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ETHER PHOSPHOLIPID METABOLISM
IN THE ADULT AWAKE RAT: A MODEL TO STUDY
THE ROLE OF ETHER PHOSPHOLIPIDS IN BRAIN

DISSERTATION

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

By

Thad A. Rosenberger

*****

The Ohio State University
1999

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ABSTRACT

Ether phospholipids represent a unique class of lipids characterized as having either a 1-0-alkyl or a 1-0-alk-l'-enyl linkage at the sn-1 position of the phospholipid moiety. This bond defines the ether phospholipid and is conserved throughout the biosynthetic process de novo. In the brain, the pathways responsible for the biosynthesis of ether phospholipid de novo display precursor-product relationships involving a sequential series of reactions. This precursor-product relationship was exploited in the development of a pulse-chase fatty alcohol model used to quantitate the biosynthetic rate and turnover of ether phospholipid synthesis following an intravenous injection of [1,1-³H]hexadecanol in the adult awake rat. The primary hypothesis used in this investigation is that ether phospholipid metabolism can be measured within the central nervous system using an intravenous infusion of [1,1-³H]hexadecanol and steady-state tracer kinetics. The greatest importance of this work is that the application of tracer kinetic analysis to ether phospholipid biosynthesis de novo allows assessment of the biological importance of these molecules within the central nervous in vivo.

To address these suppositions, whole brain analysis, quantitative autoradiography, and membrane fractionation experiments were performed using this model. Whole brain analysis showed that neither plasma or brain fatty alcohol levels were elevated by the
infusion and approximately $1.0 \pm 0.4\%$ of total labeled plasma hexadecanol was incorporated into brain. At 2.0 hours, $29.1 \pm 1.5$, $32.6 \pm 2.3$, and $31.4 \pm 1.0$ percent of total labeled brain phospholipid was found in the $1-O$-alkyl-2-acyl-$sn$-glycerol-3-phosphate, ethanolamine and choline glycerophospholipid fractions, respectively. Ethanolamine and choline glycerophospholipids contained only trace amounts of radioactivity in the diacyl fraction, indicating that the oxidation of $[1,1-^3\text{H}]$hexadecanol to palmitic acid resulted in the complete loss of the tritium atoms. The rates of synthesis and turnover of brain ether phospholipids were calculated \textit{in vivo} using tracer kinetics. The rates of plasmenylcholine, plasmanylethanolamine, plasmenylethanolamine, and plasmenylcholine synthesis were 33.6, 63.3, 1,157.4, and 2,861.6 to 533.6 nmol x gram$^{-1}$ x hour$^{-1}$, respectively. The half-lives of total brain plasmenylethanolamine and plasmenylcholine were 12.3 and 0.1 to 1.1 hours.

The time-dependent autoradiography studies show that ether phospholipids are actively synthesized in gray matter regions and are heterogeneously distributed throughout the brain. Only a small percentage of activity was found in the white matter. Gray matter regions with the highest levels of incorporation include the cingulate cortex, motor cortex, somatosensory cortex, molecular layer of the hippocampus, and the thalamic nucleus. Membrane fractionation studies support the autoradiographic analysis and show that approximately 97 percent of ether phospholipid biosynthesis \textit{de novo} occurred in gray matter regions with only a small percentage occurring in the myelin.

These studies suggests that ether phospholipids, primarily plasmenylethanolamine and plasmenylcholine, actively participate in signaling and/or membrane remodeling independent of myelin phospholipid turnover. These studies also suggests that these
phospholipids play a significant role in brain function. Application of this model to ether phospholipid biosynthesis in vivo offers an opportunity to more fully characterize the role these lipids have in brain function and provides a means to evaluate their participation in signal transduction, neuroplasticity, brain injury, as well as other diseases.
Dedicated in loving memory of my brother
Joseph A. Rosenberger
July 30, 1963 to June 19, 1999
ACKNOWLEDGMENTS

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I thank Drs. Eric J. Murphy, Akhlaq A. Farooqui, and A. David Purdon, for their stimulating discussions and comic relief which made the completion of this project and my graduate career both interesting and enjoyable. Without Dr. Murphy's assistance, I would never have gained the analytical abilities nor critical thinking needed to complete this degree in a timely manner.

I also wish to thank Dr. Stanley I. Rapoport for the opportunity to complete my graduate research at the National Institute on Aging in the section on Brain Physiology and Metabolism. His encouragement will always be appreciated.

Finally, I would like to acknowledge my family for their support, patience, and encouragement. I would also like to thank my fiancee, Dora, for her love and for helping me to explain to my family why I have been in school for so long and that being a graduate student isn't all that strange.
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PUBLICATIONS

Abstracts


Research Publications


Book Chapters


FIELDS OF STUDY

Major Field: Medical Biochemistry
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<th>Compound</th>
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<tr>
<td>ethanolamine glycerophospholipid</td>
<td>EtnGpl</td>
</tr>
<tr>
<td>plasmanylethanolamine</td>
<td>PakEtn</td>
</tr>
<tr>
<td>1-(O)-alkyl-2-acyl-(sn)-glycero-3-phosphoethanolamine</td>
<td>PakEtn</td>
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<tr>
<td>2-lysoplasmanylethanolamine</td>
<td>2-lysoPakEtn</td>
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<td>plasmenylethanolamine</td>
<td>PlsEtn</td>
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<tr>
<td>1-(O)-alk-1'-enyl-2-acyl-(sn)-glycero-3-phosphoethanolamine</td>
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<tr>
<td>2-lysoplasmenylethanolamine</td>
<td>2-lysoPakEtn</td>
</tr>
<tr>
<td>choline glycerophospholipid</td>
<td>ChoGpl</td>
</tr>
<tr>
<td>plasmanylcholine</td>
<td>PakCho</td>
</tr>
<tr>
<td>1-(O)-alkyl-2-acyl-(sn)-glycero-3-phosphocholine</td>
<td>PakCho</td>
</tr>
<tr>
<td>2-lysoplasmanylcholine, 2-lysoPAF</td>
<td>2-lysoPakCho</td>
</tr>
<tr>
<td>plasmenylcholine</td>
<td>PlsCho</td>
</tr>
<tr>
<td>1-(O)-alk-1'-enyl-2-acyl-(sn)-glycero-3-phosphocholine</td>
<td>PlsCho</td>
</tr>
<tr>
<td>2-lysoplasmenylcholine</td>
<td>2-lysoPlsCho</td>
</tr>
<tr>
<td>diradyl-(sn)-glycero-3-phosphate</td>
<td>HOGpl</td>
</tr>
<tr>
<td>1-(O)-alkyl-2-acyl-(sn)-glycero-3-phosphate</td>
<td>PakOH</td>
</tr>
<tr>
<td>Compound</td>
<td>Abbreviation</td>
</tr>
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<tr>
<td>1-O-alkyl-2-lyso-sn-glycero-3-phosphate</td>
<td>2-lysoPakOH</td>
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<tr>
<td>hexadecanol</td>
<td>16:0 (ol)</td>
</tr>
<tr>
<td>octadecanol</td>
<td>18:0 (ol)</td>
</tr>
<tr>
<td>Δ9 octadecenol</td>
<td>18:1 (ol)</td>
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<td>platelet activating factor</td>
<td>PAF</td>
</tr>
<tr>
<td>1-O-alkyl-2-acetyl-sn-glycero-3-phosphocholine</td>
<td>DhAP</td>
</tr>
<tr>
<td>dihydroxyacetonephosphate</td>
<td>acyl DHAP</td>
</tr>
<tr>
<td>1-O-alkyl dihydroxyacetonephosphate</td>
<td>1-O-alkyl DHAP</td>
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<tr>
<td>S-adenosyl methionine</td>
<td>AdoMet</td>
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Note: The six letter abbreviations of the phospholipids and the variations of these six letter abbreviations adhere to the published guidelines proposed by Professor Lloyd A. Horrocks (Horrocks, 1989a) and the International Union of Pure and Applied Chemistry-International Union of Biochemistry (IUPAC-IUB, 1978).
CHAPTER I

INTRODUCTION

In this study, the rate of biosynthesis and turnover of brain ether phospholipids in the awake adult rat were measured in vivo using a pulse-chase experimental rat model. Early indications from this work lend weight to my supposition that "brain ether phospholipids, primarily ethanolamine and choline plasmalagens, actively participate in receptor-mediated signaling events and measurements of their rate of synthesis and turnover in vivo will provide support for this hypothesis." I will also argue that there are active ether phospholipid pools within the brain, which are localized to regions associated with signal transduction and long-term potentiation. I will provide support for my hypotheses by introducing an experimental rat fatty alcohol model which utilizes an intravenous infusion of [1,1-^3H]hexadecanol for measurement of the rate of ether phospholipid synthesis de novo. This dissertation is divided into three sections: (1) chemical and mathematical model, (2) whole brain studies, and (3) autoradiographic analysis.

The primary importance of this work is that the application of tracer kinetic analysis to brain ether phospholipid biosynthesis de novo allows assessment of the
biological importance of these molecules within the central nervous system. The fundamental assumption made in this investigation is that ether phospholipid metabolism can be measured within the central nervous system using an intravenous infusion of [1,1-$^3$H]hexadecanol and steady-state tracer kinetics. Furthermore, development of an animal model to study ether phospholipid biosynthesis gives a means to efficiently test drugs and therapeutic strategies that are targeted to specific pathways involved in ether phospholipid metabolism.

The term ether phospholipids is a general name given to naturally occurring lipids characterized as having a 1-O-alkyl (plasmanyl-) or a 1-O-alk-1'-enyl (plasmenyl-) linkage at the sn-1 position of the phospholipid moiety. Ether phospholipids are widely distributed in all mammalian tissues. However, they are especially abundant in heart, kidney, muscle, and the central nervous system (Druilhet et al., 1975, Horrocks, 1972, Horrocks and Sharma, 1982). In the human brain, plasmenyl-type ether phospholipids, commonly referred to as plasmalogens, account for approximately 23% of total brain phospholipid (Panganamala et al., 1971), whereas studies using monkey spinal cord myelin show 33-35% of myelin phospholipids are plasmalogen (Horrocks, 1967). In rat brain, a similar distribution is observed in which the plasmenyl-type ether lipids account for 27% of the total brain phospholipid content and 33% in the myelin phospholipid content (Ansell, 1973, Eng and Noble, 1968). However, of all the ether phospholipids, plasmenylethanolamine (P1sEtn) has the highest content in both total brain and myelin phospholipids, making up 26% and 32% of the total, respectively (Ansell, 1973).
Although the large proportion of PIsEtn within the brain and myelin implies a structural role, evidence exists that suggests a greater physiological function.

The brain contains several different enzymes that are involved in the remodeling of ether phospholipids and the breakdown of ether phospholipids into several different biologically active molecules. A number of brain enzymes are highly selective for plasmanyl- and plasmenyl-type glycerophospholipids including both anabolic acyltransferases and phosphotransferases (Choy et al., 1997, Nixon et al., 1999, Yamashita et al., 1997), as well as catabolic lipases (Gunawan and Debuch, 1981, Gunawan et al., 1979, Hirashima et al., 1992, Van Iderstine et al., 1996, Wykle and Schremmer, 1974). The mere existence of these enzymes suggests that ether phospholipids are involved in a dynamic function such as lipid-mediated signal transduction. In particular, a 39 kDa phospholipase A₂ has been found in brain that is highly selective for plasmenylethanolamine (PIsEtn) and may be involved in plasmalogen acyl chain remodeling and/or arachidonic acid release (Hirashima et al., 1992). Unfortunately, little is known concerning the regulation of this enzyme. However, it may be involved in the initial release of arachidonic acid following ischemia, reperfusion, and spinal cord injuries (Farooqui et al., 1997). Furthermore, this enzyme provides the potential to release the reservoir of arachidonic acid contained in the ether phospholipids for prostaglandin and thromboxane formation (Farooqui et al., 1995). In heart, a similar enzyme is stimulated by IL-1 and releases arachidonic acid from plasmenylcholine (PIsCho) (McHowat and Liu, 1997). This enzyme is also stimulated during myocardial ischemia and its product, lysoplasmenylcholine (lysoPIsCho), is thought to contribute to
the electrophysiological alterations that result in the arrhythmogenesis found in the ischemic heart (McHowat et al., 1998).

The brain also contains microsomal enzymes that further metabolize the 2-lysoplasmenylphospholipid products produced by PLA$_2$ activity. These enzymes breakdown 2-lyso plasmylethanolamine (lysoPlsEtn) to produce both ethanolamine and phosphoethanolamine (Gunawan et al., 1979). This enzyme also functions to degrade 2-lysoplasmanylethanolamine (lysoPakEtn) in a similar fashion (Vierbuchen et al., 1979). The $K_m$ value for 2-lysoPakEtn is lower than that for 2-LysoPlsEtn. Thus lysoPakEtn acts as a non-competitive inhibitor of this enzyme with respect to lysoPlsEtn hydrolysis (Gunawan et al., 1979). These enzymic activities are developmentally regulated with activity decreasing shortly after peak myelination, suggesting that 1-O-alk-1'-enylglycerol may be actively recycled during the myelination process. Lysoplasmalogenase activity is also seen in rat liver homogenates (Gunawan and Debuch, 1981) and rat intestinal epithelium (Jurkowitz et al., 1999). This enzyme is localized in microsomal membranes and hydrolyzes lysoPlsEtn to free aldehyde and glycerol-3-phosphoethanolamine independent of calcium and magnesium. Lysoplasmalogenase activity also exists in brain microsomal membranes with characteristics similar to those of the liver and intestinal enzymes (Gunawan and Debuch, 1985). Substrate specificity experiments show brain lysoplasmalogenase does not hydrolyze PlsEtn, requires a free hydroxyl group at the sn-2 position, and is independent of the polar head group attached to the sn-3 position of the phospholipid moiety. The existence in the brain of these enzymic activities that hydrolyze the products from the PLA$_2$-catalyzed reactions infers that mechanisms have evolved that
protect neural membranes from the detrimental effects of 2-lyso ether phospholipid analogs following receptor-mediated activation of the plasmalogen-selective PLA$_2$. The existence of these enzymes further supports the hypothesis that the turnover of brain plasmalogens reflects neural activity.

Additional evidence for the involvement of ether phospholipids in signaling mechanisms comes from cell culture studies using a number of different cell types. In cultured C6 rat glioma cells, phorbol esters stimulate the protein kinase C (PKC) mediated activation of phospholipase D (PLD) that in turn hydrolyzes PlsEtn (Van Iderstine et al., 1996). In rat mesangial cells, several ether-linked diradylglycerols are formed in response to inflammatory cytokines and vasoactive peptides (Mandal et al., 1997). The distinct ether-linked molecular species of diradylglycerols attenuate the activation of PKC (Musial et al., 1995). This suggests that PLD-mediated release of ether-linked diradylglycerols may function in a feedback manner to inhibit PKC activation and attenuate receptor-mediated phospholipase C (PLC) activation. This is supported in studies in which a bovine brain diacylglycerol lipase was unable to hydrolyze ether-linked molecular species of diradylglycerols (Rosenberger et al., unpublished results). This suggests that receptor-mediated activation of lipases that function to stimulate PKC may also be attenuated through lipase-mediated mechanisms secondary to the original activation. Therefore, if long term potentiation and PKC activation are required for memory consolidation, then lipases that produce and metabolize different molecular species of diradylglycerols may function to modulate the learning process.
The rapid turnover and metabolism of plasmalogens further supports the hypothesis that plasmalogens are involved in receptor-mediated signaling events. In mouse brain, arachidonic acid is rapidly incorporated into PlsCho following the intraventricular administration of albumin-bound $[^3]$H]arachidonic acid (Horrocks et al., 1986). After 1 hour, the specific activity of PlsCho approximated 40 percent of the total esterified fatty acid in the phospholipid fraction despite PlsCho comprising only 0.3 percent of total arachidonate-containing phospholipid molecular species in the mouse brain (Horrocks, 1989b). Since arachidonic acid is primarily located at the sn-2 position of phospholipid moiety, these results suggest that PlsCho is actively participating in a deacylation/reacylation process. This rapid turnover of PlsCho is supported from experiments using cultured hamster brain astrocytes in which incubation with $[^1]$C]glucose resulted in the highest specific activity being present in the PlsCho fraction (Eichberg et al., 1976). Following the intracerebral injection of $[^1]$H]glucose into rat brain, the synthesis rate and turnover time of PlsEtn was measured. In this study, the rate of PlsEtn formation de novo is 1,920 nmol x gram$^{-1}$ x hour$^{-1}$, with a turnover time of 5.3% of the total brain PlsEtn pool in one hour (Masuzawa et al., 1984). Since PlsEtn is the precursor for the formation of PlsCho, these data suggest that the rapid turnover of PlsEtn and the rapid incorporation of arachidonic acid into PlsCho may be due, in part, to increased receptor-mediated activation that results in the consumption of PlsCho.

