cardiac Plasmalogenase Activity and Plasmenylethanolamine Content in Different Species

Cardiac plasmalogenase activity towards plasmenylethanolamine was determined in several mammalian species, by the spectrophotometric

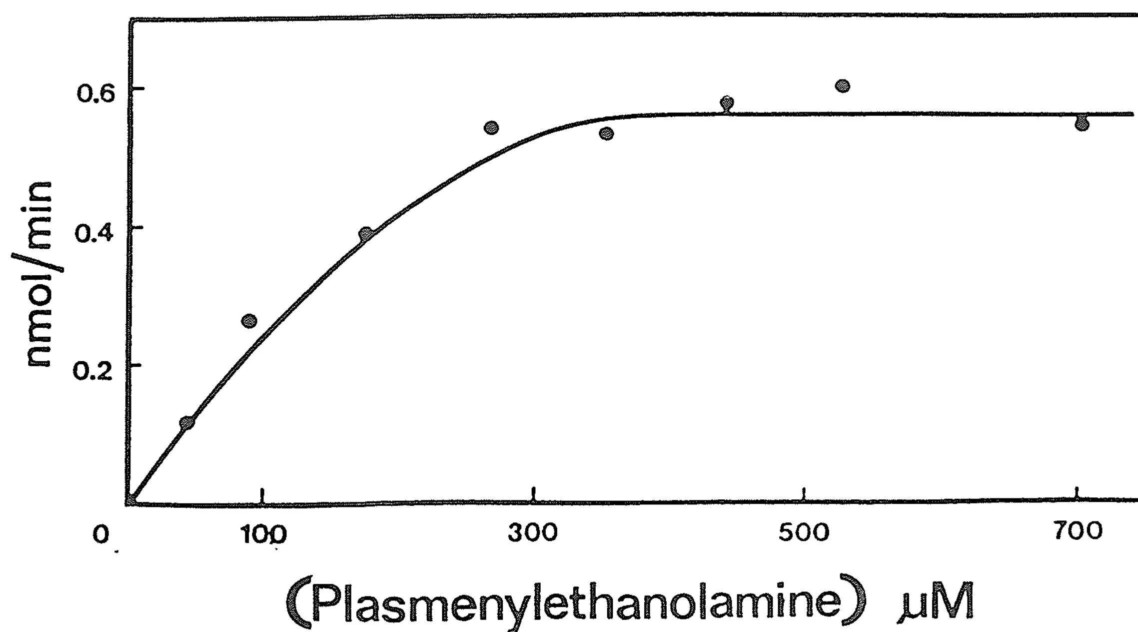


Fig. 26. The effect of substrate concentration on hamster heart microsomal plasmalogenase activity.

Hamster heart microsomal plasmalogenase was assayed at pH 8.5 with the spectrophotometric method as described in text. Each assay contained 5 µg of microsomal protein in the presence of 50-700 µM of plasmenylethanolamine. The formation of NADH was monitored at A_{340} over a 10 min period. Each point represents the mean of two separate experiments.

