METABOLIC REGULATION OF ETHANOLAMINE-CONTAINING PHOSPHOLIPIDS IN MAMMALIAN TISSUES

By

Christopher R. McMaster

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

Department of Biochemistry and Molecular Biology
University of Manitoba

1992

National Library of Canada

Bibliothèque nationale du Canada

Acquisitions and Bibliographic Services Branch

Direction des acquisitions et des services bibliographiques

395 Wellington Street
Ottawa, Ontario
K1A 0N4

395, rue Wellington
Ottawa (Ontario)
K1A 0N4

Your file Votre référence

Our file Notre référence

The author has granted an irrevocable non-exclusive licence allowing the National Library of Canada to reproduce, loan, distribute or sell copies of his/her thesis by any means and in any form or format, making this thesis available to interested persons.

L'auteur a accordé une licence irrévocable et non exclusive permettant à la Bibliothèque nationale du Canada de reproduire, prêter, distribuer ou vendre des copies de sa thèse de quelque manière et sous quelque forme que ce soit pour mettre des exemplaires de cette thèse à la disposition des personnes intéressées.

The author retains ownership of the copyright in his/her thesis. Neither the thesis nor substantial extracts from it may be printed or otherwise reproduced without his/her permission.

L'auteur conserve la propriété du droit d'auteur qui protège sa thèse. Ni la thèse ni des extraits substantiels de celle-ci ne doivent être imprimés ou autrement reproduits sans son autorisation.

ISBN 0-315-77969-1

Canada

METABOLIC REGULATION OF ETHANOLAMINE-CONTAINING PHOSPHOLIPIDS IN MAMMALIAN TISSUES

BY

CHRISTOPHER R. MCMASTER

A Thesis submitted to the Faculty of Graduate Studies of the University of Manitoba in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

© 1992

Permission has been granted to the LIBRARY OF THE UNIVERSITY OF MANITOBA to lend or sell copies of this thesis, to the NATIONAL LIBRARY OF CANADA to microfilm this thesis and to lend or sell copies of the film, and UNIVERSITY MICROFILMS to publish an abstract of this thesis.

The author reserves other publication rights, and neither the thesis nor extensive extracts from it may be printed or otherwise reproduced without the author's permission.

TO MY PARENTS

FOR EILEEN

ACKNOWLEDGEMENTS

I would like to thank Dr. Choy for his patience with a struggling student. Pat, I will leave with much more than I began. Thank you.

There are also a host of people that have supported my endeavors over the past four years. Special thanks to my colleagues in the lab that have come and gone, Grant (coach), Karmin (hoky), Monroe (Mono), Jagat (Mookie), Adrian (Ice), Rubeena, Zha, Lu, and Lore.

I wish to express my gratitude to the members of my advisory committee, Dr. Stevens, Dr. Yamada, and Dr. Man. I would like to thank Dr. Roy Baker for his critical assessment of this thesis.

Paul, I could not have made it without your support and comic relief and the occasional beer. Thanks.

Keith, thanks for Jim's, and Mark for the Port club, and Ketan for keeping me in line.

Terry and Tracy, thanks for those words of encouragement, even after I miss a two foot putt.

Dave, maybe some new clubs would help?

LIST OF CONTENTS

INTRODUCTION 1

I. Biological Membrane 1

1. Phospholipids in the membrane 1

2. Membrane proteins 5

II. Phosphatidylethanolamine Biosynthesis 6

Overview 6

1. The CDP-ethanolamine pathway 8

(1) Ethanolamine uptake 10

(2) Ethanolamine kinase 10

(3) CTP: phosphoethanolamine cytidylyltransferase 12

(4) CDP-ethanolamine: 1,2-diacylglycerol phosphoethanolamine-transferase 13

2. Phosphatidylethanolamine Biosynthesis from Phosphatidylserine 15

Overview 15

(1) Base-exchange enzyme 15

(2) Phosphatidylserine decarboxylase 16

III. Regulation of Phosphatidylethanolamine Biosynthesis 17

1. The CDP-ethanolamine pathway 17

2. Regulation of phosphatidylethanolamine biosynthesis from phosphatidylserine 18

IV. Role of Phosphatidylethanolamine in Signal Transduction 21

V. Plasmenylethanolamine Catabolism 25

Overview 25

1. Plasmalogenase 29

2. Lysoplasmalogenase 29

MATERIALS AND METHODS 31

A. MATERIALS 31

I. Experimental Animals 31

II. Chemicals 31

B. METHODS 34

I. Studies on Intracellular Ethanolamine Pool Sizes 34

1. Ethanolamine pool size analysis 34

(1) Preparation of tissue sample 34

(2) Coupling of ethanolamine with phenylisothiocyanate 35

(3) Analysis of PTCethanolamine by HPLC 35

II. Studies on the Metabolism of Phosphatidylethanolamine in the Hamster Heart 37

1. Perfusion of the isolated hamster heart 37

2. Analysis of phospholipids 38

3. Analysis of radioactivity in ethanolamine-containing metabolites 39

4. Subcellular fractionation of the heart 39

5. Synthesis of phophatidyl-[1-^{3}H]ethanolamine 40

6. Enzyme assays 41

(1) Ethanolamine kinase 41

(2) Choline kinase 41

(3) CTP: phosphoethanolamine cytidylyltransferase 42

(4) Phosphoethanolaminetransferase 43

(5) The hydrolysis of phosphatidylethanolamine 43

7. Serine and ethanolamine pool size analysis 44

III. Studies on the Catabolism of Plasmenylethanolamine 46

1. Preparation of plasmalogens for enzyme assays 46

2. Preparation of subcellular fractions of guinea pig tissues 47
3. Plasmalogenase assays 47
(1) Substrate disappearance method 47
(2) Appearance of product method 49
4. The determination of marker enzyme activities 50

IV. Analytical Procedures 51
1. Determination of lipid phosphorus 51
2. Plasmalogen (vinyl ether) determination 52
3. Protein determination 52
4. Radioactivity determination 53
5. Statistical Analysis 53

EXPERIMENTAL RESULTS 54

A. DETERMINATION OF INTRACELLULAR ETHANOLAMINE POOL SIZES 54
I. Utilization of PITC to Determine Ethanolamine Pool Sizes 54
1. Conversion of ethanolamine into its phenylthiocarbamyl derivative 54
2. Separation and identification of PTCethanolamine by HPLC 55
3. Extraction of ethanolamine from hamster tissues and analysis of 58

intracellular pool sizes

B. REGULATION OF PHOSPHATIDYLETHANOLAMINE BIOSYNTHESIS BY EXOGENOUS ETHANOLAMINE 63

I. Ethanolamine as a Precursor for Phosphatidylethanolamine Biosynthesis 63

1. Ethanolamine uptake and incorporation into phosphatidylethanolamine 63

2. Analysis of ethanolamine-containing metabolites 64

3. Pool size analysis 67

4. Effect of ethanolamine analogues on ethanolamine uptake 67

C. THE ROLE OF SERINE IN THE REGULATION OF PHOSPHATIDYLETHANOLAMINE BIOSYNTHESIS 70

I. Ability of Exogenous Ethanolamine to Modulate Phosphatidylethanolamine Biosynthesis 70

1. The inhibition of ethanolamine uptake by exogenous serine 70

2. The effect of exogenous serine on the CDP-ethanolamine pathway 74

3. Effect of serine on the enzymes of the CDP-ethanolamine pathway 77

4. Intracellular serine pool in the hamster heart 82

5. Decarboxylation of phosphatidylserine to phosphatidylethanolamine 82

6. Effect of serine on the base-exchange reaction 84

7. Effect of serine on the catabolism of phosphatidylethanolamine 86

D. PLASMENYLETHANOLAMINE CATABOLISM 90

I. Cytosolic Plasmalogenase in Guinea Pig Tissues 90

1. Plasmalogenase assays 90

2. Subcellular localization of plasmalogenase activity 92

3. Substrate specificity 92

4. Characterization of plasmalogenase activities 97

5. Gel filtration chromatography of guinea pig brain cytosol 101

DISCUSSION 104

I. Regulation of Phosphatidylethanolamine Biosynthesis by Exogenous Ethanolamine 104

II. Regulation of Phosphatidylethanolamine Biosynthesis by Serine 109

III. Plasmenylethanolamine Catabolism 113

CONCLUDING DISCUSSION 115

SUMMARY 120

REFERENCES 122

LIST OF FIGURES

Figure 1. The biological membrane. 2

Figure 2. Structure of phospholipids and head groups. 3

Figure 3. Pathways for the biosynthesis of phosphatidylethanolamine. 7

Figure 4. The CDP-ethanolamine pathway. 9

Figure 5. Proposed roles of phospholipids in signal transduction. 22

Figure 6. Structure of ethanolamine-containing phospholipids. 26

Figure 7. Pathways for the hydrolysis of plasmenylethanolamine. 28

Figure 8. Separation of PTCethanolamine by reverse-phase HPLC. 56

Figure 9. The quantitation of standard PTCethanolamine by reverse-phase HPLC. 57

Figure 10. The quantitation of ethanolamine in hamster heart. 61

Figure 11. Time course for the inhibition of ethanolamine uptake and subsequent incorporation into phosphatidylethanolamine. 72

Figure 12. Lineweaver-Burke plot of ethanolamine uptake versus ethanolamine concentration in the presence of serine. 75

Figure 13. The effect of serine on ethanolamine kinase activity. 80

Figure 14. Lineweaver-Burke plot of ethanolamine kinase activity versus ethanolamine concentration in the presence of serine. 81

Figure 15. Effect of substrate concentration on the measurement of the 91

vinyl ether content of plasmalogens.

Figure 16. Time course for the disappearance of plasmenylethanolamine and lysoplasmenylethanolamine in guinea pig brain cytosol. 96

Figure 17. Effect of substrate concentrations on guinea pig brain cytosolic plasmalogenase activity. 98

Figure 18. Effect of pH on guinea pig brain cytosolic and microsomal plasmalogenase activities. 100

Figure 19. Effect of heat treatment on plasmalogenase activities in the cytosol and microsomes of guinea pig brain. 102

Figure 20. Sepharose 6B chromatography of guinea pig brain cytosol. 103

LIST OF TABLES

Table 1. Ethanolamine recovery during pool size analysis. 60

Table 2. Pool sizes of ethanolamine in hamster tissues. 62

Table 3. Ethanolamine uptake and incorporation into phosphatidylethanolamine. 65

Table 4. Radioactivity incorporation into ethanolamine-containing metabolites. 66

Table 5. Pool sizes of ethanolamine and phosphatidylethanolamine. 68

Table 6. Effect of ethanolamine analogues on ethanolamine uptake. 69

Table 7. Effect of serine on ethanolamine uptake and incorporation into phosphatidylethanolamine. 73

Table 8. Effect of serine of the distribution of radioactivity in ethanolamine metabolites. 76

Table 9. Effect of serine on the enzymes of the CDP-ethanolamine pathway. 78

Table 10. Uptake of L-[^{3}H]serine and its incorporation into phospholipids in the hamster heart. 83

Table 11. Incorporation of [1-^{3}H]glycerol into phospholipids. 85

Table 12. Effect of serine on phosphatidylethanolamine degradation. 88

Table 13. Plasmalogenase activities in the subcellular fractions of guinea pig tissues. 93

Table 14. The distribution of microsomal marker enzyme activities in the microsomal and cytosolic fractions of guinea pig brain and heart. 94

Table 15. Effects of EDTA and cations on cytosolic plasmalogenase activity. 99

ABSTRACT

Phospholipids are required for the formation of biological membranes and as a store for the production of lipid second messengers and lipid mediators. The mechanisms regulating the synthesis and catabolism of the needed amount of the various types of phospholipid are poorly understood. Phosphatidylethanolamine is a major phospholipid in mammalian tissues. The role of exogenous precursors (ethanolamine and serine) in the regulation of phosphatidylethanolamine biosynthesis was investigated. In this study, the isolated perfused hamster heart was utilized to examine phosphatidylethanolamine synthesis via the CDP-ethanolamine pathway. At low concentrations of exogenous ethanolamine, the rate limiting step in the CDP-ethanolamine pathway was the conversion of phosphoethanolamine to CDP-ethanolamine. However, when the exogenous ethanolamine concentration was raised to physiological levels the rate limiting step was the phosphorylation of ethanolamine. Analysis of intracellular ethanolamine pool sizes revealed that the ethanolamine pool size did not change when hearts were perfused with either low or high exogenous ethanolamine concentrations. Thus it appeared that the newly imported ethanolamine pool was separate from the endogenous intracellular pool, and the imported pool was preferentially utilized for phosphatidylethanolamine biosynthesis.

To investigate if serine also played a regulatory role in the biosynthesis of phosphatidylethanolamine, hearts were perfused with physiological levels of ethanolamine in the presence of serine. Serine was found to inhibit ethanolamine uptake and the phosphorylation of ethanolamine. A 5-fold increase in the intracellular serine pool during perfusion with serine facilitated the inhibition of ethanolamine kinase *in vivo*. This increased intracellular pool of serine did not increase the synthesis of phosphatidylethanolamine by phosphatidylserine decarboxylation or the base-exchange reaction.

The hamster heart was chosen for the study of phosphatidylethanolamine biosynthesis because it contains low levels of plasmenylethanolamine. However, most mammalian hearts and brains contain a large amount of plasmenyl-ethanolamine. Plasmenylethanolamine is believed to be metabolized by either a microsomal plasmalogenase or a phospholipase A_2-lysoplasmalogenase system. An examination of the subcellular distribution of plasmalogenase activities in guinea pig and rat tissues revealed the presence of a cytosolic plasmalogenase activity. This enzyme accounted for the majority of the plasmalogenase activity in the brain, heart, and liver. Since the guinea pig brain contained the highest amount of soluble plasmalogenase, this source was chosen to further characterize its enzyme activity. The cytosolic plasmalogenase was completely inhibited by 1 mM EDTA and Mn^{2+}, but was unaffected by both Ca^{2+} and Mg^{2+}. The cytosolic

and microsomal enzyme activities had a K_m of 100-150 μM. Both enzymes had a pH optimum of 7.5, and the microsomal enzyme was slightly more heat stable than the soluble form. Sepharose 6B chromatography of the cytosolic enzyme revealed a molecular weight of 250,000, confirming the enzyme was truly soluble. The similarity in characteristics of the cytosolic and microsomal enzymes make it plausible to speculate that both activities may originate from the same protein.

INTRODUCTION

I. BIOLOGICAL MEMBRANE

1. Phospholipids in the Membrane

Biological membranes contain an astonishing variety of phospholipids (White 1973). The maintenance of the appropriate phospholipid composition within a membrane requires the concerted effort of several elaborate metabolic pathways within a cell (Vance 1985). Cellular phospholipids are assembled into bilayers which allow for the rapid lateral diffusion of lipid through the plane of the membrane, but act against the translocation of lipids from one membrane bilayer to the other (Singer and Nicolson 1972) (Fig. 1). This phospholipid bilayer forms a permeability barrier between different compartments within a cell and between the cell and the outside environment. Phospholipids assume a bilayer due to their amphipathic nature. This characteristic is due to the presence of a hydrophilic head group and a hydrophobic tail. The head group determines the type of phospholipid while the fatty acid tails affect the fluidity of the membrane. Increasing the content of unsaturated fatty acids in the tails of the phospholipids results in a bilayer with increased fluidity. The four main classes of phospholipid are phosphatidylcholine, phosphatidylethanolamine, phosphatidylserine, and phosphatidylinositol (Fig. 2). Phospholipids constitute about 50% of the mass of

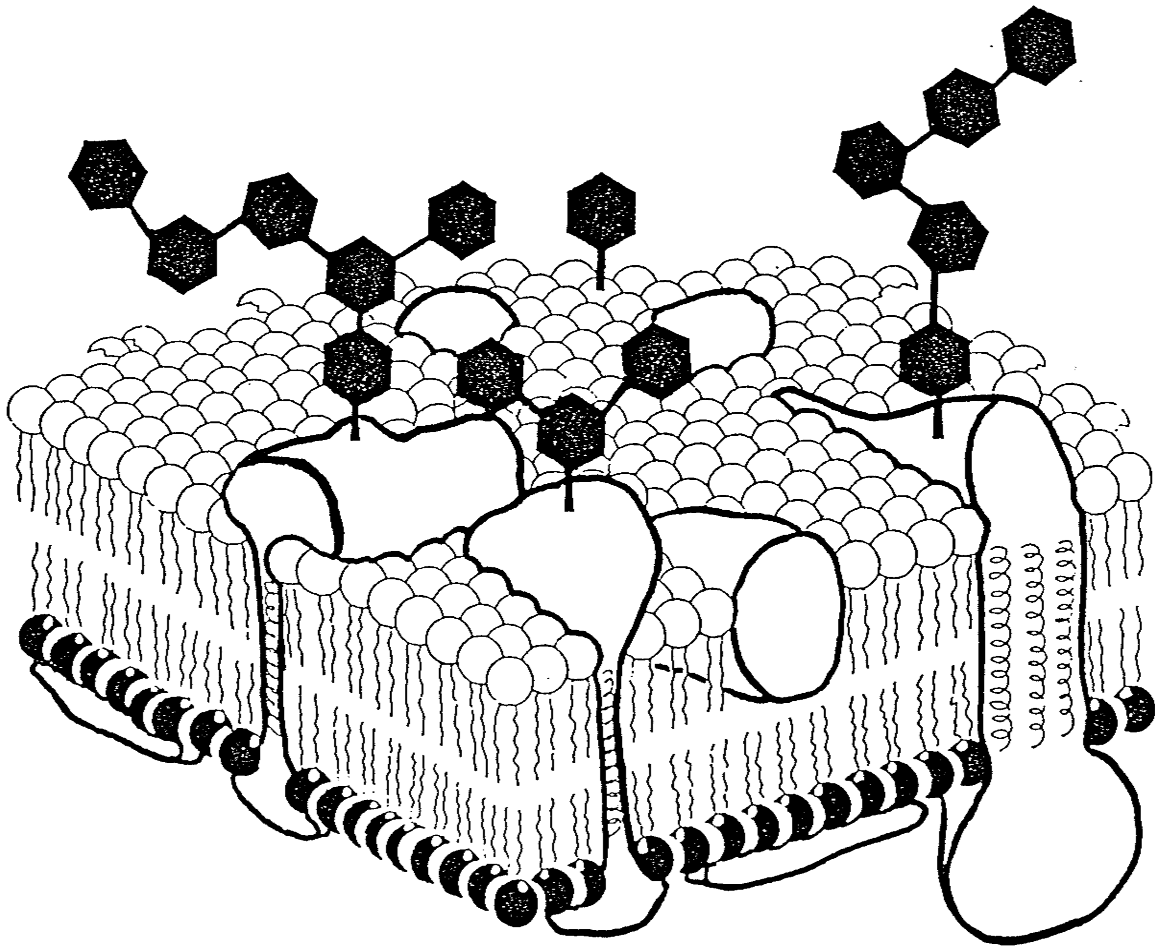
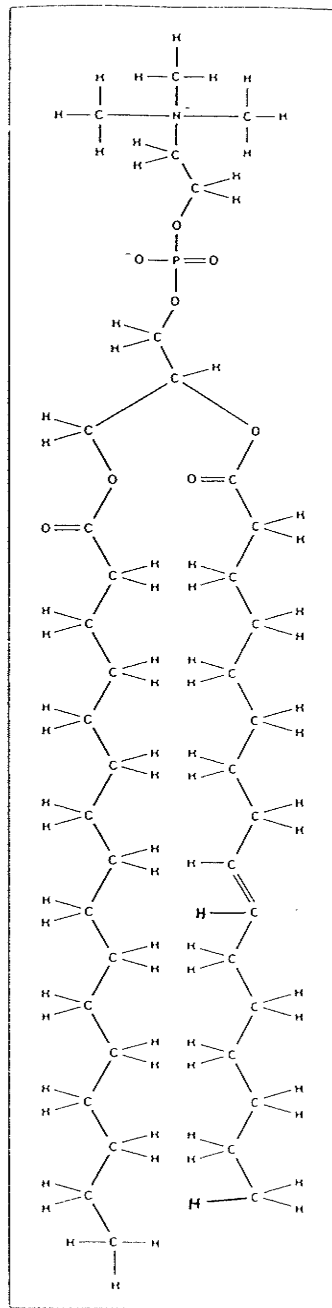
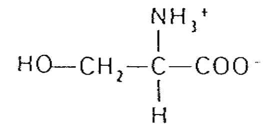


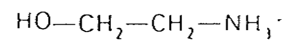
Figure 1. The Biological Membrane. The biological membrane is composed mainly of lipid, protein, and carbohydrate.



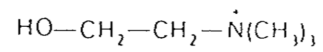
Serine



Ethanolamine



Choline



Inositol

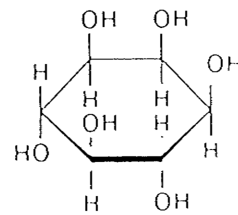


Figure 2. Structure of Phospholipids and Head Groups.

membranes in mammalian cells. Accompanying phospholipids in a biological membrane are several other classes of lipids including sphingolipids and cholesterol, as well as membrane bound proteins.

It is apparent that phospholipids are important cellular components and their metabolism is under several tiers of control (Vance 1985; Carman and Henry 1989; Bell and Coleman 1980). Alterations in the metabolism of phospholipids in the membrane may affect cellular processes such as the transport of Na^+ and K^+ (Katz and Messineo 1982). Also, phospholipids are required for the transduction of biological signals across the membrane (Berridge and Irvine 1989; Exton 1990; Pelech and Vance 1989; Berridge 1987; Gilman 1987) and the release of arachidonic acid for the production of prostaglandins and leukotrienes (Samuelsson and Funk 1989). In addition, the overproduction of lysophospholipids has been implicated as a biochemical cause for the generation of cardiac arrhythmias (Man and Choy 1982).

The turnover of phospholipids in response to extracellular mediators is well established. Upon binding of an agonist to its receptor phosphatidylinositol 4,5-bisphosphate, phosphatidylcholine, and phosphatidylethanolamine (Berridge and Irvine 1989; Exton 1990; Kiss and Anderson 1990; Hii *et al* 1991) are all broken down into their various component parts that act to generate intracellular

responses to the various extracellular signals. However, a survey of the phospholipid composition of a tissue will reveal that there is very little change in the lipid composition of cell membranes. This implies that there is a coordinate regulation of phospholipid turnover and synthesis. The understanding of the regulation of phospholipid metabolism is required to firmly grasp the role filled by the diverse array of phospholipids in the biological membrane.

2. Membrane Proteins

The other main component of a biological membrane is protein. Most proteins are noncovalently associated with the membrane bilayer (Vance 1985). Proteins can be intimately associated with the membrane by either entering or completely transversing the bilayer (Yeagle 1989). Proteins that intimately associate with membrane lipids are referred to as integral membrane proteins and contain one or more hydrophobic sequences that penetrate the lipid bilayer. These proteins can not be released from the membrane without the aid of detergents or organic solvents. Other proteins may interact with the surface of the membrane in a noncovalent manner. These proteins are referred to as peripheral membrane proteins and are released from the membrane by milder procedures including extreme pH treatment and exposure to solutions of high ionic strength. These procedures will generally leave the membrane bilayer intact. Membrane bound

proteins are known to be activated by the composition of phospholipids in the surrounding membrane (Walsh and Bell 1986a, 1986b). The most notable example being the activation of protein kinase C by phosphatidylserine (Bell and Burns 1991; Hannun *et al* 1985). Recently, several membrane bound proteins have been found to be covalently associated with the biological membrane. For example, some proteins are linked to a fatty acid embedded in the membrane, leaving the protein exposed (Gordon *et al* 1991). Other proteins are linked to the membrane via a glycosylphosphatidylinositol anchor (Doering *et al* 1990). The roles of these covalent linkages of proteins to the membrane are not truly understood.

II. PHOSPHATIDYLETHANOLAMINE BIOSYNTHESIS

Overview

Phosphatidylethanolamine is a major phospholipid in mammalian tissues and can be synthesized via the CDP-ethanolamine pathway, the decarboxylation of phosphatidylserine, and the Ca^{2+} mediated base-exchange reaction (Vance 1985) (Fig. 3). The contribution of each of these pathways for phosphatidylethanolamine formation varies from one cell type to another. The decarboxylation of phosphatidylserine is touted as the major pathway for phosphatidylethanolamine biosynthesis in BHK-21 and chinese hamster ovary cells (Voelker 1984; Miller and

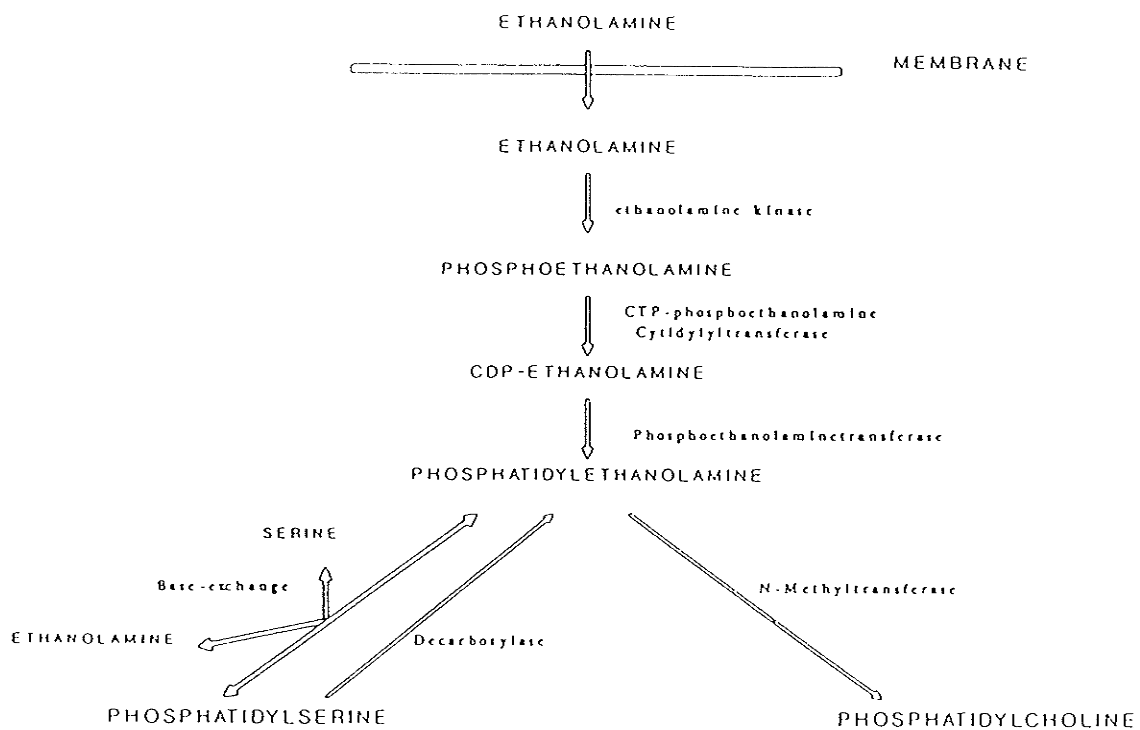


Figure 3. Pathways for the Biosynthesis of Phosphatidylethanolamine.