Furthermore, several ether phospholipid metabolites have been shown to modulate biological function. Plasmanylcholine (PakCho) is the precursor for platelet activating factor (PAF), a potent second messenger (Blank et al., 1979). Its biosynthesis is induced
by 2-lyso-plasmenylethanolamine, a plasmalogen metabolite, through the activation of
CoA-independent transacylases in human neutrophil membrane preparations (Nieto et al.,
1991). In heart, 2-lyso plasmenylcholine (lysoPlsCho) activates myocardial cAMP-
dependent protein kinase (Williams and Ford, 1997). Also, 1-O-alk-1'-enyl-2-lyso-sn-
glycerol-3-phosphate is a potent activator of the MAP kinases ERK1/2 and elicits a
mitogenic response in Swiss 3T3 fibroblasts (Liliom et al., 1998). Thus, within the central
nervous system, ether phospholipids and their metabolites may have a greater biological
function and their maintenance may be critical to intracellular signaling and neuronal
function.

Other properties associated with the physiological role of ether phospholipids
involve the unique chemical characteristics associated with the vinyl ether linkage found in
plasmalogens. PlsEtn substantially lowers the bilayer to hexagonal II phase transition
temperature (Lohner et al., 1991), implying it may facilitate the formation of critical fusion
intermediates (Hermetter et al., 1989) and promote membrane fusion as well as vesicle
formation (Glaser and Gross, 1994).

In addition to serving as a structural component, the reactivity of the vinyl ether
linkage, found in plasmalogens, with singlet oxygen and reactive oxygen species implies
these phospholipids may also function as antioxidants (Lee, 1998). Exposure of
plasmalogen-deficient variants of the murine cell line RAW 264.7 to short-term treatment
with electron transport inhibitors antimycin A or cyanide (chemical hypoxia) resulted in a
more rapid loss of viability than in the parent strain and could be rescued using the
antioxidant Trolox, an alpha-tocopherol analogue (Zoeller et al., 1999). These findings
identified the vinyl ether linkage found in plasmalogens as a crucial element in cellular protection and support the hypothesis that plasmalogens, through the vinyl ether, act as antioxidants to protect cells against reactive oxygen species. However, in studies measuring the effect of PlsEtn on lipid peroxidation in liposomal suspension of egg yolk phosphatidylcholine, PlsEtn was not an effective free radical initiator (Zommara et al., 1995). These authors demonstrated that PlsEtn exerts its ability to function as an antioxidant by trapping transition metal ions and inhibiting the free radical initiation process. Regardless, the presence of the vinyl ether linkage found in plasmalogens does serve as a possible membrane-localized antioxidant. However, the mechanism by which this occurs remains ambiguous.

Since brain lipid metabolism is tightly coupled to receptor function and membrane trafficking as well as signal transduction, there is a need to reevaluate the influence of ether phospholipid metabolism in the central nervous system. To address this issue, I have developed a pulse-chase method to study ether phospholipid biosynthesis de novo using an intravenous infusion of \([1,1-{^3}H]\)hexadecanol in the awake adult rat. The use of the tritium labeled \([1,1-{^3}H]\)hexadecanol permits measurement of only ether phospholipid synthesis because the tritium is lost during the oxidation of \([1,1-{^3}H]\)hexadecanol to palmitic acid. Furthermore, there is the stoichiometric retention of label following the desaturation step involved in plasmalogen formation allowing for the direct measure of plasmalogen biosynthesis. Application of tracer kinetics to this model permits the determination of the de novo rate and turnover of brain ether phospholipids in vivo. Again, the primary importance of this work is that application of tracer kinetic analysis to brain ether
phospholipid biosynthesis *de novo* allows assessment of the biological importance of these molecules within the central nervous system. Also, development of an animal model to study ether phospholipid biosynthesis gives a means to efficiently test drugs and therapeutic strategies that are targeted to specific pathways involved in ether phospholipid metabolism.
CHAPTER 2

THE RAT FATTY ALCOHOL MODEL

2.1 Introduction

The purpose of this chapter is to outline the accepted pathways in the brain for ether phospholipids synthesis *de novo* and to introduce a pulse-chase rat fatty alcohol model that was used to characterize this process *in vivo*. This model utilizes an intravenous infusion of [1,1-\(^{3}\)H]hexadecanol that maintains steady-state levels of all brain ether phospholipids in the adult awake rat. The use of the labeled [1,1-\(^{3}\)H]hexadecanol permits measurement of only ether phospholipid biosynthesis because the tritium atoms are lost during the oxidation of [1,1-\(^{3}\)H]hexadecanol to palmitic acid. Furthermore, the stoichiometric retention of label following the desaturation step involved in plasmalogen biosynthesis allows the direct measure of all stable ether phospholipids in the brain. This model can be used in either biochemical or autoradiographic analysis. Biochemical analysis is used to calculate the biosynthetic rate, turnover and half-lives of the individual ether phospholipids in the brain and autoradiographic analysis was used to measure regional areas of incorporation. The premise of the model is that a pulse-chase rat fatty alcohol model, which maintains steady-state levels of brain ether phospholipids, can be
used to measure the *de novo* biosynthetic rate and turnover of brain ether phospholipid *in vivo*. In this chapter, I will: (1) outline the accepted pathways of brain ether phospholipid biosynthesis *de novo*, (2) introduce a simplified diagram reflecting these pathways, (3) present an experimental model that uses the tracer [1,1-$^3$H]hexadecanol, and (4) outline the tracer kinetics used to calculate the biosynthetic rate, turnover, and half-lives of the brain ether phospholipids *in vivo*.

2.2 Ether Phospholipid Synthesis *de novo*

Except for intermediary metabolites and certain bioactive lipids, ether linkages in phospholipids of mammalian cells exist almost exclusively in the choline and ethanolamine glycerophospholipid classes. In mammalian tissue, the 1-O-alkyl and the 1-O-alk-1'-enyl substituents, found in ether phospholipids, are located at the *sn*-1 position of the phospholipid moiety and are comprised mainly of hexadecanol (16:0 (ol)), octadecanol (18:0 (ol)), and Δ9 octadecenol (18:1 (ol)) (Snyder, 1996). This bond defines this subclass of lipids and is conserved throughout ether phospholipid biosynthesis *de novo*. Although all stable ether phospholipids in the brain are acylated at the *sn*-2 position with long-chain fatty acid, the plasmalogens are more highly enriched with polyunsaturated fatty acids, especially arachidonate (Nakagawa and Horrocks, 1986). In the mouse and ox brain, the relative proportion of hexadecanol-containing molecular species in the PlsEtn subclass are 19.3 and 25.4 percent, respectively (Sun and Horrocks, 1969a). However, despite the relatively low proportion of this species, following intraventricular administration of [$^3$H]arachidonic acid in the mouse, the PlsCho
fraction of hexadecanol-containing molecular species displayed the highest rate of incorporation (Horrocks et al., 1986). This suggests that the hexadecanol-containing molecular species of PIsEtn may be rapidly remodeled to form active metabolic pools of hexadecanol-containing PIsCho. The structures of these ether phospholipids are drawn in Figure 2.1.

In the late 1960s and 70s, following the advent of efficient extraction, separation, and analytical protocols, the biosynthetic pathways of ether phospholipid synthesis were studied. Ether phospholipid biosynthesis occurs in a series of reactions involving both peroxisomal and microsomal membranes (Paltauf, 1994). The preliminary steps of ether phospholipid biosynthesis involve the formation of both fatty alcohol and acyl-dihydroxyacetone phosphate (acyl-DHAP). These reactions occur on the cytoplasmic and lumenal side of the peroxisomal membrane and are catalyzed by acyl-CoA reductase and DHAP acyltransferase, respectively (Fig 2.2, Reactions I and II) (Lee, 1998). DHAP acyltransferase catalyzes the esterification of the free hydroxyl group of DHAP and utilizes long-chain (>C10) acyl-CoA to form acyl-DHAP (Reaction II) (Hajra, 1997). Acyl-CoA reductase catalyzes the two consecutive reductions of acyl-CoA using NADPH as cofactor and forms fatty alcohol (Reaction I) (Rizzo et al., 1987). The fatty acetate formed during the initial reduction is short lived, suggesting that the fatty acetate is a reactive intermediate and not a product of the reaction.

Another source of fatty alcohol is the formation of the fatty alcohol by the β-oxidation of lignoceric acid. Studies using liver peroxisomes show lignoceric acid, and not hexadecanol or hexadecanoic acid, is the principle source of the 1-O-alkyl group found in
ether phospholipids (Hayashi and Hara, 1997). This suggests that the formation of fatty alcohol may be coupled to peroxisomal β-oxidation. However, whether the fatty alcohol is formed by this pathway directly or by the direct reduction of fatty acid is not clear. The quantitative contribution of this pathway is currently unclear. Since there is relatively low activity of the acyl-CoA reductase in mammalian tissue, the formation of the fatty alcohol is considered to be the rate limiting step in the formation of ether phospholipids (Snyder, 1972).

The committed step in ether phospholipid biosynthesis occurs on the luminal side of the peroxisomal membrane and involves the condensation of fatty alcohol with acyl-dihydroxyacetone phosphate (acyl-DHAP). This reaction is catalyzed by alkyl-DHAP synthase (Fig 2.2, Reaction III) and forms 1-O-alkyl DHAP plus free fatty acid (de Vet and van den Bosch, 1999, Van den Bosch and de Vet, 1997). The 1-O-alkyl DHAP is transferred to the cytoplasmic side of the peroxisomal membrane and reduced to 1-O-alkyl-2-lyso-sn-glycerol-3-phosphate. This reaction is catalyzed by alkyl-DHAP reductase (Fig. 2.2, Reaction IV) using NADPH as cofactor. This reaction represents the last step of ether phospholipid biosynthesis occurring in the peroxisome. Therefore, the peroxisomally catalyzed reactions involved in ether phospholipid biosynthesis function to produce 1-O-alkyl-2-lyso-sn-glycerol-3-phosphate (2-lysoPakOH) from DHAP and acyl-CoA, with the rate limiting step being the initial formation of the fatty alcohol.

The next step in ether phospholipid biosynthesis involves the conversion of 2-lysoPakOH to the plasmanyl-type glycerophospholipids, PakCho and PakEtn in the microsome. Three enzymes are involved in the biosynthesis of plasmanyl-type
glycerophospholipids. These include 1-O-alkyl-2-lyso-sm-glycerol-3-phosphate acyltransferase, 1-O-alkyl-2-acyl-sm-glycerol-3-phosphate phosphohydrolase, and the 1-O-alkyl-2-acyl-sm-glycerol : CDP-choline choline (CDP-ethanolamine ethanolamine) phosphotransferases (Fig 2.3). All of these enzymes are located in microsomes (Snyder et al., 1985). Therefore, following the peroxisomal reduction of 1-O-alkyl DHAP, the 2-lysoPakOH must be translocated across the peroxisomal membrane to the microsome. In the microsomes, the 2-lysoPakOH is acylated at the sn-2 position forming 1-O-alkyl-2-acyl-sm-glycerol-3-phosphate (PakOH). This reaction is catalyzed by 1-O-alkyl-2-lyso-sm-glycerol-3-phosphate acyltransferase and utilizes acyl-CoA (Arthur and Choy, 1986, Choy et al., 1997). The product, PakOH, serves as precursor for the formation of both plasmanylethanolamine (PakEtn) and plasmanylcholine (PakCho).

The final step in plasmanyl-type glycerophospholipid synthesis requires CDP-choline or CDP-ethanolamine, magnesium, 1-O-alkyl-2-acyl-sm-glycerol and the respective 1-O-alkyl-2-acyl-sm-glycerol : CDP-choline choline (CDP-ethanolamine ethanolamine) phosphotransferase (McMaster and Bell, 1997a, McMaster and Bell, 1997b, Radominska-Pyrek and Horrocks, 1972, Radominska-Pyrek et al., 1977, Snyder, 1997). This reaction forms PakEtn and PakCho from PakOH. First PakOH is dephosphorylated by 1-O-alkyl-2-acyl-sm-glycerol-3-phosphate phosphohydrolase forming the diradylglycerol, 1-O-alkyl-2-acyl-sm-glycerol, and inorganic phosphate. The diradylglycerol then becomes the substrate for the 1-O-alkyl-2-acyl-sm-glycerol : CDP-choline choline (CDP-ethanolamine ethanolamine) phosphotransferase and forms PakCho and PakEtn, respectively. It is unclear at this point whether there are separate choline and ethanolamine
phosphotransferases or whether the same enzyme catalyzes both reactions. Similar pH optima, thermolabilities, and inhibition by dithiothreitol exhibited by diacylglycerol and 1-\(O\)-alkyl-2-acyl-\(sn\)-glycerol choline phosphotransferases suggests that one enzyme may be responsible for catalyzing the formation of both phosphatidyl and plasmanyl-type choline glycerophospholipids (Lee et al., 1982). A CDP-ethanolamine ethanolamine phosphotransferase has also recently been purified from bovine liver that exhibits both ethanolamine phosphotransferase and choline phosphotransferase activity (Mancini et al., 1999). Therefore, it has been postulated that the availability of the diradylglycerols and the turnover rate of ether-linked lipids are important factors in controlling the levels of plasmanyl- and plasmenyl-type choline glycerophospholipids (Jamil et al., 1992, Mancini et al., 1999).

The formation of plasmenyl-type glycerophospholipids is initiated by insertion of a \textit{cis}-double bond between C1 and C2 of the fatty alcohol located at the \(sn\)-1 position of PakEtn and is catalyzed by the \(\Delta1\) alkyl desaturase complex (Fig. 2.4). This microsomal electron transport system contains three components; NADH : cytochrome b5 reductase, cytochrome b5, and a cyanide sensitive \(\Delta1\) alkyl desaturase. The complex requires NADH, molecular oxygen, and PakEtn and forms PlsEtn and water. Interestingly, this desaturase complex shows a high degree of stereospecificity in that it cleaves the hydrogen in the (1S, 2S) configuration forming a \textit{cis}-double bond (Stoffel and LeKim, 1971) and also demonstrates substrate specificity in that it only catalyzes the formation of ethanolamine plasmalogen (PlsEtn) from PakEtn. This hypothesis is supported by experiments in which 1-\(O\)-[\textsuperscript{3}H]alkyl- 2-acyl-\(sn\)-glycero-3-phospho[\textsuperscript{14}C]ethanolamine was injected intracerebrally.
into young rats and the $[^{3}H/^{14}C]$ ratio found in the labeled PlsEtn was equal to that found in the substrate (Paltauf, 1983). Also, time course experiments using rat myocytes labeled with 1-O-$[^{3}H]$alkyl-2-lyso-$sn$-glycero-3-phosphocholine show that PlsCho is not formed directly from PakCho, but through a mechanism that involves the formation of PakEtn (Lee et al., 1991). From these and other experiments, it is widely accepted that the formation of choline plasmalogen is preceded by the formation of PakEtn and PlsEtn.

Due to the substrate specificity of the $\Delta 1$ alkyl desaturase complex, the formation of PlsCho from PlsEtn may potentially comprise several complex metabolic pathways. A detailed explanation of these is well beyond the scope of this dissertation. The topic has been reviewed recently (Lee, 1998, Paltauf, 1994, Snyder, 1996). However, the possible metabolic reactions involved in the biosynthesis of PlsCho from PlsEtn are divided into two major groups. The first group, involving only polar-head group modification (Fig. 2.5), encompasses a series of parallel reactions that directly modifies the polar-head group found at the $sn$-3 position of the phospholipid moiety. Of these, the most direct pathways for the formation of PlsCho from PlsEtn are the reactions involving either base-exchange (Gaiti et al., 1976) or N-methyltransferase (Horrocks et al., 1986, Vance et al., 1997) (Fig 2.5, Reactions 1 and 2, respectively). These pathways directly modify PlsEtn by either exchanging the ethanolamine moiety with choline or by successively methylating ethanolamine using S-adenosyl methionine (AdoMet) as a methyl donor. Other pathways that involve only polar-head group modification in the formation of PlsCho include phospholipase C hydrolysis of PlsEtn and the reverse reaction of ethanolamine phosphotransferase (Fig 2.5, Reactions 3 and 4, respectively) (Goracci et al., 1985, Strum
and Daniel, 1993). Both form the diradylglycerol, 1-O-alk-1'-enyl-2-acyl-sn-glycerol, which can be used as substrate for the choline phosphotransferase enzyme forming PlsCho (Fig. 2.5, Reaction 7). Alternatively, phospholipase D can hydrolyze PlsEtn to 1-O-alk-1'-enyl-2-acyl-sn-glycerol-3-phosphate (PlsOH) (Fig. 2.5, Reaction 5) (Mohn et al., 1992). The PlsOH is then dephosphorylated by a phosphohydrolase forming 1-O-alk-1'-enyl-2-acyl-sn-glycerol that is subsequently made into PlsCho by a choline phosphotransferase catalyzed reaction (Fig 2.5, Reaction 6 and 7, respectively).