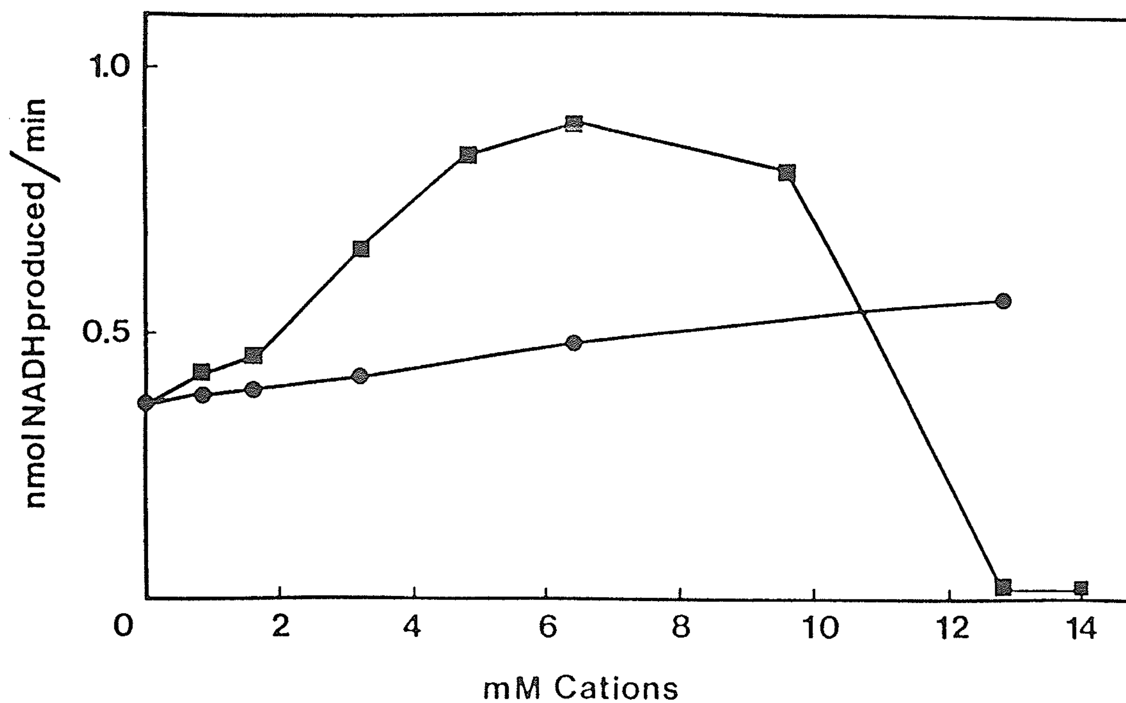


Fig. 27. The effects of Mg^{2+} and Ca^{2+} on hamster heart plasmalogenase activity.

Enzyme activity was assayed as described in Fig.26 in the presence of 0.5-14 mM of Ca^{2+} (■) and Mg^{2+} (●). Each assay contained 3.5 µg of microsomal protein. Each point represents the mean of three separate experiments.

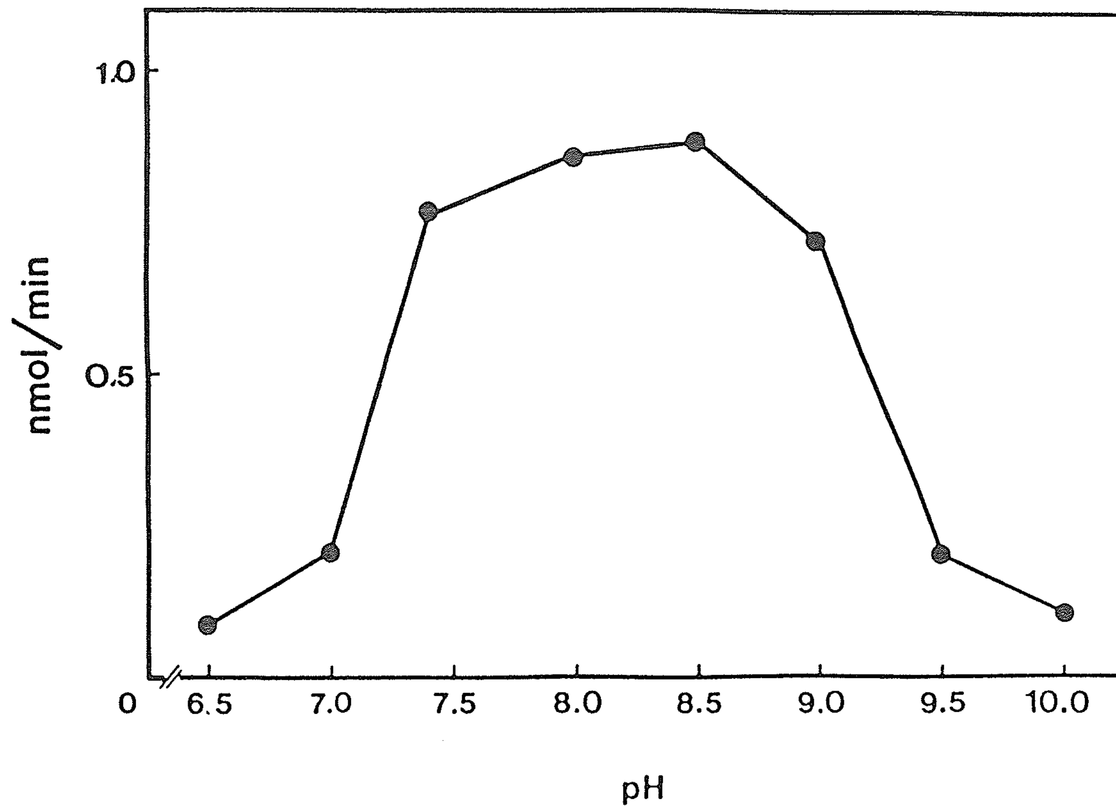


Fig. 28. The effect of pH on hamster heart plasmalogenase activity.

Hamster heart microsomal plasmalogenase activity was determined spectrophotometrically by the procedure described in the text. Each assay contained 8 µg of microsomal protein. Each point represents the means of two separate experiments.

assay developed (Table 20). Microsomal plasmalogenase activity was determined for each sample at a pH of 8.5, with 8 µg of microsomal protein. Ethanolamine glycerophospholipid fractions from individual heart lipid extracts were analyzed for their plasmenylethanolamine content as described previously. There was no direct correlation between cardiac plasmenylethanolamine content and plasmalogenase activity in the different species.