Kent 1986), whereas the CDP-ethanolamine pathway is regarded as the main route for phosphatidylethanolamine formation in most mammalian tissues (Zelinski and Choy 1982b; Sundler and Akesson 1975b; Arthur and Page 1991; Xu *et al* 1991). The presence of alternate pathways implies that the amount of phosphatidylethanolamine synthesized by a particular pathway is not exactly defined.

1. The CDP-ethanolamine Pathway

In this pathway, ethanolamine transported into the cell is rapidly phosphorylated into phosphoethanolamine by ethanolamine kinase. Phosphoethanolamine is then converted into CDP-ethanolamine by CTP: phosphoethanolamine cytidylyltransferase. The CDP-ethanolamine formed is condensed with diacylglycerol by CDP-ethanolamine: 1,2-diacylglycerol phosphoethanolamine-transferase for the formation of phosphatidylethanolamine (Fig. 4). The conversion of phosphoethanolamine to CDP-ethanolamine, catalyzed by CTP:phospho-ethanolamine cytidylyltransferase, is usually the rate-limiting step in this pathway (Zelinski and Choy 1982b; Sundler and Akesson 1975b). Although there is no known dietary deficiency for ethanolamine (Tijburg *et al* 1988), how mammalian cells make ethanolamine is still a basic question requiring an answer. The only known route for the generation of intracellular ethanolamine is via the base-

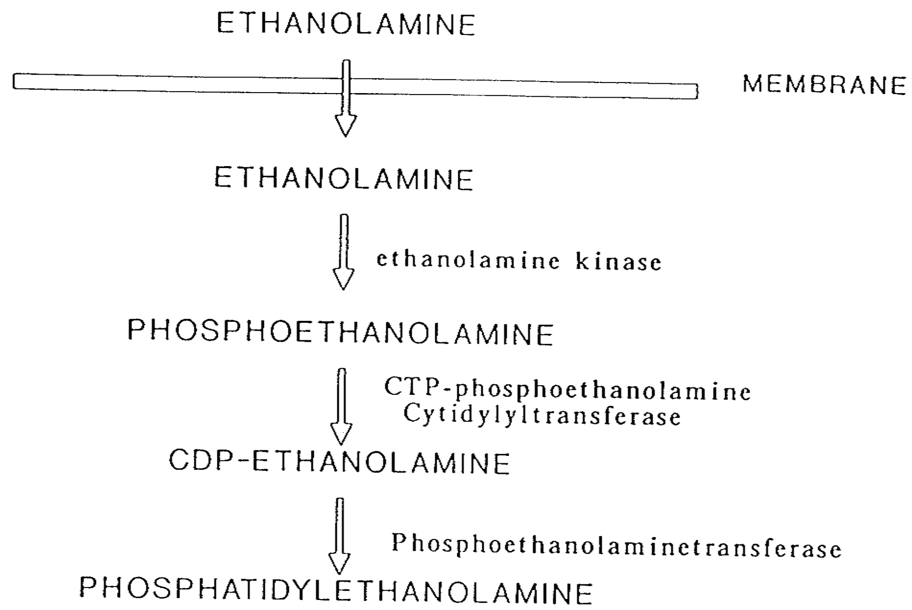


Figure 4. The CDP-ethanolamine Pathway.

exchange pathway, however, it has not been demonstrated that the cell can utilize this intracellular pool of ethanolamine.

(1) Ethanolamine Uptake

In the hamster heart, only one uptake system for ethanolamine was identified. Ethanolamine uptake was linear from 0.1-100 μM exogenous ethanolamine with a K_m for ethanolamine of 170 μM. This single uptake system (Zelinski and Choy 1982b) is different from that observed in cell culture where ethanolamine is taken up by both low and high affinity uptake mechanisms (Yorek *et al* 1985; Pu and Anderson 1984). The high and low affinity systems had K_m values for ethanolamine of 40 μM and 2.0 mM, respectively. The high uptake system appeared to be partially dependent on Na^+ (Yorek *et al* 1985). At present, only limited information is available on the modulation of ethanolamine uptake in mammalian tissues. However, the supply of exogenous ethanolamine was shown to alter the contributions of the decarboxylation of phosphatidylserine and the CDP-ethanolamine pathway to the net synthesis of phosphatidylethanolamine (Miller and Kent 1986; Yorek *et al* 1985).

(2) Ethanolamine Kinase (EC 2.7.1.82)

Ethanolamine kinase activity is recovered from the cytosol and catalyzes the phosphorylation of ethanolamine by ATP. Ethanolamine kinase catalyzes the committed step of the CDP-ethanolamine pathway. Whether the kinase that phosphorylates ethanolamine is the same enzyme that phosphorylates choline is a matter of debate. Attempts at purifying ethanolamine kinase generally result in the resolution of two separate ethanolamine kinase activities, one which contains appreciable choline kinase activity and one that does not (Brophy *et al* 1977). Kinetic studies on partially purified and purified preparations that contain both kinase activities reveal that ethanolamine and choline are mutually competitive inhibitors indicating that choline and ethanolamine share the same active site (Porter and Kent 1990; Brophy *et al* 1977). The molecular weight of a purified mammalian ethanolamine/choline kinase from mammalian liver was 47,000 (Porter and Kent 1990). An ethanolamine/choline kinase has also been purified from rat kidney with a molecular weight of 80,000 (Ishidate *et al* 1984). These kinase activities are induced to the same extent by hepatotoxic compounds and also appear to be immunologically identical (Ishidate *et al* 1985). Additionally, an ethanolamine/choline kinase has been cloned from yeast (Hosaka *et al* 1989) and was composed of 582 amino acids with a predicted molecular weight of 66,316. Disruption of this gene locus in yeast resulted in the concomitant loss of both choline kinase and ethanolamine kinase activities from yeast. However, the loss of ethanolamine kinase activity was not as complete as that for choline kinase.

Additionally, the K_m for ethanolamine is generally in the low mM range for these ethanolamine/choline kinase preparations while the K_m for choline is in the low μM range (Uchida and Yamashita 1990; Porter and Kent 1990) indicating choline is by far the preferred substrate for this enzyme. This is in comparison to a purified ethanolamine kinase from soya bean that has a Km of 8 μM for ethanolamine. The enrichment of ethanolamine kinase activity over that of choline kinase has recently been reported in human liver (Draus *et al* 1990). This enzyme appeared to exist as a dimer with a total molecular weight of 87,000. Additionally, partially purified preparations of the kinase (Brophy *et al* 1977) indicate the presence of separate enzymes responsible for the phosphorylation of ethanolamine and choline respectively.

(3) CTP: Phosphoethanolamine Cytidylyltransferase (EC 2.7.7.14)

The conversion of phosphoethanolamine and CTP to CDP-ethanolamine and PP_i by CTP: phosphoethanolamine cytidylyltransferase is found in the cytosol and is generally regarded as the rate limiting step in the synthesis of phosphatidylethanolamine via the CDP-ethanolamine pathway. This was established on the basis of both the distribution of radioactivity after labelled ethanolamine uptake as well as the ratio of pool sizes for the metabolites within the CDP-ethanolamine pathway (Sundler and Akesson 1974; Sundler and Akesson

1975b). Unlike phosphocholine cytidylyltransferase, phosphoethanolamine cytidylyltransferase does not appear to translocate to the membrane to regulate its activity. In fact, how phosphoethanolamine cytidylyltransferase activity is regulated within a cell still remains to be resolved. The enzyme has been purified from rat liver (Sundler 1975) and is composed of two subunits that from a dimer of molecular weight 100,000. The enzyme has a pH optimum of 7.8 and an apparent Km for CTP of 65 μM and for phosphoethanolamine of 53 μM. Enzyme kinetics studies on the purified enzyme revealed a sequential reaction mechanism in which CTP binds to the enzyme first followed by the binding of phosphoethanolamine. Subsequent to the release of the product PP_i is release of CDP-ethanolamine (Sundler 1975).

(4) CDP-Ethanolamine:1,2-Diacylglycerol Phosphoethanolaminetransferase (EC 2.7.8.1)

Phosphoethanolaminetransferase is an integral membrane protein thought to reside on the cytoplasmic side of the endoplasmic reticulum (Vance *et al* 1977; Ballas and Bell 1980). Vance and Vance (1988) have reported significant phosphoethanolaminetransferase activity in the Golgi apparatus. This enzyme is now known to be different from the phosphocholinetransferase for the biosynthesis of phosphatidylcholine. Phosphoethanolaminetransferase and

phosphocholinetransferase activities were separated by DEAE-sepharose column chromatography from the hamster liver (O *et al* 1989). A chinese hamster ovary cell line defective in phosphoethanolaminetransferase activity but with intact phosphocholinetransferase activity was the first genetic evidence for separate enzymes (Polokoff *et al* 1981). More recently, Hjelmstad and Bell (1987; 1988; 1990) have cloned the phosphoethanolaminetransferase and phosphocholinetransferase genes from yeast. The phosphoethanolaminetransferase gene product was also capable of utilizing CDP-choline as a substrate (Hjelmstad and Bell 1988). However, the phosphocholinetransferase gene product could not utilize CDP-ethanolamine (Hjelmstad and Bell 1987; Hjelmstad and Bell 1990; Hjelmstad and Bell 1991a). Phosphoethanolaminetransferase requires phosphatidylcholine for full activity and this activation of activity was competitively inhibited by phosphatidylethanolamine suggesting specific interaction points for phospholipids within the enzyme (Hjelmstad and Bell 1991). Another role for phosphoethanolaminetransferase has been proposed in the biosynthesis of the vinyl ether linked phospholipid, plasmenylethanolamine (Arthur and Page 1991; Morikawa *et al* 1987; Yorek *et al* 1985; Xu *et al* 1991). *In vitro* studies on the substrate specificity of phosphoethanolaminetransferase from rat brain indicated the enzyme was active towards diacylglycerol and alkenylglycerol (Ansell and Metcalfe 1971) indicating the enzyme may catalyze both reactions. Apart from the contributions to the understanding of phosphoethanolaminetransferase at a genetic

level, the enzyme has been difficult to solubilize (O *et al* 1989; Vecchini *et al* 1987) and a significant purification has not been achieved.

2. Phosphatidylethanolamine Biosynthesis from Phosphatidylserine

Overview

In addition to the CDP-ethanolamine pathway, phosphatidylethanolamine can be synthesized from phosphatidylserine by either the decarboxylation of phosphatidylserine or via base-exchange between phosphatidylserine and phosphatidylethanolamine (Fig. 3). The role of base-exchange in the net synthesis of phosphatidylethanolamine is believed to be $<10\%$ (Sundler *et al* 1974; Zelinski and Choy 1982b). Recent studies have demonstrated that in the absence of ethanolamine, the decarboxylation of phosphatidylserine is a major route for phosphatidylethanolamine biosynthesis in a baby hamster kidney cell line (Voelker 1984) as well as in chinese hamster ovary cells (Miller and Kent 1986).

(1) Base-Exchange Enzyme

There are believed to be two main base-exchange activities in mammalian cells (Tijburg *et al* 1989b). One activity appears to exchange serine with either

ethanolamine or choline (Kuge *et al* 1986a, 1986b, 1991), while the other is exclusive to ethanolamine. The phosphatidylethanolamine-serine base exchange activity is believed to be located on the cytoplasmic side of the endoplasmic reticulum (Bell *et al* 1981) and has been purified from rat brain microsomes (Suzuki and Kanfer 1985). This enzyme required Ca^{2+} and had a pH optimum of 7. The K_m values for ethanolamine and serine were 20 μm and 110 μM respectively. Kinetic results suggest an identical binding site for ethanolamine and serine on the enzyme (Suzuki and Kanfer 1985).

(2) Phosphatidylserine Decarboxylase (EC 4.1.1.65)

Phosphatidylserine decarboxylase is located on the inner membrane of the mitochondria (Voelker 1989a) but has not been extensively purified from mammalian sources. However, a cDNA clone encoding phosphatidylserine decarboxylase has been isolated from Chinese hamster ovary cells (Kuge *et al* 1991). This gene successfully complemented a previously identified phosphatidylserine/phosphatidylethanolamine biosynthetic defect in these cells (Nishijima *et al* 1986; Kuge *et al* 1986a, 1986b). The clone coded for a protein containing 370 amino acid residues and this clone had significant sequence homology with *Escherichia coli* phosphatidylserine decarboxylase (Li and Dowhan 1988).

III. REGULATION OF PHOSPHATIDYLETHANOLAMINE BIOSYNTHESIS

1. The CDP-ethanolamine Pathway

Although the regulation of phosphatidylcholine biosynthesis has been studied extensively over the years, there was little knowledge concerning the biosynthesis of phosphatidylethanolamine until the work of Sundler and Akesson in the mid 1970's. From initial studies on intact animals (Sundler 1973; Sundler and Akesson 1975a) and the later studies utilizing isolated hepatocytes (Sundler and Akesson 1975b), it became apparent that phosphoethanolamine cytidylyltransferase was the rate limiting step in CDP-ethanolamine pathway in the liver. In the isolated hamster heart, cytidylytransferase was also found to be rate-limiting at low levels of exogenous ethanolamine (Zelinski and Choy 1982b). Sundler and Akesson (1975a) proposed that that there are separate pools of phosphoethanolamine in the liver and that there is no rapid equilibration between the endogenous phosphoethanolamine pool and the phosphoethanolamine derived from newly imported ethanolamine. The CDP-ethanolamine pathway has been shown to be regulated by the exogenous supply of various metabolites. Increasing extracellular choline concentrations result in an increase in the intracellular concentration of choline in the isolated hamster heart (Zelinski and Choy 1982a) and in human retinoblastoma cells (Yorek *et al* 1986). This increased intracellular choline pool

is able to inhibit ethanolamine kinase resulting in decreased phosphatidylethanolamine biosynthesis.

2. Regulation of Phosphatidylethanolamine Biosynthesis from Phosphatidylserine

Phosphatidylethanolamine can be synthesized from phosphatidylserine by Ca^{2+} mediated base-exchange and by the decarboxylation of phosphatidylserine. The only known route for the synthesis of phosphatidylserine in mammalian cells occurs via base-exchange activities. However, the contribution of base-exchange towards phosphatidylethanolamine biosynthesis is believed to be quite small in both the liver and the heart (Sundler *et al* 1974; Zelinski and Choy 1982b). Ethanolamine was found to inhibit phosphatidylserine synthesis via base-exchange in rat hepatocytes (Bjerve 1985) and bovine aortic endothelial cells (Lipton *et al* 1990). The contribution of phosphatidylserine decarboxylation towards phosphatidylethanolamine formation could be increased by increasing the amount of exogenous serine (Bjerve 1985). The decarboxylation of phosphatidylserine is not considered a main route for phosphatidylethanolamine biosynthesis in mammalian tissues (Arthur and Page 1991; Xu *et al* 1991; Tijburg *et al* 1989a; Yorek *et al* 1985; Zelinski and Choy 1982b). However in a cultured baby hamster kidney cell line (Voelker 1984) and in Chinese hamster ovary cells (Miller and Kent 1986), phosphatidylserine decarboxylase is touted as the main route for

phosphatidylethanolamine biosynthesis. Addition of increasing concentrations of ethanolamine to Chinese hamster ovary cells (Miller and Kent 1986) and human retinoblastoma cells (Yorek *et al* 1985) resulted in an increased contribution of the CDP-ethanolamine pathway for phosphatidylethanolamine biosynthesis at the expense of the phosphatidylserine decarboxylase pathway. This is in contrast to baby hamster kidney cells where ethanolamine supply did not affect the roles of phosphatidylserine decarboxylase in the synthesis of phosphatidylethanolamine (Voelker 1984).

For phosphatidylethanolamine biosynthesis to occur via phosphatidylserine, phosphatidylserine must first be formed by base-exchange activities on the endoplasmic reticulum (Voelker 1985). Phosphatidylserine must then be transported to the inner mitochondrial membrane to be decarboxylated to phosphatidylethanolamine. In order for this process to occur rapidly enough to significantly contribute to phosphatidylethanolamine formation there must be a gradient of phosphatidylserine transport from the endoplasmic reticulum to the mitochondria. Vance (1991) has recently demonstrated that newly made phosphatidylserine is preferentially translocated between rat liver mitochondria and endoplasmic reticulum *in vitro*. This process did not require cytosolic proteins including phospholipid transfer proteins and was found to be slightly stimulated by ATP (Vance 1991). This transfer is proposed to occur via a collision-based

transfer of phosphatidylserine between mitochondrial and endoplasmic reticulum membranes. Analogous work utilizing rat liver subcellular fractions (Voelker 1989a) and permeabilized Chinese hamster ovary cells (Voelker 1989b) describes a process whereby ATP is not required for translocation but is required to place phosphatidylserine in an environment that will allow the collision-based transfer of phosphatidylserine from the endoplasmic reticulum to the mitochondria. Additional work in yeast has revealed that new phosphatidylethanolamine made by phosphatidylserine decarboxylation is preferentially transferred from the inner to the outer mitochondrial membrane (Simbeni *et al* 1990) for subsequent preferential transport back to the endoplasmic reticulum (Vance 1991). The rate-limiting step in this process is believed to be the collision-based transport of phospholipids from one organelle to another. This work is in contrast to genetic studies in Chinese hamster ovary cells expressing a cloned phosphatidylserine decarboxylase. Increasing the expression of phosphatidylserine decarboxylase was found to increase the contribution of this pathway to the biosynthesis of phosphatidylethanolamine indicating that the decarboxylation of phosphatidylserine may be the rate-limiting step in this pathway (Kuge *et al* 1991).

IV. ROLE OF PHOSPHATIDYLETHANOLAMINE IN SIGNAL TRANSDUCTION

There is a significant body of work devoted to the roles of phosphatidylinositol (Berridge 1987; Berridge and Irvine 1989) and phosphatidylcholine (Exton 1990; Pelech and Vance 1989) in signal transduction. Generally, an extracellular signalling molecule binds to a plasma membrane receptor resulting in a conformational change in the cytoplasmic domain of the receptor. This change in conformation is thought to activate a G-protein (Gilman 1987) by displacing GDP from the G-protein with the subsequent binding of GTP. The G-protein is inactivated by an intrinsic GTPase activity resulting in the conversion of GTP back to GDP. An activated G-protein will stimulate the activity of phospholipase C or D specific for phosphatidylinositol 4,5-bisphosphate or phosphatidylcholine (Fig. 5). Most signalling molecules, including vasopressin, acetylcholine, platelet-derived growth factor, and thrombin (Pelech and Vance 1989) stimulate the hydrolysis of phosphatidylinositol 4,5-bisphosphate into inositol 1,4,5-triphosphate and diacylglycerol. Inositol triphosphate mobilizes Ca^{2+} from intracellular stores while diacylglycerol, in concert with Ca^{2+}, activates a phospholipid dependent protein kinase, protein kinase C (Bell and Burns 1991). Protein kinase C phosphorylates a multitude of cellular proteins and thereby controls many cellular processes. The increase in intracellular Ca^{2+} is believed to result in the stimulation of both

phospholipase C and D activities specific for phosphatidylcholine. This process is thought to extend the signalling response by allowing for the continued release of diacylglycerol from the much larger phosphatidylcholine pool (Exton 1990; Billah and Anthes 1990).

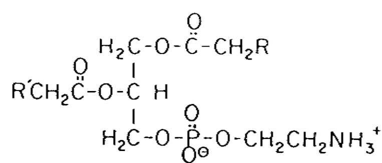
Although most of the attention has been focused on the roles of phosphatidylinositol 4,5-bisphosphate and phosphatidylcholine in signal transduction, the role of phosphatidylethanolamine has not been extensively explored. Prolactin is a polypeptide hormone that binds to receptors that are believed to act via cell signalling. Neither phosphatidylinositol 4,5-bisphosphate nor phosphatidylcholine are metabolized upon prolactin binding to its receptor, however in NB_2 lymphoma cells, phosphatidylethanolamine is turned over very rapidly. The resulting products are a mixture of phosphatidic acid and ethanolamine as well as diacylglycerol and phosphoethanolamine (Hafez and Costlow 1989). The role of these products in signal transduction was not pursued. Kiss and Anderson (1990) demonstrated that ATP stimulates the hydrolysis of phosphatidylethanolamine by phospholipase D in NIH 3T3 cells. ATP also stimulates the phospholipase C mediated hydrolysis of phosphatidylinositol 4,5-bisphosphate and the phospholipase D mediated hydrolysis of phosphatidylcholine. Sphingosine was found to have a potentiating effect on phosphatidylethanolamine hydrolysis. However, the hydrolysis of phosphatidylethanolamine by a

phospholipase D mediated mechanism could only be demonstrated in isolated cell membranes and was not detectable in intact cells (Kiss and Anderson 1990). In contrast to ATP, sphingosine was an effective stimulator of phosphatidylethanolamine hydrolysis in intact cells and this effect was stimulated by phorbol esters. This additive effect was in contrast to the hydrolysis of phosphatidylcholine where sphingosine inhibited the stimulatory effect of phorbol esters on phosphatidylcholine hydrolysis (Kiss and Anderson 1990). Phorbol esters have also been found to stimulate the hydrolysis of both phosphatidylcholine and phosphatidylethanolamine by a phospholipase D mediated activity in HeLa cells (Hii *et al* 1991). The hydrolysis of phosphatidylethanolamine by phospholipase D results in the release of phosphatidic acid and ethanolamine. The roles of these molecules in the generation of intracellular messages is undefined apart from the hydrolysis of phosphatidic acid to diacylglycerol for the stimulation of protein kinase C activity (Billah and Anthes 1990).

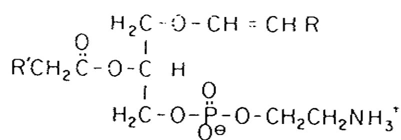
V. PLASMENYLETHANOLAMINE CATABOLISM

Overview

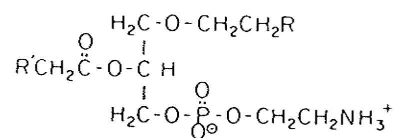
Phospholipids containing an *O*-alkenyl group at the C-1 position (plasmalogens) are abundant in many mammalian tissues (Horrocks and Sharma 1982) (Fig. 6). The most widely distributed plasmalogens are the 1-alkenyl-2-acyl-*sn*-glycero-3-phosphoethanolamine (plasmenylethanolamine) species (Horrocks and Sharma 1982). Despite their ubiquitous distribution, only limited information is available on the metabolism or function of these phospholipids (Paltauf 1984). Plasmenylethanolamine is most abundant in mammalian brains and hearts (Horrocks and Sharma 1982), however rat and hamster hearts are an exception with their cardiac tissue containing less than 7% plasmenylethanolamine compared to 30-50% found in the ethanolamine-containing phospholipids of other mammalian species. The high concentrations of plasmenylethanolamines in electrically active tissues such as the brain and the heart imply that they are involved in ion transport across membranes (Gross 1984). Plasmalogens may also serve as reservoirs for prostaglandin precursors due to the large proportion of arachidonic acid at the C-2 position (Gross 1985; Wykle *et al* 1973; Horrocks and Fu 1978). Recently, a 1-alkenyl-2-acetyl-*sn*-glycero-3-



phosphatidylethanolamine



plasmenylethanolamine



plasmanylethanolamine

Figure 6. Structure of Ethanolamine Containing Phospholipids.

phosphoethanolamine analogue of the platelet activating factor has been identified in human neutrophils (Tessner and Wykle 1987). The importance of plasmalogens in protecting cell membranes from oxidative stress with the 1-alkenyl bond functioning as an oxygen radical scavenger has been postulated (Morand *et al* 1988).

The 1-alkenyl bond of plasmenylethanolamine can be hydrolysed via two separate catabolic pathways (Fig. 7). In the mammalian brain and heart a microsomal plasmalogenase has been identified that cleaves plasmenylethanolamine to lysophosphatidylethanolamine and a fatty aldehyde (D'Amato *et al* 1975; Arthur *et al* 1985). An alternate route for the cleavage of the vinyl ether bond of plasmenylethanolamine involves the action of a putative phospholipase A_2 followed by a lysoplasmalogenase. A lysoplasmalogenase activity has been characterized in both liver (Alexander-Jurkowitz *et al* 1989) and brain (Gunawan and Debuch 1982) microsomes. In view of the irreversible damage to both cerebral and cardiac tissues by oxidative stresses during and after ischemia (Katz and Messineo 1981) and the putative protective role plasmalogens play in this process (Morand *et al* 1988), the identification and characterization of plasmalogenase activities in these tissues is highly desirable.