The second group of pathways that form PlsCho from PlsEtn include reactions that modify both the sn-2 position and the polar-head group. The salient feature of this paradigm is that PlsCho formation is coupled to mechanisms that involve acyl-chain remodeling. These can function to enrich plasmalogens with polyunsaturated fatty acid as well as provide intermediates that have potent biological activity as outlined in Chapter one. The initial step in this model is the PLA₂ catalyzed reaction that forms lysoPlsEtn and free fatty acid (Fig 2.6, Reaction 1). The lysoPlsEtn can be used as substrate for three separate reactions forming either the re-acylated PlsEtn, 1-O-alk-1'-enyl-2-lyso-sn-glycerol, or 1-O-alk-1'-enyl-2-lyso-sn-glycerol-3-phosphate (Fig. 2.6, Reactions 2, 3, and 4, respectively). The re-acylation of lysoPlsEtn is catalyzed by a CoA-independent transacylase that transfers the fatty acid located at the sn-2 position, independent of any cofactors, from diacyl-type phospholipids to lyso-ether phospholipids (Yamashita et al., 1997). The type of acyl-chains transferred by this reaction are restricted to both n-6 and n-3 polyunsaturated fatty acids (PUFA) with chain length between C20 and C22.

Interestingly, both lysoPlsEtn and lysoPlsCho can act as fatty acid acceptors in this
reaction, suggesting that this pathway may function to maintain the PUFA enriched molecular species of both PlsEtn and PlsCho. The product of the PLA_2 catalyzed reaction, lysoPlsEtn, can also be hydrolyzed by either lysophospholipase C (lysoPLC) (Strum and Daniel, 1993) or lysophospholipase D (lysoPLD) (Wykle and Schremmer, 1974). These reactions form either 1-0-alk-1'-enyl-2-lyso-sn-glycerol or 1-0-alk-1'-enyl-2-lyso-sn-glycero-3-phosphate (Fig. 2.6, Reactions 3 and 4), respectively (Wykle and Schremmer, 1974). The lysoPLC and lysoPLD pathways merge upon phosphorylation of the lysoPLC product, 1-0-alk-1'-enyl-2-lyso-sn-glycerol forming 1-0-alk-1'-enyl-2-lyso-sn-glycero-3-phosphate (Fig. 2.6, Reaction 5). The 1-0-alk-1'-enyl-2-lyso-sn-glycero-3-phosphate is then acylated at the sn-2 position by acyl-CoA acyltransferase forming 1-0-alk-1'-enyl-2-acyl-sn-glycero-3-phosphate (PlsOH). Similar to the phospholipase D reaction outlined in Figure 2.5, the re-acylated PlsOH is used to form PlsCho by reactions involving both phosphohydrolase and choline phosphotransferase enzymes (Fig. 2.6, reactions 7 and 8, respectively).

Whether the brain maintains the ability to catalyze all of the reactions outlined in the two paradigms listed above remains unknown. Therefore, the quantitative contribution and regulation of the pathways that form PlsCho remains unknown. Regardless, the de novo biosynthesis of PlsCho must occur through a mechanism that is preceded by the formation of PlsEtn. Also, the extent in the overlap of the pathways involved in PlsCho formation suggests that evolutionary mechanisms have evolved that maintain a high degree of variability to sustain not only the molecular species composition, but also the levels of both PlsEtn and PlsCho.
In summary, the primary step in ether phospholipid biosynthesis involves the formation of the glycerol-ether bond following the condensation of fatty alcohol with acyl DHAP. This bond defines the ether phospholipids and is maintained throughout the series of reactions that ultimately results in the formation of both PakCho and PlsCho (Fig 2.7). The complex biochemical pathways involved in ether phospholipid biosynthesis de novo in the brain have developed to supply five metabolically active ether phospholipid pools. These active metabolic pools include PakOH, PakCho, PakEtn, PlsEtn, and PlsCho. Following the condensation of labeled fatty alcohol with acyl-DHAP in the brain, the first stable ether phospholipid formed is PakOH. The stable brain PakOH pool is then used as a substrate in the formation of both PakCho and PakEtn pools as outlined in Figure 2.3. The PakEtn then becomes the substrate for a series of reactions resulting in the formation of a stable brain PlsEtn pool. This PlsEtn compartment then serves as substrate for the reactions outlined in Figures 2.5 and 2.6 and results in the formation of the brain PlsCho. This substrate-product relationship is the basis for this fatty alcohol model of ether phospholipid biosynthesis and has been exploited to design a pulse-chase fatty alcohol model in the awake adult rat.

2.3 Experimental Model

In this model, 1.75 mCi x kg⁻¹ of albumin-bound [1,1-³H]hexadecanol is intravenously infused, in the awake adult male Sprague-Dawley rat over a period of five minutes. All experiments were performed in accordance with the NIH Guidelines for the Care and Use of Laboratory Animals (NIH publication no. 80-23). Briefly, male
Sprague-Dawley rats (150-280 g, Charles River Breeding Laboratory, Wilmington, MA) were anesthetized with 2-3% Halothane (Halocarbon Labs, River Edge, NJ) and polyethylene catheters (PE 50, Becton Dickinson, Sparks, MD) filled with 100 IU sodium heparin were placed in the right femoral artery and vein. The wounds were closed with clips and Lidocain (1.0% solution) was applied as a local anesthetic. The animals were wrapped in fast setting plaster body casts, taped to wooden blocks, and allowed to recover from anesthesia for at least 3.0 hours in a thermostatically controlled heating environment set to maintain body temperature at 36.5°C.

The awake rats were then intravenously infused with a saline solution containing 0.875 mCi x kg\(^{-1}\) x mL\(^{-1}\) \([1,1'-\text{H}]\text{hexadecanol suspended in 0.06 mg BSA for 5.0 min at a rate of 0.4 mL x min}^{-1}\) (Harvard Apparatus, South Natick MA). Arterial blood samples were collected throughout the experiment to determine the specific activity of blood and plasma components. At 5, 10, 20, 30, 60, and 120 minutes following the start of infusion, animals were killed using an overdose of intravenously infused sodium pentobarbital (100 mg x kg\(^{-1}\)). At death, their brains were prepared for either biochemical analysis or quantitative autoradiography. For biochemical analysis, the rats were subject to head-focused microwave irradiation (5.5 kw, 3.4 sec., Cober Electronics, Stamford, CT) immediately following death and their brains were excised, and frozen on dry ice. Animals used for quantitative autoradiography were immediately decapitated following death and their brains were excised and frozen in 2-methylbutane at -50°C. All brains were stored at -80°C until use.
Operational equations, derived by the model and applied to the biochemical analysis data, provide the unidirectional transfer coefficients that represent the movement of labeled hexadecanol from one stable ether phospholipid pool to the next. Under steady-state conditions, these transfer coefficients are used to calculate the individual flux, turnover, and half-life of the ether phospholipid pools in the brain. The steady-state flux calculations represent the unidirectional transfer of mass per gram of brain from PakOH into the individual ether phospholipid pools as a function of time. The steady-state turnover and half-life calculations represent the metabolic utilization of these ether phospholipid pools. Although, autoradiographic studies do not discriminate between individual ether phospholipid pools, regional incorporation and utilization information can be obtained. This model incorporates several assumptions and experimental conditions that are outlined below.

2.4 Assumptions of the Model

To establish the time-dependent specific activities in the central nervous system for each stable ether lipid compartment following the intravenous infusion of [1,1-\(^3\)H]hexadecanol, a number of assumptions and experimental conditions need to be addressed. The first, is that the oxidation of [1,1-\(^3\)H]hexadecanol to palmitic acid results in the complete loss of the tritium atoms. Since the tracer used in these experiments is labeled at the C1 carbon of hexadecanol, oxidation to palmitic acid will result in the replacement of the label with a non-labeled carbonyl group and results in the formation of tritiated water. Thus only ether phospholipids are labeled following the incorporation of
[1,1-\textsuperscript{3}H]hexadecanol into brain lipid compartments. This is supported from pulse-chase experiments using perfused rabbit hearts that found [1-\textsuperscript{2}H]hexadecanol only labeled ether phospholipids and fatty alcohol-containing neutral lipid species (Ford and Gross, 1994).

The second assumption is that infusion of 1.75 mCi x kg\textsuperscript{-1} [1,1-\textsuperscript{3}H]hexadecanol is at tracer levels allowing the concentration of stable brain ether phospholipids to remain constant. The specific activity of the tracer used was 55 mCi x nmol\textsuperscript{-1} and the rats weighed between 150 and 185 grams. At the dosage listed above, approximately 14 to 18 pmol of hexadecanol was infused over a period of five minutes. This is well below plasma levels of hexadecanol that were measured at 670 nmol x mL\textsuperscript{-1}. Hence, this concentration of tracer did not alter plasma or brain levels of hexadecanol (Chapter 3). Also, in human fibroblast cells cultured in fatty acid free medium, [1-\textsuperscript{14}C]hexadecanol is oxidized to [1-\textsuperscript{14}C]palmitic acid in a manner dependent on the concentration of exogenous palmitic acid as well as fatty acyl-CoA (Rizzo et al., 1987). These studies found that a balance exists between cellular free fatty acid and free fatty alcohol that acts to regulate the levels of cellular free fatty alcohol. This suggests that cellular mechanisms are in place that maintain steady-state levels of cellular fatty alcohol.

The third assumption is that all tracer found in the central nervous system originates from movement of free [1,1-\textsuperscript{3}H]hexadecanol from plasma across the blood brain barrier. Little is known concerning the dynamics of the plasma hexadecanol-albumin complex. However analogies based on the structural and H\textsubscript{2}O-monolayer partition coefficient of palmitic acid and hexadecanol suggests that the hexadecanol-albumin complex will have similar characteristic as the palmitic acid-albumin complex. In plasma,
free fatty acids normally are more than 99.99% bound to circulating plasma proteins (Goodman, 1958, Goodman and Shafir, 1959). At normal plasma protein concentrations, the equilibrium between unbound and bound unacylated palmitic acid is 0.007% (Spector et al., 1971, Wosilait and Soler-Argilaga, 1975). There is no evidence that the fatty acid-albumin complex can cross the blood-brain barrier, however 5% of radiolabeled palmitic acid is extracted in a single pass of blood through the rat brain (Pardridge and Mietus, 1980). Therefore, the fatty acid-albumin complex must dissociate as blood passes through the cerebral capillary bed allowing unbound fatty acid to be available for diffusion. By comparison, the same should be true for the fatty alcohol-albumin complex. Similar brain incorporation levels between albumin-bound [1,1-{3}H]hexadecanol and albumin-bound [9,10-{3}H]palmitic acid (Grange et al., 1995) following intravenous infusion support this conclusion.

The fifth assumption is that extraperoxisomal fatty alcohol can be used as a substrate for the biosynthesis of ether phospholipids in the brain. The initial step in ether phospholipid biosynthesis begins in the peroxisome following the condensation of fatty alcohol with acyl-DHAP (Fig 2.2). In liver peroxisomes, it has been shown that the 1-O-alk-1'-enyl group of PlsEtn derives mainly from fatty alcohol synthesized de novo following β-oxidation of long-chain acyl-CoA, but not from extraperoxisomal fatty alcohol (Hayashi and Hara, 1997). However, these studies did show that approximately 10-12% of exogenous hexadecanol was incorporated as an aldehyde into the ethanolamine glycerophospholipid fraction without being β-oxidized or elongated. Also, [1-{3}H]hexadecanol does incorporate into heart ether phospholipids as demonstrated in the
perfused rabbit myocardium (Ford and Gross, 1994). Although extraperoxisomal fatty alcohol may not be the endogenous substrate for ether phospholipid biosynthesis, studies show that it can be used as substrate by the peroxisomal alkyl-DHAP synthase. The incorporation of [1,1-\(^3\)H]hexadecanol into brain ether phospholipids found in these experiments supports this conclusion.

The sixth assumption is that following diffusion of tracer into the brain, the peroxisomally catalyzed condensation of fatty alcohol with acyl-DHAP coupled to reduction and acylation of the sn-2 position is much more rapid than the oxidation of [1,1-\(^3\)H]hexadecanol to palmitic acid. Once an unbound plasma fatty alcohol molecule crosses the blood-brain barrier and enters the brain, it is subject to one of three metabolic possibilities. The fatty alcohol can return to the blood, it can be oxidized to palmitic acid or it can be used in the formation of 1-0-alkyl-DHAP. Experimentally, [1,1-\(^3\)H]hexadecanol displays a plasma half-life of 1.5 minutes and the specific activity of the plasma hexadecanol approaches zero at ten minutes. This suggests that the diffusion of tracer from tissue into plasma is minimal. The oxidation of [1,1-\(^3\)H]hexadecanol to palmitic acid results in the loss of tritium from the tracer. However, the extent to which the brain maintains the ability to oxidize hexadecanol to palmitic acid remains unknown. Experimentally, the rate at which the specific activity of brain hexadecanol falls compared to the change in the specific activity of the brain PakOH suggests that the oxidation of hexadecanol to palmitic acid is much slower than the formation of PakOH. This is important since the rate-limiting step in the biosynthesis of ether phospholipids is thought to be the formation of the fatty alcohol. Since the oxidation of hexadecanol to palmitic
acid is slower than the condensation of fatty alcohol with acyl-DHAP, an equilibrium can be established between free brain $[1,1^{-2}\text{H}]$hexadecanol and the ether phospholipid precursor pool, PakOH. This equilibrium will provide a labeled ether phospholipid precursor pool of high enough specific activity to allow measurement of the formation of all subsequent brain ether phospholipid species. This relationship will remain true as long as the specific activity of the brain $[1,1^{-2}\text{H}]$hexadecanol pool remains well above the specific activity of the brain $[1,1^{-2}\text{H}]$PakOH pool. Once the specific activity of the brain $[1,1^{-2}\text{H}]$hexadecanol pools falls below that of the $[1,1^{-2}\text{H}]$PakOH, the efflux rate of radioactivity will no longer equal the influx rate and the tracer kinetic analysis will fail.

The last assumption is that ether phospholipid biosynthesis de novo occurs in a unidirectional manner consistent with the accepted pathways outlined in section 2.2 of this Chapter and simplified in Figure 2.7. The unidirectional incorporation of tracer into ether phospholipids assumes there is negligible loss of tracer due to catabolism of intermediate ether phospholipids and that ether phospholipid biosynthesis de novo depends upon the substrate-product relationship outlined previously. The accepted pattern of ether phospholipid biosynthesis de novo requires the initial formation of PakOH that becomes part of the metabolically active brain PakOH pool. This metabolically active brain PakOH pool serves as the substrate for the choline and ethanolamine phosphotransferases that form both the brain PakCho and PakEtn pools, respectively. The Δ1 alkyl desaturase complex, which only catalyzes the formation of PIsEtn from PakEtn, converts the metabolically active PakEtn pool to an active brain PIsEtn pool. The PIsEtn pool then is
used as substrate to form PlsCho by either the sn-2 and/or polar-head group modification pathways.

2.5 Unidirectional transfer coefficients (\( k_{br}^{*} \)) and incorporation coefficients (\( k^{*} \))

Since the formation of the ether-linkage at the sn-1 position of the phospholipid moiety defines the ether phospholipid and is conserved throughout the entire biosynthetic process, pulse-labeling with radioactive [1,1-\(^3\)H]hexadecanol will provide a means to measure the biosynthetic rate and turnover of the both the ethanolamine and choline ether phospholipids in the rat brain. As stated above, the rat fatty alcohol model is based on the substrate-product relationship found in ether phospholipid biosynthesis \textit{de novo} and measures the unidirectional incorporation of [1,1-\(^3\)H]hexadecanol. The model has been applied to two paradigms; biochemical analysis and quantitative autoradiography. Biochemical analysis provides the biosynthetic rate \textit{de novo}, turnover, and half-life based upon transfer coefficients (\( k_{br}^{*} \)) and quantitative autoradiography provides regional incorporation data based on incorporation coefficients (\( k^{*} \)). Although there are many similarities between \( k_{br}^{*} \) and \( k^{*} \), their derivation is different and the discussion of these terms will be dealt with separately.