Our interest in the guinea pig heart as a model for mammalian plasmenylcholine biosynthesis, led us to further investigate the plasmalogenase activity present in the various tissues of the guinea pig (Table 21). Microsomal plasmalogenase activity and plasmenylethanolamine content were determined as described above. As before, it was noted that there was a lack of correlation between plasmenylethanolamine content and the plasmalogenase activity found in the various tissues.

III. Quantitation of Lysophosphatidylcholine

a) Acetylation of Lysophosphatidylcholine

A procedure for the determination of small amounts of lysophosphatidylcholine in cardiac tissues was developed. Lysophosphatidylcholine (LPC) was separated from the major phospholipids by column chromatography and then acetylated with labeled acetic anhydride. In a preliminary study, 10 nmol of LPC was acetylated with [^{3}H]acetic anhydride (2000 dpm/nmol) and the resulting product was isolated by thin layer chromatography. The acetylated LPC migrated as a single band which was

TABLE 20

Plasmenylethanolamine content and plasmalogenase activity in mammalian hearts.

Microsomal plasmalogenase activity was determined spectrophotometrically as described in the text. Plasmenylethanolamine was isolated after alkaline hydrolysis and quantitated by alkenyl content, as described in "Materials and Methods". Each value represents the mean of at least three separate experiments.

Species	Plasmenylethanolamine content	Plasmalogenase activity
	% ethanolamine phosphoglyceride	umol NADH formed per min/mg protein
Pig	43.0	0.129
Guinea pig	34.5	0.082
Dog	31.1	0.198
Rat	12.7	0.451
Hamster	8.7	0.166

TABLE 21

Plasmenylethanolamine content and plasmalogenase activity in guinea pig tissues.

Microsomal plasmalogenase activity was determined spectrophotometrically as described in the text. Plasmenylethanolamine was isolated after alkaline hydrolysis and quantitated by alkenyl content as described in "Materials and Methods". Each value represents the mean of at least three separate experiments.

Tissue	Plasmenylethanolamine content	Plasmalogenase activity
	% ethanolamine phosphoglyceride	umol NADH formed per min/mg protein
Brain	55.3	0.169
Lung	44.2	0.213
Spleen	36.8	0.231
Heart	34.5	0.087
Kidney	33.9	0.152
Liver	20.4	0.084

found to correspond to the standard 1-acyl, 2-acetyl-sn-glycerophosphocholine (Rf = 0.11). The site of acetylation of LPC was investigated by treatment of the acetylated LPC with phospholipase C. The reaction was terminated and the radioactivity in the aqueous and organic phases were determined. No radioactivity was detected in the aqueous phase which indicates that the phosphocholine moiety in LPC was not acetylated. Over 98% of the original radioactivity was found in the organic phase. Analysis of the organic phase by thin layer chromatography revealed that all of the radioactivity in this fraction migrated close to the solvent front, which indicates that the acetylated LPC was completely hydrolyzed by phospholipase C and the lipid moiety of LPC after hydrolysis was acetylated. In order to demonstrate that the acyl groups were not acetylated, the acyl groups of LPC before and after acetylation were methylated and analyzed by gas-liquid chromatography. Comparison of the methyl esters showed identical acyl contents before and after acetylation, and no significant radioactivity was detected in the long-chain methyl esters. From the results obtained, it is clear that the acyl group of the LPC was not acetylated, and there was no significant ^{3}H exchanged onto the acyl group.

Acetylation of LPC was shown to be linear from 0.5-100 nmol of LPC (Fig.29) and the yield was greater than 90% of the theoretical yield as calculated based on the specific radioactivity of the [^{3}H]acetic anhydride. Identical results were obtained with [1-^{14}C]acetic anhydride. Both pyridine and perchloric acid were required for the reac-