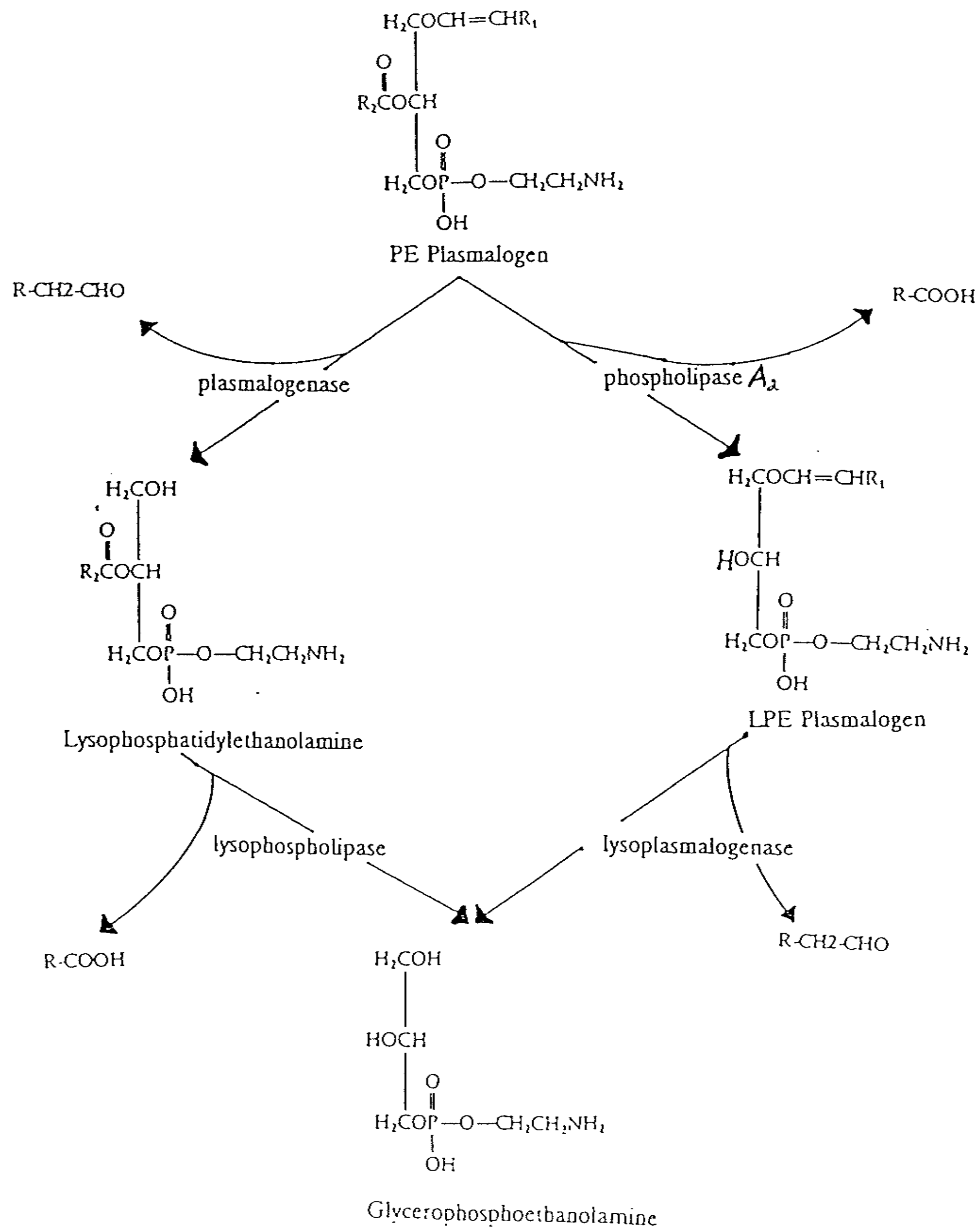


Figure 7. Pathways for the Hydrolysis of Plasmenylethanolamine.

1. Plasmalogenase

Since mammalian brain and heart are the most abundant source of plasmenylethanolamine, plasmalogenase activities have been studied most extensively in these tissues. Plasmalogenase for the hydrolysis of plasmenylethanolamine was initially characterized as a microsomal enzyme (Ansell and Spanner 1965; Horrocks and Fu 1978). The plasmalogenase activity from hamster heart microsomes was found to have a pH optimum of 8.5 and did not require cations for activity (Arthur *et al* 1985). Microsomal plasmalogenase activity has also been characterized in brain (Ansell and Spanner 1968; Dorman *et al* 1977; Horrocks and Fu 1978). It was initially believed that plasmalogenase required Mg^{2+} (Ansell and Spanner 1968) but this dependence was not substantiated by further investigation (Dorman *et al* 1977; D'Amato *et al* 1975). The plasmalogenase activity from rat (Ansell and Spanner 1968) and bovine (D'Amato *et al* 1975) brain was partially purified by obtaining an acetone-dried extract of whole brain. This enzyme activity was found to have similar properties to the microsomal enzyme.

2. Lysoplasmalogenase

An alternative route for the hydrolysis of the vinyl ether bond of

plasmenylethanolamine is believed to be via the action of a putative phospholipase A_2 followed by a lysoplasmalogenase. Lysoplasmalogenase activity has been identified in low amounts in the brain (Gunawan and Debuch 1982), although this is believed to be minor in comparison to the plasmalogenase activity. However, a significant amount of lysoplasmalogenase activity has been described in rat liver microsomes (Jurkowitz-Alexander 1989; Hirashima *et al* 1989). The enzyme was purified 200-fold and was found to utilize both lysoplasmenylethanolamine (K_m 42 μM) and lysoplasmenylcholine (K_m 5.5 μM) as substrates. This is in contrast to the plasmalogenase enzymes that utilize exclusively plasmenylethanolamine and do not hydrolyse plasmenylcholine (Arthur *et al* 1985). The optimal pH for lysoplasmalogenase for both substrates was 7 and the enzyme was not activated by divalent cations.

MATERIALS AND METHODS

A. MATERIALS

I. Experimental Animals

Male Syrian golden hamsters (125 ± 25 g) were used throughout the studies on phosphatidylethanolamine metabolism. Male guinea pigs (275 ± 25 g) were utilized for the study of plasmenylethanolamine catabolism. Both hamsters and guinea pigs were maintained on the appropriate Purina chow and tap water, *ad libitum*, in a light- and temperature-controlled room.

II. Chemicals

[1-^{3}H]Ethanolamine hydrochloride, [2-^{14}C]phosphoethanolamine, and [1-^{3}H]glycerol were products of Amersham International Limited (Oakville, Ontario). CDP-[1,2-^{14}C]ethanolamine was purchased from ICN Biomedicals (Costa Mesa, CA). [Methyl-^{3}H]choline chloride and L-[^{3}H(G)]serine were purchased from Dupont-NEN Research Products (Dorval, Quebec). Phosphatidyl-[1-^{3}H]ethanolamine was synthesized as described by McMaster and Choy (1992c). Ethanolamine, phosphoethanolamine, CDP-ethanolamine, serine, choline, glycine,

alanine, ATP, CTP, 0.2% ninhydrin spray, activated charcoal, aldehyde dehydrogenase, NAD^+, glutathione (reduced form), NADPH, cytochrome c (reduced form), iodine, potassium iodide, butylated hydroxytoluene, 2',7'-dichlorofluorescein, Triton QS-15, and polyoxyethylene sorbitan monolaurate (Tween 20) were obtained from Sigma Chemical Company (St. Louis, MO). Monomethylethanolamine and dimethylethanolamine were the products of Aldrich Chemical Company, Inc. (Milwaukee, WI). Phenylisothiocyanate and standard amino acid mixtures were purchased from Pierce Chemical Company (Rockville, IL). Pyridine, triethylamine and thin layer chromatography plates (Sil-G25) were purchased from Fisher Scientific (Ottawa, Ontario). Celite was obtained from Supelco Inc. (Oakville, Ontario). 1-Amino-2-naphthol-4-sulfonic acid was obtained from ICN Biomedicals (Cleveland, OH). AG 1-X8 anion exchange resin (100-200 mesh) and silicic acid (BIO-SIL A) for column chromatography were purchased from Bio-Rad Laboratories (Mississauga, Ontario). Phosphatidylcholine (pig liver), phosphatidylethanolamine (pig liver), phosphatidylserine (pig liver), and lysophosphatidylethanolamine (pig liver) were products of Serdary Research Laboratories (London, Ontario). BDH Limited (Poole, England) provided the 2% dimethyldichlorosilane in 1,1,1-trichloroethane solution. Sepharose 6B for column chromatography and a SuperPac Spherisorb 3μm ODS2 column (4 x 125 mm) equipped with a 3 μm ODS guard cartridge for HPLC were products of Pharmacia LKB Biotechnology (Uppsala, Sweden). Acetonitrile (HPLC-grade) and all other

chemicals were of the highest available grade and were acquired from the Canlab division of Baxter Diagnostics Corporation (Mississauga, Ontario).

B. METHODS

I. Studies on Intracellular Ethanolamine Pool Sizes

1. Ethanolamine pool size analysis

(1) Preparation of tissue sample.

Syrian golden hamsters weighing 120 ± 20 g were used, and sacrificed by decapitation. The heart, kidney, and liver (0.25 g) were removed and homogenized in 10 ml chloroform/methanol (1:1, v/v). In some experiments, 1 μCi of labelled ethanolamine was added to the homogenate. The homogenate was allowed to sit at room temperature for 30 min and was then centrifuged at 1,000 x *g* for 10 min. The supernatant was decanted and the pellet was extracted twice with 5 ml of chloroform/methanol (2:1, v/v) each time. The extracts were pooled, and the ratio of chloroform/methanol/0.9% KCl in the extract was adjusted to 4:2:3 (v/v/v). The mixture was centrifuged at 250 x *g* to facilitate phase separation. An aliquot (5 ml) of the aqueous phase was evaporated to dryness under a stream of nitrogen, and the sample was dissolved in 0.5 ml of 10 mM sodium phosphate buffer, pH 7.4. The sample solution was applied to a 2 x 0.5 cm charcoal/celite (1:2, w/w) column equilibrated with 10 mM sodium phosphate solution (pH 7.4)

containing 2% ethanol. Ethanolamine was eluted from the column with 10 ml of the same buffer. The volume of the eluent was reduced by evaporation.

(2) Coupling of ethanolamine with phenylisothiocyanate (PITC).

Coupling was conducted by a modified method of Heinrikson and Meredith (1984). The buffer in the sample was removed *in vacuo* with a Savant (Farmingdale, NY) SC100 vacuum concentrator, and 200 μl coupling buffer containing acetonitrile/pyridine/triethyl-amine/water (10:5:2:3, by vol.) was added. The solvent in the mixture was removed *in vacuo* and the sample was redissolved in another 200 μl of coupling buffer. The coupling reaction was started by the addition of 5-20 μl PITC and the reaction mixture was allowed to sit at room temperature for 15 min. The solvent in the reaction mixture was evaporated *in vacuo* and the resulting products were dissolved in 250 μl of water/acetonitrile (7:2, v/v).

(3) Analysis of PTCethanolamine by HPLC.

A Pharmacia LKB (Uppsala, Sweden) SuperPac Spherisorb 3μm ODS2 column (4 x 125 mm) equipped with a 3 μm ODS guard cartridge was used in this study. The column was equilibrated with 12.5 mM potassium phosphate, pH 6.4 at a

constant flow rate of 1.0 ml/min. After sample application (20 μl), the column was washed for 5 min with the equilibration buffer, followed by 30 min wash with a linear gradient of 0-40% acetonitrile. After the completion of the run, the column was washed with acetonitrile for 10 min and re-equilibrated with the equilibration buffer. The absorbance of the eluant was monitored at 254 nm by an Isco (Lincoln, NE) UV detector equipped with an HPLC cell. Data were collected in the first 30 min of the run, and the area of each peak was analyzed using a Beckman (Mississauga, Ontario) 450 Data System.

II. Studies on the Metabolism of Phosphatidylethanolamine in the Hamster Heart

1. Perfusion of the Isolated Hamster Heart

The isolated hamster heart was perfused in the Langendorff mode with Krebs-Henseleit buffer saturated with 95%O_2/5%CO_2 (Zelinski and Choy 1982b; Neely and Rovetto 1975). The isolated heart was stabilized by perfusion with Krebs-Henseleit buffer for 10 min, followed by perfusion with 0.04-1000 μM [1-^{3}H]ethanolamine (6.67 x 10^7 dpm/μmol) or 50 μM [1-^{3}H]glycerol (1.00 x 10^8 dpm/μmol) for 5-60 min. In some perfusions, 0.01 to 10 mM serine was added to the perfusate. Alternatively, some hamster hearts were perfused with 1 mM L-[^{3}H(G)]serine in the absence or presence of 50 μM ethanolamine. The pressure at the canula was maintained at 80 mm Hg with a flow rate of 4.0-4.5 ml/min. After the assigned period of perfusion, the radioactivity in the vascular and intercellular space of the heart was removed by perfusion with 10 ml of Krebs-Henseleit buffer followed by 5 ml of air. The heart was cut open, blotted dry, and the wet weight determined. Subsequently, the heart was homogenized in $CHCl_3/CH_3OH$ (1:1, by vol.) with two 20 sec bursts of a Polytron homogenizer (Brinkmann Instruments; Rexdale, Ontario) equipped with a PT-30 probe. The homogenate was centrifuged at 2,000 *g* to yield a clear tissue extract. The pellet was washed twice with $CHCl_3/CH_3OH$ (2:1, by vol) and the supernatants were

pooled to the original extract. An aliquot of the pooled extract was taken for radioactivity determination. Chloroform and water were added to the pooled extract to cause separation into aqueous and organic phases.

2. Analysis of Phospholipids

Phosphatidylethanolamine, phosphatidylserine and phosphatidylcholine from the organic phase were separated from other lipid metabolites by thin-layer chromatography in a solvent system containing $CHCl_3/CH_3OH/NH_4OH/H_2O$ (70:30:4:2, by vol.). Lysophosphatidylethanolamine and phosphatidylethanolamine were also separated from other phospholipids by thin-layer chromatography with the solvent system containing $CHCl_3/CH_3OH/NH_4OH$ (65:25:5, by vol.). The identity of lipid samples was determined utilizing known standards. For the determination of radioactivity, the lipid fractions in the chromatogram were visualized by iodine staining and the appropriate bands were removed and the radioactivity determined by scintillation counting. For the determination of pool sizes, the lipids were visualized with 0.25% 2',7'-dichlorofluorescein in ethanol (w/v) under UV light. Lipid samples were removed and the lipids were eluted by washing the silica gel three times with $CHCl_3/CH_30H/H_2O/CH_3COOH$ (50:39:10:1; by vol.). The extracts were pooled and 4 ml of 4M NH_4OH was added to allow for phase separation. Recovery of phospholipids from the thin-layer

chromatography plate was over 95% based on the recovery of radioactivity and lipid phosphorous from known standards. The organic phase was dried under nitrogen and the lipids were dissolved in $CHCl_3$. An aliquot was utilized for lipid phosphorus determination by the method of Bartlett (1959).

3. Analysis of Radioactivity in Ethanolamine-containing Metabolites

An aliquot of the aqueous phase was dried under nitrogen and resuspended in water. The ethanolamine-containing metabolites were separated by thin-layer chromatography with a solvent containing ethanol/2% ammonium hydroxide (1:2, by vol.) (Sundler and Akesson 1975b) and visualized by spraying the chromatogram with a 0.2% ninhydrin solution. The appropriate fractions were removed from the chromatogram and the radioactivity in each fraction was determined by scintillation counting.

4. Subcellular Fractionation of the Heart

Hamsters were sacrificed and the hearts were excised and rinsed in ice cold 0.25M sucrose in 10 mM Tris-HCl (pH 7.4). The hearts were cut into small pieces and homogenized with two 20 sec bursts of a Polytron probe (PT-30) to yield a 15% homogenate. The homogenate was centrifuged at 10,000 x g for 20 min. The

resulting supernatant was recentrifuged at 100,000 x g for 60 min. The supernatant obtained from the final centrifugation was designated the cytosolic fraction. The microsomal pellet was resuspended in 0.25M sucrose in 10 mM Tris-HCl (pH 7.4) with a Dounce homogenizer utilizing a type A pestle. Ethanolamine kinase, choline kinase, and CTP:phosphoethanolamine cytidylyltransferase activities were determined in the cytosolic fraction. Ethanolaminephosphotransferase activity was determined in the microsomal fraction.

5. Synthesis of Phosphatidyl-[1-^{3}H]ethanolamine

The labelled phosphatidylethanolamine used for *in vitro* enzyme assays was synthesized *in vivo* in hamster tissues by interperitoneal injection of the hamster with labelled ethanolamine (100 μCi) in 1 ml saline. The hamster was sacrificed 16 hours after injection, and the liver, kidney, and spleen were removed. The organs were homogenized in chloroform/methanol (1:1, by vol.) and water and chloroform were added to facilitate phase separation. Phosphatidylethanolamine was purified from the lipid extract by silicic acid chromatography (Sweeley 1969). The specific radioactivity of the phosphatidylethanolamine obtained was 2.29 x 10^5 dpm/μmol.

6. Enzyme Assays

(1) Ethanolamine kinase (EC 2.7.1.82)

Ethanolamine kinase activity was determined in the cytosol according to the method of Schneider and Vance (1978). The reaction mixture contained 80 mM sodium glycylglycine (pH 8.5), 1 mM [1-^{3}H]ethanolamine, 3 mM $MgCl_2$, 3 mM ATP, and heart cytosol in a final volume of 100 μl. The reaction mixture was incubated for 30 min at 37°C. The reaction was stopped by placing the tubes in a boiling water bath for 5 min. The protein was pelleted by centrifugation and 50 μl of the supernatant was applied to a thin-layer chromatography plate with phosphoethanolamine as carrier. The plate was developed in ethanol/2% ammonium hydroxide (1:2; v/v) and the phosphoethanolamine band was visualized by ninhydrin spray. This band was removed and radioactivity was determined by scintillation counting.

(2) Choline kinase (EC 2.7.1.32)

Choline kinase activity was determined in the cytosol according to the method of Weinhold and Rethy (1974). A reaction mixture contained 50 mM Tris-HCl (pH 8.0), 5 mM ATP, 5 mM [Methyl-^{3}H]choline, and heart cytosol protein in a final

volume of 200 μl. The reaction mixture was incubated at 37°C for 15 min and was stopped by placing the tubes in a boiling water bath for 5 min. The reaction mixture was placed on an AG 1-X8 anion exchange column (0.5 cm x 3.0 cm) and washed with 10 ml water. The column was subsequently washed with 1.5 ml of 1.0 M NaOH and 1.5 ml of 0.1 M NaOH for the elution of phosphocholine from the resin. Radioactivity associated with the phosphocholine fraction was determined by scintillation counting.

(3) CTP:phosphoethanolamine cytidylyltransferase (EC 2.7.7.14)

CTP:phosphoethanolamine cytidylyltransferase activity was determined in the cytosol according to the method of Sundler (1975). The reaction mixture contained 20 mM Tris-HCl (pH 7.8), 10 mM $MgCl_2$, 2 mM CTP, 0.5 mM [2-^{14}C]phosphoethanolamine and heart cytosol in a final volume of 100 μl. The reaction was incubated at 37°C for 20 min and was stopped by placing the tubes in a boiling water bath for 5 min. The protein was pelleted by centrifugation and an aliquot of the supernatant was applied to a thin-layer chromatography plate with CDP-ethanolamine carrier. The same developing solvent as that for ethanolamine kinase was used. The CDP-ethanolamine fraction was visualized with ninhydrin spray and this band was removed and the radioactivity determined by scintillation counting.

(4) Phosphoethanolaminetransferase (EC 2.7.8.1)

Phosphoethanolaminetransferase activity was assayed as described by O *et al* (1989). The reaction mixture contained 100 mM Tris-HCl (pH 8.5), 10 mM $MnCl_2$, 1 mM EDTA, 1 mM diacylglycerol (prepared in 0.015% Tween 20 (w/v) by sonication), 0.2 mM CDP-[1,2-^{14}C]ethanolamine and microsomal protein in a final volume of 1.0 ml. The reaction was incubated at 37°C for 15 min and terminated by the addition of 3 ml of $CHCl_3$:CH_3OH (2:1, v/v) to the reaction mixture. Phase separation was obtained by the addition of 0.5 ml water. The organic phase was washed twice with 2 ml of 40% methanol and the radioactivity in the lower phase was determined by scintillation counting. Analysis of the radioactivity in the organic phase by thin-layer chromatography revealed that over 98% of the radioactivity was associated with the phosphatidylethanolamine fraction.

(5) The hydrolysis of phosphatidylethanolamine

The hydrolysis of phosphatidylethanolamine was determined in the post-mitochondrial fraction (Cao *et al* 1987). The reaction mixture contained 1 μmol of phosphatidyl-[1-^{3}H]ethanolamine, 20 mM Tris/HCl (pH 7.4), 10 mM $CaCl_2$ and

the enzyme in a total volume of 0.5 ml. The reaction was incubated at 37°C for 15 min and terminated by the addition of 1.5 ml $CHCl_3/CH_3OH$ (2:1, by vol). Water (0.25 ml) was added to the mixture to facilitate phase separation. The radioactivity associated with the lysophosphatidylethanolamine fraction in the organic phase and the phosphoethanolamine, ethanolamine and glycerophosphoethanolamine fractions in the aqueous phase were determined by thin-layer chromatography as described in previous sections.

7. Serine and Ethanolamine Pool Size Analyses

Serine and ethanolamine pool sizes were determined by reverse-phase HPLC (McMaster and Choy 1992b). Briefly, a tissue extract was applied to a charcoal/celite (1:2, by wt.) column for the removal of nucleotides and other aromatic molecules. Serine and ethanolamine were eluted from the column (0.5 x 3 cm) with 15 ml of 10 mM sodium phosphate buffer (pH 7.4) containing 2% ethanol. An aliquot of the sample was reacted with phenylisothiocyanate (Heinrikson and Meredith 1984) and the phenylthiocarbamyl derivatives were separated from other metabolites by reverse-phase HPLC with a Pharmacia LKB Spherisorb ODS2 column (4 x 125 mm). The column was equilibrated with 10 mM sodium phosphate (pH 6.4) and subsequent to sample application, a linear gradient of 0-40% acetonitrile was applied over 30 min. Sample detection was

monitored at 254 nm with an ISCO UA-5 absorbance detector equipped with a 190 μl HPLC cell. Phenylthiocarbamylserine was eluted as a single symmetrical peak at 6 min after sample application and its identity was confirmed by the addition of authentic standards to some samples. Phenylthiocarbamylethanolamine eluted as a single symmetrical peak with a retention time of 17.5 min as noted in previous sections. Peak areas were analyzed by a Beckman 450 Data Control System.

III. Studies on the Catabolism of Plasmenylethanolamine

1. Preparation of Plasmalogens for Enzyme Assays.

Plasmalogens were prepared as previously described (Arthur *et al* 1985). Briefly, lipids were extracted from porcine hearts by the method of Folch *et al.* (1957) in the presence of 0.5% butylated hydroxytoluene (w/v). The volume was reduced *in vacuo* and the lipid sample was dissolved in chloroform and applied to a silicic acid column. The individual phospholipids were eluted from the column with increasing amounts of methanol in chloroform (Sweeley 1969). Fractions from the column were analyzed by thin-layer chromatography with a solvent containing $CHCl_3/CH_3OH/CH_3COOH/H_2O$ (70/30/2/4; by vol). The fractions containing diradylglycerophosphoethanolamine were pooled. Phosphatidylethanolamine in the pooled diradylglycerophosphoethanolamine fraction was destroyed by the preferential hydrolysis of the ester bonds of phosphatidylethanolamine by mild alkaline hydrolysis with 0.35 M NaOH in 96% methanol for 20 min as described by Renkonnen (1963). Lysoplasmenylethanolamine was prepared by hydrolysing the diradyl-glycerophosphoethanolamine fraction in 0.35 M NaOH in 96% methanol for 45 min. The plasmenylethanolamine and lysoplasmenylethanolamine obtained after alkaline hydrolysis were repurified by silicic acid column chromatography. The purity of the plasmalogen fractions was assessed by

determining the ratio of the vinyl ether content and the total phosphorus content in the samples. Only preparations with purity greater than 96% were used for enzyme assays.

2. Preparation of Subcellular Fractions from Guinea Pig Tissues.

Guinea pig tissues were removed and placed on ice. The tissue was homogenized in 0.25 M sucrose in 10 mM Tris-HCl (pH 7.4) with two 20 sec bursts of a Polytron probe (PT-30) at a speed setting of six. Alternatively, brain tissue was also homogenized in a Potter-Elvehjem homogenizer equipped with a teflon pestle. The tissue homogenates were centrifuged at 10,000 x *g* for 20 min and the supernatant was centrifuged again at 100,000 x *g* for 60 min. The supernatant obtained from the last centrifugation was removed with a Pasteur pipette and designated as the cytosolic fraction. The precipitate containing the microsomal pellet was dispersed in the homogenizing buffer with a Dounce homogenizer equipped with a type A pestle.