The transfer coefficients (\( k_{br}^{*} \)) following infusion of [1,1-\(^3\)H]hexadecanol are based on the time-dependent change in the specific activity of the brain ether phospholipids as determined through biochemical analysis. To account for the individual rates of entry of labeled hexadecanol into each of the metabolically active brain ether
phospholipid compartments \((i = 1...6\), Figure 2.8\), we write the net brain [1,1-

\(^3\)H]hexadecanol concentration at time \(T\) \((C_{br,i}^\cdot(T))\) as the sum of the tracer concentrations

\((C_{br,i}^\cdot(T))\) in each of these compartments (equation 2.1), in which \(C_{br,i}^\cdot(T)\), is equal to the

\[
C_{br,i}^\cdot(T) = \sum_{i=1}^{6} C_{br,i}^\cdot(T) \quad \text{(Equation 2.1)}
\]

specific activity of the ether phospholipid compartment \((i)\). Therefore, the time-dependent
change in the specific activity in a given product compartment \((i)\), can be used to calculate
the incorporation coefficient, \(k_{br,i}^\cdot\), of labeled hexadecanol into this compartment from its
substrate compartment \((i-1)\) as outlined in equation 2.2. Since the changes in the specific

\[
dC_{br,i}^\cdot / dt = k_{br,i}^\cdot C_{br,i-1}^\cdot \quad \text{(Equation 2.2)}
\]

activity of both the product and substrate compartments are measured experimentally,
equation 2.2 can be solved for \(k_{br,i}^\cdot\), as outlined in equations 3 and 4, respectively. Thus,

\[
C_{br,i}^\cdot(t_2) - C_{br,i}^\cdot(t_1) = k_{br,i}^\cdot \int_{t_1}^{t_2} C_{br,i-1}^\cdot dt \quad \text{(Equation 2.3)}
\]

\[
k_{br,i}^\cdot = \frac{C_{br,i}^\cdot(t_2) - C_{br,i}^\cdot(t_1)}{\int_{t_1}^{t_2} C_{br,i-1}^\cdot dt} \quad \text{(Equation 2.4)}
\]

the transfer coefficient of labeled hexadecanol from the substrate compartment \((i-1)\) to the
product compartment \((i)\) is equal to the change in the specific activity of the product
compartment divided by the integrated change in the specific activity of the substrate
compartment. At steady-state, the cold concentration of the individual ether
phospholipids do not change following infusion. Therefore, the transfer coefficient
represents the flux of labeled hexadecanol from substrate compartments into product
compartments independent of mass flow. The derivation of the transfer coefficients for
the ether phospholipid compartments \((i = 3 \ldots 6)\) are listed in Figure 2.9.

The regional brain incorporation coefficients \((k^i)\) following infusion of \([1,1-\text{H}]\)hexadecanol are based on the time-dependent change in the specific activity of all brain
precursor and stable ether phospholipid compartments as determined using quantitative
autoradiography. Since quantitative autoradiography does not discriminate between brain
\([1,1-\text{H}]\)hexadecanol and ether phospholipid subclasses, the incorporation coefficient, \(k^i\),
is defined as the total brain radioactivity within all stable compartments at time \(T\).

Therefore, by comparison, the change in the specific activity of labeled brain
compartments is equal to the incorporation coefficient \((k^i)\) multiplied by the specific
activity of the plasma hexadecanol (equation 2.5). Since the change in the specific activity

\[
d\dot{C}_{br}^i(T)/dt = d \sum_{j=1}^{n} \dot{C}_{br}(T)/dt = k^i \cdot C_{pl}^i \tag{Equation 2.5}
\]

of the label is measured using quantitative autoradiography and the plasma specific activity
is measured biochemically, equation 2.5 can be solved for the incorporation coefficient
(equation 2.6). Therefore, the incorporation coefficient represents the flux of labeled

\[
k^i = \dot{C}_{br}(T)/ \int_0^T \dot{C}_{pl} dt = \sum_{j=1}^{n} \dot{C}_{br}(T)/ \int_0^T \dot{C}_{pl} dt = \sum_{i=1}^{6} k^i \tag{Equation 2.6}
\]

hexadecanol from plasma into brain regions independent of the tracer form and is also
independent of mass.
Therefore, the values of $k_i^-$ are obtained from tracer concentrations in individual stable ether phospholipid compartments following chemical analysis, and $k^*$ values are derived from autoradiographic studies used to determine regional brain incorporation. Consequently, the transfer coefficients reflect the transfer of the labeled hexadecanol from the plasma into the individual ether phospholipid pools, whereas the incorporation coefficients represent flux from plasma into specific brain regions.

2.6 Biosynthetic rate, turnover, and half-lives of brain ether phospholipid pools

Incorporation of the labeled fatty alcohol into a stable metabolically active brain ether phospholipid compartment ($i = 3 \ldots 6$) occurs from substrate pools ($i - 1$) or for the PakOH compartment from the brain [1, 1-$^3$H]hexadecanol pool (Fig. 2.8). At steady-state, the transfer coefficients outlined in section 2.5 reflect the mass transfer from substrate pools into product pools of stable brain ether phospholipid compartments. The steady-state contingency also allows for the calculation of the flux, turnover, and half-lives of the individual ether phospholipid pools.

At steady-state, the mass transfer into the stable ether phospholipid compartments must equal the mass transfer out since the levels of each stable brain ether phospholipid compartment are not changed throughout the duration of the experiment. This experimental condition allows the calculation of the biosynthetic rate of the individual ether phospholipid pools. The flux ($J_{br,i}$, nmol x gram$^{-1}$ x min$^{-1}$) into the product ether phospholipid compartments is calculated by multiplying the transfer coefficient ($k^-_{br,i}$)
(units = min⁻¹) by the cold concentration of the substrate pool ($c_{br,-1}$, nmol x gram⁻¹) (equation 2.7). The flux values represent the mass transfer of the ether linkage from

$$J_{br} = k_{br}c_{br,-1} \text{ (Equation 2.7)}$$

substrate compartments ($i-1$) into product compartments ($i$) and is equivalent to the biosynthetic rate required to maintain the metabolically active brain ether phospholipid pools.

The steady-state conditions also allow estimation of the turnover and the half-lives of the individual ether phospholipid pools. Again, at steady-state, the flux into the stable ether phospholipid compartments must equal the flux out since the level of each stable brain ether phospholipid compartment is not changed throughout the experiment. This experimental condition allows the calculation of the turnover rate of the individual ether phospholipid pools. Consequently, division of the flux ($J_{br}$) by the cold concentration of the product pool ($c_{br,i}$) provides the turnover of the product pool ($i$) in units of

$$\text{turnover}_{br} = J_{br} / c_{br,i} = k_{br}c_{br,-1} / c_{br,i} \text{ (Equation 2.8)}$$

minutes⁻¹ (equation 2.8). Since the equations used to calculate the transfer coefficients assume first-order kinetics, the half-lives of the individual metabolically active ether phospholipid pools can be calculated by equating the inverse of the turnover rate ($\text{turnover}^{-1}$) with 0.693 (equation 2.9).

$$\text{half-life}_{br,i} = 0.693 / \text{turnover}_{br} \text{ (Equation 2.9)}$$
2.8 Summary

In summary, I have outlined the accepted patterns of brain ether phospholipid biosynthesis \textit{de novo} and developed both an analytical and mathematical model to describe these reactions. In this chapter, a pulse-chase fatty alcohol model has been described that utilizes the intravenous administration of $[1,1$-$^3$H]$\text{hexadecanol}$ in the adult awake rat. Experimentally, this model maintains steady-state levels of brain ether phospholipids that allow the application of tracer kinetic analysis. Under the experimental conditions outlined above, the time-dependent transfer of labeled $[1,1$-$^3$H]$\text{hexadecanol}$ between stable brain ether phospholipid pools is used to calculate the transfer coefficients of tracer between brain ether phospholipid compartments. These transfer coefficients are then used to measure the biosynthetic rate \textit{de novo}, turnover, and half-lives of brain ether phospholipids \textit{in vivo}. This model can be applied to biochemical or autoradiographic analysis to determine the individual biosynthetic rates or localized regions of incorporation in the adult rat brain. Since brain lipid metabolism is tightly coupled to receptor function and membrane trafficking as well as signal transduction, the biosynthetic and turnover rates of brain ether phospholipids will allow assessment of the biological importance of these molecules within the central nervous system.
Figure 2.1: Structures of the major ether phospholipids found in the brain including names and six letter abbreviations in parentheses.
Peroxisomal Membranes

Cytoplasmic Side

I. \[ \text{RCSCoA (fatty acyl-CoA)} \]
   \[ 2 \text{NADPH} \]
   \[ \text{Acyl-CoA Reductase} \]
   \[ 2 \text{NADP}^+ \]
   \[ \text{ROH (fatty alcohol)} \]

Lumenal Side

II. \[ \text{RCSCoA (fatty acyl-CoA)} \]
   \[ \text{DHAP acyltransferase} \]
   \[ \text{DHAP} \]
   \[ \text{CoA} \]
   \[ \text{Acyl-DHAP} \]

IV. \[ \text{Alkyl-DHAP} \]

III. \[ \text{Alkyl-DHAP reductase} \]

\[ \text{ROH (fatty alcohol)} \]

\[ \text{Alkyl-DHAP synthase} \]

\[ \text{Acyl-DHAP} \]

\[ \text{Alkyl-DHAP} \]

\[ \text{1-O-alkyl-2-lyso-sn-glycerol-3-phosphate} \]

Figure 2.2: Peroxisomal enzymes participating in the steps responsible for introducing the ether-linkage into phospholipids.
Figure 2.3: Microsomal enzymes involved in the biosynthesis of 1-O-alkyl-2-acyl-sn-glycero-3-phosphocholine (phosphoethanolamine).
Figure 2.4: Microsomal electron transport system that contains the Δ1 alkyl desaturase complex.
Figure 2.5: Metabolic pathways involved in the biosynthesis of choline plasmalogen from ethanolamine plasmalogen including only polar-head group modification. Reactions include: (1) base-exchange, (2) N-methyltransferase, (3) phospholipase C, (4) reverse reaction of ethanolamine phosphotransferase, (5) phospholipase D, (6) phosphohydrolase, and (7) CDP-choline choline phosphotransferase enzymes.
Figure 2.6: Metabolic pathways involved in the biosynthesis of choline plasmalogen from ethanolamine plasmalogen including both sn-2 and polar-head group modification. Reactions include: (1) phospholipase A2, (2) CoA-independent transacylase, (3) lysophospholipase C, (4) lysophospholipase D, (5) phosphotransferase, (6) acyl-CoA acyltransferase, (7) phosphohydrolase, and (8) CDP-choline choline phosphotransferase enzymes.
Figure 2.7: General outline of ether phospholipid biosynthesis showing the reactive brain ether phospholipid pools and the flow of tritium label (bold type) during the course of the experiment.
Figure 2.8: Schematic diagram of ether phospholipid biosynthesis de novo representing the incorporation coefficient \( k^* \) and the transfer coefficients \( k_{i-1}^* \) for each of the metabolically active brain ether phospholipid compartments \((i = 1 \ldots 6)\).
Figure 2.9: Derivation of the individual transfer coefficients of the brain ether phospholipid compartments ($i = 3...6$).
CHAPTER 3

WHOLE BRAIN ANALYSIS

3.1 Introduction

In this chapter, I will describe experiments that were performed using the pulse-chase fatty alcohol model presented in Chapter two. These experiments were performed on whole brain samples and used to measure the transfer coefficient, biosynthetic rate, turnover, and half-lives of ethanolamine and choline ether phospholipids in the adult awake rat brain in vivo. The fundamental assumption made in this investigation is that the rapid turnover of ether phospholipids within the central nervous system indicates an active participation in the neurochemistry involved in maintaining neurological function. Thus, the primary importance of this work is that the application of tracer kinetic analysis to ether phospholipid biosynthesis de novo will allow assessment of the biological importance of these molecules within the central nervous system post-myelination. This chapter will include four sections: (1) introduction, (2) materials and methods, (3) results, and (4) discussion.

Several methods have been developed that study phospholipid metabolism and turnover in brain using in vivo techniques. These methods utilize phospholipid precursors
and fatty acids labeled with radioactive atoms (Masuzawa et al., 1984, Miller et al., 1977, Robinson et al., 1992, Schmid and Takahashi, 1970). Unfortunately, the absolute rates at which these phospholipids are reported to turnover are sometimes conflicting. These discrepancies are due, in part, to several variables including the age of the animal, the type of tracer used, the route the tracer is administered, and the way the rates are calculated.

In organ systems, phospholipids are differentially distributed among various membranes and involve complex phospholipid class distribution and contain multiple different molecular species (Ansell, 1973, Horrocks, 1972). In the central nervous system, phospholipid distribution becomes particularly complicated due to the differing cell types as well as having a multitude of specialized structures. As a general rule, phospholipids are rapidly metabolized in the brain (Porcellati et al., 1983). However the extent to which this occurs depends on many factors including the age and state of myelination of the animal being studied.

Age is a principal factor to consider when interpreting phospholipid turnover data in the central nervous system. The rat, whose central nervous system undergoes a considerable amount of development postnatally, does not begin to form myelin until approximately 10-12 days following the formation of oligodendroglial precursor cells (Dewille and Horrocks, 1992). This period of active myelination peaks at 20 days and declines until the animal reaches 6 months of age. At peak myelination, myelin accumulates in the rat at a rate of approximately 3.5 mg x day⁻¹ (Morell et al., 1994). At six months of age, approximately 60 mg of myelin can be isolated from the rat and represents an increase of 1,500 percent over the 4 mg found in the 15 day old rat (Morell
et al., 1994). Since approximately 45 percent of the dry weight of myelin is phospholipid (Dewille and Horrocks, 1992), there is a degree of developmentally-linked metabolic regulation involved in the formation of myelin phospholipid.

This developmental regulation of phospholipid metabolism is shown very well in experiments that measured myelin PlsEtn turnover as a function of age. Experiments measuring \[^{14}\text{C}]\text{ethanolamine incorporation in the myelinating rat, between 40 days and five months of age, found that the half-life of myelin PlsEtn increased as myelination decreased. Initially, the half-life for PlsEtn is 1.6 days at 7 days of age and increased to 20 days at 24 months of age (Porcellati et al., 1983). Also, the developing rat brain undergoes fatty acid compositional changes, with the percentage of palmitic acid decreasing while the percentage of stearic and oleic acids increase with age (Marshall et al., 1966). However, despite this decrease in palmitic acid, there is a two-fold increase in the percent composition of hexadecanol-containing ether phospholipids in rat between the ages of 5 and 40 days (Natarajan and Schmid, 1977). Hence, the biosynthetic rate and turnover of brain phospholipid metabolism as well as the fatty acid composition is dependent on the age of the animal being studied. Therefore, the interpretation of the biosynthetic rates and turnover values of ether phospholipid metabolism is highly dependent on the age of the animal being studied. The metabolic data concerning ether phospholipid metabolism in myelinating and non-myelinating rats apparently reflect different physiological roles of these phospholipids.

The precursor used to measure ether phospholipid biosynthesis can also lead to decidedly different interpretations of the metabolic nature of these phospholipids. Because
the turnover of phospholipids is necessary for the repair and maintenance of neural membranes, different turnover rates may reflect different functional roles for individual phospholipid classes (Burton, 1971). Also, since phospholipid precursors are taken up, released, and recycled by different pathways in the central nervous system (Lee, 1998), the nature of the tracer and how it is labeled is very important. The type of tracer used directly impacts the pathway being studied. Therefore, when designing a tracer to study ether phospholipid metabolism, five structural groups in the basic phospholipid moiety can be targeted. These include the glycerol backbone, the fatty alcohol attached to the sn-1 position, the fatty acid attached to the sn-2 position, the sn-3 phosphate, and the sn-3 polar-head group. Studies focused on remodeling pathways often use labeled fatty acids or bases that target acyl-chain remodeling or polar head-group remodeling. The most direct way to study ether phospholipid biosynthesis de novo is through targeting either the glycerol backbone or the sn-1 fatty alcohol. The latter two approaches have been used extensively to measure brain ether phospholipid metabolism in vivo.