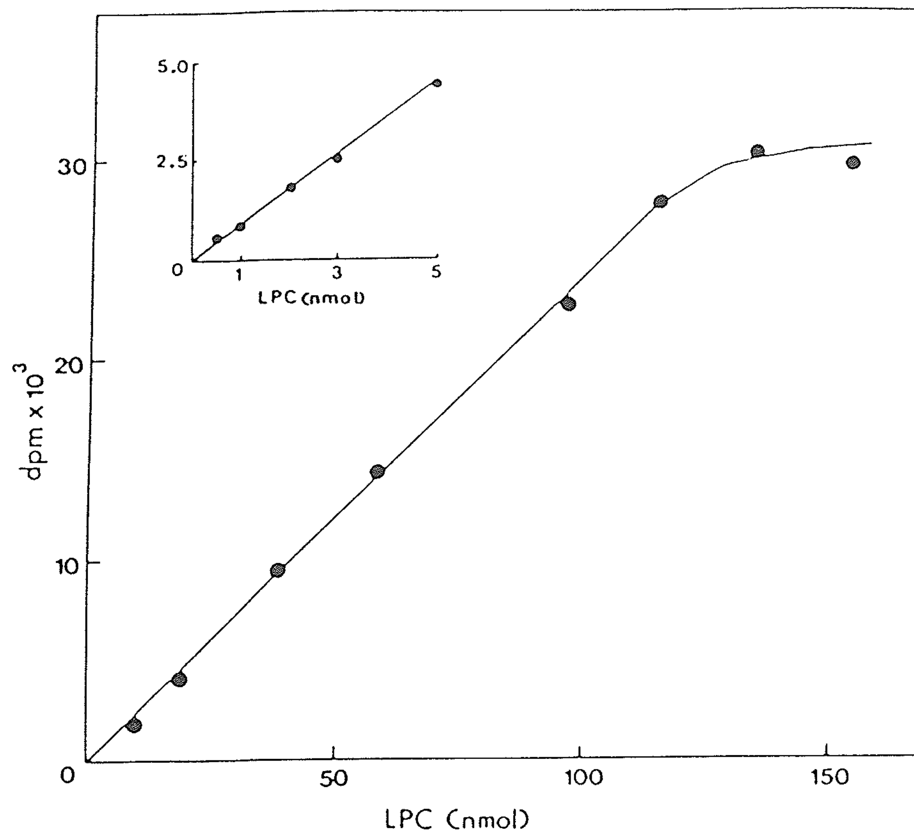


Fig. 29. Quantitation of lysophosphatidylcholine by acetylation.

The formation of 1-acyl, 2-[^{3}H]acetyl-sn-glycerophosphocholine (acetylated LPC) with 0.5-160 nmol of LPC was determined as described in "Materials and Methods". Specific radioactivity of [^{3}H]acetic anhydride was 500 dpm/nmol for each assay except in the inset where 2,000 dpm/nmol was used. Each point represents the mean of triplicate determinations.

tion and the ratio of acetic anhydride-pyridine-perchloric acid was optimized at 6:4:0.1 (v/v). The optimum reaction volume for 20 nmol of LPC was 60 µl and further increases in volume did not improve the yield. The reaction was essentially completed after a 60 min incubation at 70°C, but the yield was dramatically reduced when the reaction was carried out at 37°C, regardless of the length of incubation.

Since acetic anhydride has the ability to acetylate other lysophospholipids and lipids containing a hydroxyl group, the LPC fraction must be isolated from the total tissue lipid extract prior to the acetylation reaction. Isolation by silicic acid column chromatography was rapid and provided a good yield. When 1-[1-^{14}C]palmitoyl glycerophosphocholine was added to the total lipid extract prior to column chromatography, 92% of the radioactivity was recovered in the LPC fraction. Analysis of this fraction by thin layer chromatography showed that it contained only lysophosphatidylcholine, sphingomyelin and phosphatidylcholine. Although the sphingomyelin was also acetylated during the reaction, its acetylated product migrated differently (Rf = 0.19) and was readily separated from the acetylated product of LPC by thin layer chromatography. No radioactivity was detected in phosphatidylcholine after the reaction, which indicates that it was not acetylated and there was no isotope exchanged onto the acyl chains.

b) Lysophosphatidylcholine Content of Normal and Ischemic Cardiac Tissue

LPC content in control and ischemic canine heart tissue was deter-

mined by the acetylation method. The amount of radioactivity associated with the acetylated LPC was used to determine the LPC content from a standard curve. The data obtained are shown in Table 22. In a control experiment, an internal standard of 10 nmol of unlabeled LPC was added to each sample prior to the acetylation. With the aid of this internal standard, the yield of the acetylation reaction was determined to be better than 90%. The results obtained from the acetylation reaction were compared with those obtained by determination of lipid-phosphorus content (Table 22). A 2.5-fold increase in LPC content in the ischemic as compared to normal cardiac tissue was observed by both methods.

TABLE 22

Lysophosphatidylcholine (LPC) content in control and ischemic canine heart.

Ischemic tissue from canine heart was obtained by a Harris two-stage occlusion of the left anterior coronary artery. The levels of LPC in both control and ischemic tissues were assayed by the acetylation procedure and the lipid-P determination, as described in "Materials and Methods".

Mode of LPC determination	LPC content		Ratio
	Control (C) tissue	Ischemic (I) tissue	
	nmol/g wet weight		I/C
Acetylation	92[a] ± 4[b] (15)[c]	229 ± 26 (9)	2.5
Lipid-P	103 ± 7 (30)	255 ± 25 (6)	2.5

[a] Mean
[b] Standard error of the mean
[c] Number of experiments

Discussion

I. The Formation of Phosphatidylcholine and Plasmenylcholine from Choline in the Guinea Pig Heart

This study was aimed at identifying the pathways for the incorporation of choline into phosphatidylcholine and plasmenylcholine in the guinea pig heart. The contributions of each known pathway to the *de novo* synthesis of phosphatidylcholine and plasmenylcholine were assessed. The guinea pig heart was the preferred animal model since a high amount (34%) of the total choline phosphoglycerides are in the form of plasmenylcholine.