3. Plasmalogenase Assays.

(1) Substrate disappearance method

All glassware including test tubes were treated with a 2% dimethyldichlorosilane in 1,1,1-trichloroethane solution to minimize the adherence of lipids to glass containers. Plasmalogenase activity was monitored by the disappearance of the substrate and the result obtained from this assay was later confirmed by the spectrophotometric method which was based on the appearance of aldehyde in the reaction (D'Amato *et al* 1975; Arthur *et al* 1985). In the assay based on the disappearance of the substrate, purified plasmenylethanolamine (2 μmol) was suspended in 1 ml of 10 mM Tris-HCl (pH 7.4) containing 0.05% Tween 20. The mixture was sonicated in a water bath until translucent. The reaction mixture (1.5 ml) contained 300 nmol dispersed plasmalogen, 50 mM Tris-HCl (pH 7.4) and an enzyme preparation containing 0.75 - 1.25 mg of protein. The reaction was initiated by the addition of the enzyme and the mixture was incubated at 37°C for 15 min. Control tubes contained either no enzyme or enzyme that had been incubated at 100°C for 5 min. At 0 and 15 min of incubation, 600 μl was removed from the reaction mixture and placed into a tube containing 1.5 ml $CHCl_3/CH_3OH$ (2/1, v/v). Water and chloroform were added to cause phase separation. The upper phase was removed and an aliquot of the lower phase was assayed for vinyl ether content spectrophotometrically at 355 nm (Gottfried and Rapport 1962). Plasmalogenase activity was calculated from the difference in vinyl ether content between the 0 and 15 min time-points. Total enzyme activity was calculated from the product of the specific activity of the enzyme and the total

protein content in the subcellular fraction.

(2) Appearance of product method

The production of aldehyde from the plasmalogenase reaction was monitored by a coupled enzyme assay. In brief, the long-chain aldehyde released from the reaction was converted to fatty acid by an aldehyde dehydrogenase with the concomitant production of NADH being monitored spectrophotometrically (Arthur *et al* 1985). The substrate (2 μmol of plasmenylethanolamine) was suspended in 1 ml of 0.2% Triton QS-15 by sonication. The reaction mixture (1 ml) contained 50 mM Tris-HCl (pH 8.0), 100 mM KCl, 7 mM glutathione, 10 mM NAD^+, 2 units aldehyde dehydrogenase and 300 nmol plasmenylethanolamine. The reaction was initiated by the addition of 10-50 μg of protein from the appropriate subcellular fraction and incubated at 37°C with the absorbance of the mixture monitored at 340 nm for at least 5 min. The reference cuvette contained all the components in the sample cuvette except the enzyme. Triton QS-15 was used in this assay since aldehyde dehydrogenase activity was found to be severely inhibited by Tween-20. However, plasmalogenase activity in both cytosolic and microsomal fractions was only slightly inhibited by Triton QS-15 (10-20%).

4. The Determination of Marker Enzyme Activities.

The degree of microsomal contamination in the cytosolic fraction was determined by the activities of known microsomal marker enzymes in the cytosol. NADPH-cytochrome c reductase (Masters *et al* 1967) and phosphoethanolaminetransferase activities (O *et al* 1989) were used as microsomal markers in the brain. Phosphoethanolaminetransferase and 5'nucleotidase activities (Bers 1979) were used as microsomal markers in the heart.

IV. Analytical Procedures

1. Determination of Lipid Phosphorus

The lipid phosphorus content in organic phases was determined by the method of Bartlett (1959). An aliquot of the sample was removed and evaporated under nitrogen. Inorganic phosphorus was used as standard (0-10 μg/tube). Inorganic phosphorous standard was prepared by dissolving 0.011 g of KH_2PO_4 in 250 ml water (10 μg phosphorous/ml). 1.1 ml Perchloric acid (70%, w/w) was added to each sample tube and the mixture was incubated at 160°C for 2 hrs. The mixture was allowed to cool and the appropriate amount of inorganic phosphorous was added to the standard tubes in a volume of 1 ml. Subsequently, the volume of each tube was brought up to 9 ml with water and 750 μl of 5% ammonium molybdate (w/v) was added and the tubes were vortexed. A 250 μl solution of ANSA (1-amino-2-naphthol-4-sulfonic acid) was added and the tubes were vortexed. The tubes were placed in a boiling water bath for 10 min and then allowed to cool to room temperature. Absorbance was subsequently monitored at 830 nm. ANSA was prepared by dissolving 11.7 g sodium sulfite (anhydrous), 0.2 g sodium metabisulfite, and 0.25 g of ANSA in 100 ml of water. This solution could be stored in the dark at room temperature for up to two months.

2. Plasmalogen (vinyl ether) Determinations

Plasmalogen (vinyl ether content) was assessed by the iodine determination method of Gottfried and Rapport (1962). Five identical samples, along with five blank tubes were evaporated under nitrogen and redissolved in 0.5 ml methanol. The tubes were sonicated briefly to disperse the lipid. The tubes were warmed at 65°C for 2 min and mixed well. To three of the five tubes, 0.5 ml of freshly prepared 0.3 mM iodine in 3% KI (w/v) was added. To the other two tubes, 0.5 ml of freshly prepared 3% KI (w/v) was added. The tubes were vortexed and allowed to stand at room temperature for 20 min. Subsequently, 4 ml of ethanol was added and the tubes were mixed well. Absorbance was read at 355 nm.

3. Protein Determination

Protein concentrations of subcellular fraction were determined by the modified method of Lowry *et al* (1951). Bovine serum albumin (1 mg/ml) was used as standard (0-100 μg). To each tube was added 0.1 ml 5% sodium deoxycholate (w/v) and water to bring the volume of each tube to 1 ml. Solution A was made by dissolving equal volumes of 1% copper sulfate (w/v) and 2% potassium sodium tartarate (w/v). Solution B was made by mixing 1 ml of solution A with 50 ml of 2% sodium carbonate (w/v) in 0.1 M NaOH. Subsequently, 4 ml of solution B was

added to each tube and the tubes were mixed and allowed to stand at room temperature for 10 min, and then 0.5 ml phenol reagent was added and mixed immediately. Tubes were incubated at 60°C for 10 min. Asorbance was read at 730 nm.

4. Radioactivity Determination

Radioactivity was determined using an LKB liquid scintillation counter (1211 MiniBeta) and the counting efficiency was calculated by the channels' ratio calibration method.

5. Statistical Analysis

The student's t-test was used for all statistic analyses. The minimum significance level was set at $p < 0.05$. All results in these studies are expressed as the mean $\pm$ standard deviation of at least three separate experiments except where otherwise indicated. The points on all figures have standard deviations of less than 20% of the mean.

EXPERIMENTAL RESULTS

A. DETERMINATION OF INTRACELLULAR ETHANOLAMINE POOL SIZES

I. Utilization of PITC To Determine Ethanolamine Pool Sizes

The regulatory role of ethanolamine kinase in the CDP-ethanolamine pathway was established quite early in our studies on phosphatidylethanolamine biosynthesis. One reason for ethanolamine kinase to become rate-limiting clould be due to an increase in intracellular ethanolamine pool size. It was therefore necessary to establish a method for the determination of intracellular ethanolamine levels. A survey of the literature revealed that there were no methods readily available for the determination of ethanolamine levels in mammalian tissues. To this end, we developed a procedure that utilized phenylisothiocyanate (PITC) as a precolumn derivatization step for the subsequent separation, identification, and quantitation of ethanolamine. This procedure was employed for subsequent studies due to its simplicity, sensitivity and ease in sample detection, as well as the stability of the derivatized products.

1. Conversion of Ethanolamine to its Phenylthiocarbamyl Derivative

The conversion of ethanolamine into its phenylthiocarbamyl (PTC) derivative was investigated. Known amounts (0.1-2.5 μmol) of ethanolamine were used to react with PITC to form PTCethanolamine. After the reaction, the unreacted PITC was removed by evaporation *in vacuo* and the amount of PTCethanolamine formed was determined spectrophotometrically. Based on the molar extinction coefficient of PTCamino acids at 254 nm (Heinrikson and Meredith 1984), over 98% of the ethanolamine in the reaction mixture was converted into PTCethanolamine.

2. Separation and Identification of PTCethanolamine by HPLC

The ability to separate and identify PTCethanolamine by reverse-phase HPLC was examined (McMaster and Choy 1992b). Under the conditions outlined in Materials and Methods, PTCethanolamine was eluted as a single peak with a retention time of 17.5 min (Fig. 8a). When a PTCamino acid mixture (derived from a standard amino acid mixture) was applied to the column, none of the amino acid derivatives displayed a retention time between 16.5-18.5 min (Fig. 8b). When a mixture of PTCethanolamine and PTCamino acids was applied to the column, PTCethanolamine was eluted as a single peak with a retention time of 17.5 min (Fig. 8c).

The amount of PTCethanolamine applied *vs* peak area (Fig. 9) obtained by reverse-

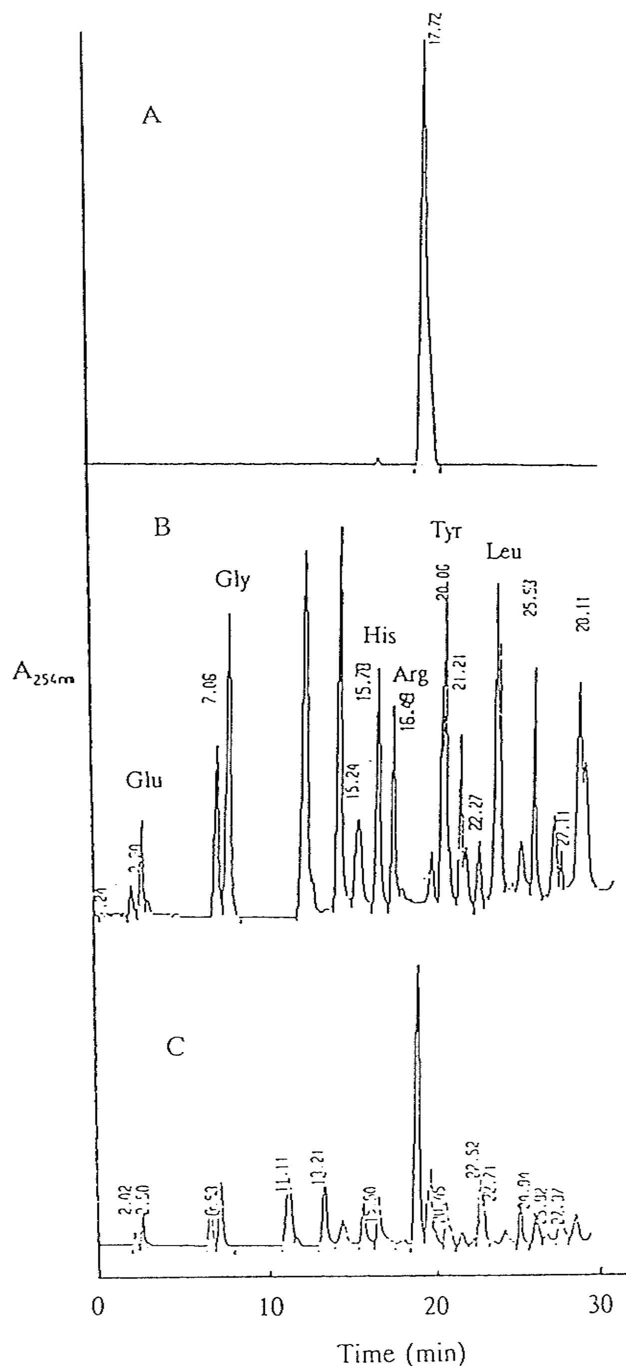


Figure 8. Separation of PTCethanolamine by reverse-phase HPLC. Subsequent to sample application (20 μL), the column was washed for 5 min with 12.5 mM potassium phosphate buffer, pH 6.4, followed by 30 min wash with a linear gradient of 0-40% acetonitrile. The flow rate was 1 ml per min, and the numbers shown are the retention times for each peak. (a) PTCethanolamine, (b) PTCamino acids, (c) mixture of PTCethanolamine and PTCamino acids.

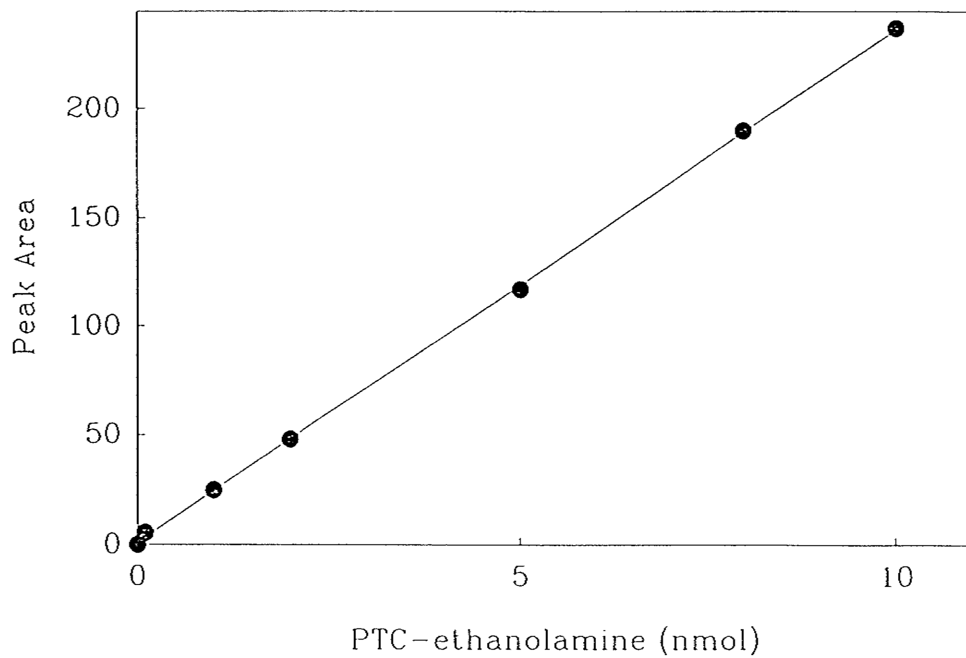


Figure 9. The quantitation of standard PTCethanolamine by reverse-phase HPLC. Known amounts of PTCethanolamine were applied to the column and the corresponding peak areas detected at 254 nm are depicted. Each point is the mean of two separate determinations.

phase HPLC was examined. The quantitation of PTCethanolamine by peak area was found to be linear when 0.10-10.0 nmol PTCethanolamine was applied to the column. The sensitivity of the determination could be further enhanced by an increase in the sensitivity of the detector unit.

3. Extraction of Ethanolamine from Hamster Tissues and Analysis of Intracellular Pool Sizes

A chloroform/methanol mixture was used for the extraction of ethanolamine from the tissue. The tissue was homogenized in 40 volumes of chloroform/methanol (1:1, v/v) and the homogenate was centrifuged to obtain a clear extract. The pellet was re-extracted twice with chloroform/methanol (2:1, v/v). A 0.9% KCl solution was added to the pooled extract to cause phase separation. When labelled ethanolamine was added to the tissue homogenate, 99% of the radioactivity was recovered in the aqueous phase. The partition of ethanolamine into the aqueous phase eliminated the contamination of lipid material in the sample. However, the aqueous phase contained nucleotides and other metabolites whose absorbance at UV range might interfere with the analysis of PTCethanolamine. The majority of these metabolites were removed by charcoal chromatography. The efficiency of the column to remove these contaminants (99.5%) was monitored by determining the absorbance of the sample at 260 nm and 280 nm before and after charcoal chromatography. The yield

of ethanolamine was 93% (Table 1).

For the analysis of ethanolamine content, two aliquots were taken from the aqueous phase of each sample. A known amount of ethanolamine (100 nmol), which served as an internal standard, was added to one of the aliquots. Both aliquots were reacted with PITC to obtain the PTC derivatives. After the reaction, the preparation was resuspended in 250 μl of water/acetonitrile (7:2, v/v) and a 20 μl fraction was analyzed by reverse-phase HPLC. A typical chromatogram is shown in Fig. 10a. The chromatogram of an identical sample containing the internal standard is depicted in Fig. 10b. The internal standard resulted in an increase in the size of the peak corresponding to the authentic PTCethanolamine standard with a retention time of 17.5 min. The increased peak area of the sample containing the internal standard was 8 nmol (Fig. 10) which corresponded to the amount of internal standard we added to the sample. Our results indicated that all the ethanolamine in the sample was being derivatized to PTCethanolamine. In some samples, labelled ethanolamine was added to the tissue homogenate and the sample was processed and derivatized as per Materials and Methods. The eluant from the HPLC was collected into 0.5 ml fractions and the radioactivity in each fraction was determined. The fraction corresponding to the 17.5 min time point contained the radioactivity which further confirms this peak as PTCethanolamine. The pool sizes of ethanolamine in hamster heart, liver and kidney are shown in Table 2.

TABLE 1

Ethanolamine Recovery During Pool Size Analysis[a]

Procedure	% Yield[b]
Tissue homogenate	100
Aqueous phase	99
Charcoal chromatography	93

[a][1-^{14}C]Ethanolamine (1 μCi) was added to the tissue homogenate, and the yield after each step was calculated from the amount of radioactivity recovered.

[b]The values are the mean of three separate experiments.

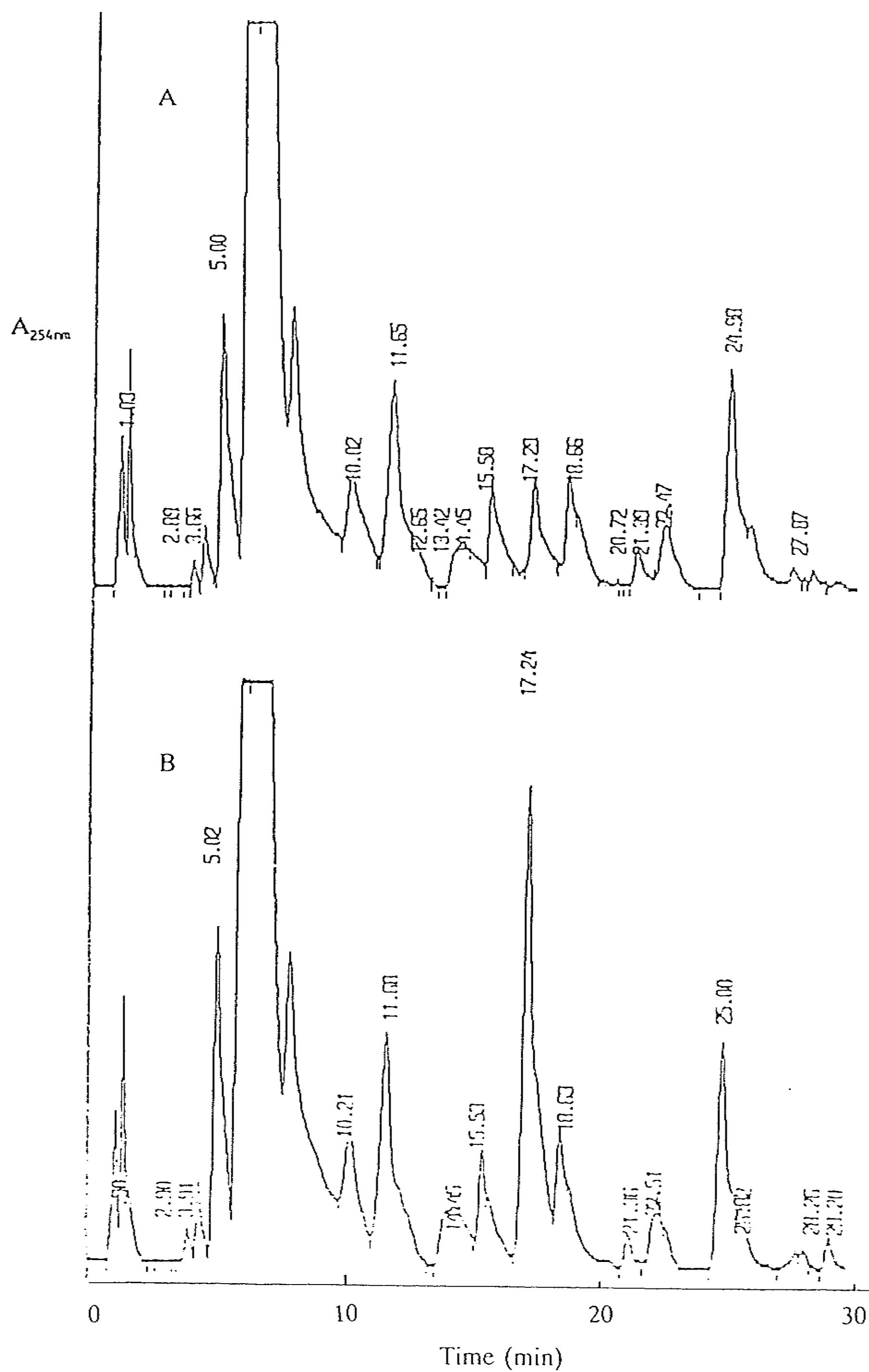


Figure 10. The quantitation of ethanolamine in hamster heart. Ethanolamine was extracted from the tissue and derivatized to PTCethanolamine. The derivatized tissue extract was analyzed by reverse-phase HPLC. (a) The elution profile of the tissue extract, and (b) the elution profile of the tissue extract containing an internal standard of ethanolamine.

TABLE 2

Pool Sizes of Ethanolamine in Hamster Tissues[a]

Tissue	Pool size of ethanolamine
Heart	1.07 ± 0.07
Liver	0.92 ± 0.15
Kidney	1.11 ± 0.32

[a]Each value represents the mean ± the standard deviation of three separate sets of experiments, each determined in duplicate. The pool sizes of ethanolamine in hamster tissues are expressed as μmol/g wet weight.

B. REGULATION OF PHOSPHATIDYLETHANOLAMINE BIOSYNTHESIS BY EXOGENOUS ETHANOLAMINE

I. Ethanolamine as a Precursor for Phosphatidylethanolamine Biosynthesis

Since the majority of phosphatidylethanolamine is synthesized via the CDP-ethanolamine pathway in the hamster heart (Zelinski and Choy 1982b), we postulated that the exogenous supply of ethanolamine could affect phosphatidylethanolamine biosynthesis. The only known intracellular supply of ethanolamine arises from base-exchange activity between phosphatidylethanolamine and other phospholipids, most notably phosphatidylserine. However, base-exchange activity is low in most mammalian tissues including the mammalian heart (Zelinski and Choy 1982b; Arthur and Page 1991), and this activity is generally believed to be too low to supply the cell's ethanolamine needs (Vance 1985). Although ethanolamine is required for the growth of cultured hybridoma cells (Murakami *et al* 1982), there is no known dietary deficiency of ethanolamine in mammals. How mammalian cells supply their ethanolamine needs in the absence of an exogenous supply of ethanolamine is still a basic question in the metabolism of phospholipids (Vance 1985).

1. Ethanolamine Uptake and its Incorporation into Phosphatidylethanolamine

In hamster hearts, the uptake of ethanolamine was linear between 0.04-50 μM ethanolamine in the perfusate (Table 3). However, the efficiency to incorporate the labelled ethanolamine into phosphatidylethanolamine was not linear with ethanolamine uptake. When hearts were perfused with 0.04 μM ethanolamine, 70% of the labelled ethanolamine was incorporated into phosphatidylethanolamine. At 0.4 μM ethanolamine in the perfusate, the efficiency of incorporation was decreased to 40%. Further decreases in the efficiency of incorporation were observed at higher exogenous ethanolamine concentrations (Table 3).

2. Analysis of Ethanolamine-containing Metabolites

The distribution of radioactivity throughout the metabolites of the CDP-ethanolamine pathway was examined (Table 4). At low concentrations of ethanolamine in the perfusate (0.04 and 0.1 μM), the majority of labelled material was found in the phosphoethanolamine fraction. At higher ethanolamine concentrations (0.4-1,000 μM), the majority of the radioactivity was accumulated in the ethanolamine fraction, with a corresponding decrease in radioactivity in the phosphoethanolamine fraction. The percentage distribution of CDP-ethanolamine remained relatively constant under all experimental conditions.

TABLE 3

Ethanolamine Uptake and Incorporation into Phosphatidylethanolamine

Hamster hearts were perfused with [1-^{3}H]ethanolamine in Krebs-Henseleit buffer for 30 min at 37°C. The specific radioactivity of ethanolamine was 3.33 x 10^6 dpm/nmol (#) or 3.33 x 10^4 dpm/nmol (*). The uptake of ethanolamine was estimated from the total tissue extract after perfusion. Each value represents the mean ± standard deviation of four separate experiments.

[Ethanolamine] in perfusate	Uptake of [1-^{3}H] ethanolamine	Radioactivity in phosphatidylethanolamine
	(dpm x 10^{-6}/g heart)	
0.04 μM #	0.20 ± 0.09	0.14 ± 0.02
0.1 μM #	0.49 ± 0.15	0.35 ± 0.05
0.4 μM #	1.66 ± 0.31	0.66 ± 0.22
50 μM *	1.94 ± 0.43	0.58 ± 0.08
250 μM *	3.60 ± 0.69	1.00 ± 0.15
1000 μM *	6.72 ± 1.34	1.46 ± 0.16

TABLE 4

Radioactivity Incorporation into Ethanolamine-containing Metabolites

Hamster hearts were perfused with [1-^{3}H]ethanolamine and the radioactivities incorporated into the ethanolamine-containing metabolites were determined. The experimental conditions and symbols used are the same as described in Table 1. The percentage of radioactivity associated with each of the ethanolamine-containing metabolites are indicated in parentheses.