Another variable that can result in conflicting metabolic data involves the route of tracer delivery. Several methods have been employed in the past. These include intraperitoneal (Freysz et al., 1969), intracerebral (Miller et al., 1977, Schmid and Takahashi, 1970, Sun and Horrocks, 1969b, Sun and Horrocks, 1971, Sun and Horrocks, 1973), and intravenous injection (Dhopseshwarkar and Mead, 1969, Dhopseshwarkar and Mead, 1970, Grange et al., 1995, Mukherjee et al., 1980, Robinson et al., 1992) of labeled phospholipid precursors. Experimentally however, intravenous and intraperitoneal injection of labeled phospholipid precursors results in low levels of incorporation into the
central nervous system. This is due mainly to whole body utilization of the phospholipid precursor. When [1-\textsuperscript{14}C]octadecenol was injected into the tail vein, the majority of radiotracer was incorporated into the heart, lung, and liver with less than 5 percent entering the brain (Mukherjee et al., 1980). Furthermore, intraperitoneal and intravenous injection can also lead peripheral metabolism of the phospholipid precursor as well as the difficulty of crossing the blood brain barrier (Das et al., 1992). Therefore, in the past, the method of choice was to introduce labeled phospholipid precursors directly into the brain through intracerebral injection, circumventing many of the difficulties found with peripheral administration of tracer. However, intracerebral injection does pose several problems including the possibility of injury, increased stress, and most notably uneven distribution of the tracer. Since the brain maintains relatively low levels of phospholipid precursors, intracerebral injection can cause regions where the precursor levels are disproportionate to that found in the normal brain. This non-physiological distribution of phospholipid precursors can force mass-balance shifts in the normal rate of ether phospholipid synthesis and create regions where steady-state kinetics are no longer applicable. Therefore, the route by which the phospholipid precursor is given can affect the biosynthetic rate and turnover of ether phospholipids and give variable results.

Finally, another factor that can affect the absolute rate at which ether phospholipids turnover involves the way the rates are calculated. In the past, the half-life of labeled ether phospholipid pools in the brain were calculated by estimating the efflux rate of radioactivity from the individual ether phospholipid pools as a function of time. These calculations resulted in the half-life of microsomal PIsEtn being 4-20 days and
myelin PIsEtn being 11-34 days (Horrocks, 1972, Miller et al., 1977, Porcellati et al., 1983). However, a singular study was performed on the myelinating rat in which the biosynthetic rate and half-life of brain PIsEtn was calculated using steady-state kinetics. PIsEtn was synthesized at a rate of 1,920 nmol x gram\(^{-1}\) x hour\(^{-1}\) and had a half-life of 5.3 hours (Masuzawa et al., 1984). This half-life was much less than that previously reported using the efflux method. However, the discrepancy between the half-life values calculated using efflux methods and steady-state kinetics can be explained. Efflux rate calculations depend on the influx of radioactively labeled precursors and recycling of the labeled phospholipid pool to be negligible. However, recycling of the injected label will occur after long periods of time (Miller et al., 1977). Therefore, the apparent half-lives measured using efflux calculations will be much longer than those calculated using steady-state kinetics and may represent processes other than the direct utilization of the ether phospholipid pool in question.

In summary, a method to study ether phospholipid biosynthesis de novo using intravenous infusion of \([1,1-^{2}\text{H}]\text{hexadecanol}\) in the awake adult rat has been employed. In this study, the use of labeled \([1,1-^{3}\text{H}]\text{hexadecanol}\) permits measurement of only ether phospholipid synthesis because the tritium atoms are lost during the oxidation of \([1,1-^{3}\text{H}]\text{hexadecanol}\) to palmitic acid. Furthermore, there is stoichiometric retention of label following the desaturation step involved in plasmalogen formation, allowing for the direct measure of plasmalogen biosynthesis. Steady-state tracer kinetics was applied to this model to determine the de novo rate and turnover of whole brain ether phospholipids in vivo.
3.2 Material and Methods

[1,1-\textsuperscript{3}H]Hexadecanol (50 mCi x mmol\textsuperscript{-1}, \geq 97\% pure) was purchased from Moravek Biochemicals (Brea, CA). Phospholipid and neutral lipid standards were from Nu-Chek-Prep Inc. (Elysian, MN) and fatty alcohol standards and “essentially fatty acid free” bovine serum albumin were from the Sigma Chemical Co. (St. Louis, MO). Acetic anhydride, anhydrous pyridine, and all TLC plates were purchased from Alltech Associates (Deerfield, IL). HPLC grade hexane and isopropyl alcohol were from EM Science (Gibbstown, NJ) and chloroform, methanol, and other chemicals used were of reagent grade from Mallinckrodt Inc. (Paris, KY) unless noted otherwise. Beckman Ready-Safe\textsuperscript{®} scintillation cocktail (Fullerton, CA) containing 1.0\% glacial acetic acid was used to determine the radioactivity of all samples. All extracts were stored in HPLC grade hexane/isopropyl alcohol (3:2 (v/v) + 5.5\% H\textsubscript{2}O) under nitrogen at -20\°C until use unless noted otherwise.

Surgical preparation. Experiments were performed in accordance with the NIH Guidelines for the Care and Use of Laboratory Animals (NIH publication no. 80-23). Male Sprague-Dawley rats (150-280 g, Charles River Breeding Laboratory, Wilmington, MA) were anesthetized with 2-3\% Halothane (Halocarbon Labs, River Edge, NJ) and polyethylene catheters (PE 50, Becton Dickinson, Sparks, MD) filled with 100 IU sodium heparin were placed in the right femoral artery and vein. The wounds were closed with clips and Lidocain (1.0\% solution) was applied as a local anesthetic. The animals were wrapped in fast setting plaster body casts, taped to wooden blocks, and allowed to recover.
from anesthesia for at least 3.0 hours in a thermostatically controlled heating environment set to maintain body temperature at 36.5°C.

**Infusion of [1,1-³H]hexadecanol.** Awake rats were intravenously infused for 5.0 min at a rate of 0.4 mL x min⁻¹ with a saline solution containing 0.875 mCi x kg⁻¹ x mL⁻¹ [1,1-³H]hexadecanol suspended in 0.06 mg BSA (Harvard Apparatus, South Natick MA). Arterial blood samples were collected throughout the experiment to determine the specific activity of blood and plasma components. At various times following the start of infusion, all animals were killed using an overdose of intravenously infused sodium pentobarbital (100 mg x kg⁻¹) followed by head-focused microwave irradiation (5.5 kw, 3.4 sec., Cober Electronics, Stamford, CT). Rats brains were excised, frozen on dry ice, and stored at -80°C until use.

**Whole brain and plasma extraction.** Whole brains were extracted using a modified two-phase method. Briefly, brains were weighed and homogenized in 10 mL of methanol (MeOH) followed by the addition of 20 mL chloroform (CHCl₃). Ten milliliters of 0.1 M KCl was added to the extract to form two phases and separation was promoted by centrifugation (1000 x g) at room temperature for 15.0 minutes. The lower CHCl₃ phase containing the lipids was removed and the upper aqueous phase was re-extracted using a second 20 mL aliquot of CHCl₃. Both CHCl₃ extracts were combined, concentrated in vacuo (Savant Inst., Hicksville, NY) and filtered using a 0.2 micron Nylon filter. All plasma and blood samples were extracted using the Folch method (Folch et al., 1957).
Brain phospholipid separation. Standards and samples were applied to 20 x 20 cm Whatman silica gel 60A LK6 TLC plates and separated into classes using a solvent system of CHCl₃: MeOH: glacial acetic acid (HOAc): H₂O (50:37.5:3:2, by vol.) (Jolly et al., 1997). Bands corresponding to ethanolamine glycerophospholipid (EtnGpl), choline glycerophospholipid (ChoGpl), diradyl-sn-glycerol-3-phosphate (HOGpl), and neutral lipids were scraped off the TLC plates and stored in HIP (3:2) + 5.5% H₂O.

Chemical modification of EtnGpl and ChoGpl. The EtnGpl and ChoGpl fractions were extracted from the silica gel using HIP (3:2) + 5.5% H₂O and concentrated under a steady stream of N₂ at 40°C. Samples were incubated in 2.0 mL of methanol containing 0.5 M KOH at 30°C for 15 minutes. At 15.0 minutes, 1.0 mL ethyl formate (Sigma Chem. Co., St. Louis, MO) was added followed by the addition of 4.0 mL CHCl₃:n-butanol (BuOH) (4:2 (v/v)). Two-phases were formed by the addition of 2.0 mL 0.1 M KCl and phase separation was promoted by centrifugation at 1000 x g (Ansell and Spanner, 1963). The lower solvent phase containing the lyso-ether phospholipid analogs (1-0-alkyl-2-lyso-sn-glycero-3-phosphoethanolamine (2-lysoPakEtn) + 1-0-alk-1'-enyl-2-lyso-sn-glycero-3-phosphoethanolamine (2-lysoPPlsEtn) or 1-0-alkyl-2-lyso-sn-glycero-3-phosphocholine (2-lysoPakCho) + 1-0-alk-1'-enyl-2-lyso-sn-glycero-3-phosphocholine (2-lysoPPlsCho)) were then transferred into conical screw-top test tubes and stored in 10 mL HIP (3:2) + 5.5% H₂O.

Separation of 2-lysoPakEtn - 2-lysoPPlsEtn and 2-lysoPakCho - 2-lysoPPlsCho classes. The lyso-ether phospholipid extracts were concentrated under a steady stream of N₂ at 40°C and dissolved in 100 µL CHCl₃. An aliquot of each sample (25 µL) was
spotted onto a 10 x 10 cm Analtech silica gel G TLC plate and the lipid classes were separated using CHCl₃ : MeOH : ammonium hydroxide (NH₄OH) (65 : 25 : 4, by vol.). On a separate 10 x 10 cm Analtech silica gel G TLC plate, the remaining 75 µL of sample was spotted. The second TLC plate was exposed to HCl fumes for 15 minutes and separated using the solvent system described above. Bands corresponding to 2-lysoPakEtnc + 2-lysoPlsEtnc, and 2-lysoPakCho + 2-lysoPlsCho (plate #1) or 2-lysoPakEtnc, and 2-lysoPakCho (plate #2) were scraped off the TLC plates and transferred into acid-washed 16 x 120 mm test tubes. These fractions on silica were then solvated in 1.0 mL HIP 3:2 + 5.5% H₂O and a portion of the sample was used to quantitate radioactivity (250 µL). The remainder of the sample was assayed for lipid phosphorus (Rouser et al., 1969).

Quantitation of brain and plasma hexadecanol and brain 1-O-alkyl-2-acyl-sn-glycerophosphate (PakOH). The neutral lipids and brain HOGpl from the phospholipid separation were extracted from the silica gel using HIP 3:2 + 5.5% H₂O and transferred into conical screw-top test tubes. Brain neutral lipids or plasma lipid extracts were concentrated under a steady-stream of N₂ at 40°C and dissolved in 100 µL CHCl₃. Standards and samples were applied to 20 x 20 cm Analtech silica gel G TLC plates and the neutral lipids separated using a solvent system of heptane: isopropyl ether : HOAc (60:40:4, by vol.) (Breckenridge and Kuksis, 1968). Bands corresponding to fatty alcohol (brain/plasma) or HOGpl (brain) were transferred into separate acid washed tubes and dissolved in 1.0 mL HIP 3:2 + 5.5% H₂O. 250 µL of each sample was used to quantitate radioactivity and the remainder was used to calculate concentration. The specific activity of the 1-O-alkyl-2-lyso-sn-glycerol-3-phosphate (2-lysoPakOH) was determined form the
lipid phosphorus content (Rouser et al., 1969) following methanolic KOH hydrolysis of the HOGpl and separation via TLC as described above for the chemical modification of EtnGpl and ChoGpl. The fatty alcohol was quantitated by gas liquid chromatography (GLC) following acetate derivitization. A schematic diagram outlining the procedures used to separate and quantitate the lipids is presented in Figure 3.1.

GLC analysis of fatty alcohol. Acetate derivatives of the brain and plasma fatty alcohols were separated by GLC using a Hewlett-Packard model 5890 series II gas chromatograph (King of Prussia, PA) equipped with a Supelco (Bellefonte, PA) capillary column (SP 2330; 30 m x 0.32 mm i.d.) and a flame ionization detector. The flow rate of the helium carrier gas was 1.0 mL x min⁻¹. Injection and detection ports were set at 250°C. Sample runs were initiated at 150°C with a temperature gradient to 220°C over 40.0 minutes. Acetylated fatty alcohol standards were used to establish relative retention times and relative correction factors. The internal standard used for this calculation was methyl heptadecanoate dissolved in 2,2,4-trimethylpentane (HPLC grade, Sigma Chem. Co., St. Louis, MO). The detector response was linear within the sample concentration range for all fatty acetate standards of varying chain length and degree of unsaturation with correlation coefficients of 0.990 or greater.

Calculations. The specific activity (dpm x nmol⁻¹) of the labeled brain ether phospholipids and labeled brain hexadecanol were directly measured from tissue of rats killed at 5, 10, 20, 30, 60, and 120 minutes following the start of the infusion. The specific activity of the brain hexadecanol pool was then corrected for vascular influence by subtracting the specific activity (dpm x nmol⁻¹ x mL⁻¹) of hexadecanol found in the residual
cerebral blood volume. The residual cerebral blood volume for this size and species of rat is $2.3 \pm 0.2 \times 10^2$ (mL x g$^{-1}$, mean ± SEM) (Grange et al., 1995).

The corrected specific activity of the brain hexadecanol ($C_{brain\text{-}hexadecanol}^*$ in dpm x nmol$^{-1}$) and the specific activity of whole blood hexadecanol ($C_{blood\text{-}hexadecanol}^*$ in dpm x nmol$^{-1}$ x mL$^{-1}$) for each experimental time point was then used to calculate the integrated specific activity of hexadecanol from the start of the infusion ($t_1 = 0$) to the time of death ($t_2 = 120$) using the "trapezoidal rule" (SigmaPlot Windows, Version 3 03, SPSS, Chicago, IL). These values were used to calculate the ratio of labeled hexadecanol ($\Psi$) which entered the brain during infusion as outlined in equation 1. The ether phospholipid precursor pool values were calculated using the time-dependent specific activity of the individual ether phospholipids and are equal to the numerator found in the derivation of the transfer coefficients outlined in Figure 2.9. The transfer coefficients, flux values, turnover rates, and half-lives of the individual ether phospholipids were calculated as outlined in Chapter two using the specific equations listed in Figure 2.9.

3.3 Results

*Distribution of Plasma Activity.* Intravenous infusion of [1,1-$^3$H]hexadecanol produced a transient increase of tritiated plasma species in the organic extract peaking at 5.0 minutes then leveling above baseline at twenty minutes (Figure 3.2). Counts found in the volatile aqueous extract, denoting tritiated H$_2$O, increased linearly from the start of
infusion to 10 minutes, then remained constant throughout the remainder of the
experiment. Counts from the nonvolatile aqueous phase were minimal and represented
approximately 6.4 ± 1.0% of total plasma radioactivity. Assays of plasma hexadecanol
following infusion (Figure 3.3) show that labeled hexadecanol mirrors the total plasma
radioactivity. However, labeled plasma hexadecanol returns to baseline by twenty minutes
having an unbound plasma half-life of 1.8 ± 0.3 minutes. The integrated specific activity
of plasma hexadecanol represents 23.3 ± 4.0% of the total plasma radioactivity suggesting
this amount was available to the rat for the manufacture of hexadecanol-containing
species. The oxidation of [1,1-3H]hexadecanol was linear between the start of infusion
and 30 minutes (Figure 3.3, inset). The apparent rate of oxidation was 2.9 % x min⁻¹ and
accounted for 84 % of the initial amount of radioactivity administered.

Radioactivity in the plasma lipid components was found in the fatty alcohol,
phospholipid, triradylglycerol, and diradylglycerol fractions (Figure 3.4). These fractions
accounted for, on average, 91.2 ± 11.7% of total plasma radioactivity found in the organic
extract throughout the duration of the experiment (Table 3.1). The plasma distribution of
counts into the plasmanyl and plasmenyl fractions was not determined due to limited
sample size. The proportion of labeled plasma phospholipid increased throughout the
duration of the experiment. The influence of the total plasma labeled phospholipid on
brain specific activity, considering a residual cerebral blood volume of 2.3 ± 0.2 (mL x
gram⁻¹), accounted for 0.1 ± 0.1% at 5.0 minutes and increased to 2.9 ± 0.6% at 120
minutes.
Assay of the total plasma and brain fatty alcohol showed that the infusion of 1.75 mCi x kg$^{-1}$ [1,1-$^{3}$H]hexadecanol did not increase the cold concentration of plasma or brain hexadecanol despite a 300-fold concentration gradient of hexadecanol in the plasma (Table 3.2). Both the plasma and brain levels remained at 667.3 ± 87.6 nmol/mL and 3.7 ± 0.8 nmol/gram, respectively throughout the duration of the experiment.