The structure of plasmenylcholine was discovered in the late 1950's but today there is still very little information available on the metabolism of this ether lipid. Indeed, the biosynthetic pathway and the physiological function(s) of plasmenylcholine in mammalian tissue were not known. This lack of information may be due to the difficulties involved in the isolation, quantitation and handling of this phospholipid. The major problem encountered is the unavailability of suitable methods to separate plasmenylcholine from phosphatidylcholine. Currently, it is necessary to hydrolyze or derivatize one or both of these lipid classes prior to their separation. Thus, it is not yet possible to separate these two phospholipids from each other in their native form.

This study is the first successful attempt to elucidate the biosynthetic route of plasmenylcholine from choline in a mammalian heart.

The formation of plasmenylcholine inevitably requires choline, thus a logical approach to the study of the formation of this ether lipid was to determine if it could be labeled with radioactive choline. As seen in the study with the hamster heart (13), the guinea pig heart has the ability to metabolize choline. Following perfusion with labeled choline, the three metabolites of the CDP-choline pathway (choline, phosphocholine and CDP-choline) were found to be labeled. Radioactivity was also found in the phosphatidylcholine and plasmenylcholine fractions. In addition, small amounts of radioactivity were found to be associated with lysophosphatidylcholine and sphingomyelin. The above labeled metabolites accounted for 99% of the total radioactivity incorporated into the guinea pig heart and no other labeled metabolites were detected. The results of this study indicate that the major pathway for the biosynthesis of phosphatidylcholine and plasmenylcholine from choline in the guinea pig heart is the CDP-choline pathway.

A distinct lag in the incorporation of labeled choline into phosphatidylcholine and plasmenylcholine was found during the first 30 min of perfusion. This finding is similar to that reported previously from our laboratory (13) with hamster heart, which indicates that the base-exchange reaction is not a major route for the formation of phosphatidylcholine and plasmenylcholine. The exact contribution of the base-exchange reaction to the formation of phosphatidylcholine and plasmenylcholine was not determined in this study. However, the lag phase is evidence for the minor contribution of this reaction to *de novo* phosphatidylcholine and plasmenylcholine synthesis (222). The contribution of progressive methylation of phosphatidylethanolamine and

plasmenylethanolamine to the formation of phosphatidylcholine and plasmenylcholine is very minor in the guinea pig heart. However, this does not rule out a possible role for the methylation pathway in the transmission of biological signals across the membrane or in other important physiological functions. Such functions are presumed to be localized in the membrane (229,230) and were recently found to require the conversion of only small amounts of phosphatidylethanolamine to phosphatidylcholine.

In the pulse-label study, choline was labeled within 15 min. Subsequently, a small decline in label was observed. It appears that the decrease was caused by a small but steady loss of labeled choline in the perfusate. However, such a decrease of labeled material in the perfusate probably does not have any major effect on the validity of the pulse-label studies since the amount of labeled choline left in the perfusate after 120 min of perfusion was still greater than 85% of the amount at the beginning of the perfusion. The incorporation of label into phosphocholine and CDP-choline levels out at 60 min indicating that an equilibrium was reached after this time period.

One essential requirement for demonstrating that the CDP-choline pathway is the major route for the biosynthesis of plasmenylcholine is the detection of an enzyme activity for the condensation of CDP-choline with 1-alkenyl-2-acyl-glycerol. In this study, we clearly demonstrate the existence of two phosphocholinetransferase activities in the guinea pig heart which utilized 1,2-diacylglycerols or 1-alkenyl-2-acyl-glycerols as the substrate. There was a 5-fold differ-

ence between these two activities. The higher activity for 1,2-diacylglycerols is not defined and it is not known if these two activities belong to the same or different enzymes responsible for the synthesis of phosphatidylcholine and plasmenylcholine. If there were two enzymes, one may be specific for the substrate 1,2-diacylglycerol and the other enzyme for 1-alkenyl-2-acyl-glycerol. Alternatively, if there is only one phosphocholinetransferase enzyme and it preferentially catalyzes the synthesis of phosphatidylcholine from CDP-choline and 1,2-diacylglycerol, it may also be able to act on 1-alkenyl-2-acyl-glycerol to form plasmenylcholine. This may be the case in rat liver and brain (163) where it was found that the same ethanolamine and choline phosphotransferases were used for the synthesis of alkylacylglycerophospholipids and diacylglycerophospholipids. In the harderian gland of rabbits (168), the microsomal ethanolamine phosphotransferase was found to be more active with diacylglycerols than with alkylacylglycerols and the choline phosphotransferase was found to be equally active with both glycerol derivatives. In the same study (168), it was also found that the incorporation of [^{14}C]CDP-ethanolamine and [^{14}C]CDP-choline into plasmenylethanolamine and plasmenylcholine respectively, was increased several fold by the addition of alkylacylglycerols but not by the addition of diacylglycerols.