[Ethanolamine] in perfusate	ethanolamine	phospho-ethanolamine	CDP-ethanolamine
		(dpm x 10^{-6}/g heart)	
0.04 μM #	0.019 ± 0.005 (30%)	0.035 ± 0.004 (56%)	0.009 ± 0.002 (14%)
0.1 μM #	0.051 ± 0.015 (38%)	0.071 ± 0.010 (53%)	0.013 ± 0.002 (9%)
0.4 μM #	0.511 ± 0.076 (54%)	0.310 ± 0.062 (33%)	0.115 ± 0.005 (13%)
50 μM *	0.577 ± 0.162 (50%)	0.452 ± 0.093 (38%)	0.143 ± 0.088 (12%)
250 μM *	1.275 ± 0.105 (51%)	0.929 ± 0.132 (37%)	0.294 ± 0.008 (12%)
1000 μM *	3.257 ± 0.832 (58%)	1.797 ± 0.133 (32%)	0.562 ± 0.090 (10%)

3. Pool Size Analysis

The pool sizes of ethanolamine and phosphatidylethanolamine in the isolated hamster heart perfused with various concentrations of ethanolamine were determined. There was no significant change in the pool size of ethanolamine when hearts were perfused with 0.1-1,000 μM ethanolamine. No significant change in the total amount of phosphatidylethanolamine in the perfused hamster heart was detected under all experimental conditions (Table 5).

4. Effect of Ethanolamine Analogues on Ethanolamine Uptake

Hamster hearts were perfused with 50 μM [1-^{3}H] ethanolamine in the presence of various ethanolamine analogues. The presence of 0.5 mM monomethylethanolamine or 1mM L-serine in the perfusate significantly inhibited ethanolamine uptake. However, the ethanolamine uptake in the heart was not affected by similar concentrations of dimethylethanolamine, glycine, or L-alanine in the perfusate (Table 6).

TABLE 5

Pool Sizes of Ethanolamine and Phosphatidylethanolamine

Ethanolamine concentration in the perfusate	ethanolamine (μmol/g heart)	phosphatidylethanolamine (μmol/g heart)
Unperfused	1.07 ± 0.07	11.46 ± 0.60
0.1 μM	1.14 ± 0.05	12.13 ± 0.47
50 μM	1.17 ± 0.23	12.30 ± 1.20
250 μM	1.17 ± 0.24	11.41 ± 0.54
1000 μM	1.27 ± 0.18	12.43 ± 1.05

TABLE 6

Effect of Ethanolamine Analogues on Ethanolamine Uptake

Hamster hearts were perfused with 50 μM [1-^{3}H] ethanolamine in the presence of ethanolamine analogues for 30 min. The specific radioactivity of ethanolamine in the perfusate was 3.33 x 10^{4} dpm/nmol. Values are the mean ± standard deviation of three separate experiments done in duplicate.

Ethanolamine analogue in the perfusate	Uptake of ethanolamine (dpm x 10^{-6}/g heart)
Control	2.06 ± 0.38
0.5 mM Monomethylethanolamine	1.26 ± 0.19 *
0.5 mM Dimethylethanolamine	1.96 ± 0.10
1.0 mM Glycine	2.24 ± 0.34
1.0 mM L-Alanine	2.00 ± 0.24
1.0 mM L-Serine	1.21 ± 0.17 *

* $p < 0.02$

C. THE ROLE OF SERINE IN THE REGULATION OF PHOSPHATIDYLETHANOLAMINE BIOSYNTHESIS

I. Ability of Exogenous Serine to Modulate Phosphatidylethanolamine Biosynthesis

Phosphatidylethanolamine can be synthesized via three different routes: The CDP-ethanolamine pathway, the decarboxylation of phosphatidylserine, and by Ca^{2+} mediated base-exchange. The exogenous precursors for these pathways are ethanolamine for phosphatidylethanolamine biosynthesis via the CDP-ethanolamine pathway, and L-serine for both the decarboxylation and base-exchange pathways. The regulatory role of exogenous ethanolamine had been established in our earlier studies (McMaster and Choy 1992a). However, the contribution of these various pathways varies from one cell type to another, and the presence of alternate pathways implies that the amount of phosphatidylethanolamine synthesized by a particular pathway is not exactly defined. Hence, we examined if the contribution of each pathway could be affected by the supply of their exogenous precursors, ethanolamine and serine.

1. The Inhibition of Ethanolamine Uptake by Exogenous Serine

Hamster hearts were perfused with 50 μM [1-^{3}H]ethanolamine with or without 1

mM serine for 5-60 min. Subsequent to perfusion, hearts were homogenized in $CHCl_3/CH_3OH$ (1:1, by vol.) and radioactivity was determined in the extract for total ethanolamine uptake. As depicted in Fig. 11, the uptake of ethanolamine was linear from 5 to 30 min of perfusion, and less than 10% of the radioactivity in the perfusate was taken up by the heart at all time points. However, the linear part of the uptake curve did not extrapolate to zero. One likely explanation is that additional time (2-3 min) was required for the removal of the labeled perfusate from the vascular and intercellular space after perfusion, and hence the time of exposure of the cardiac cells to radioactivity was slightly longer than the assigned time point. The uptake of ethanolamine and the labelling of phosphatidylethanolamine were found to be inhibited ($p<0.01$) by 1 mM serine at all time points of perfusion.

The effect of serine concentrations on the uptake of ethanolamine and phosphatidylethanolamine labelling was investigated. At 60 min of perfusion the presence of 0.01 mM serine did not affect ethanolamine uptake. However, the uptake of ethanolamine was inhibited by higher concentrations (0.05-10 mM) of serine in the perfusate (Table 7). Similarly, the labelling of phosphatidylethanolamine was not affected by 0.01 mM serine, but reductions in the labelling of the phospholipid were detected at higher serine concentrations. The ability of other amino acids to inhibit ethanolamine uptake and

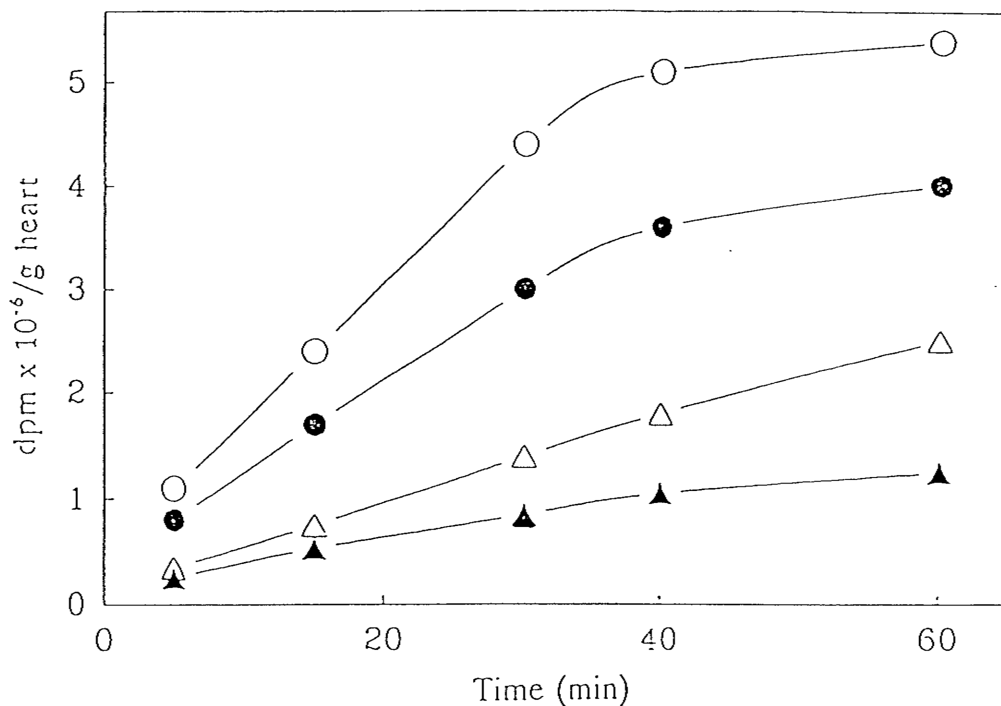


Figure 11. Time course for the inhibition of ethanolamine uptake and subsequent incorporation into phosphatidylethanolamine. Hamster hearts were perfused with 50 μM [1-^{3}H]ethanolamine in the absence or presence of 1 mM serine as indicated in "Materials and Methods". The radioactivity associated with the tissue homogenate after perfusion was used to determine ethanolamine uptake (○,●). Subsequent to phase separation, phosphatidylethanolamine in the organic phase was isolated by thin layer chromatography (△ ▲). The open symbols represent values obtained from perfusions in the absence of serine and the closed symbols are in the presence of 1 mM serine. The values are the mean of four different experiments.

TABLE 7

Effect of Serine on Ethanolamine Uptake and Incorporation into Phosphatidylethanolamine

Hamster hearts were perfused with 50 μM [1-^{3}H]ethanolamine in the absence (control) and presence of various concentrations of serine for 60 min. Values are the mean ± standard deviation of three to six separate experiments performed in duplicate.

[Serine] (mM)	Ethanolamine Uptake (dpm x 10^{-6}/g heart)	Phosphatidylethanolamine (dpm x 10^{-6}/g heart)
Control	5.28 ± 0.43	2.84 ± 0.30
0.01	5.30 ± 0.47	2.89 ± 0.25
0.05	4.27 ± 0.69 [a]	2.32 ± 0.33 [a]
0.10	3.79 ± 0.55 [b]	1.56 ± 0.32 [b]
1.00	3.85 ± 0.38 [b]	1.24 ± 0.07 [b]
10.00	3.85 ± 0.76 [b]	1.15 ± 0.02 [b]

[a] $p < 0.05$ as compared to control

[b] $p < 0.01$ as compared to control

phosphatidylethanolamine labelling was also examined. The presence of 1 mM glycine or alanine in the perfusate did not cause any inhibition of ethanolamine Since the uptake of ethanolamine was linear up to 30 min of perfusion, the nature of inhibition of ethanolamine uptake by serine was examined under this condition. Hamster hearts were perfused with different ethanolamine concentrations in the presence or absence of 1 mM serine. The double reciprocal plot between ethanolamine uptake and ethanolamine concentration in the perfusate is depicted in Fig. 12. The results indicate that the inhibition of ethanolamine uptake by serine was essentially non-competitive.

2. The Effect of Exogenous Serine on the CDP-Ethanolamine Pathway

The distribution of radioactivity in the metabolites of the CDP-ethanolamine pathway in hearts perfused with different serine concentrations was examined. As shown in Table 8, the majority of the label was associated with the ethanolamine fraction regardless of the concentration of exogenous serine in the perfusate. At higher concentrations of serine in the perfusate (0.1 - 10 mM), significant increases in the labelling of ethanolamine were observed with corresponding decreases in the amount of label associated with phosphoethanolamine. No significant change in radioactivity in the CDP-ethanolamine fraction was detected under all experimental conditions. The distribution of radioactivity within the ethanolamine-containing

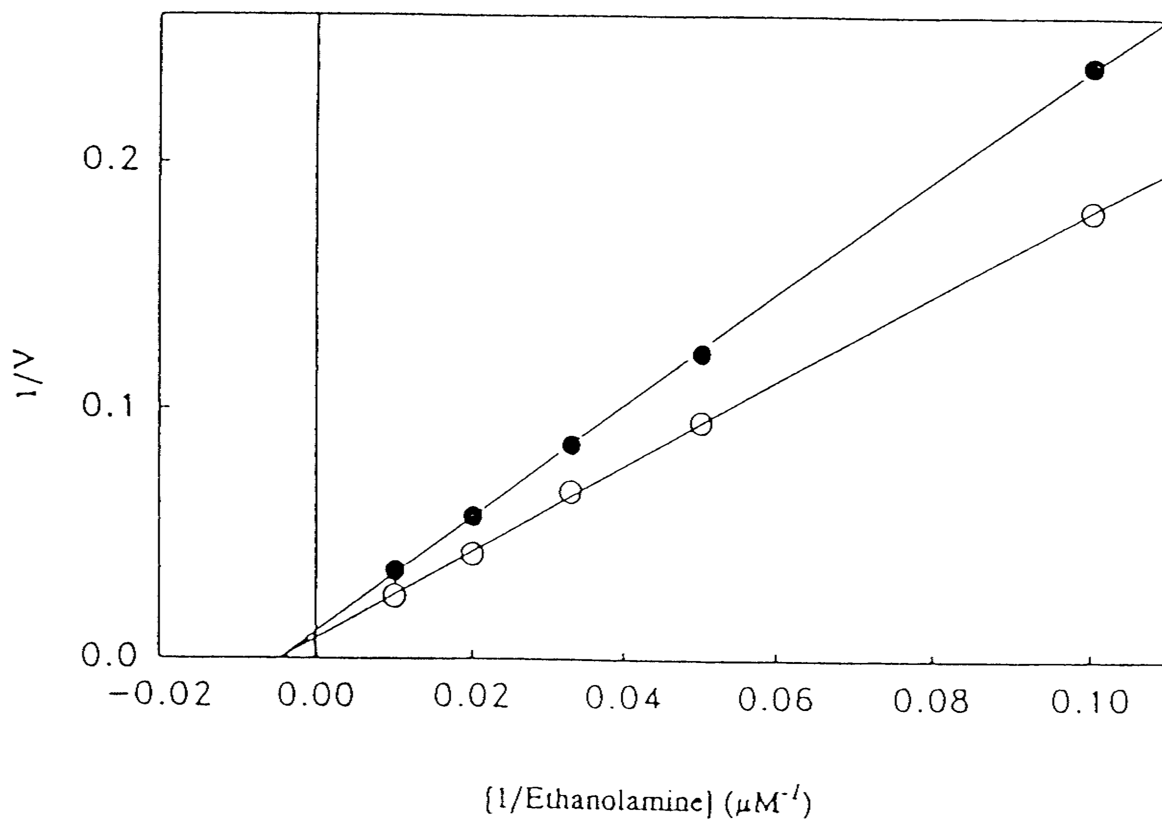


Figure 12. Lineweaver-Burke plot of ethanolamine uptake versus ethanolamine concentration in the presence of serine. Hearts were perfused as described in "Materials and Methods" for 30 min at various concentrations of ethanolamine in the absence (○) or presence (●) of 1 mM serine. Each value represents the mean of four separate experiments.

TABLE 8

Effect of Serine on the Distribution of Radioactivity in Ethanolamine Metabolites

Hamster hearts were perfused for 60 min with 50 μM [1-^{3}H]ethanolamine in the absence (control) and presence of various concentrations of serine. Values are the mean ± standard deviation of three to six experiments performed in duplicate.

[Serine] (mM)	Ethanolamine	Phosphoethanolamine	CDP-ethanolamine
		(dpm x 10^{-6}/g heart)	
Control	1.53 ± 0.17	0.51 ± 0.03	0.31 ± 0.04
0.01	1.57 ± 0.38	0.52 ± 0.04	0.33 ± 0.03
0.05	1.59 ± 0.19	0.45 ± 0.04 [a]	0.30 ± 0.05
0.10	1.83 ± 0.17 [a]	0.41 ± 0.04 [b]	0.30 ± 0.06
1.00	1.95 ± 0.18 [a]	0.36 ± 0.02 [b]	0.32 ± 0.03
10.00	2.26 ± 0.21 [b]	0.35 ± 0.06 [b]	0.32 ± 0.04

[a] $p < 0.05$ as compared to control

[b] $p < 0.01$ as compared to control

metabolites indicated that higher concentrations of serine were affecting ethanolamine metabolism as well as ethanolamine uptake. The rate of phosphatidylethanolamine biosynthesis via this pathway was assessed from the average specific radioactivity of CDP-ethanolamine (Zelinski and Choy 1982b). In the presence of 50 μM ethanolamine the pool size of CDP-ethanolamine in the heart was 1.5 $\pm$ 0.3 μmol/g heart and was not significantly changed by the addition of serine to the perfusate. The rate of phosphatidylethanolamine biosynthesis was estimated to be 225 nmol/min/g heart, and was decreased to 98 nmol/min/g heart by perfusion with 1 mM serine.

3. Effect of Serine on the Enzymes in the CDP-Ethanolamine Pathway

Changes in the labelling of the ethanolamine-containing metabolites might result from the direct modulation of the enzymes in the CDP-ethanolamine pathway by serine. Hence, the activities of these enzymes were assayed in the presence and absence of 1 mM serine and the results are depicted in Table 9. The presence of serine did not affect CTP:phosphoethanolamine cytidylyltransferase or CDP-ethanolamine:diacylglycerol phosphoethanolaminetransferase activities in the hamster heart, but the activity of ethanolamine kinase was significantly inhibited. Interestingly, the activity of choline kinase was not inhibited by serine (data not shown). The inhibition of ethanolamine kinase activity is in general agreement with

TABLE 9

Effect of Serine on the Enzymes of the CDP-ethanolamine Pathway

Enzyme	Activity (nmol/min/mg)
Ethanolamine kinase	0.200 ± 0.011 (4)
Ethanolamine kinase + 1 mM serine	0.151 ± 0.009 [a] (4)
Phosphoethanolamine cytidylyltransferase	1.012 ± 0.010 (4)
Phosphoethanolamine cytidylyltransferase + 1 mM serine	1.030 ± 0.026 (4)
Phosphoethanolaminetransferase	0.104 ± 0.006 (4)
Phosphoethanolaminetransferase + 1 mM serine	0.105 ± 0.004 (4)

[a] $p < 0.01$ as compared to enzyme activity without 1 mM serine

the observed increase in the labelling of ethanolamine and the corresponding decrease in phosphoethanolamine labelling when hamster hearts were perfused with labelled ethanolamine in the presence of serine (Table 8).

The nature of inhibition of ethanolamine kinase activity by serine was further investigated. As depicted in Fig. 13, the inhibition of enzyme activity by serine was dose-dependent but in a non-linear fashion. The efficiency of inhibition was greatest at lower serine concentrations (0.025-0.5 mM) but rather modest at higher serine concentrations (2-50 mM). For example, 0.1 mM serine caused a 21% reduction in enzyme activity whereas a 100-fold increase in serine (10 mM) resulted in only a 36% reduction of enzyme activity. The nature of inhibition of ethanolamine kinase activity by serine was examined. Enzyme activities were assayed at various concentrations of ethanolamine in the presence of 0.1 and 10 mM serine and the results obtained were expressed in a Lineweaver-Burke plot (Fig. 14). Similar to the values reported elsewhere (Zelinski and Choy 1982a), the K_m for ethanolamine was estimated to be 5 mM. The presence of serine caused changes in the K_m and V_{max} values and the inhibition appears to be the "mixed type" which suggests that the action of serine may not be confined to the binding of the enzyme-substrate complex.

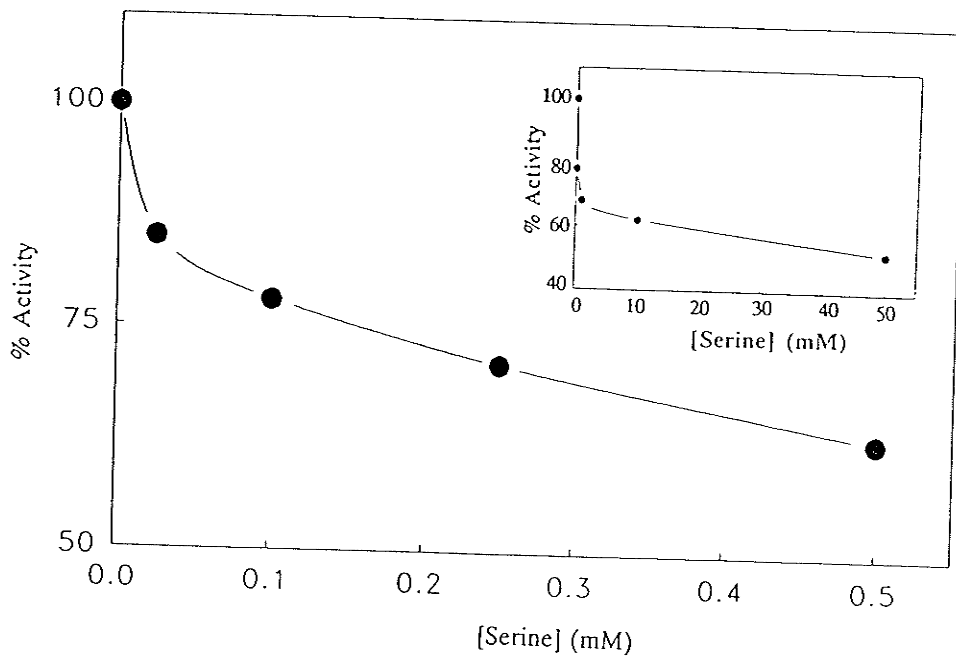


Figure 13. The effect of serine concentration on ethanolamine kinase activity. Ethanolamine kinase was assayed as described by Schneider and Vance (1978). Enzyme activity is expressed as % control (in the absence of serine). Each value represents the mean of two separate experiments performed in duplicate.

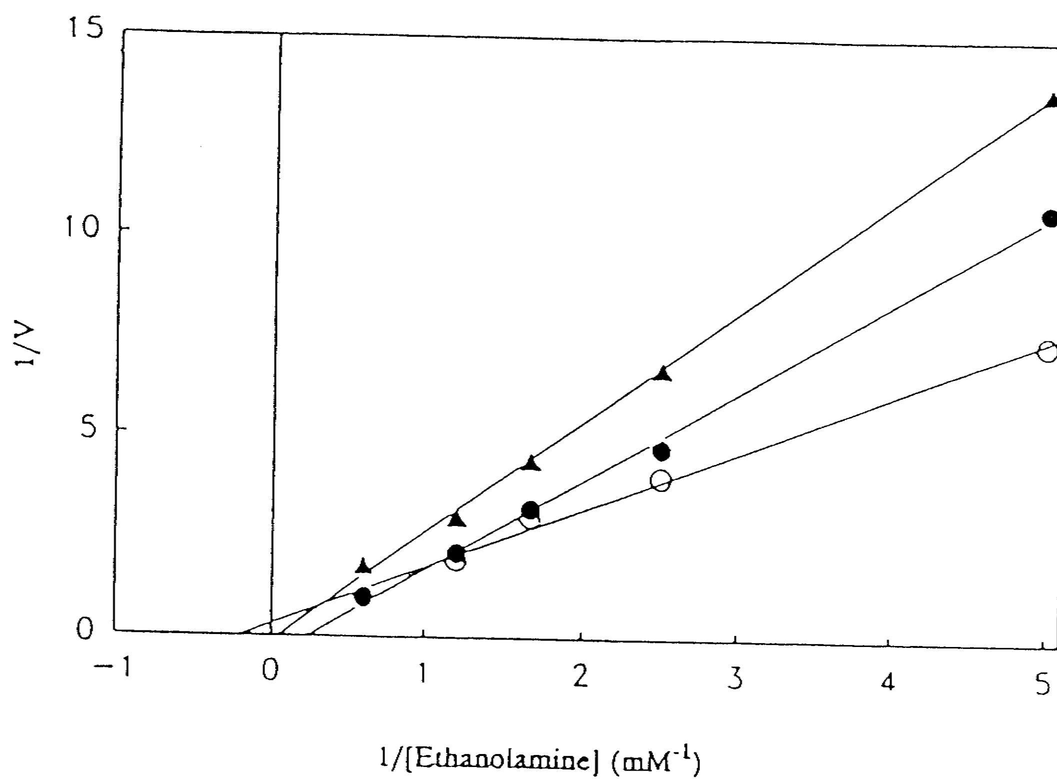


Figure 14. Lineweaver-Burke plot of ethanolamine kinase activity versus ethanolamine concentration in the presence of serine. Ethanolamine kinase activity was assayed as described in Fig. 13. Each value is the mean of three separate experiments performed in duplicate in the absence (○) and presence of 0.01 mM (●) and 10 mM (▲) serine.

4. Intracellular Serine Pool in the Hamster Heart

If perfusion with serine was to affect the enzymes of the CDP-ethanolamine pathway *in vivo*, an increase in the intracellular pool size of serine would be expected. Hence, the intracellular serine pools were determined. The serine pool in the control heart was estimated to be 52 ± 15 nmol/g heart (wet weight). The value obtained from this study is similar to that reported for feline organs (Tallan *et al* 1954). When hamster hearts were perfused with 1 mM serine for 60 min, the level of serine in the cardiac tissue was 258 ± 57 nmol/g heart which represents a 5-fold increase over the value obtained from control hearts.

5. Decarboxylation of Phosphatidylserine to Phosphatidylethanolamine

The contribution of phosphatidylserine decarboxylation to the formation of phosphatidylethanolamine was assessed by perfusing hearts with 1 mM labelled serine in the absence and presence of 50 μM ethanolamine for 60 min. As depicted in Table 10, ethanolamine did not affect serine uptake or its incorporation into phosphatidylserine. The subsequent incorporation of labelled material into phosphatidylethanolamine was also not affected by ethanolamine. Phosphatidylethanolamine formation by phosphatidylserine decarboxylation was calculated from the average specific radioactivity of phosphatidylserine and was

TABLE 10

Uptake of L-[^{3}H]Serine and its Incorporation into Phospholipids in the Hamster Heart

Hamster hearts were perfused with 1 mM L-[^{3}H]serine in the absence (control) and presence of 50 μM ethanolamine for 60 min. Subsequent to perfusion, the total uptake of radioactivity and the radioactivity in the phospholipid fractions were determined. The results are expressed as the mean ± standard deviation of three separate experiments performed in duplicate.

	Control	50 μM Ethanolamine
	(dpm x 10^{-5}/g heart)	
Total uptake	226.0 ± 38.2	246.4 ± 15.5
Phosphatidylserine	4.21 ± 0.64	3.90 ± 0.52
Phosphatidylethanolamine	0.51 ± 0.10	0.52 ± 0.05
Phosphatidylcholine	0.04 ± 0.01	0.04 ± 0.01

estimated to be 8.3 nmol/min/g heart. The value obtained is similar to those obtained previously by this lab when the hamster heart was perfused with a much lower exogenous serine concentration (Zelinski and Choy 1982b).