Distribution of [1,1-$^{3}$H]hexadecanol in Brain. Radioactivity from whole brain extracts was distributed into four compartments: free fatty alcohol, 1-$O$-alkyl-2-acyl-$sn$-glycerol-3-phosphate (PakOH), EtnGpl, and ChoGpl. These fractions accounted for 96.4 ± 1.4% of the total radioactivity in the brain organic fraction (Table 3.3). Analysis of class distribution found no activity in the diacyl glycerophospholipids, indicating the oxidation of labeled hexadecanol to palmitic acid resulted in the complete loss of the tritium atoms. The distribution of counts in the PakOH fraction increased to 66.9 ± 2.0% at 10 minutes, remained constant through 30 minutes, then dropped to 44.8 ± 5.4 and 29.1 ± 1.5% at 60 and 120 minutes, respectively. The counts in the EtnGpl and ChoGpl increased steadily from 3.0 ± 0.6 and 3.2 ± 0.4% at five minutes to 32.6 ± 2.3 and 31.4 ± 1.0% at 120 minutes, respectively.

Brain Specific Activity. The specific activity of the brain hexadecanol pool peaked at five minutes and steadily decreased through 120 minutes (Figure 3.5) and resulted in 1.0 ± 0.4% of total labeled plasma hexadecanol entering the brain during the experiment (Equation 3.1). The specific activity of the PakOH was 104.0 ± 26.2 dpm x nmol$^{-1}$ at 5.0 minutes, decreased to 84.3 ± 22.4 dpm x nmol$^{-1}$ at 10 minutes, and remained above 60.5 ± 22.7 dpm x nmol$^{-1}$ throughout the experiment. The specific activity of the stable ether
phospholipid pools increased steadily throughout the duration of the experiment. The ether phospholipids with the highest specific activity were the PakCho and PlsCho fractions, which increased to 96 and 78 dpm x nmol\(^{-1}\) at 120 minutes (Figure 3.6). The PakEtn and PlsEtn fractions also increased throughout the duration of the experiment and were 16 and 3 dpm x nmol\(^{-1}\) at 120 minutes. PakEtn is the precursor for both the PlsEtn and PlsCho pools. The plasmanylcholine (PakCho) fraction also increased throughout the duration of the experiment.

**Brain Precursor Pool Specific Activity.** The specific activity of the individual ether phospholipids were used to calculate the precursor pool specific activity and are equivalent to the numerator in the transfer coefficient equations (Figure 2.9). Since these values are dependent on the incorporation coefficient, these calculations were made starting with the PlsCho followed by PlsEtn, PakEtn, and finally PakCho. The time-dependent changes in the precursor pool specific activity of the labeled brain ether lipid fractions demonstrate the appropriate precursor-product relationship, in that the specific activity of the substrate pools remains higher than that of the product pools throughout the duration of the experiment (Figure 3.7 and 3.8). The specific activity of the PakOH precursor pool remained relatively constant between 30 and 120 minutes ranging from 150 ± 5 to 160 ± 22 dpm x nmol\(^{-1}\), respectively. The specific activity of the brain hexadecanol fell sharply throughout the duration of the experiment. However, at 120 minutes the specific activity of the brain hexadecanol precursor pool remained 1000-fold higher than the specific activity to the PakOH pool. The specific activity of the PakCho and PakEtn precursor pools also gradually increase from zero to 96 ± 6 and 97 ± 6 dpm x nmol\(^{-1}\),
while the PlsCho and PlsEtn pools increased from zero to 78 ± 5 and 81 ± 5 dpm x nmol\(^{-1}\), respectively. This relationship adheres to the accepted substrate-product relationship for ether lipid biosynthesis \textit{de novo} as outlined in Chapter 2 (Figure 2.7).

\textit{Rate Calculations}. Using the simplified substrate product relationship for ether phospholipid biosynthesis as outlined in Figure 2.7, the transfer coefficients of labeled hexadecanol from substrate pools \((i - 1)\) to product pools \((i)\) were calculated by measuring the change in the specific activity of the product precursor pools between 30 and 120 minutes divided by the integrated specific activity of the substrate precursor pools as outlined in equation 2.4 and specified in Figure 2.9. The transfer coefficients of labeled \([1,1-^3\text{H}]\text{hexadecanol}\) into PakCho, PakEtn, PlsEtn, and PlsCho were 0.096, 0.180, 0.611, and 0.235 h\(^{-1}\), respectively (Table 5). The slower PakCho and PakEtn pools resulted in the flux values (equation 2.7) of hexadecanol from the substrate pools (PakOH, \(i - 1\)) into product pools (PakCho and PakEtn, \(i\)) at rates of 33.6 ± 2.9 and 63.3 ± 5.9 (nmol x gram\(^{-1}\) x h\(^{-1}\)), respectively. The half-life of the hexadecanol-containing molecular species of PakCho and PakEtn pools were 8.4 ± 0.7 and 20.6 ± 1.8 hours, respectively, using equation 2.9.

The flux of labeled hexadecanol into the PlsEtn and PlsCho was 1,157.4 ± 47.6 and 533.6 ± 41.2 (nmol x gram\(^{-1}\) x h\(^{-1}\), equation 2.7) and resulted in the turnover rate of hexadecanol-containing PlsEtn and PlsCho pools at a rate of 5.6 ± 0.2 and 64.9 ± 5.0 percent per hour (equation 2.8). The half-life of PlsEtn and PlsCho pools in the awake adult rat, using equation 2.9, were 12.3 ± 0.5 and 1.1 ± 0.1 hours, respectively.
3.4 Discussion

This study examines the rate of incorporation of a labeled fatty alcohol into brain ether phospholipids using a pulse-chase experimental design followed by tracer kinetic analysis. The results demonstrate that [\(1,1^{-2}\text{H}\)]hexadecanol is rapidly incorporated into both plasmanyl and plasmenyl-type choline and ethanolamine phospholipids in a manner consistent with accepted patterns of ether phospholipid biosynthesis \textit{de novo} (Paltauf, 1994). Our laboratory has published similar methods measuring the turnover and half-life of brain phospholipids \textit{in vivo} utilizing an intravenous infusion of both labeled palmitic acid (Grange et al., 1995) and arachidonic acid (Washizaki et al., 1994). However, the intravenous infusion of \([1,1^{-2}\text{H}]\)hexadecanol to measure brain ether phospholipid biosynthesis involves a decidedly different rationale, unlike the fatty acid model (Robinson et al., 1992), which depends on the dilution of the labeled brain acyl-CoA pool (\(\lambda\)) coupled to fatty acid recycling. This model relies on the initial labeling of the brain PakOH pool followed by the sequential labeling of the PakCho, PakEtn, PlsEtn, and PlsCho pools (Figure 2.7).

The salient feature of this model is that a tracer level of labeled hexadecanol was infused intravenously that maintained steady-state levels of all ether phospholipids in the brain throughout the duration of the experiment. Therefore, tracer kinetic analysis allowed the calculation of the transfer coefficients (\(k_{esr}^*\)) of PakCho, PakEtn, PlsEtn, and PlsCho. Accordingly, the transfer coefficient reflects the sequential flow \textit{de novo} of tracer from one stable ether phospholipid pool to the next, independent of mass. Computations were then performed which allowed the calculation of the net unidirectional flux, turnover,
and half-life of the brain PakCho, PakEtn, PlsEtn, and PlsCho pools by equating the cold concentration of each with its transfer coefficient as outlined in equations 2.7 through 2.9.

The infusion of [1,1-\textsuperscript{3}H]hexadecanol resulted in a significant proportion of the tritium label being lost to tritiated H\textsubscript{2}O as demonstrated by the presence of label found in the volatile aqueous phase of the plasma extracts. However, the direct measure of plasma hexadecanol showed that approximately 23.0 ± 4.0% of total labeled hexadecanol was not lost to oxidation and was utilized by the animal for the production of hexadecanol-containing species. This is in good agreement with experiments performed on clofibrate-fed rats in which [1-\textsuperscript{14}C]hexadecanol was infused through the femoral artery resulting in 80-85% of total fatty alcohol oxidized to the fatty acid and esterified into liver PtdEtn and 2-lysoPtdEtn (Hayashi and Hara, 1997). Due to the nature of the tracer used in our experiments, the rate of oxidation of hexadecanol to palmitic acid within the brain cannot be determined. Nonetheless, the decrease in the specific activity of brain hexadecanol compared to the specific activity of brain PakOH suggest that the brain can oxidize hexadecanol. The alkyl-DHAP synthase has no preference for fatty alcohol chain length or degree of saturation as determined by biochemical analysis (Hajra, 1983). This suggests that hexadecanol, if present in the peroxisome, can be utilized for the production of alkyl-DHAP. Our data supports this conclusion.

The formation of alkyl-DHAP and acylation to form PakOH is catalyzed on inner and outer luminal surfaces of the peroxisomal fractions (Hajra, 1983). The resulting ethanolamine and choline plasmanyl-type phospholipids are largely formed in microsomal fractions by CDP-ethanolamine ethanolamine and CDP-choline choline.
phosphotransferases (Paltauf, 1983). In our study, PakEtn and PakCho syntheses occur at a rate of 63.3 ± 5.9 and 33.6 ± 2.9 nmol x gram⁻¹ x h⁻¹, respectively. These rates are in good agreement with studies using brain microsomal preparations in which the synthesis rates for PakCho and PakEtn were reported as 47.7 and 11.9-22.7 (nmol x gram⁻¹ x h⁻¹), respectively (Radominska-Pyrek and Horrocks, 1972, Radominska-Pyrek et al., 1977).

However, the half-life of the PakCho pool is approximately 12 hours less then PakEtn being 8.4 ± 0.8 hours. This suggests that the brain PakCho pool is metabolically active and supports the role of PakCho as an intermediate in the formation of platelet activating factor (Horrocks, 1989c, Snyder, 1999).

The specific activity of the PIsEtn precursor pool remained higher then that of the PIsCho pool supporting evidence that PIsEtn is the precursor of PIsCho. However, the route of brain PIsCho synthesis remains ambiguous. Several theories have been postulated in which polar head group and/or sn-2 group modification may be involved. Interestingly, the rates of PIsEtn and PIsCho formation in this study were 1,157.4 ± 47.6 and 533.6 ± 41.2 nmol x gram⁻¹ x min⁻¹, respectively. These values are much higher then those published using microsomal preparations 3.5-80.0 and 9.7-14.8 (PIsEtn and PIsCho, respectively) (Ansell and Metcalfe, 1971, Radominska-Pyrek and Horrocks, 1972, Radominska-Pyrek et al., 1977). However, our values for flux and turnover of PIsEtn are strikingly close to those from steady-state kinetics experiments in which [1⁻³H]glycerol was injected intracerebrally. This study reported total flux values of 1,920 nmol x gram⁻¹ x hour⁻¹ and a turnover of 5.3 hours⁻¹ (Masuzawa et al., 1984). No comparative data is available for PIsCho formation in the brain using either efflux or steady-state calculations.
The discrepancy between studies using microsomal preparations and our in vivo measurements suggests there may be a cytosolic influence on choline as well as ethanolamine plasmalogen formation. The rapid formation of labeled PlsCho following the intracerebral injection of [3H]arachidonic acid (Horrocks, 1989c, Horrocks et al., 1986) also supports the idea of a cytosolic reacylation processes being involved in PlsCho formation.

The discrepancies between the specific activities of the individual ether phospholipids and those calculated for the ether phospholipid precursor pools suggests that there are localized ether phospholipid pools within the brain that are not actively turning over. This is supported by autoradiography studies that show [1,1-3H]hexadecanol is strongly incorporated into gray matter regions including cortical, hippocampal, and thalamic regions and is not strongly incorporated into white matter (Chapter 4). The occurrence of approximately 40 percent of the brain PlsEtn pool in the myelin (Horrocks, 1967) may account for this discrepancy. Since the flux and turnover values are dependent upon the cold concentration of the active precursor and product pools, the active PlsEtn precursor pool was approximated using the turnover value listed in table 3.5 to approximate the rate of PlsCho formation. The value was estimated to be 1,136.5 nmol x gram\(^{-1}\). Since the white matter contributions of PakEtn and PakCho are low, being approximately 6.3 and 16.7 percent of the total brain pool, respectively (Ansell, 1973), estimations were not made for these ether phospholipid subclasses.

In summary, we have measured the de novo rate and turnover of brain ether phospholipid synthesis in the awake adult rat brain in vivo. Utilization of an intravenous
infusion of [1,1-\(^{3}\text{H}\)]hexadecanol and tracer kinetics allowed specific assessment of all brain ethanolamine and choline ether phospholipids including the complete analysis of plasmalogen formation throughout the duration of the experiment. The rates of formation of both PakEtn and PakCho are in good agreement with published values using microsomal preparations. However, the rates of synthesis and turnover of PlsEtn and PlsCho are an order of magnitude greater than those values observed using microsomal assays and efflux calculations. However, the flux and turnover of PlsEtn is supported from steady-state experiments in which intracerebral injection of [1-\(^{3}\text{H}\)]glycerol was done. The rates of plasmalogen synthesis and turnover reported here suggest that plasmalogens may have a greater function within the central nervous system than previously thought. In all, the rapid turnover of brain plasmalogens suggests that their study warrants further investigation.
<table>
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<tr>
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<th>5 min (n=5)</th>
<th></th>
<th>10 min (n=5)</th>
<th></th>
<th>20 min (n=5)</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>% distribution</td>
<td>sd</td>
<td>% distribution</td>
<td>sd</td>
<td>% distribution</td>
<td>sd</td>
</tr>
<tr>
<td>TG</td>
<td>12.3</td>
<td>5.3</td>
<td>35.7</td>
<td>12.1</td>
<td>31.5</td>
<td>8.0</td>
</tr>
<tr>
<td>DG</td>
<td>8.9</td>
<td>1.4</td>
<td>10.7</td>
<td>1.6</td>
<td>24.3</td>
<td>1.0</td>
</tr>
<tr>
<td>16:0 (ol)</td>
<td>72.8</td>
<td>5.2</td>
<td>36.8</td>
<td>0.9</td>
<td>12.5</td>
<td>2.5</td>
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<tr>
<td>PL</td>
<td>2.7</td>
<td>0.4</td>
<td>4.8</td>
<td>0.2</td>
<td>11.1</td>
<td>2.2</td>
</tr>
<tr>
<td>Total</td>
<td>96.7</td>
<td>88.0</td>
<td>79.4</td>
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<table>
<thead>
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<th></th>
<th>60 min (n=5)</th>
<th></th>
<th>120 min (n=5)</th>
<th></th>
</tr>
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<td>sd</td>
<td>% distribution</td>
<td>sd</td>
<td>% distribution</td>
<td>sd</td>
</tr>
<tr>
<td>TG</td>
<td>37.3</td>
<td>5.7</td>
<td>34.1</td>
<td>12.8</td>
<td>34.2</td>
<td>11.5</td>
</tr>
<tr>
<td>DG</td>
<td>15.0</td>
<td>3.5</td>
<td>7.4</td>
<td>1.7</td>
<td>6.6</td>
<td>2.0</td>
</tr>
<tr>
<td>16:0 (ol)</td>
<td>14.4</td>
<td>1.6</td>
<td>9.5</td>
<td>2.0</td>
<td>6.6</td>
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<tr>
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<td>1.5</td>
<td>31.5</td>
<td>7.1</td>
<td>67.1</td>
<td>11.2</td>
</tr>
<tr>
<td>Total</td>
<td>85.8</td>
<td>82.5</td>
<td>114.5</td>
<td></td>
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</tbody>
</table>

Average Yield = 91.2%

Table 3.1: Distribution of radioactivity found in the plasma organic extract following the infusion of [1,1-\textsuperscript{3}H]hexadecanol in the awake adult rat. Values represent the means ± sd (n = 5) in units of percent distribution. Abbreviations are: triradylglycerol (TG), diradylglycerol (DG), hexadecanol (16:0 (ol)), and phospholipid (PL). Average yield was calculated from the sum of each fraction at the experimental time points listed above.
### Table 3.2: Levels of plasma and brain hexadecanol following the infusion of [1,1-\(^{3}\)H]hexadecanol in the adult awake rat

Values represent the means ± sd and are expressed in units of nmol x mL\(^{-1}\) for plasma and nmol x gram\(^{-1}\) for brain. The relative standard deviation was calculated from the mean and sd of individual samples (n).