No other pathway is known for the formation of the alkenyl bond other than the desaturation of plasmanylethanolamine to form plasmenylethanolamine (119,120). Thus, it is proposed that the lipid precursor 1-alkenyl-2-acyl-glycerol probably arises from the catabo-

lism of plasmenylethanolamine by an enzyme with phospholipase C-like activity, which would remove the polar head group. The amount of 1-alkyl-2-acyl-sn-glycero-3-phosphocholine in neutrophils and platelets largely exceeds that which is needed for the synthesis of 1-alkyl-2-acetyl-sn-glycero-3-phosphocholine (platelet activating factor) (227). However, the function of the remaining 1-alkyl-2-acylglycerophosphocholine is not known, since the conversion of 1-alkyl-2-acylglycerophosphocholine to 1-alkenyl-2-acylglycerophosphocholine has not been described (122,153). Our data indicate that 1-alkenyl-2-acyl-glycerol is present in the guinea pig heart but our results obtained from the study of the incorporation of choline into plasmenylcholine do not allow us to distinguish whether the labeled choline was transferred to pre-existing 1-alkenyl-2-acyl-glycerol (derived from plasmenylethanolamine) or to newly synthesized 1-alkenyl-2-acyl-glycerol.

The pool size of phosphocholine in the guinea pig heart was found to be similar to that of choline. This finding is different from the results obtained by others using rat liver (225) and HeLa cells (226) where the pool of phosphocholine is much larger than the pool of choline. However, the pool sizes of these metabolites in the guinea pig heart are of the same order of magnitude as those reported in the hamster heart (13). In addition, the amount of CDP-choline in the guinea pig heart is very similar to that found in the hamster heart (13). From the average pool size and specific radioactivity of CDP-choline at 90 to 120 min of perfusion, the rate of plasmenylcholine formation in the guinea pig heart via the CDP-choline pathway was estimated to be 0.18 nmol/min/g heart. The rate of formation of phos-

phatidylcholine in the guinea pig heart was calculated to be 2.69 nmol/min/g heart. In a previous report from our laboratory, the rate of phosphatidylcholine formation in the hamster heart was estimated to be 39 nmol/min/g heart. This discrepancy in the rate of phosphatidylcholine formation may be due to the inherent differences between these two species, as each has a distinct metabolic heart rate and a characteristic turnover rate for cardiac phosphatidylcholine.

There is evidence in the guinea pig heart for two rate-limiting steps in the CDP-choline pathway. Pulse-chase experiments with labeled choline show that the initial rate-limiting step is found at the conversion of choline to phosphcholine by choline kinase. The labeled choline required 30 min of chase to remove it from the choline pool before it reached an equilibrium level. Since the pool sizes of choline and phosphocholine are similar, a rate determining step for the phosphorylation of choline is indicated. If this step were not rate-limiting, then one would expect the pool size of choline to be smaller than the phosphocholine pool. The second rate determining step occurs at the conversion of phosphocholine to CDP-choline by cytidylyltransferase. After 20 min of chase, the radioactivity in phosphocholine became maximum and then was seen to gradually disappear from this pool, into the CDP-choline pool. The pool size of phosphocholine was much larger than the pool of CDP-choline illustrating that a bottleneck occured at this stage.

The existence of two rate-limiting steps in the CDP-choline pathway of the guinea pig heart is very intriguing. For many years, it has

been generally accepted that the step catalyzed by CTP-phosphocholine cytidylyltransferase is usually the regulated step in the biosynthesis of phosphatidylcholine (13,225,226). This conclusion is based mainly on the observation that the pool size of phosphocholine is usually several times larger than the pool size of choline or CDP-choline. The results of the present study indicate that the reaction catalyzed by choline kinase may play a regulatory role in the CDP-choline pathway in the guinea pig heart. The phosphorylation of choline is the first committed step of the pathway and this suggests that this is a most logical place for a rate-determining step (231). As in the present study, pulse-chase studies with Ehrlich ascites cells show that radioactive choline is not immediately phosphorylated but accumulates as choline (232). In rooster liver, a stimulation of choline kinase activity by diethylstibestrol is observed simultaneously with an increase in phosphatidylcholine biosynthesis (233). Thus, it seems that the reaction catalyzed by choline kinase may play a more active role in the regulation of the biosynthesis of phosphatidylcholine than has been noted from previous studies in rat liver (225) and HeLa cells (234). Purification of choline kinase from the guinea pig heart is necessary in order to investigate the role that this enzyme may play as a regulator of the CDP-choline pathway.