6. Effect of Serine on the Base-exchange Reaction

A known pathway for the biosynthesis of phosphatidylserine is by a base-exchange reaction where serine is exchanged with the head group of another phospholipid (Vance 1985; Kuge *et al* 1986b). An elevated intracellular serine level might cause the enhancement of this reaction with phosphatidylethanolamine, thus reducing the amount of labelled phosphatidylethanolamine in the heart. In order to test this possibility, hamster hearts were perfused with 50 μM labelled glycerol and 50 μM ethanolamine in the presence and absence of 1 mM serine for 60 min. Subsequent to perfusion, the labelling of phospholipids in the hearts were analyzed and the results are shown in Table 11. No significant changes in the labelling of phosphatidylserine, phosphatidylcholine or lysophosphatidylethanolamine were detected. In the presence of serine, a 56% reduction in the labelling of phosphatidylethanolamine was observed. Since there was no change in the radioactivity associated with phosphatidylserine when serine was added to the perfusate, enhanced base-exchange activity for phosphatidylserine biosynthesis did not seem to occur. Incidentally, no change in the size of either the

TABLE 11

Incorporation of [1-^{3}H] Glycerol into Phospholipids

Hamster hearts were perfused with 50 μM [1-^{3}H]glycerol and 50 μM ethanolamine in the absence (control) and presence of 1 mM serine. Each value is the mean ± standard deviation of three separate experiments performed in duplicate.

Phospholipid	control	1 mM serine
	(dpm x 10^{-6}/g heart)	
Phosphatidylethanolamine	1.93 ± 0.29	0.85 ± 0.17 [a]
Phosphatidylserine	0.74 ± 0.16	0.77 ± 0.15
Phosphatidylcholine	1.23 ± 0.20	1.08 ± 0.21
Lysophosphatidylethanolamine	0.06 ± 0.01	0.05 ± 0.01

[a] $p < 0.01$ as compared to control

phosphatidylethanolamine, phosphatidylserine or phosphatidylcholine pool sizes were detected under any perfusion conditions (data not shown).

7. Effect of Serine on the Catabolism of Phosphatidylethanolamine

The inhibition of the ethanolamine kinase activity by serine would provide a viable explanation to the reduction in labelling of phosphatidylethanolamine. However, the reduced labelling of phosphatidylethanolamine might also arise from the enhancement of its catabolism. In order to examine the effect of serine on the turnover of phosphatidylethanolamine, hamster hearts were pulse labelled with 1 μM [1-^{3}H]-ethanolamine (8.00 x 10^9 dpm/μmol) for 15 min and chased with 50 μM unlabelled ethanolamine for 240 min. The purpose of the pulse-chase experiment was to place all the labelled material into phosphatidylethanolamine. Analysis of the radioactivity distribution after the chase revealed that over 90% of the radioactivity in the heart was located in the phosphatidylethanolamine fraction. Subsequent to the chase, hearts were perfused in the presence or absence of 1 mM serine for another 60 min. After perfusion, the phosphatidylethanolamine and lysophosphatidylethanolamine fractions of the hearts were isolated and analyzed for radioactivity. No significant difference in radioactivity was detected in these fractions between the experimental and control hearts (data not shown). The result of this study is in general agreement with the [^{3}H]glycerol study (Table 11) which

indicates that the labelling of lysophosphatidylethanolamine was not changed by the presence of serine.

The direct effect of serine on the degradation of phosphatidylethanolamine was also examined. Phosphatidyl-[^{3}H]ethanolamine was incubated with a post-mitochondrial fraction for 15 min at 37 °C in the presence or absence of serine. Subsequent to incubation, the radioactivity in the ethanolamine-containing metabolites was determined. As depicted in Table 12, no significant change was detected in the presence or absence of 1 mM serine.

TABLE 12

Effect of Serine on Phosphatidylethanolamine Degradation

Phosphatidyl-[1-^{3}H]ethanolamine was incubated with hamster heart post-mitochondrial fraction for 15 min at 37°C in the absence (control) and presence of serine. Lysophosphatidylethanolamine (100 nmol) was added to the incubation mixture for the determination of radioactivity in lysophosphatidylethanolamine.

Metabolite	Control	1 mM Serine
	(dpm x 10^{-4}/hr/mg protein)	
Lysophosphatidylethanolamine	0.526 ± 0.059 (3)	0.534 ± 0.035 (3)
Glycerophosphoethanolamine	1.742 ± 0.437 (5)	1.561 ± 0.207 (5)
Phosphoethanolamine	0.462 ± 0.039 (5)	0.456 ± 0.032 (5)
Ethanolamine	0.566 ± 0.015 (5)	0.596 ± 0.116 (5)

D. PLASMENYLETHANOLAMINE CATABOLISM

I. Cytosolic Plasmalogenase in Guinea Pig Tissues

The hamster heart was chosen for studies on phosphatidylethanolamine metabolism due to its low (7%) levels of plasmenylethanolamine. However, most mammalian hearts contain a significant amount of plasmenylethanolamine (Horrocks and Sharma 1982). To this extent, the guinea pig was chosen to study the catabolism of plasmenylethanolamine. The mammalian brain and heart contain the highest amount of plasmenylethanolamine, with about 40-50% of the ethanolamine-containing phospholipids being in plasmenylethanolamine form in these tissues (Horrocks and Sharma 1982). Plasmenylethanolamine is believed to be catabolized by either a microsomal plasmalogenase or a microsomal phospholipase-lysoplasmalogenase system (Arthur *et al* 1985; Alexander-Jurkowitz *et al* 1989). Our studies revealed the existence of a previously unrecognized cytosolic plasmalogenase activity. This activity was shown to be truly soluble and its characteristics are described (McMaster *et al* 1992b).

1. Plasmalogenase Assays.

The disappearance of plasmenylethanolamine was monitored by the loss of the vinyl

ether bond. A typical assay resulted in the disappearance of 10-20 nmol of the vinyl ether bond of plasmenylethanolamine over the 15 min incubation period. This change in vinyl ether content resulted in an increase in absorbance of 0.040-0.080 A.U. at 355 nm (Fig. 15) which could be easily detected by a modern spectrophotometer. Enzyme activity was linear with protein concentration up to 1.5 mg of protein from guinea pig brain cytosol or microsomes, however, utilizing less than 0.2 mg of protein was the minimum with which activity could be detected by the substrate disappearance method.

The appearance of fatty aldehyde was used as a confirmatory assay for plasmalogenase activity in the guinea pig brain and heart subcellular fractions. In a typical assay, an increase in absorbance ranging from 0.005-0.020 A.U./min was obtained for a 5 min incubation period. An increase in absorbance of greater than 0.040 A.U./min resulted in the loss of linearity, probably due to the capacity of the aldehyde dehydrogenase to turn over the long chain aldehydes. No significant change in absorbance was detected when NAD^+, aldehyde dehydrogenase, or substrate was eliminated from the reaction mixture. However, this assay is not suitable for the assay of plasmalogenase in the liver. This fact has been reported by other investigators (Jurkowitz-Alexander *et al* 1989).

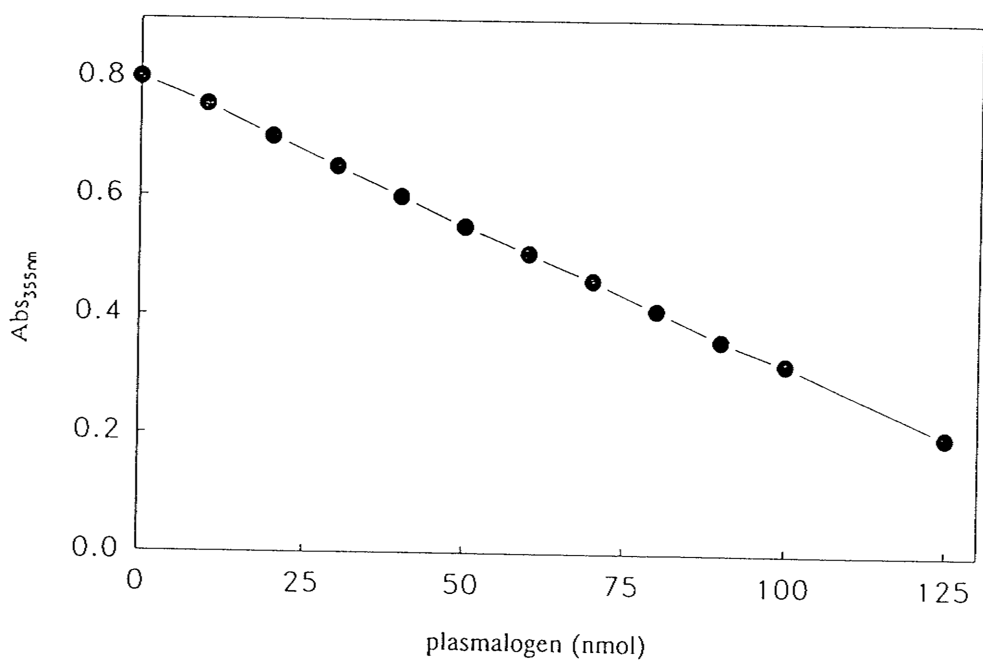


Figure 15. Effect of substrate concentration on the measurement of the vinyl ether content of plasmalogens. Plasmenylethanolamine was assayed by the iodine disappearance method as described in Materials and Methods. Each point is the mean of four separate experiments. Virtually an identical curve was obtained when lysoplasmenylethanolamine was used as the vinyl ether source.

2. Subcellular Localization of Plasmalogenase Activity.

The distribution of plasmalogenase activities in the subcellular fractions of guinea pig brain, heart and liver was investigated. The enzyme activity was originally assessed by the disappearance of the substrate during the reaction. A small amount of plasmalogenase activity was found in the mitochondrial fraction of the brain and heart (data not shown), however, the vast majority of the enzyme activity (>90%) was located in the microsomal and cytosolic fractions. As depicted in Table 13, approximately 80% of the total enzyme activity was found in the cytosolic fraction in the brain, heart, and liver. The plasmalogenase activities in the brain and heart were also confirmed by the continuous spectrophotometric method. The distribution of enzyme activity between the cytosol and microsomes was not affected by the mode of tissue homogenization. Plasmalogenase activity was very low in the liver.

It could be argued that the plasmalogenase activity in the brain and heart cytosol might arise from contamination by microsomal particles. Hence, the microsomal marker enzyme activities in the cytosolic fractions were assessed (Table 14). The result shows that the contamination of the cytosolic fraction by microsomal enzyme markers did not exceed 12% in all cases. Hence, the plasmalogenase activities in the cytosolic fraction of the brain and heart could not arise solely from microsomal contamination.

TABLE 13

Plasmalogenase Activities in the Subcellular Fractions of Guinea Pig Tissues[a]

Tissue		Specific Activity	Total Activity[b]	
		Plasmenylethanolamine disappearance	NADH appearance	% distribution
		(nmol/h/mg protein)		
Brain	Cytosol[c]	89.8 ± 7.3	77.1 ± 6.6	83%
	Microsome[c]	66.1 ± 3.6	55.3 ± 7.4	17%
	Cytosol[d]	84.2 ± 7.9	---	78%
	Microsome[d]	77.8 ± 13.1	---	22%
Heart	Cytosol	47.6 ± 5.8	41.6 ± 6.2	68%
	Microsome	57.0 ± 8.1	56.7 ± 6.2	32%
Liver	Cytosol	6.5 ± 2.5	---	75%
	Microsome	5.1 ± 1.5	---	25%

[a]Plasmalogenase activities were determined by both substrate disappearance and coupled enzyme assays. Each value represents the mean ± standard deviation of at least four different experiments for each assay method separately.

[b]Total activity was calculated from the product specific activity of the enzyme and the amount of protein in each subcellular fractions. A similar distribution was also observed in rat brain and heart.

[c]Prepared by homogenization with a Polytron

[d]Prepared by homogenization with a Potter-Elvehjem homogenizer

TABLE 14

The Distribution of Microsomal Marker Enzyme Activities in the Microsomal and Cytosolic Fractions of Guinea Pig Brain and Heart.

Enzyme		Brain	Heart
		Percentage Distribution[a]	
NADPH-cytochrome c reductase	Cytosol	12%	--
	Microsome	88%	--
5'nucleotidase	Cytosol	--	2%
	Microsome	--	98%
Phosphoethanolamine transferase	Cytosol	6%	N.D.[b]
	Microsome	94%	100%

[a]Each value is the average of three separate experiments.

[b]N.D.-not detectable

3. Substrate Specificity

In order to show that the hydrolysis of the vinyl ether group of plasmenylethanolamine was a direct action of the plasmalogenase and not the combined action of phospholipase A_2-lysoplasmalogenase, the hydrolysed phospholipid products of the reaction were isolated and examined after incubation with guinea pig brain cytosol. Subsequent to 15 min of incubation, 22 nmol of plasmenylethanolamine was hydrolyzed and the radylglycerophosphoethanolamine product in the reaction mixture was isolated by thin-layer chromatography and assayed for both lipid phosphorus and vinyl ether contents. In a typical set of experiments, the radylglycerophosphoethanolamine fraction was undetectable for both lipid phosphorus and vinyl ether content at time 0. However, after the 15 min incubation it was found to contain 14 ± 2 nmol of lipid phosphorus, but the vinyl ether content was undetectable. Since the lysophosphatidylethanolomine fraction did not contain the vinyl ether group, these results imply that the plasmenylethanolamine was hydrolysed by the plasmalogenase but not the phospholipase A_2-lysoplasmalogenase system.

Time courses for the hydrolysis of plasmenylethanolamine and lysoplasmenylethanolamine in guinea pig brain cytosol are shown in Fig. 16. The hydrolysis of plasmenylethanolamine was linear up to 30 min. However, the brain

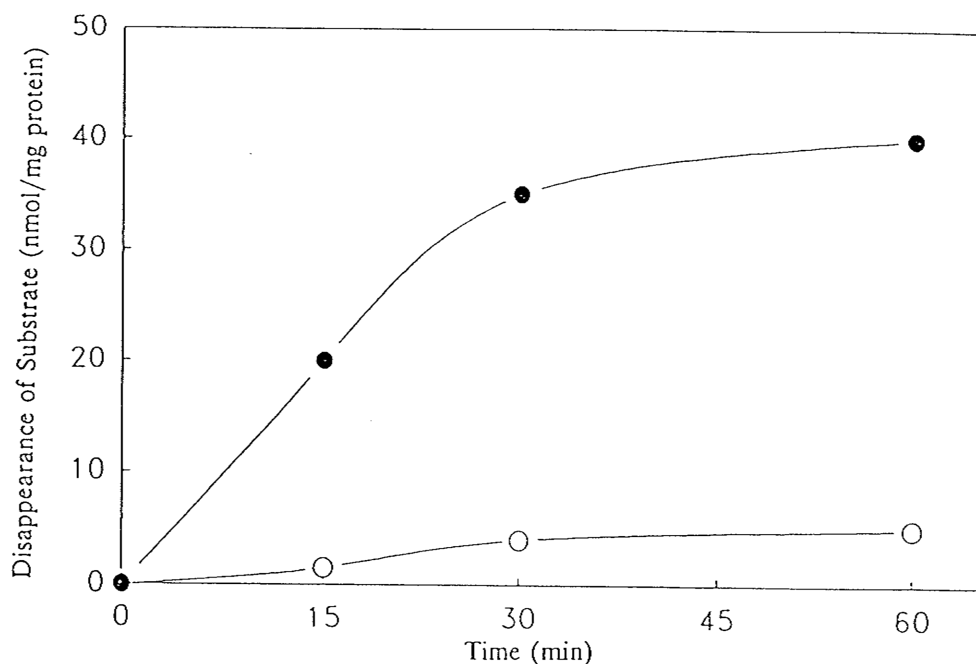


Figure 16. Time course for the disappearance of plasmenylethanolamine and lysoplasmenylethanolamine in guinea pig brain cytosol. Cytosol was incubated at 37°C in the presence of 300 μM plasmenylethanolamine (●) or lysoplasmenylethanolamine (○) and enzyme activity was assayed by the substrate disappearance method as described in Materials and Methods. Each point represents the mean of three separate experiments.

cytosol had very limited ability to hydrolyse lysoplasmenylethanolamine. The results obtained in this study support the notion that the hydrolysis of plasmenylethanolamine in the brain cytosol resulted from the direct action of the plasmalogenase.

4. Characterization of Plasmalogenase Activities.

In view of the abundance of soluble plasmalogenase in the brain, its cytosol was employed for further characterization of the enzyme activity. The effect of substrate concentrations on plasmalogenase activity was investigated (Fig. 17). From the double reciprocal plot of enzyme activity *vs* plasmenylethanolamine concentrations, the K_m of the enzyme for plasmenylethanolamine was estimated to be 154 μM. The K_m of plasmenylethanolamine for the cytosolic enzyme is comparable to the microsomal enzyme (105 μM) and also the enzyme obtained from an acetone extract of the bovine brain (285 μM) (D'Amato *et al* 1975). Similar to the microsomal enzyme, the cytosolic enzyme from the brain was also inhibited by 1 mM EDTA and 1 mM Mn^{2+} (data not shown). However, the presence of 1 mM Ca^{2+} or Mg^{2+} had very little effect on both enzyme activities (Table 15) (Arthur *et al* 1985). Taken together, our results show that plasmalogenase activity in the cytosol may require a minimum level of metallic cation(s) for full activity. The pH profiles of the brain cytosolic and microsomal plasmalogenase activities were

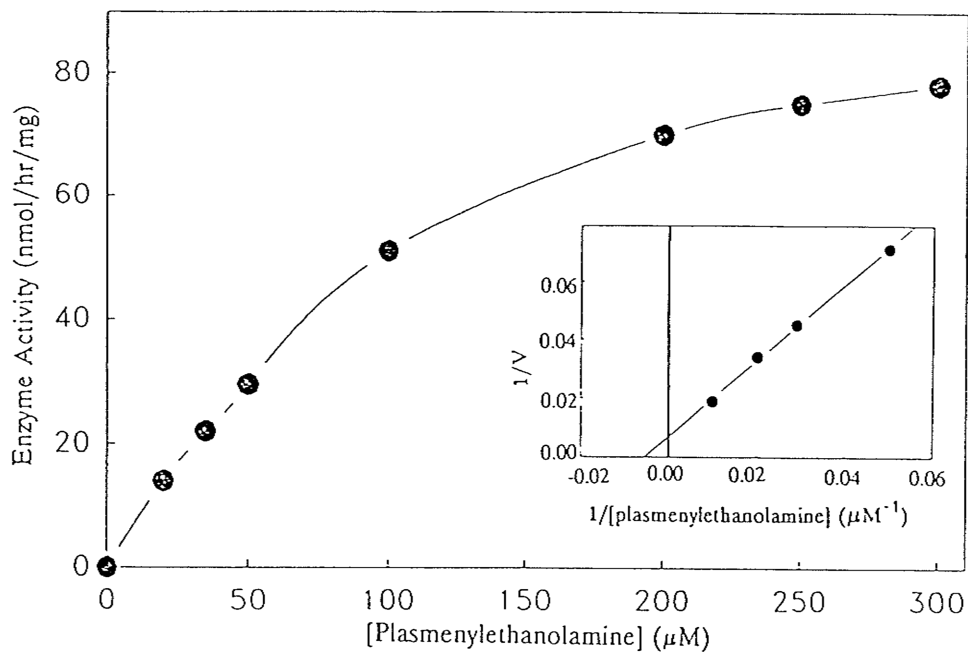


Figure 17. Effect of substrate concentrations on guinea pig brain cytosolic plasmalogenase activity. Plasmenylethanolamine was suspended in 0.05% Tween 20 by sonication. Aliquots were removed and used to determine plasmalogenase activity. Enzyme activity was assayed by the substrate disappearance method at 0-300 μM plasmenylethanolamine over 15 min at 37°C as described in Materials and Methods. Inset is the Lineweaver-Burke plot of the same data. Each point represents the mean of three separate determinations.

TABLE 15

Effects of EDTA and Cations on Cytosolic Plasmalogenase Activity[a]

Cation (1 mM)	Enzyme Activity (% control)
Control	100%
Ca^{2+}	85%
Mg^{2+}	100%
Mn^{2+}	N.D.[b]
EDTA	N.D.[b]

[a]Plasmalogenase activities were assayed by substrate disappearance method as described Materials and Methods in the absence (control) or presence of 1 mM of various cations or EDTA. Each value is the average of three separate experiments.

[b]N.D.-not detectable

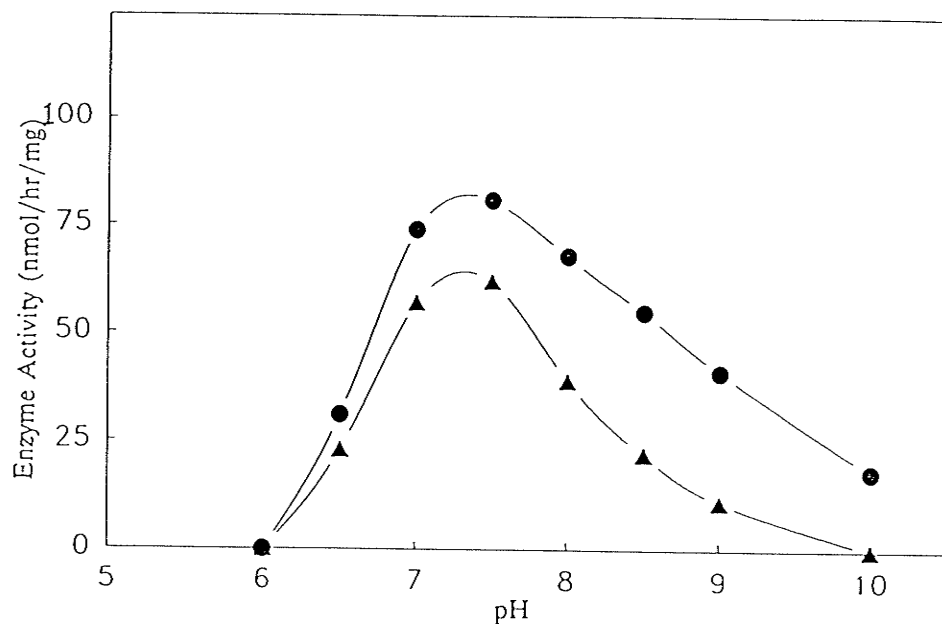


Figure 18. Effect of pH on guinea pig brain cytosolic and microsomal plasmalogenase activities. The cytosolic (●) and microsomal (▲) enzyme activities were assayed by the disappearance of vinyl ether content at 37°C for 15 min and confirmed by the appearance of NADH as outlined in the coupled aldehyde dehydrogenase method in the Materials and Methods section. Tris-succinate buffer was used from pH 6-7, and Tris-HCl was used from pH 7-10. No significant difference was detected in enzyme activity at pH 7 when either buffer was utilized. Each point represents the mean of four separate experiments.

determined (Fig. 18). Both enzymes displayed similar pH profiles with optima at pH 7.5. When the enzymes from the microsomal and cytosolic fractions were incubated at 50°C and 55°C (Fig. 19), the microsomal enzyme was more stable than the cytosolic form.

5. Gel Filtration Chromatography of Guinea Pig Brain Cytosol.

Gel filtration chromatography was employed to confirm the true solubility of the brain cytosolic plasmalogenase. The brain cytosol (1 ml) was applied to a Sepharose 6B column (2 x 65 cm) equilibrated with 0.1 M Tris-HCl (pH 7.5). Subsequent to sample application, the column was washed with the same buffer and fractions of 0.35 ml were collected and assayed for enzyme activity (Fig. 20). Plasmalogenase activity was eluted as a broad peak away from the void volume of the column. The fraction with the highest enzyme activity had an apparent molecular weight of 250,000. The broad and asymmetrical peak of enzyme activity implies that the enzyme was eluted as multimeric proteins or in aggregation with other cytosolic proteins. An alternate explanation is that the enzyme might still be associated with some lipid molecules present in the cytosol. The fact that none of the enzyme activity was eluted near the void volume of the column suggests that it was not complexed with microsomal or large liposomal particles.

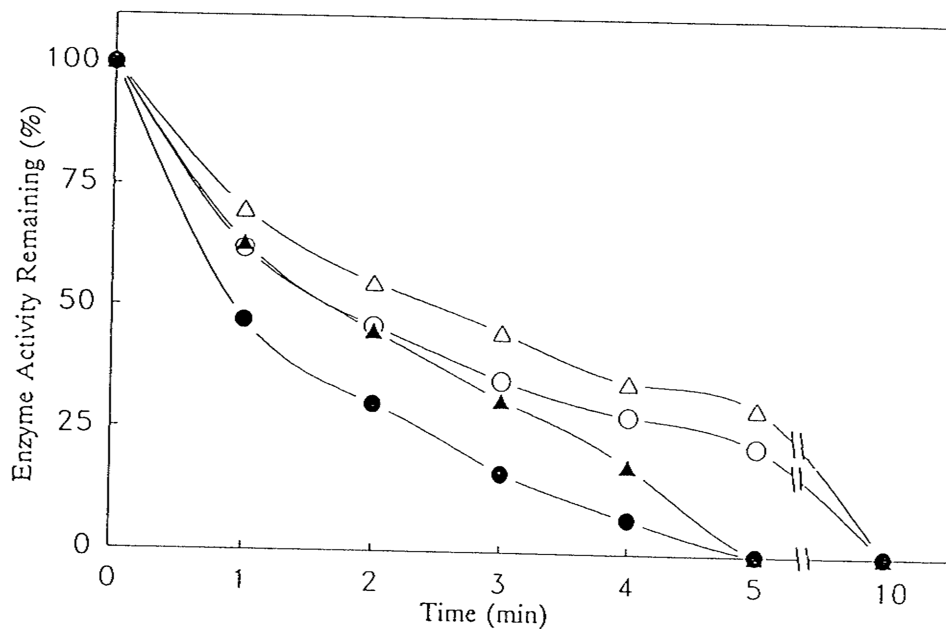


Figure 19. Effect of heat treatment on plasmalogenase activities in the cytosol and microsomes of the guinea pig brain. Guinea pig brain cytosol (○,●) and microsomes (△,▲) were incubated at 50°C (open symbols) or 55°C (closed symbols) for 0-5 min. Subsequent to incubation, enzyme activities were determined by disappearance of substrate and confirmed by the appearance of NADH as outlined in Materials and Methods. Each point is the mean of three separate experiments using the substrate disappearance method for the determination of enzyme activity.