<table>
<thead>
<tr>
<th>Time</th>
<th>nmol/mL</th>
<th>sd</th>
<th>n=5</th>
<th></th>
<th>Time</th>
<th>nmol/gram</th>
<th>sd</th>
<th>n=5</th>
</tr>
</thead>
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<tr>
<td>0</td>
<td>662.6</td>
<td>99.4</td>
<td>n=5</td>
<td></td>
<td>0</td>
<td>ND</td>
<td>ND</td>
<td>n=5</td>
</tr>
<tr>
<td>5</td>
<td>692.1</td>
<td>97.9</td>
<td>n=5</td>
<td></td>
<td>5</td>
<td>3.56</td>
<td>0.44</td>
<td>n=5</td>
</tr>
<tr>
<td>10</td>
<td>683.6</td>
<td>71.4</td>
<td>n=5</td>
<td></td>
<td>10</td>
<td>2.68</td>
<td>0.22</td>
<td>n=4</td>
</tr>
<tr>
<td>20</td>
<td>635.7</td>
<td>95.8</td>
<td>n=5</td>
<td></td>
<td>20</td>
<td>3.61</td>
<td>1.08</td>
<td>n=5</td>
</tr>
<tr>
<td>30</td>
<td>584.2</td>
<td>95.3</td>
<td>n=5</td>
<td></td>
<td>30</td>
<td>3.77</td>
<td>0.99</td>
<td>n=5</td>
</tr>
<tr>
<td>60</td>
<td>610.9</td>
<td>63.5</td>
<td>n=5</td>
<td></td>
<td>60</td>
<td>3.90</td>
<td>0.59</td>
<td>n=5</td>
</tr>
<tr>
<td>120</td>
<td>660.2</td>
<td>60.2</td>
<td>n=5</td>
<td></td>
<td>120</td>
<td>3.50</td>
<td>0.36</td>
<td>n=5</td>
</tr>
<tr>
<td>Total</td>
<td>667.3</td>
<td>87.6</td>
<td>n=60</td>
<td></td>
<td>Total</td>
<td>3.71</td>
<td>0.85</td>
<td>n=29</td>
</tr>
<tr>
<td>RSD</td>
<td>0.13</td>
<td></td>
<td></td>
<td></td>
<td>RSD</td>
<td>0.23</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Table 3.3: Distribution of radioactivity found in the brain organic extract following the infusion of [1,1-\(^3^H\)]hexadecanol in the awake adult rat. Values represent the means ± sd (n = 4 to 5). Abbreviations are: hexadecanol (16:0 (ol)), 1-O-alkyl-2-acyl-sn-glycerol-3-phosphate (PakOH), ethanolamine glycerophospholipid (EtnGpl), and choline glycerophospholipid (ChoGpl) at all experimental time points. Average yield was calculated from the total of each experimental time point.
<table>
<thead>
<tr>
<th></th>
<th>nmol/gram</th>
<th>sd</th>
<th>RSD</th>
<th>% Dist.</th>
<th>sd</th>
<th>RSD</th>
</tr>
</thead>
<tbody>
<tr>
<td>PtdEtn</td>
<td>10,369.6</td>
<td>692.8</td>
<td>0.067</td>
<td>31.9</td>
<td>2.3</td>
<td>0.072</td>
</tr>
<tr>
<td>PlsEtn</td>
<td>20,294.9</td>
<td>1,710.1</td>
<td>0.084</td>
<td>62.3</td>
<td>2.4</td>
<td>0.039</td>
</tr>
<tr>
<td>PakEtn</td>
<td>1,861.2</td>
<td>236.6</td>
<td>0.127</td>
<td>5.7</td>
<td>0.7</td>
<td>0.123</td>
</tr>
<tr>
<td>EtnGpl</td>
<td>32,525.8</td>
<td>1,936.6</td>
<td>0.060</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PtdCho</td>
<td>17,261.4</td>
<td>1,101.0</td>
<td>0.064</td>
<td>93.4</td>
<td>0.7</td>
<td>0.007</td>
</tr>
<tr>
<td>PlsCho</td>
<td>822.7</td>
<td>96.8</td>
<td>0.118</td>
<td>4.5</td>
<td>0.5</td>
<td>0.111</td>
</tr>
<tr>
<td>PakCho</td>
<td>401.4</td>
<td>64.0</td>
<td>0.159</td>
<td>2.2</td>
<td>0.4</td>
<td>0.182</td>
</tr>
<tr>
<td>ChoGpl</td>
<td>18,485.4</td>
<td>1,125.2</td>
<td>0.061</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 3.4: Levels and percent distribution of rat brain ethanolamine and choline glycerophospholipids. Values represent the mean ± sd, including the relative standard deviation (RSD) (n = 25). Abbreviations are: phosphatidylethanolamine (PtdEtn), plasmenylethanolamine (PlsEtn), plasmanylethanolamine (PakEtn), ethanolamine glycerophospholipid (EtnGpl), phosphatidylcholine (PtdCho), plasmenylcholine (PlsCho), plasmanylcholine (PakCho), and choline glycerophospholipid (ChoGpl).
Table 3.5: Transfer coefficient, flux, turnover rate and half-life of brain ether phospholipids following the infusion of [1,1-\(^3\)H]hexadecanol in the adult awake rat. Values are expressed as hours\(^{-1}\) (transfer coefficients), nmol x gram\(^{-1}\) x hour\(^{-1}\) (flux, mean ± sd), percent x hour\(^{-1}\) (turnover rate, mean ± sd), and hours (half-life, mean ± sd).

Flux value calculated using approximated active PlsEtn pool size of 1,136.5 nmol x gram\(^{-1}\) (5.6 percent of total). Abbreviations are: plasmanylcholine (PakCho), plasmanylethanolamine (PakEtn), plasmenylethanolamine (PlsEtn), and plasmenylcholine (PlsCho).

<table>
<thead>
<tr>
<th>Transfer Coefficient (h(^{-1}))</th>
<th>Flux of labeled hexadecanol from i-1 to i (nmol x gram(^{-1}) x h(^{-1}))</th>
<th>Turnover Rate (% h(^{-1}))</th>
<th>Half-life (hour)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PakCho</td>
<td>0.096</td>
<td>33.6 ± 2.9</td>
<td>8.4 ± 0.7</td>
</tr>
<tr>
<td>PakEtn</td>
<td>0.180</td>
<td>63.3 ± 5.9</td>
<td>3.4 ± 0.3</td>
</tr>
<tr>
<td>PlsEtn</td>
<td>0.611</td>
<td>1,157.4 ± 47.6</td>
<td>5.6 ± 0.2</td>
</tr>
<tr>
<td>PlsCho</td>
<td>0.235</td>
<td>4,769.3 ± 368.2</td>
<td>579.7 ± 44.6</td>
</tr>
<tr>
<td>*PlsCho</td>
<td>0.235</td>
<td>533.6 ± 41.2</td>
<td>64.9 ± 5.0</td>
</tr>
</tbody>
</table>
Figure 3.1: Diagrammatic representation of the separation techniques involved in measuring the specific activity of brain ether phospholipids including: extraction, separation, and chemical modification.
Filtered Extract (5.0 mL HIP (3:2) + 5.5% H₂O)

1/50

NL

C/M/HOAc/H₂O

EtmGpl

0.5 M Methanolic KOH

Phase Separation
(CHCl₃/BuOH/H₂O)

2-lysoPakEttn

+ 2-lysoPisEttn

or

2-lysoPakCho

+ 2-lysoPisCho

Heptane/diethyl Ether/HOAc

Falc

PtdOH

Methylation + TLC

PakOH

3/4 Quantitate via Phos.
1/4 Count

3/4 Quantitate via GLC
1/4 Count

Figure 3.1
Figure 3.2: Time course of total plasma radioactivity from Folch organic (filled circles), aqueous (open circle), and dried aqueous (filled triangles) extracts. Values represent the mean ± sd (bars) in units of dpm x mL$^{-1}$ ($n = 5$).
Figure 3.3: Time course of labeled plasma hexadecanol following intravenous infusion of [1,1-$^3$H]hexadecanol. Figure inset is the apparent oxidation rate of [1,1-$^3$H]hexadecanol between 0 and 30 minutes. Values represent the mean ± sd (bars) in units of dpm x mL$^{-1}$ ($n = 5$).
Figure 3.4: Time course of labeled plasma lipids following intravenous infusion of \([1,1-\textsuperscript{3}H]\)hexadecanol. Values represent the mean ± sd (bars) in units of dpm x mL\(^{-1}\) (n = 5).
Figure 3.5: Time course of the brain specific activity of hexadecanol and 1-O-alkyl-2-acyl-sn-glycerol-3-phosphate (PakOH) following the intravenous infusion of [1,1-\(^3\)H]hexadecanol. Values represent the mean ± sd (bars) in units of dpm x nmol\(^{-1}\) (n = 5).
Figure 3.6: Time course of the brain specific activity of 1-O-alkyl-2-acyl-sn-glycerol-3-phosphate (PakOH), plasmanylcholine (PakCho), plasmenylethanolamine (PakEtn), plasmenylethanolamine (PlsEtn), and plasmenylcholine (PlsCho) following the intravenous infusion of [1,1-³H]hexadecanol. Values represent the mean ± sd (bars) in units of dpm x nmol⁻¹ (n = 5).
Figure 3.7: Time course in the brain of the calculated precursor pool specific activity of hexadecanol and 1-O-alkyl-2-acyl-sn-glycerol-3-phosphate (PakOH) following the intravenous infusion of [1,1-³H]hexadecanol. Values represent the mean ± sd (bars) in units of dpm x nmol⁻¹ (n = 5).
Figure 3.8: Time course in the brain of the calculated precursor pool specific activity of 1-\(O\)-alkyl-2-acyl-\(sn\)-glycerol-3-phosphate (PakOH), plasmanylcholine (PakCho), plasmanylethanolamine (PakEtn), plasmenylethanolamine (PlsEtn), and plasmenylcholine (PlsCho) following the intravenous infusion of \([1,1^{-3}\text{H}]\text{hexadecanol}\). Values represent the mean ± sd (bars) in units of dpm x nmol\(^{-1}\) (n = 5).
Figure 3.9: Time course in the brain of the calculated precursor pool specific activity of plasmanylcholine (PakCho), plasmanylethanolamine (PakEtn), plasmenylethanolamine (PlsEtn), and plasmenylcholine (PlsCho) following the intravenous infusion of [1,1-3H]hexadecanol. Values represent the mean ± sd (bars) in units of dpm x nmol⁻¹ (n = 5).
4.1 Introduction

In this chapter, I will discuss the results from autoradiographic and membrane fractionation experiments performed on rat brains following the intravenous infusion of $^{[1,1-^3H]}$hexadecanol. The observed differences between the specific activities of the individual ether phospholipids and those calculated for the ether phospholipid precursor pools, as outlined in Chapter 3, suggest that there are localized phospholipid pools within the brain that are not actively turning over. Due to this observation, the metabolically active PlsEtn pool was approximated based on its turnover rate. The calculated active PlsEtn pool is 1,143.5 nmol x gram$^{-1}$. This value was used to calculate the biosynthetic rate of PlsCho formation. To validate this correction, autoradiographic and membrane fractionation experiments were performed on brains from rats infused with $^{[1,1-^3H]}$hexadecanol. These measurements were performed to determine the regional incorporation coefficients ($k^*$) of tracer into different gray and white matter regions in the brain and to determine the incorporation levels and the distribution of tracer into the different membrane fractions. The hypothesis behind these studies, is that there are fast
and slow metabolic ether phospholipid pools in the mature brain that result in heterogeneity in both the incorporation of [1,1-$^3$H]hexadecanol, and in biosynthesis of ether phospholipid de novo. The primary importance of this work is that steady-state tracer kinetic analysis performed on the adult awake rat results in the quantification of ether phospholipid biosynthesis in vivo independent of myelination and that the rate constants provided by this analysis can be used to estimate the biological importance of these phospholipids in the brain. This chapter is divided into four sections: (1) introduction, (2) material and methods, (3) results, and (4) discussion.

The nervous system is composed of lipids that are differently distributed among various membranes, possesses many cell types, and has multiple subcellular structures. It has been proposed that the biosynthetic rate and turnover of lipids varies widely among the different membranes and that it is also possible that different classes of lipids within the same membrane may turnover at differing metabolic rates (Porcellati et al., 1983). Evidence for differential turnover in a single membrane is supported in experiments measuring the receptor-mediated polyphosphoinositide turnover. It has also been suggested based on increased labeling of brain PIsCho following receptor-stimulation that N-methylation of PIsEtn forming PIsCho may be linked to β-adrenergic stimulation and that a number of other receptors can initiate the hydrolysis of PIsCho (Horrocks et al., 1986). Receptor-mediated changes in ether phospholipid metabolism may result in the release of arachidonic acid, alter the intracellular levels of bioactive lipid metabolites, increase membrane disorder, modulate intracellular ions levels, and change the levels of
cyclic nucleotides. Therefore, when measuring brain phospholipid metabolism *in vivo*, it is important to quantitate the regional differences in tracer uptake.

Several studies have quantified brain fatty acid uptake in whole brain (Dhopeshwarkar and Mead, 1969, Dhopeshwarkar and Mead, 1970, Miller et al., 1977, Schmid and Takahashi, 1970, Sun and Horrocks, 1969b, Sun and Horrocks, 1971), individual brain regions (Kimes et al., 1983, Noronha et al., 1990, Rabin et al., 1998, Robinson et al., 1992, Washizaki et al., 1994), and subcellular fractions (Jones et al., 1997, Sun and Horrocks, 1973). However, only the quantification of *in vivo* imaging of tracer uptake into the individual brain regions directly links the labeled phospholipid to its physiological function. *In vivo* imaging has shown that arachidonic acid turnover is increased following transient ischemia in the awake gerbil (Rabin et al., 1998) and a 6-hydroxydopamine lesion in the awake rat (Hayakawa et al., 1998). Arachidonic acid turnover has also been shown using *in vivo* imaging to be specifically decreased in chronic lithium-treated rats (Chang et al., 1996). In an extension of recent studies (Contreras et al., 1999), this method has shown the dose-dependent decrease of palmitic acid incorporation in all brain regions following administration of anesthesia (unpublished results). Therefore, to get an idea of the physiological function of phospholipid metabolism, *in vivo* imaging experiments must be performed on subjects following tracer infusion. Until now, no one has reported the regional localization of fatty alcohol uptake in the adult rat brain. Therefore an effort was made to develop a procedure that measures the incorporation of plasma hexadecanol into the brain of the awake rat, and to determine if regional differences in incorporation do exist.
The method utilized to quantitate brain fatty alcohol incorporation involves quantitative autoradiography. This method has been used extensively in our laboratory to quantitate arachidonic, palmitic, and docosahexaenoic acid incorporation in the rat brain of adult awake rats (Kimes et al., 1983, Noronha et al., 1990, Rabin et al., 1998, Robinson et al., 1992, Washizaki et al., 1994) and has been extended to positron emission tomography (PET) studies using primate (Chang et al., 1997) and human subjects (Rapoport, 1997, Rapoport et al., 1997). This method allows the examination of regional brain phospholipid metabolism in the awake animal and can identify net changes in incorporation of the radiolabeled ether phospholipid precursor into localized brain regions. These measurements can be used to calculate the regional incorporation coefficients of tracer from plasma into brain as outlined in Chapter 2. However, quantitative autoradiography can only identify net changes in incorporation, whereas the exact rate of incorporation into the specific phospholipid requires the additional chemical analysis as outlined in Chapter 3. Together, both chemical and autoradiographic analysis can lend insight into the physiological role of ether phospholipids in the central nervous system.

4.2 Material and Methods

[1,1-3H]Hexadecanol (50 mCi x mmol⁻¹, ≥97% pure) was purchased from Moravek Biochemicals (Brea, CA). Phospholipid and neutral lipid standards were from Nu-Chek-Prep Inc. (Elysian, MN) and fatty alcohol standards and “essentially fatty acid free” bovine serum albumin were from the Sigma Chemical Co. (St. Louis, MO). HPLC grade hexane and isopropyl alcohol were from EM Science (Gibbstown, NJ) and
chloroform, methanol, and other chemicals used were of reagent grade from Mallinckrodt Inc. (Paris, KY) unless noted otherwise. Beckman Ready-Safe® scintillation cocktail (Fullerton, CA) containing 1.0% glacial acetic acid was used to determine the radioactivity of all samples. All extracts were stored in HPLC grade hexane: isopropyl alcohol (3:2 (v/v) + 5.5% H2O) under nitrogen (N2) at -80°C until use unless noted otherwise.

Surgical preparation. Experiments were performed in accordance with the NIH Guidelines for the Care and Use of Laboratory Animals (NIH publication no. 80-23). Male Sprague-Dawley rats (150-280 g, Charles River Breeding Laboratory, Wilmington, MA) were anesthetized with 2-3% Halothane (Halocarbon Labs, River Edge, NJ) and polyethylene catheters (PE 50, Becton Dickinson, Sparks, MD) filled with 100 IU sodium heparin were placed in the right femoral artery and vein. The wounds were closed with clips and Lidocain (1.0% solution) was applied as a local anesthetic. The animals were wrapped in fast setting plaster body casts, taped to wooden blocks, and allowed to recover from anesthesia for at least 3.0 hours in a thermostatically controlled heating environment set to maintain body temperature at 36.5°C.