II. Plasmalogenase in Mammalian Hearts

Enzymes capable of hydrolyzing the alkenyl bond of plasmalogens have not been previously described in cardiac tissue. This study was the first to demonstrate the existence of this enzyme in mammalian

heart with plasmenylethanolamine as substrate. Since the action of plasmalogenases on plasmalogens produces long-chain aldehydes (184), a coupled reaction for the conversion of aldehydes to another metabolite with the reduction of NAD^+ or $NADP^+$ would provide a facile approach for the determination of this enzyme activity. The conversion of the aldehydes to alcohols with the oxidation of NADH to NAD^+ by alcohol dehydrogenase had been used to measure plasmalogenase activity (184). However, several difficulties were encountered with this approach. Due to the inhibitory effect of alcohol on alcohol dehydrogenase, the amount of plasmalogen used in the assay had to be limited to suboptimal concentrations (184). When higher concentrations of plasmalogens were used, a linear reaction rate could not be obtained, affecting the accuracy of measurements (184). Our results indicate that the present method is superior to the coupled reaction with alcohol dehydrogenase. The reaction rate obtained by the coupled reaction with aldehyde dehydrogenase (100 nmol of product formed/min/mg protein) were similar to those obtained by monitoring substrate disappearance. However, our spectrophotometric assay is at least 25-fold more sensitive. The control experiments in our study showed quite conclusively that our assay is specific for plasmalogenase activity. We also showed that plasmenylethanolamine but not lysoplasmenylethanolamine was utilized as substrate, which suggests the absence of lysoplasmalogenase activity in the hamster heart microsomes. Both ethanolamine plasmalogenases and lysoplasmalogenase activities have been described in the brain (181,183,186).

The Mg^{2+} requirement for plasmalogenase activity in the brain has been reported (181). However, this cation had very little effect on the enzyme activity in the hamster heart even at high concentrations (up to 12.8 mM). At present, we do not know if the inhibition observed after initial activation by Ca^{2+} is due to a direct effect of Ca^{2+} on the enzyme or whether a cation-substrate interaction is responsible for the decreased activity. The optimum pH of the heart plasmalogenase activity was estimated to be 8.5 compared to 7.4 for the brain ethanolamine plasmalogenase. In addition, the heart enzyme had a much broader pH profile and was quite active at pH 7.4. It is likely that the brain ethanolamine plasmalogenase is kinetically different from the enzyme found in the hamster heart microsomes.

The phospholipid content of the hamster heart was estimated to be 29 µmol/g heart. Plasmenylethanolamine and plasmenylcholine accounted for 4.2%. This value is very low compared to those reported for rabbit, bovine, human, sheep and canine hearts (38,69). It is, however, comparable to that found in the rat heart (38). Our results for the plasmenylethanolamine content of the hearts of several mammalian species shows that the pig has the highest content, at 43% of the total ethanolamine phosphoglycerides and the hamster has the lowest at 8.7%. The cardiac plasmalogenase activity present in these species show that the rat has the highest activity and the guinea pig has the lowest activity. Hence, it was noted that there is no apparent correlation between the cardiac plasmalogenase activity and the content of cardiac plasmenylethanolamine from these different species. Plasmenylethanolamine content and plasmalogenase activities in different tissues of

the guinea pig reveal that the highest plasmenylethanolamine content exists in the brain and the highest plasmalogenase activity resides in the spleen. The lack of correlation between the cardiac or other tissue plasmenylethanolamine content and the plasmalogenase activity suggests that this enzyme is not responsible for the tissue or species differences in plasmenylethanolamine content observed in various mammals.

Recently in our laboratory, the catabolism of plasmenylcholine has been investigated. The absence of plasmalogenase activity for plasmenylcholine was demonstrated in the guinea pig heart (6) and by other investigators in the canine heart (177). Plasmenylcholine was hydrolyzed by phospholipase A2 activities in cardiac fractions of microsomes, mitochondria and cytosol of the guinea pig (6). The phospholipase A2 activities have a high specificity for plasmenylcholine with linoleoyl or oleoyl at the 2-position of the glycerol moiety (6). Lysoplasmalogenase activity (cleaves the alkenyl bond) for lysoplasmenylcholine was also detected and characterized in the microsomal and mitochondrial fractions of the guinea pig heart (6). Thus, it has been postulated that the major pathway (Fig.30) for the catabolism of plasmenylcholine involves the hydrolysis of the fatty acid at the 2-position first, by phospholipase A2 and then, hydrolysis of the alkenyl group of the resultant lysoplasmenylcholine by lysoplasmalogenase (6). Alternatively, the lysoplasmenylcholine formed can also be reacylated to form the parent phospholipid by acyl-CoA : 1-alkenyl-GPC acyltransferase (190).