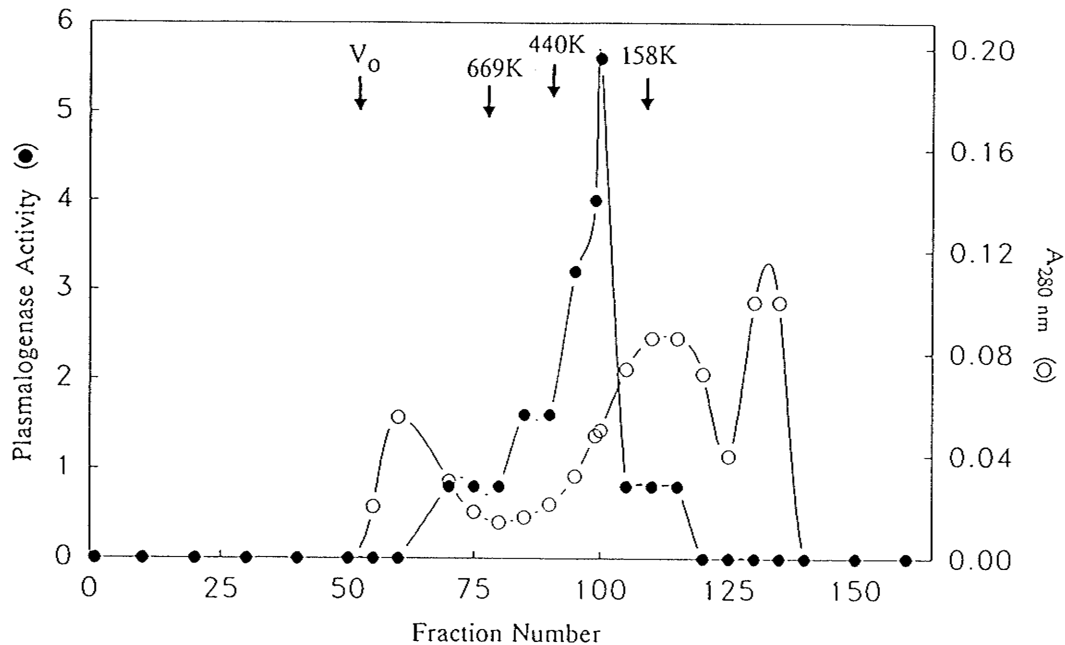


Figure 20. Sepharose 6B chromatography of guinea pig brain cytosol. Guinea pig brain cytosol (1 ml) containing 5 mg of protein was applied to a Sepharose 6B column (2.0 x 65 cm) equilibrated with 100 mM Tris-HCl (pH 7.5) and 0.35 ml fractions were collected. Enzyme activity was assayed by the coupled aldehyde dehydrogenase method and the activity is expressed as nmol NADH formed/h/ml. The total yield of plasmalogenase activity form the column was 20-25% of the total added. Molecular weight markers are noted with arrows.

DISCUSSION

I. Regulation of Phosphatidylethanolamine Biosynthesis by Exogenous Ethanolamine

The ability to determine the intracellular ethanolamine pool sizes of mammalian tissues enabled the prediction of separate ethanolamine pools within the hamster heart (McMaster and Choy 1992a). Ethanolamine pool sizes were measured by utilizing PITC derivatization followed by separation by reverse phase HPLC (McMaster and Choy 1992b). There was very little difference in the ethanolamine pools of the hamster liver, heart, and kidney. The value obtained from hamster liver was only slightly higher than the reported value (0.3 μmol/g) for feline liver (Tallan *et al* 1954), but the value for the hamster heart was significantly lower than that reported earlier (5.2 μmol/g) (Zelinski and Choy 1982b). The difference in liver ethanolamine could be explained by a slight variation between species. The difference in ethanolamine level in the heart may arise from the methods of quantitation. In the earlier study (Zelinski and Choy 1982b), the amount of ethanolamine was determined by the calibration factor of an amino acid, whereas internal and external standards of ethanolamine were used for calibration in the present study.

Recently, the determination of ethanolamine in bovine aortic endothelial cells

(Lipton *et al* 1990) and fetal bovine serum (Lipton *et al* 1990) by HPLC equipped with a C_{18} column was reported. Ethanolamine was converted to 2,4-dinitrobenzyl-ethanolamine and the product was quantitated by a reverse phase HPLC procedure. However, the sensitivity and specificity of this assay was not defined to allow comparison with the present study. The determination of ethanmolamine levels by PITC derivitization and subsequent reverse-phase HPLC is a facile and rapid procedue to examine the pool sizes of ethanolamine in mammalian tissues. The sensitivity of this procedure should be high enough for the determination of ethanolamine in biopsy and tissue culture samples. This procedure was important for the determination of separate ethanolamine pools in the hamster heart (McMaster and Choy 1992a), an intracelluar store of ethanolamine and a newly imported pool that is tightly coupled to phosphatidylethanolamine biosynthesis.

The ability to determine ethanolamine pool size provided us with the required capability to study ethanolamine uptake and phosphatidylethanolamine biosynthesis in the hamster heart. In earlier studies, it was shown that only one uptake system for ethanolamine was present in the hamster heart. This single uptake system (Zelinski and Choy 1982b) is different from that observed in cell culture where ethanolamine is taken up by both low and high affinity uptake mechanisms (Yorek *et al* 1985; Pu and Anderson 1984). However, the efficiency of labelled ethanolamine incorporated into phosphatidylethanolamine was not proportional to ethanolamine uptake. The

decrease in the efficiency of labelled ethanolamine incorporation into phosphatidylethanolamine was not accompanied by a concomitant decrease in phosphatidylethanolamine pool size. This disparity was further investigated by analyzing the distribution of radioactivity in the ethanolamine-containing metabolites.

Analysis of the ethanolamine-containing metabolites in the aqueous extract revealed a shift in the rate-limiting step in the CDP-ethanolamine pathway. At low concentrations of ethanolamine in the perfusate (0.04 and 0.1 μM), the majority of the radioactivity was associated with the phosphoethanolamine fraction. This observation is in agreement with previous findings that the reaction catalyzed by CTP:phosphoethanolamine cytidylyltransferase was rate-limiting (Zelinski and Choy 1982b; Sundler and Akesson 1975b). At higher concentrations of ethanolamine in the perfusate (0.4-1000 μM), the majority of the label was associated with the ethanolamine fraction. The accumulation of labelled ethanolamine suggests that the conversion of ethanolamine to phosphoethanolamine had become rate-limiting with increased ethanolamine uptake. The shift in the rate-limiting step could be caused by an increase in the intracellular ethanolamine pool which might saturate the ethanolamine kinase reaction for the conversion of ethanolamine to phosphoethanolamine. However, this was not the case since no significant change in the intracellular ethanolamine pool was detected. Another explanation is that the uptake of ethanolamine was tightly coupled to the ethanolamine kinase which would

cause a preferential phosphorylation of the ethanolamine taken up by the heart. The limited ability of ethanolamine kinase to phosphorylate the newly imported ethanolamine would result in the accumulation of labelled ethanolamine in the heart at high rates of ethanolamine uptake. Indeed, the reduced ability to phosphorylate the newly imported ethanolamine in the hamster heart may explain the reduced efficiency for the incorporation of labelled ethanolamine into phosphatidylethanolamine observed at high exogenous ethanolamine concentrations. In a previous study, the existence of separate pools of ethanolamine has been postulated (Sundler 1973).

In the hamster heart, the majority of phosphatidylethanolamine is synthesized via the CDP-ethanolamine pathway and the phosphorylation of ethanolamine is the first committed reaction in this pathway. Since the uptake of ethanolamine is not highly regulated, the ethanolamine kinase reaction may play an important role in discriminating the appropriate amount of ethanolamine for phosphatidylethanolamine biosynthesis. The fluctuating concentrations of ethanolamine (0.01-0.9 mM) in the serum (Zelinski and Choy 1982b; Baba *et al* 1984; Milakofsky *et al* 1985) would inevitably change the rate of ethanolamine uptake by the heart. The rate-limiting role of ethanolamine kinase in the phosphorylation of ethanolamine during high ethanolamine uptake may provide the heart with an additional mechanism for maintaining the rate of phosphatidylethanolamine biosynthesis (McMaster and Choy

1992a).

Although it was demonstrated that extracelluar ethanolamine could regulate the biosyntheisis of phosphatidylethanolamine (McMaster and Choy 1992a), there is very little known about the ethanolamine transporter (Lipton *et al* 1990; Pu and Anderson 1984; Yorek *et al* 1985). To this end, the effects of various ethanolamine analogues on ethanolamine uptake were examined. Monomethylethanolamine and dimethylethanolamime are ethanolamine analogues with modified amino terminals while glycine, alanine and serine contain modified carboxy moieties. Of the amino terminal analogues, only monomethylethanolamine significantly inhibited ethanolamine uptake. Additionally, previous reports have indicated choline does not inhibit ethanolamine uptake (Zelinski and Choy 1982a). This suggests that the N-group of ethanolamine can not be modified beyond the addition of one methyl group before the molecule is not recognized by the ethanolamine transporter (McMaster *et al* 1992a). Neither glycine nor alanine inhibited ethanolamine uptake, however, serine did inhibit ethanolamine uptake. Thus, the recognition of the carboxyl terminus does not appear to be as rigorous as that for the amino terminus. The inhibition of ethanolamine uptake by serine must be due to the nature of the prosthetic group since it is similar to both alanine and glycine in all other respects. This inhibition of ethanolamine uptake was particularly interesting since serine is the other metabolite responsible for the synthesis of phosphatidylethanolamine.

II. Regulation of Phosphatidylethanolamine Biosynthesis by Serine

It is clear from this study that exogenous serine plays an important role in the regulation of phosphatidylethanolamine biosynthesis in the hamster heart. Two modes of regulation have been identified: (1) the inhibition of ethanolamine uptake and (2) the modulation of ethanolamine kinase.

We reported earlier that the uptake of ethanolamine by the hamster heart was not inhibited by choline (Zelinski and Choy 1982a). The present study showed that ethanolamine uptake was inhibited only by serine but not glycine or alanine. Taken together, the selective nature of inhibition of ethanolamine uptake in the hamster heart infers that the uptake site is highly specific in its recognition of structural analogues of ethanolamine. The non-competitive mode of inhibition of ethanolamine uptake by serine suggests that serine was not co-transported into the cell via the same site.

Beyond its role in the attenuation of ethanolamine uptake, serine also modulates the biosynthesis of phosphatidylethanolamine. We have shown that the CDP-ethanolamine pathway is the principal pathway for the biosynthesis of phosphatidylethanolamine in the hamster heart and the step catalyzed by CTP:phosphoethanolamine cytidylyltransferase is rate-limiting (Zelinski and Choy

1982b). In addition, the conversion of ethanolamine to phosphoethanolamine has been shown to become rate-limiting at 0.5 μM or higher concentrations of ethanolamine in the perfusate (McMaster and Choy 1992a). These findings, together with the results obtained from the present study, clearly indicate that an increase in the intracellular pool of serine results in the inhibition of the ethanolamine kinase activity and subsequently, phosphatidylethanolamine biosynthesis. Interestingly, the activity of choline kinase was not inhibited by serine. The ability to inhibit ethanolamine kinase but not choline kinase makes serine a specific modulator of phosphatidylethanolamine biosynthesis. Analysis of the ethanolamine-containing metabolites in the hamster heart after perfusion with serine (0.1-10 mM) were consistent with the enzyme inhibition studies. However, at 0.05 mM serine in the perfusate, a cursory view of the distribution of radioactivity did not appear to be consistent with the ethanolamine kinase inhibition, since no significant increase in the labelling of ethanolamine was detected. One explanation for this apparent discrepancy is that the inhibition of ethanolamine uptake by serine would cause a reduction in the labelling of ethanolamine. Such a reduction was counterbalanced by the accumulation of labelled ethanolamine which was caused by the inhibition of the ethanolamine kinase reaction. We postulate that at 0.05 mM serine in the perfusate, these two factors appear to exert similar effects on the overall labelling of ethanolamine. At higher concentrations of serine in the perfusate, the increase in the intracellular pool of serine inhibited the ethanolamine kinase in a fashion which

offset the reduction in ethanolamine uptake and caused a significant increase in the labelling of ethanolamine.

Another point of interest is that serine perfusion did not affect the pool size and the specific radioactivity of CDP-ethanolamine in the heart. The maintenance of the CDP-ethanolamine pool in spite of a reduction in the *de novo* phosphatidylethanolamine biosynthesis was probably due to the transient nature of the CDP-ethanolamine. In an earlier study, the enhancement of phosphatidylethanolamine biosynthesis by exogenous fatty acid did not elicit any changes in the labeling or the pool size of CDP-ethanolamine (Sundler and Akesson 1975b). The results of these studies suggest that the radioactivity incorporated into phosphatidylethanolamine is a reflection of the labeling of phosphoethanolamine. The modulation of phosphatidylethanolamine biosynthesis by exogenous serine may be physiologically important since the circulating level of serine (0.03-0.4 mM) is affected by nutritional and pathophysiological conditions (Milakofsky *et al* 1985; Tallan *et al* 1954;).

An increase in the degradation of phosphatidylethanolamine and/or the enhancement of the base-exchange reaction could also contribute to the reduction in the labelling of phosphatidylethanolamine. Hence, the degradation of phosphatidylethanolamine was examined by both *in vitro* and *in vivo* approaches. Our studies clearly

demonstrated that the degradation of phosphatidylethanolamine was not stimulated by exogenous serine. Although an increase in the intracellular serine pool was produced by exogenous serine in the isolated heart, the rate of base-exchange between phosphatidylethanolamine and other phospholipids was not altered. It is clear that exogenous serine had no effect on the catabolism of phosphatidylethanolamine or its conversion into other phospholipids via the base-exchange reactions.

The utilization of serine as a precursor for phosphatidylethanolamine biosynthesis has been demonstrated in BHK-21 and CHO cells, rat liver, cultured glioma cells, human Y79 retinoblastoma cells and aortic endothelial cells (Voelker 1984; Miller and Kent 1986; Arthur and Page 1991; Xu *et al* 1991; Yorek *et al* 1985; Lipton *et al* 1990). Although this pathway is present in the hamster heart, its contribution to the overall phosphatidylethanolamine biosynthesis was estimated to be modest in comparison to the CDP-ethanolamine pathway (Zelinski and Choy 1982b). We have shown in the present study that the rate of decarboxylation of phosphatidylserine was not altered by higher exogenous serine concentrations. In addition, the inhibition of phosphatidylethanolamine biosynthesis in the CDP-ethanolamine pathway does not elicit any stimulation of phosphatidylserine biosynthesis for the ultimate production of phosphatidylethanolamine. It is clear that transient changes in circulating serine concentrations have an immediate effect on the rate of phosphatidylethanolamine

biosynthesis via the CDP-ethanolamine pathway, but do not display any direct or immediate effect on the contribution of the decarboxylation pathway for phosphatidylethanolamine formation in the hamster heart (McMaster and Choy 1992c).

III. Plasmenylethanolamine Catabolism

In a previous study, the existence of a metabolite in the rat brain cytosol for the elimination of the vinyl ether bond of plasmenylethanolamine was reported (Yavin and Gatt 1972a). The material was thermostable at 100°C, had a low molecular weight and was found to be a non-protein entity which was later identified as ascorbic acid (Yavin and Gatt 1972b). Hence, our study is the first identification of a truly soluble plasmalogenase from mammalian sources. In the last two decades, the existence of plasmalogenase activity in the brain has been a matter of debate. Plasmalogenase activity was identified in the microsomes of rat brain (D'Amato *et al* 1975), in neuronal perikarya, astroglia, and oligodendroglia from bovine brain (Dorman *et al* 1977), and from the brains of rats and monkeys (Ansell and Spanner 1968). Using another approach, it was shown that the rat brain had no ability to catabolize radiolabelled plasmalogens but low levels of lysoplasmalogenase activity were detected in the organ (Gunawan and Debuch 1982). Our results support the existence of considerable amounts of plasmalogenase activity in the brain and also

the presence of low levels of lysoplasmalogenase activity. At present, the reason for the discrepancy in the identification of plasmalogenase activity is not entirely clear. One explanation is that the activity of the plasmalogenase is highly dependent on the source of the plasmalogen and the method of suspension in the buffer. The assay procedure in this study had been optimized to provide a high plasmalogenase activity (McMaster *et al* 1992b).

The similarity in characteristics between the cytosolic and microsomal enzyme makes it plausible to speculate that both enzymes may originate from the same protein. However, the ability to obtain the same distribution of enzyme activity between the two compartments by different methods of homogenization confirms that the cytosolic enzyme was not mechanically detached from the microsomes during tissue homogenization. The identification of a truly soluble plasmalogenase makes the guinea pig brain cytosol an ideal source of the enzyme for its subsequent purification. At present, the physiological significance of the distribution of the enzyme in two subcellular compartments remains undefined.

CONCLUDING DISCUSSION

Phospholipids not only form the bilayer of cellular and organellar membranes but function as reservoirs for the production of lipid second messengers and/or lipid mediators. The molecular mechanisms regulating and coordinating the synthesis of the needed amounts of individual phospholipids to meet these needs is poorly understood. Phosphatidylethanolamine is a major phospholipid in mammalian tissues. Phosphatidylethanolamine can be synthesized via either the CDP-ethanolamine pathway, the decarboxylation of phosphatidylserine, or by base-exchange with other phospholipids. In this work, initial studies utilizing the isolated perfused hamster heart confirmed that at low exogenous ethanolamine concentrations the rate-limiting step in the CDP-ethanolamine pathway was the conversion of phosphoethanolamine to CDP-ethanolamine by CTP: phosphoethanolamine cytidylyltransferase. However, it was discovered that when extracellular ethanolamine concentrations were raised to physiological levels, the rate-limiting step in this pathway was the phosphorylation of ethanolamine by ethanolamine kinase. To determine if the shift in the rate-limiting step was due to an increase in the intracellular pool size of ethanolamine, a procedure was required for the determination of tissue ethanolamine pool sizes. Since no standard methods were available for the determination of ethanolamine levels in mammalian tissues, a procedure utilizing phenylisothiocyanate (PITC) derivitization of a tissue extract followed by fractionation by reverse-phase high

performance liquid chromatography was developed. The ease of PITC derivitization coupled with picomole levels of detection made this method a facilitative procedure for the determination of ethanolamine pool sizes from tissue samples. When the intracellular levels of ethanolamine were determined subsequent to heart perfusion with low and high concentrations of exogenous ethanolamine, there was no change detected in the pool sizes under all perfusion conditions. It was hence postulated that the newly imported ethanolamine was preferentially utilized for phosphatidylethanolamine biosynthesis over the endogenous intracellular ethanolamine pool.

Although the CDP-ethanolamine pathway is generally regarded as the main route for phosphatidylethanolamine biosynthesis in most mammalian tissues, the presence of alternate pathways implies that the contribution of each pathway is not exactly defined. Whether the supply of the exogenous precursors for phosphatidylethanolamine synthesis (ethanolamine and serine) would regulate phosphatidylethanolamine biosynthesis was investigated. Hearts were perfused with physiological levels of circulating ethanolamine (50 μM) in the absence and presence of various concentrations of serine for 5-60 min revealed an inhibition of ethanolamine uptake and its subsequent incorporation into phosphatidylethanolamine at all time points. The nature of this inhibition of ethanolamine uptake was found to be noncompetetive suggesting ethanolamine and serine do not share the same

transporter. Analysis of the ethanolamine-containing metabolites in the CDP-ethanolamine pathway revealed an accumulation of label in the ethanolamine fraction with a corresponding decrease in the phosphoethanolamine fraction when hearts were perfused with serine. These results are consistent with an inhibition of ethanolamine kinase by serine. Examination of the direct effect of serine on the enzymes of the CDP-ethanolamine pathway revealed serine did indeed inhibit ethanolamine kinase *in vitro*. The nature of this inhibition was of the mixed type. Utilization of the HPLC method developed for the determination of intracellular ethanolamine pool sizes revealed that the inhibition of ethanolamine kinase by serine did not change the intracellular pool size of ethanolamine. Additionally, this method was also able to determine intracellular serine pool sizes and a 5-fold increase in the intracellular serine pool size was detected when hearts were perfused with 1 mM serine for 60 min. This increase in intracellular serine could facilitate the apparent inhibition of ethanolamine kinase observed *in vivo*. However, the increase in intracellular serine pool size could also facilitate increased synthesis of phosphatidylserine by base-exchange reactions leading to increased phosphatidylserine decarboxylation for the synthesis of phosphatidylethanolamine. Hearts were perfused with either labelled serine or labelled glycerol to test this hypothesis and it was revealed that there was no increase in the synthesis of phosphatidylserine or its decarboxylation to phosphatidylethanolamine when intracellular serine pool sizes were elevated. Increased catabolism of

phosphatidylethanolamine could also contribute to decreased phosphatidylethanolamine labelling and increased ethanolamine labelling in hearts perfused with serine. Hearts were perfused pulse-chased with radioactive ethanolamine to label the phosphatidylethanolamine fraction of the heart. Subsequent addition of serine did not elicit increased turnover of phosphatidylethanolamine. These results were confirmed by *in vitro* enzyme assays.

Most mammalian hearts have a large proportion of their ethanolamine-containing phospholipids in the vinyl ether linked plasmenylethanolamine form. The hamster heart was specifically chosen for the study of phosphatidylethanolamine biosynthesis due to the low amounts of plasmenylethanolamine in its membranes. The role of ether linked phospholipids in membranes is poorly understood, however, their importance is revealed by the debilitating genetic disease Zellweger's syndrome in which there is a almost a complete absence of plasmenylethanolamine synthesis. One function of plasmenylethanolamine is believed to be to protect cells against oxidative stresses. Due to the extreme damage done to cardiac and cerebral tissues during ischemia, the regulation of plasmenylethanolamine metabolism is highly desirable. Plasmenylethanolamine is turned over by plasmalogenase, an enzyme that cleaves the vinyl ether linkage releasing a fatty aldehyde and lysophosphatidylethanolamine. This enzyme was believed to be microsomal, however, in determining the subcellular examination of plasmalogenase activities in guinea pig and rat tissues a cytosolic

plasmalogenase activity was revealed. This enzyme accounted for the majority of the plasmalogenase activity in the brain, heart, and liver. Determination of the subcellular localization of known marker enzymes revealed that the cytosolic activity could not arise from microsomal contamination. Since the guinea pig brain was the richest source of the enzyme, plasmalogenase was characterized in the subcellular fractions of this tissue. The cytosolic plasmalogenase was completely inhibited by 1 mM EDTA and Mn^{2+}, but was essentially unaffected by Ca^{2+} and Mg^{2+}. The cytosolic and microsomal enzymes had a K_m of 100-150 μM. Both enzymes had a pH optimum of 7.5, and the microsomal enzyme was slightly more stable to heat treatment at 50°C and 55°C. Sepharose 6B chromatography of the cytosolic enzyme revealed that plasmalogenase activity eluted far from the void volume at a molecular weight of 250,000, confirming the enzyme exists in a truly soluble form.

SUMMARY

The main route for phosphatidylethanolamine biosynthesis in mammalian tissues is the CDP-ethanolamine pathway. This pathway utilizes one molecule of ATP and one molecule of CTP to synthesize one phosphatidylethanolamine molecule. Despite this drain on the energy available to the cell, phosphatidylethanolamine is constantly being synthesized in order to maintain the appropriate phospholipid composition within the cell membranes. As the biological roles of phosphatidylethanolamine become more apparent, the regulation of its metabolism will require a greater understanding. The recent discovery of release of second messenger molecules from increased turnover of phosphatidylethanolamine will require counterbalancing effects in terms of up-regulating its synthesis to maintain the required levels of phosphatidylethanolamine within a cell. This study has revealed some of the mechanisms that operate in cardiac tissue in response to varied precursor supplies for the biosynthesis of phosphatidylethanolamine. The supply of ethanolamine was found to affect the rate of phosphatidylethanolamine biosynthesis by two mechanisms: the rate of ethamolamine uptake; and the triggering of ethanolamine kinase as a second rate-limiting step for phosphatidylethanolamine biosynthesis when ethanolamine uptake is high. Serine can also be used as a precursor for phosphatidylethanolamine synthesis. Serine was found to modulate phosphatidylethanolamine biosynthesis by altering the rate of ethanolamine uptake

and by inhibiting the ethanolamine kinase reaction of the CDP-ethanolamine pathway. However, serine was not utilized as an alternate precursor for the synthesis of phosphatidylethanolamine by either base-exchange or decarboxylation pathways when the CDP-ethanolamine pathway was inhibited.

Most mammalian cells possess a significant amount of vinyl ether linked ethanolamine-containing phospholipid. The role of plasmenylethanolamine in mammalian tissues is generally ignored, with most studies not bothering to discern between the different types of ethanolamine-containing phospholipids within a cell. The hamster heart contains $<7\%$ plasmenylethanolamine in its ethanolamine-containing phospholipids and was ideal for the study of phosphatidylethanolamine biosynthesis in the mammalian heart. However, most mammalian hearts contain 25-50% plasmenylethanolamine in their ethanolamine-containing phospholipids. Hence, Guinea pig tissues were chosen for the study of plasmenylethanolamine since the composition of plasmenylethanolamine in its tissues is quite high. This study lead to the first identification of a soluble plasmalogenase capable of catabolizing plasmenylethanolamine. The work presented has taken care to highlight the roles of the separate types of ethanolamine-containing phospholipids in mammalian tissues to further our understanding of their metabolism.

REFERENCES

Ansell, G.B., and Metcalfe, R.F. 1971.
Studies on the CDP-ethanolamine-1,2-diglyceride ethanolaminephosphotransferase of rat brain.
J. Neurochem. 18: 647-665.

Ansell, G.B., and Spanner S. 1982.
Phosphatidylserine, phosphatidylethanolamine and phosphatidylcholine.
In Phospholipids. *Edited by* J.N. Hawthorne and G.B. Ansell. Elsevier Biomedical Press, Amsterdam, pp. 1-49.

Ansell, G.B., and Spanner, S. 1968.
Plasmalogenase activity in normal and demyelinating tissue of the central nervous system.
Biochem. J. 108: 207-209.

Arthur, G., Covic, L., Wientzek, M., and Choy, P.C. 1985.
Plasmalogenase in hamster heart.
Biochim. Biophys. Acta 833: 189-195.

Arthur, G., and Page L. 1991.
Synthesis of phosphatidylethanolamine and ethanolamine plasmalogen by the CDP-ethanolamine and decarboxylase pathways in rat heart, kidney and liver.
Biochem. J. 273: 121-125.

Baba, S., Watanabe, Y., Gejyo, F., Arakawa, M. 1984.
High-performance liquid chromatographic determination of serum aliphatic amines in chronic renal failure.
Clin. Chim. Acta 136: 49-56.

Ballas, L., and Bell, R.M. 1980.
Topography of phosphatidylcholine, phosphatidylethanolamine, and triacylglycerol biosynthetic enzymes in rat liver microsomes.
Biochim. Biophys, Acta 602: 578-590.

Bartlett, G.R. 1959.
Phosphorus assay in column chromatography.
J. Biol. Chem. 234: 466-468.

Bell, R.M., Ballas, L.M., and Coleman, R.A. 1981.
Lipid topogenesis.
J. Lipid Res. 22: 391-403.

Bell, R.M., and Coleman, R.A. 1980.
Enzymes of glycerophospholipid synthesis in eukaryotes.
Ann. Rev. Biochem. 49: 459-487.

Bell, R.M., and Burns, D.J. 1991.
Lipid activation of protein kinase C.
J. Biol. Chem. 266: 4661-4664.

Berridge, M.J. 1987.
Inositol triphosphate and diacylglycerol: Two interacting second messengers.
Ann. Rev. Biochem. 56: 159-193.

Berridge, M.J., and Irvine, R.F. 1989.
Inositol phosphates and cell signalling.
Nature 341: 197-205

Bers, D.M. 1979.
Isolation and characterization of cardiac sarcolemma.
Biochim. Biophys. Acta 555: 131-146.

Billah, M.M., and Anthes, C. 1990.
The regulation of and cellular functions of phosphatidylcholine hydrolysis.
Biochem. J. 269: 281-291.

Borkenhagen, L.F., Kennedy, E.P., and Fielding, L. 1961.
Enzymatic formation and decarboxylation of phosphatidylserine.
J. Biol. Chem. 236: PC28-30.

Bjerve, K.S. 1985.
The biosynthesis of phosphatidylserine and phosphatidylethanolamine from L-[3-^{14}C]serine in isolated rat hepatocytes.
Biochim, Biophys. Acta 833: 396-405.

Brophy, P.J., Choy, P.C., Toone, J.R., and Vance, D.E. 1977.
Choline kinase and ethanolamine kinase are separate, soluble enzymes in rat liver.
Eur. J. Biochem. 78: 491-495.

Carman, G.M., and Henry, S.A. 1989.
Phospholipid biosynthesis in yeast.
Ann. Rev. Biochem. 58: 635-669.

Cao, Y-z., Tam, S.W., Arthur, G.A., Chen, H., and Choy, P.C. 1987.
The purification and characterization of a phospholipase A in hamster heart cytosol for the hydrolysis of phosphatidylcholine.
J. Biol. Chem. 262: 16927-16935

D'Amato, R.A., Horrocks, L.A., and Richardson, K.E. 1975.
Kinetic properties of plasmalogenase from bovine brain.
J. Neurochem. 24: 1251-1255.

Dorman, R.V., Toews, A.D., and Horrocks, L.A. 1977.
Plasmalogenase activities in neuronal perikarya, astroglia, and oligodendroglia isolated from bovine brain.
J. Lipid Res. 18: 115-117.

Doering, T.L., Masterson, W.J., Hart, G.W., and Englund, P.T. 1990.
Biosynthesis of glycosylphosphatidylinositol membrane anchors.
J. Biol. Chem. 265: 611-614.

Draus, E., Niefind, J., Vietor, K., and Havsteen, B. 1990.
Isolation and characterization of the human liver ethanolamine kinase.
Biochim. Biophys. Acta 1045: 195-204.

Exton, J.E. 1990.
Signalling through phosphatidylcholine breakdown.
J. Biol. Chem. 265: 1-4.

Folch, J., Lees, M., and Sloane-Stanley, G.H. 1957.
A simple method for the isolation and purification of total lipides from animal tissues.
J. Biol. Chem. 226: 497-506.

Gilman, A.G. 1987.
G proteins: Transducers of receptor-generated signals.
Ann. Rev. Biochem. 56: 615-649.

Gordon, J.I., Duronio, R.J., Rudnick, D.A., Adams, S.P., and Gokel, G.W. 1991.

Protein N-myristoylation.
J. Biol. Chem. **266**: 8647-8650.

Gottfried, E.L., and Rapport, M.M. 1962.
The biochemistry of plasmalogens.
J. Biol. Chem. **237**: 329-333.

Gross, R.W. 1984.
High plasmalogen and arachidonic acid content of canine myocardial sarcolemma: A fast atom bombardment mass spectroscopic and gas chromatography-mass spectroscopic characterization.
Biochemistry **23**: 158-165.

Gross, R.W. 1985.
Identification of plasmalogens as the major phospholipid constituent of cardiac sarcoplasmic reticulum.
Biochemistry **24**: 1662-1668.

Gunawan, J., and Debuch, H. 1982.
Lysoplasmalogenase - A microsomal enzyme from rat brain.
J. Neurochem. **39**: 693-699.

Hafez, M.M., and Costlow, M.E. 1989.
Phosphatidylethanolamine turnover is an early event in the response of NB_2 lymphoma cells to prolactin.
Exp. Cell Res. **184**: 37-43.

Hannun, Y.A., Loomis, C.R., and Bell, R.M. 1985.
Activation of protein kinase C by Triton X-100 mixed micelles containing diacylglycerol and phosphatidylserine.
J. Biol. Chem. **260**: 10039-10043.

Hatch, G.M., O, K., and Choy, P.C. 1989.
Regulation of phosphatidylcholine metabolism in mammalian hearts.
Biochem. Cell Biol. **67**: 67-77.

Heinrikson, R.L., and Meridith, S.C. 1984.
Amino acid analysis by reverse-phase high-performance liquid chromatography: precolumn derivatization with phenylisothiocyanate.
Anal. Biochem. **136**: 65-74.

Hii, C.S., Edwards, Y.S., and Murray, A.W. 1991.
Phorbol ester-stimulated hydrolysis of phosphatidylcholine and phosphatidylethanolamine by phospholipase D in HeLa cells.
J. Biol. Chem. 266: 20238-20243.

Hjelmstad, R.H., and Bell, R.M. 1987.
Mutants of *Saccharomyces cerevisiae* defective in *sn*-1,2-diacylglycerol cholinephosphotransferase.
J. Biol. Chem. 262: 3909-3917.

Hjelmstad, R.H., and Bell, R.M. 1988.
The *sn*-1,2-diacylglycerol ethanolamine phosphotransferase activity of *Saccharomyces cerevisiae.*
J. Biol. Chem. 263: 19748-19757.

Hjelmstad, R.H., and Bell, R.M. 1990.
The *sn*-1,2-diacylglycerol cholinephosphotransferase of *Saccharomyces cerevisiae.*
J. Biol. Chem. 265: 1755-1764.

Hjelmstad, R.H., and Bell, R.M. 1991a.
sn-1,2-Diacylglycerol choline- and ethanolaminephosphotransferases in *Saccharomyces cerevisiae.*
J. Biol. Chem. 266: 4357-4365.

Hjelmstad, R.H., and Bell, R.M. 1991b.
sn-1,2-Diacylglycerol choline- and ethanolaminephosphotransferases in *Saccharomyces cerevisiae.*
J. Biol. Chem. 266: 5094-5103.

Horrocks, L.A., and Fu, S.U. 1978.
Pathway for the hydrolysis of plasmalogens in brain.
Adv. Exp. Med. Biol. 101: 397-406.

Horrocks, L.A., and Sharma, M. 1982.
Plasmalogens and *O*-alkyl glycero-phospholipids.
In Phospholipids. *Edited by* J.N. Hawthorne and G.B. Ansell. Elsevier Biomedical Press, Amsterdam. pp. 51-93.

Hosaka, K., Kodaki, T., and Yamashita, S. 1989.
Cloning and characterization of the yeast *CKI* gene encoding choline kinase

and its expression in *Escherichia coli*.
J. Biol. Chem. 264: 2053-2059.

Hyman, B.Y., and Spector, A.A. 1982.
Choline uptake in cultured human Y79 retinoblastoma cells: Effect of polyunsaturated fatty acid compositional modifications.
J. Neurochem. 38: 650-656.

Ishidate, K., Nakagomi, K., and Nakazawa, Y. 1984.
Complete purification of choline kinase from rat kidney and preparation of rabbit antibody against rat kidney choline kinase.
J. Biol. Chem. 259: 14706-14710.

Ishidate, K., Furusawa, K., and Nakazawa, Y. 1985.
Complete co-purification of choline kinase and ethanolamine kinase from rat kidney and immunological evidence for both kinase activities residing on the same enzyme protein(s) in rat tissues.
Biochim. Biophys. Acta 836: 119-124.

Jurkowitz-Alexander, M., Ebata, H., Mills, J.S., Murphy, E.J., and Horrocks, L.A. 1989.
Solubilization, purification and characterization of lysoplasmlogen alkenylhydrolase (lysoplasmalogenase) from rat liver microsomes.
Biochim. Biophys. Acta 1002: 203-212.

Katz, A.M., and Messineo, F.C. 1981.
Lipid-membrane interactions and the pathogenesis of ischemic damage in the myocardium.
Circ. Res. 48: 1-16.

Katz, A.M., and Messineo, F.C. 1982.
Myocardial membrane function and drug action in heart failure.
Am. Heart J. 102: 491-500.

Kiss, Z., and Anderson, W.B. 1990.
ATP stimulates the hydrolysis of phosphatidylethanolamine in NIH 3T3 cells.
J. Biol. Chem. 265: 7345-7350.

Kruse, T., Reiber, H., and Neuhoff, V. 1985.
Amino acid transport across the human blood-CSF barrier.
J. Neurol. Sci. 70: 129-138.

Kuge, O., Nishijima, M., and Akamatsu, Y. 1986a.
Phosphatidylserine biosynthesis in cultured chinese hamster ovary cells.
J. Biol. Chem. **261**: 5790-5794.

Kuge, O., Nishijima, M., and Akamatsu, Y. 1986b.
Phoshatidylserine biosynthesis in cultured chinese hamster ovary cells.
J. Biol. Chem. **261**: 5795-5798.

Kuge, O. Nishijima, M., and Akamatsu, Y. 1991.
A cloned gene encoding phosphatidylserine decarboxylase complements the phosphatidylserine biosynthetic defect of a chinese hamster ovary cell mutant.
J. Biol. Chem. **266**: 6370-6376.

Le Dur, A., Chaby, R., and Szabo, L. 1978.
Colorimetric estimation of ethanolamine with *p*-benzoquinone in the nanomole range. Anal. Biochem. **88**: 285-295.

Li, Q-X., and Dowhan, W. 1988.
Structual characterization of *Escherichia coli* phosphatidylserine decarboxylase.
J. Biol. Chem. **263**: 11516-11522.

Lipton, B.A., Davidson, E.P., Ginsburg, B.H., Yorek, M.A. 1990.
Ethanolamine metabolism in cultured bovine aortic endothelial cells.
J. Biol. Chem. **265**: 7195-7201.

Lowry, O.H., Rosebrough, N.J., Farr, A.L., and Randall, R.J. 1951.
Protein measurement with the Folin phenol reagent.
J. Biol. Chem. **193**: 265-275.

Man, R.Y.K., and Choy, P.C. 1982.
Lysophosphatidylcholine causes cardiac arrhythmia.
J. Mol. Cell. Cardiol. **14**: 173-175.

Masters, B.S.S., Williams, Jr., C.H., and Kamin, H. 1967.
The preparation and properties of microsomal TPNH-cytochrome *c* reductase from pig liver.
Methods Enzymol. **10**: 565-569.

McMaster, C.R., and Choy, P.C. 1992a.
Newly imported ethanolamine is preferentially utilized for phosphatidylethanolamine bioynthesis in the hamster heart.

Biochim. Biophys. Acta **1124**: 13-16.

McMaster, C.R., and Choy, P.C. 1992b.
The determination of tissue ethanolamine levels by reverse-phase high-performance liquid chromatography.
Lipids **27**: (in press).

McMaster, C.R., and Choy, P.C. 1992c.
Serine regulates phosphatidylethanolamine biosynthesis in the hamster heart.
J. Biol. Chem. **267**: (in press).

McMaster, C.R., Tardi, P., and Choy, P.C. 1992a.
Modulation of phosphatidylethanolamine biosynthesis by exogenous ethanolamine and analogues in the hamster heart.
Mol. Cell. Biochem. **112**: (in press).

McMaster, C.R., Lu, C-Q., and Choy, P.C. 1992b.
The existence of a soluble plasmalogenase in guinea pig tissues.
Lipids (submitted).

Milakofsky, L., Hare, T.A., Miller, J.M., and Vogel, W.H. 1985.
Rat plasma levels of amino acids and related compounds during stress.
Life Sci. **36**: 753-761.

Miller, M.A., and Kent, C. 1986.
Charcterization of the pathways for phosphatidylethanolamine biosynthesis in chinese hamster ovary mutant and parental cell lines.
J. Biol. Chem. **261**: 9753-9761.

Morand, O.H., Zoeller, R.A., and Raetz, C.R.H. 1988.
Disappearance of plasmalogens from membranes of animal cells subjected to photosensitized oxidation.
J. Biol. Chem. **263**: 11597-11606.

Morakami, H., Taniguchi, S., Fujii, K., Mori, H., Kumada, K., Fujiwara, M., and Fujiwara, M. 1987.
Preferential synthesis of diacyl and alkenylacyl ethanolamine and choline phosphoglycerides in rabbit platelet membranes.
J. Biol. Chem. **262**: 1213-1217.

Murakami, H., Masui, H., Sato, G., Sueoka, N., Chow, T.P. and Kano-Sueoka,

T. 1982.
Growth of hybridoma cells in serum-free medium: Ethanolamine is an essensial component.
Proc. Natl. Acad. Sci. USA 79: 1158-1162.

Neely, J.R., and Rovetto, M.J. 1975.
Techniques for perfusing isolated rat hearts.
Methods Enzymol. 29: 43-60.

Nishijima, M., Kuge, O., and Akamatsu, Y. 1986.
Phosphatidylserine biosynthesis in cultured chinese hamster ovary cells.
J. Biol. Chem. 261: 5784-5789.

O, K-M., Siow, Y.L., and Choy, P.C. 1989.
Hamster liver cholinephosphotransferase and ethanolamine phosphotransferase are separate enzymes.
Biochem. Cell Biol. 67: 680-687.

O, K-M., and Choy. 1990.
Solubilization and partial purification of cholinephosphotransferase in hamster tissues.
Lipids 25: 122-124.

Paltauf, F. 1984.
Ether lipids as substrates for lipolytic enzymes.
In Ether Lipids. *Edited by* H.K. Mangold and F.Paltauf. Academic Press, New York. pp. 211-230.

Pelech, S.L., and Vance, D.E. 1989.
Signal tranduction via phosphatidylcholine cycles.
Trends Biochem. Sci. 14: 28-30.

Polokoff, M.A., Wing, D.C., and Raetz, C.R. 1981.
Isolation of somatic cell mutants in the biosynthesis of phosphatidylethanolamine.
J. Biol. Chem. 256: 7687-7690.

Porter, T.J., and Kent, C. 1990.
Purification and characterization of choline/ethanolamine kinase from rat liver.
J. Biol. Chem. 265: 414-422.

Pu, G.A-W. and Anderson, R.E. 1984.
Ethanolamine accummulation by photoreceptor cells of the rabbit retina.
J. Neurochem. 42: 185-191.

Renkonnen, O. 1963.
Isolation of native plasmalogens.
Acta Chem. Scand. 17: 634-640.

Samuelsson, B., and Funk, C.D. 1989.
Enzymes involved in the biosynthesis of leukotriene B_4.
J. Biol. Chem. 264: 19469-19472.

Schneider, W.J., and Vance, D.E. 1978.
Effect of choline deficiency on the enzymes that synthesize phosphatidylcholine and phosphatidylethanolamine in rat liver.
Eur. J. Biochem. 85: 181-187.

Singer, S.J., and Nicolson, G.L. 1972.
The fluid mosaic model of the structure of cell membarnes.
Science 175: 720-731.

Simbeni, R., Paltauf, F., and Daum, G. 1990.
Intramitochondrial transfer of phospholipids in the yeast, *Saccharomyces cerevisiae*.
J. Biol. Chem. 265: 281-285.

Stein, W.H., and Moore, S. 1954.
The free amino acids of human blood plasma.
J. Biol. Chem. 211: 915-926.

Sundler, R. 1973.
Biosynthesis of rat liver phosphatidylethanolamines from intraportally injected ethanolamine.
Biochim. Biophys. Acta 306: 218-226.

Sundler, R., Akesson, B., and Nilsson, A. 1974.
Quantitative role of base exchange in phosphatidylethanolamine synthesis in isolated rat hepatocytes.
Febs Lett. 43: 303-307.

Sundler, R. 1975.

Ethanolamine cytidylyltransferase.
J. Biol. Chem. 250: 8585-8590.

Sundler, R. and Åkesson, B. 1975a.
Biosynthesis of phosphatidylethanolamines and phosphatidylcholines from ethanolamine and choline in rat liver.
Biochem. J. 146: 309-315.

Sundler, R., and Åkesson, B. 1975b.
Regulation of phospholipid biosynthesis in isolated rat hepatocytes.
J. Biol. Chem. 250: 3359-3367.

Suzuki, T.T., and Kanfer, J,N. 1985.
Purification and properties of an ethanolamine-serine base exchange enzyme of rat brain microsomes.
J. Biol. Chem. 260: 1394-1399.

Sweeley, C.C. 1969.
Chromatography on columns of silicic acid.
Methods Enzymol. 14: 254-267.

Tallan, H.H., Moore, S., and Stein, W.H. 1954.
Studies on the free amino acids and related compounds in the tissues of the cat.
J. Biol. Chem. 211: 927-939.

Tessner, T.G., and Wykle, R.L. 1987.
Stimulated neutrophils produce an ethanamine plasmalogen analogue of platelet-activating factor.
J. Biol. Chem. 262: 12660-12664.

Tijburg, L.B.M., Houweling, M., Geelen, M.J.H., and Van Golde, L.M.G. 1988.
Effect of dietary conditions on the pool sizes of precursors of phosphatidylcholine and phosphatidylethanolamine synthesis in rat liver.
Biochim. Biophys. Acta 959: 1-8.

Tijburg, L.B.M., Geelen, M.J.H., and Van Golde, L.M.G. 1989a.
Biosynthesis of phosphatidylethanolamine via the CDP-ethanolamine route is an important pathway in isolated rat hepatocytes.
Biochem. Biophys. Res. Comm. 160: 1275-1280.

Tijburg, L.B.M., Geelen, M.J.H., and Van Golde, L.M.G. 1989b.
Regulation of the biosynthesis of triacylglycerol, phosphatidylcholine and phosphatidylethanolamine in the liver.
Biochim. Biophys. Acta 1004: 1-19.

Uchida, T., and Yamashita, S. 1990.
Purification and properties of choline kinase from rat brain.
Biochim. Biophys. Acta 1043: 281-288.

Van Sande, M., and Van Camp, K. 1979.
Alkaline phosphatase, phosphoethanolamine, and ethanolamine content in benign prostatic hypertrophy.
Invest. Urol. 16: 445-446.

Vance, D.E. 1985.
Phospholipid metabolism in eucaryotes.
In Biochemistry of Lipids and Membranes. *Edited by* D.E. Vance and J.E. Vance. The Benjamin/Cummings Publishing Company, Inc., Menlo Park, California. pp. 242-270.

Vance, D.E., Choy, P.C., Farren, S.B., Lim, P.H., and Schneider, W.J. 1977.
Asymmetry of phospholipid biosynthesis.
Nature 270: 268-269.

Vance, J.E. 1991.
Newly made phosphatidylserine and phosphatidylethanolamine are preferentially translocated between rat liver mitochondria and endoplasmic reticulum.
J. Biol. Chem. 266: 89-97.

Vance, J.E., and Vance, D.E. 1988.
Does rat liver Golgi have the capacity to synthesize phospholipids for lipoprotein secretion?.
J. Biol. Chem. 263: 5898-5909.

Vecchini, A., Roberti, R., Freysz, L., and Binaglia, L. 1987.
Partial purification of ethanolaminephosphotransferase from rat brain microsomes.
Biochim. Biophys. Acta 918: 40-47.

Voelker, D.R. 1984.

Phosphatidylserine functions as the major precursor of phosphatidylethanolamine in cultured BHK-21 cells.
Proc. Natl. Acad. Sci. USA **81**: 2669-2673.

Voelker, D.R. 1985.
Disruption of phosphatidylserine translocation to the mitochondria in baby hamster kidney cells.
J. Biol. Chem. **260**: 14671-14676.

Voelker, D.R. 1989a.
Reconstitution of phosphatidylserine import into rat liver mitochondria.
J. Biol. Chem. **264**: 8019-8025.

Voelker, D.R. 1989b.
Phosphatidylserine translocation to the mitochondria is an ATP-dependent process in permeabilized animal cells.
Proc. Natl. Acad. Sci. USA **86**: 9921-9925.

Walsh, J.P., and Bell, R.M. 1986a.
sn-1,2-Diacylglycerol kinase of *Escherichia coli*.
J. Biol. Chem. **261**: 6239-6247.

Walsh, J.P., and Bell, R.M. 1986b.
sn-1,2-Diacylglycerol kinase of *Escherichia coli*.
J. Biol. Chem. **261**: 15062-15069.

White, D.A. 1973.
The phospholipid composition of mammalian tissues.
In Form and Function of Phospholipids. *Edited by* G.B. Ansell, J.N. Hawthorne, and R.M.C. Dawson. Elsevier Scientific, Amsterdam. pp. 441-482.

Wykle, R.L., Blank, M.L., and Snyder, F. 1973.
The enzymatic encorporation of arachidonic acid into ether-containing choline and ethanolamine phosphoglycerides by deacylation-reacylation reactions.
Biochim. Biophys. Acta **326**: 26-33.

Xu, Z., Byers, D.M., Palmer, F.B.S.C., Spence, M.W., and Cook, H.W. 1991.
Serine utilization as a precursor of phosphatidylserine and alkenyl-(plasmenyl)-, alkyl-, and acylethanolamine phosphoglycerides in cultured glioma cells.
J. Biol. Chem. **266**: 2143-2150.

Yavin, E., and Gatt, S. 1972a.
Oxygen-dependent cleavage of the vinyl-ether linkage of plasmalogens.
Eur. J. Biochem. 25: 431-436.

Yavin, E., and Gatt, S. 1972b.
Oxygen-dependent cleavage of the vinyl-ether linkage of plasmalogens.
Eur. J. Biochem. 25: 437-446.

Yeagle, P.L. 1989.
Lipid regulation of cell membrane structure and function.
FASEB J. 3: 1833-1842.

Yorek, M.A., Rosario, R.T., Dudley, D.T., and Spector, A.A. 1985.
The utilization of ethanolamine and serine for ethanolamine phosphoglyceride synthesis by human Y79 retinoblastoma cells.
J. Biol. Chem. 260: 2930-2936.

Yorek, M.A., Dunlap, J.A., Spector, A.A., and Ginsberg, B.H. 1986.
Effect of ethanolamine on choline uptake and incorporation into phosphatidylcholine in human Y79 retinoblastoma cells.
J. Lipid Res. 27: 1205-1213.

Zelinski, T.A., and Choy, P.C. 1982a.
Choline regulates phosphatidylethanolamine biosynthesis in the isolated hamster heart.
J. Biol. Chem. 257: 13201-13204.

Zelinski, T.A., and Choy, P.C. 1982b.
Phosphatidylethanolamine biosynthesis in the isolated hamster heart.
Can. J. Biochem. 60: 817-823.

Zelinski, T.A., and Choy, P.C. 1984.
Ethanolamine inhibits choline uptake in the isolated hamster heart.
Biochim. Biophys. Acta 794: 326-332.