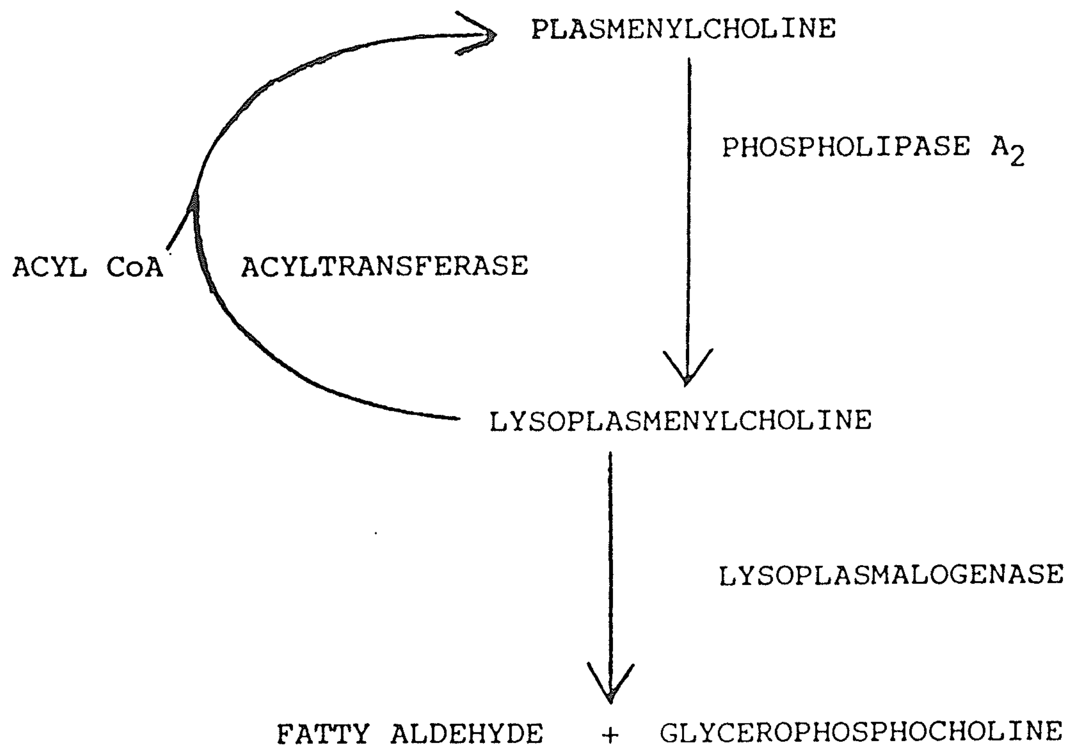


Fig. 30. Proposed major pathway for the catabolism of plasmenylcholine.

III. Quantitation of Lysophosphatidylcholine

The possible involvement of lysophosphatidylcholine (LPC) in several molecular events during arrhythmia after myocardial ischemia (78-81,197,198) has put a focus on the need to accurately and specifically quantitate this lysolipid in order to examine its physiological role in the genesis of cardiac arrhythmia. In the last several years, the concentration of LPC in cardiac tissues has been subjected to much debate (84). The discrepancies were probably caused by 1) mode of extraction and 2) mode of isolation and quantitation after isolation. It has been generally agreed that extraction of tissue LPC in an acidic medium will cause hydrolysis of the parent phospholipids, which will artificially cause the generation of LPC during extraction (84). Hence, LPC was extracted in this study with a neutral solvent, which has been shown to be a preferred mode of extraction (228). Although the method of isolation by thin layer chromatography and the quantitation of the LPC fraction by lipid-P content have been used in all previous studies, there are a number of difficulties with this mode of determination. The quantitation of LPC by lipid-P content is subject to interference from other phosphate-containing substances and a substantial amount of tissue (200 mg wet weight) is required for each determination because of the lack of sensitivity of the lipid-P assay (20-50 nmol) (228). With the present method, as little as 10 mg of cardiac tissue (0.5-1.0 nmol of LPC) suffice to accurately determine the amount of LPC and the sensitivity of the assay can be further enhanced by using labeled acetic anhydride with higher specific activ-

ity. The specificity of the assay alleviates the need for extensive purification of LPC, thus allowing the rapid isolation of the lipid by column chromatography.

The tissue LPC content obtained by lipid-P determinations is slightly but consistantly higher than that obtained by the radioactive labeling method. Although the difference is not statistically significant in all cases, the higher mean values obtained by lipid-P analysis are probably caused by small amounts of other phosphate-containing compounds that may co-chromatograph with LPC. The specificity of the present assay eliminates this possibility and allows an accurate assessment of the changes in LPC levels during cardiac ischemia and other cardiac disorders. The sensitivity of the assay also provides the ability to determine the lysolipid content in small biopsy samples and in small volumes of ischemic blood.

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