Infusion of [1,1-3H]hexadecanol. Awake rats were intravenously infused for 5.0 min at a rate of 0.4 mL x min⁻¹ with a saline solution containing 0.875 mCi x kg⁻¹ x mL⁻¹ [1,1-3H]hexadecanol suspended in 0.06 mg BSA (Harvard Apparatus, South Natick MA). Arterial blood samples were collected throughout the experiment to determine the specific activity of blood and plasma components. At various times following the start of infusion, all animals were killed using an overdose of intravenously infused sodium pentobarbital
(100 mg x kg⁻¹). At death, all animals were immediately decapitated and their brains were excised and frozen in 2-methylbutane at -50°C. Brains were stored at -80°C until use.

**Autoradiography.** Brains were analyzed as previously described (Hayakawa et al., 1998, Noronha et al., 1990). Briefly, brains used for autoradiography were secured to mounts using imbedding media (Corber Scientific) and cut into 20 μm slices using a model OTF cryostat (Bright Inst. Co.) at -20°C. Slices were transferred onto glass cover slips and allowed to dry at 60-70°C for 45 minutes. Dry slides were attached to 8 x 10 inch boards with double-sided tape, inserted into film cassettes beside radioactive tissue standards (Amersham, Corp.), covered with RPN-12 Hyperfilm™ (Amersham Corp.), and allowed to expose for 20-24 weeks. At that time, films were developed, then fixed before being analyzed. Regional brain radioactivity was measured in sextuplet from coronal brain sections by digital quantitative densitometry using the public domain analysis program NIH Image (Version 1.55, created by Wayne Rasband, NIH). These values were used to calculate the regional incorporation coefficients as outlined in Chapter 2.

**Membrane fractionation.** Subcellular localization experiments were performed using a modified method of Sun and Horrocks (Sun and Horrocks, 1973). All solutions and centrifugation steps were maintained at 4°C. Briefly, brains were homogenized using a glass Tenbroeck homogenizer in 50 mM Tris buffer, pH 7.5 containing 0.32 M sucrose. The myelin, synaptosomal, and mitochondrial membranes were separated from the plasma membranes, microsomal membranes, and cytosol using low-speed centrifugation (9,000 RPM, 12,000 x g) of the brain dispersions for 10 minutes (Figure 4.1). The plasma and microsomal membranes fraction was separated from cytosol by high-speed centrifugation.
(40,000 RPM, at 100,000 x g) for 60 minutes. The myelin was separated from the synaptosomal and mitochondrial membranes using density centrifugation at intermediate-speed (19,000 RPM, 40,000 x g) for 60 minutes. Myelin membranes were pelleted by diluting the myelin suspension with equal volumes of ice cold water followed by intermediate-speed centrifugation (19,000 RPM, 40,000 x g) for 60 minutes. All membrane fractions and plasma samples were extracted using a two-phase extraction method (Folch et al., 1957) and stored in 2.0 mL HIP 3:2 + 5.5% H₂O under N₂ at -80°C until use.

Membrane fraction phospholipid separation. Standards and samples were applied to 20 x 20 cm Whatman silica gel 60A LK6 TLC plates and separated into classes using a solvent system of CHCl₃ : MeOH : glacial acetic acid (HOAc) : H₂O (50 : 37.5 : 3 : 2, by vol.) (Jolly et al., 1997). Bands corresponding to ethanolamine glycerophospholipid (EtNGp), choline glycerophospholipid (ChoGp), diradyl-sn-glycerol-3-phosphate ( Hogp), and neutral lipids were scraped off the TLC plates and stored in HIP (3:2) + 5.5% H₂O.

Chemical modification of EtNGp and ChoGp. The EtNGp and ChoGp fractions were extracted from the silica gel using HIP (3:2) + 5.5% H₂O and concentrated under a steady stream of N₂ at 40°C. Samples were incubated in 2.0 mL of methanol containing 0.5 M KOH at 30°C for 15 minutes. At 15.0 minutes, 1.0 mL ethyl formate (Sigma Chem. Co., St. Louis, MO) was added followed by the addition of 4.0 mL CHCl₃ : n-butanol (BuOH) (4 : 2 (v/v)). Two-phases were formed by the addition of 2.0 mL 0.1 M KCl and phase separation was promoted by centrifugation at 1000 x g (Ansell and Spanner, 1963).
The lower solvent phase containing the lyso-ether phospholipid analogs (1-O-alkyl-2-lyso-
\(sn\)-glycerol-3-phosphoethanolamine (2-lysoPakEtn) + 1-O-alkyl-1'-enyl-2-lyso-\(sn\)-glycerol-3-
phosphoethanolamine (2-lysoPlsEtn) or 1-O-alkyl-2-lyso-\(sn\)-glycerol-3-phosphocholine (2-
lysoPakCho) + 1-O-alkyl-1'-enyl-2-lyso-\(sn\)-glycerol-3-phosphocholine (2-lysoPlsCho)) were
then transferred into conical screw-top test tubes and stored in 1.0 mL HIP (3:2) + 5.5% 
H\(_2\)O.

*Separation of 2-lysoPakEtn - 2-lysoPlsEtn and 2-lysoPakCho - 2-lysoPlsCho* 

classes. The lyso-ether phospholipid extracts were concentrated under a steady stream of
N\(_2\) at 40°C and dissolved in 100 \(\mu\)L CHCl\(_3\). Each sample was spotted onto a 10 x 10 cm 
Analtech silica gel G TLC plate and the lipid classes were separated using CHCl\(_3\) : MeOH 
: ammonium hydroxide (NH\(_4\)OH) (65 : 25 : 4, by vol.). Bands corresponding to 2-
lysoPakEtn + 2-lysoPlsEtn, and 2-lysoPakCho + 2-lysoPlsCho were scraped off the TLC 
plates and used to quantitate radioactivity. Aliquots of total sample extract (100 \(\mu\)L) were 
assayed to measure total lipid phosphorus (Rouser et al., 1969) and total membrane 
radioactivity.

*Quantitation of membrane plasma hexadecanol and 1-O-alkyl-2-acyl-\(sn\)-
glycerophosphate (PakOH).* The neutral lipids and HOGpl from the phospholipid 
separation were extracted from the silica gel using HIP 3:2 + 5.5% H\(_2\)O and transferred 
into conical screw-top test tubes. Membrane neutral lipids or plasma lipid extracts were 
concentrated under a steady-stream of N\(_2\) at 40°C and dissolved in 100 \(\mu\)L CHCl\(_3\).
Standards and samples were applied to 20 x 20 cm Analtech silica gel G TLC plates and 
the neutral lipids separated using a solvent system of heptane: isopropyl ether : HOAc
(60:40:4, by vol.) (Breckenridge and Kuksis, 1968). Bands corresponding to fatty alcohol were transferred into scintillation vials for quantitation of radioactivity. The activity of the 1-O-alkyl-2-lyso-sn-glycerol-3-phosphate (2-lysoPakOH) was determined using liquid scintillation counting following the methanolic KOH hydrolysis of the HOGpl and separation via TLC as described above for the chemical modification of EtnGpl and ChoGpl.

**Calculations.** The incorporation coefficients were calculated as described in Chapter 2. Plasma hexadecanol integrals were calculated for each animal from the start of the infusion (t₁ = 0) to the time of death (t₂ = 30, 60, or 240) using the “trapezoidal rule” (SigmaPlot Windows, Version 3.03, SPSS, Chicago, IL). The specific activities of the membrane extracts were calculated by dividing the total counts in each fraction by the total level of phosphorus in the same fraction. Statistics performed were a one-way ANOVA and a Tukey-Kramer multiple comparison post-hoc test using the computer program InStat2 (GraphPad, San Diego, CA).

### 4.3. Results

**Autoradiography.** Rat brains, following the intravenous infusion of [1,1-³H]hexadecanol, were used to produce autoradiographs as presented in Figures 4.2 to 4.4. At 30 minutes, autoradiographs show a homogenous distribution of tracer throughout all sections analyzed with the exception of the choroid plexus found in the ventricles. All sections analyzed show dark ventricular staining despite being thoroughly dried prior to film exposure. At 60 and 240 minutes, localization appears with the loss of tracer-
reactivity in the white matter tracts. As determined using quantitative autoradiography of 60 minute time-points, heterogeneous localization is found throughout the cortical (3.6-4.5 sec\(^{-1}\) x 10\(^4\)), hippocampal (3.3-4.5 sec\(^{-1}\) x 10\(^4\)) and mid-brain regions (3.7-4.2 sec\(^{-1}\) x 10\(^4\)) (Table 4.1). At 60 minutes, areas with the highest activity are the hippocampal molecular layer 4.5 sec\(^{-1}\) x 10\(^4\), frontal (4.3 sec\(^{-1}\) x 10\(^4\)), and cingulate (4.2 sec\(^{-1}\) x 10\(^4\)) cortical regions. At 240 minutes, the cingulate cortex, motor cortex, somatosensory cortex, and hippocampal molecular layer show the highest reactivity being 3.4, 3.2, 3.0, and 3.2 sec\(^{-1}\) x 10\(^4\), respectively. Comparison of the 240-minute autoradiographs with the 30 minutes autoradiographs show that the white matter loses a significant proportion of reactivity in all tracts analyzed. At 60 minutes, only the corpus callosum and external capsule regions are significantly different from 30 minutes.

**Membrane Fractionation.** As expected, membrane fraction experiments show the total radioactivity in the myelin fraction to decrease with time being 54,128 ± 3,609 dpm x gram\(^{-1}\) at 30 minutes and decreasing to 6,183 ± 2,001 dpm x gram\(^{-1}\) at 240 minutes (Table 4.2). Both the synaptosomal and plasma membrane fractions also decrease as does the myelin fraction. However, both of the former are approximately 14-16 times greater than the myelin fraction at 240 minutes. Interestingly, the counts found in the myelin fraction remain largely in the hexadecanol fraction throughout the duration of the experiment occupying between 77.0 ± 6.5 and 70.3 ± 4.8 percent of the total labeled fraction (Table 4.3). This suggests that the myelin is relatively inert with respect to ether phospholipid biosynthesis *de novo*. On the other hand, the percent of the labeled ether phospholipid increase as compared to the labeled hexadecanol fraction throughout the experiment in
both the synaptosomal and mitochondrial membrane fraction, and the plasma and microsomal membrane fraction. These fractions increase from approximately 18.0 percent at 30 minutes to 23.4-32.4 percent in the synaptosomal and mitochondrial membrane fractions at 240 minutes. In the plasma membrane and microsomal membrane fraction the proportion of labeled ether phospholipids increases from approximately 17.2-21.5 percent at 30 minutes to 20.5-37.2 percent at 240 minutes.

4.4. Discussion

As outlined in Chapter 3, a large discrepancy was found between the specific activity of the individual brain ether phospholipids and the specific activity of the ether phospholipid precursor pools. However, both autoradiography and membrane fractionation experiments show that the myelin found in the white tracts and myelin membrane fractions incorporate a very small percentage of tracer into the PakOH, PakEtn, PakCho and PlsCho phospholipid subclasses compared to the synaptosomal and mitochondrial membranes, and the plasma and microsomal membranes. This suggests that the whole brain contribution of myelin ether phospholipid biosynthesis de novo is small compared to the contribution made by other membrane fractions.

Since the brain myelin fraction contains approximately 40 percent of the total brain PlsEtn (Horrocks, 1967), the very small percentage of the total ether phospholipids biosynthesis occurring in this region partially justifies the correction made in the rate calculations for PlsCho from whole brain experiments (Chapter 3). The estimated size of the active PlsEtn pool is 1,135.5 nmol x gram⁻¹. However, the myelin contribution of
PlsEtn is approximately 8,117.9 nmol x gram⁻¹, 40 percent of the total, resulting in approximately 12,177 nmol x gram⁻¹ being non-myelin PlsEtn. This suggests that the synaptosomal and mitochondrial membrane fractions, and the plasma and microsomal membrane fractions also maintain high levels of non-metabolically active PlsEtn pools. Based on these estimations, the active brain PlsEtn pool is between 12,176 and 1,137 nmol x gram⁻¹. Therefore, the steady-state biosynthetic rate of brain PlsCho formation in the brain is between 2,861.5 and 533.6 nmol x gram⁻¹ x hour⁻¹ and has a half-life between 0.1 and 1.1 hours in vivo.

In summary, both autoradiographic and membrane fractionation experiments show that there are localized regions of [1,1-³H]hexadecanol incorporation in the adult awake rat. These studies show that approximately 97 percent of the ether phospholipid biosynthesis de novo occurring in the brain can be found in gray matter regions. This suggests that in the adult rat, myelin membrane maintenance is principally supported through recycling mechanisms and not ether phospholipid biosynthesis de novo. These studies support the hypothesis that there is heterogeneity in the incorporation of fatty alcohol and in ether phospholipid biosynthesis de novo in the brain. The localized incorporation of tracer into gray matter regions supports the hypothesis that ether phospholipid metabolism has a greater biological importance within the central nervous system than previously thought and that their rate of turnover suggests that they are actively involved in maintaining neurological function.
Table 4.1: Regional time-dependent changes in the incorporation coefficient following the intravenous infusion of [1,1-\textsuperscript{3}H]hexadecanol in different brain regions in the adult awake rat. Values represent the means ± sd (n = 4) in units of sec\textsuperscript{-1}. The * indicates statistical significance from 30 minute time point p < 0.05, ** indicates statistical significance from 60 minute time point p < 0.05.
Table 4.2: Specific activity of counts found in the brain membrane fractions following the intravenous infusion of [1,1-\(^3\)H]hexadecanol. Values represent the means ± sd in units of dpm x gram\(^{-1}\) (n = 4). Abbreviations are: myelin membrane fraction (myelin), synaptosomal membrane and mitochondrial membrane (SM + mito.), and plasma membrane and microsomal membrane (PM + MM).

<table>
<thead>
<tr>
<th>Fraction</th>
<th>30 min average</th>
<th>sd</th>
<th>60 min average</th>
<th>sd</th>
<th>240 min average</th>
<th>sd</th>
</tr>
</thead>
<tbody>
<tr>
<td>myelin</td>
<td>54,128</td>
<td>3,609</td>
<td>20,149</td>
<td>5,037</td>
<td>6,183</td>
<td>2,001</td>
</tr>
<tr>
<td>SM + mito.</td>
<td>319,539</td>
<td>14,361</td>
<td>224,564</td>
<td>39,397</td>
<td>162,564</td>
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<td>PM + MM</td>
<td>112,448</td>
<td>14,509</td>
<td>63,381</td>
<td>5,761</td>
<td>56,224</td>
<td>1,171</td>
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Table 4.2: Specific activity of counts found in the brain membrane fractions following the intravenous infusion of [1,1-\(^3\)H]hexadecanol. Values represent the means ± sd in units of dpm x gram\(^{-1}\) (n = 4). Abbreviations are: myelin membrane fraction (myelin), synaptosomal membrane and mitochondrial membrane (SM + mito.), and plasma membrane and microsomal membrane (PM + MM).
### Myelin membrane fraction

<table>
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<tr>
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<th></th>
<th>60 min</th>
<th></th>
<th>240 min</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>% dist.</td>
<td>sd</td>
<td>% dist.</td>
<td>sd</td>
<td>% dist.</td>
<td>sd</td>
</tr>
<tr>
<td>16:0 (ol)</td>
<td>77.0</td>
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<td>73.6</td>
<td>7.2</td>
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<tr>
<td>PakOH</td>
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<td>9.9</td>
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<td>6.0</td>
<td>2.3</td>
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<tr>
<td>PakEtn + PIsEtn</td>
<td>7.5</td>
<td>2.9</td>
<td>8.5</td>
<td>3.1</td>
<td>13.7</td>
<td>4.1</td>
</tr>
<tr>
<td>PakCho + PIsCho</td>
<td>9.6</td>
<td>4.2</td>
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<td>2.2</td>
<td>10.0</td>
<td>1.9</td>
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### Synaptosomal membrane and mitochondrial membrane

<table>
<thead>
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<th>60 min</th>
<th></th>
<th>240 min</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
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<td>sd</td>
<td>% dist.</td>
<td>sd</td>
<td>% dist.</td>
<td>sd</td>
</tr>
<tr>
<td>16:0 (ol)</td>
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<td>1.3</td>
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<td>23.4</td>
<td>3.2</td>
<td>21.2</td>
<td>1.4</td>
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### Plasma membrane and microsomal membrane

<table>
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<tr>
<th>Lipid</th>
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<th></th>
<th>60 min</th>
<th></th>
<th>240 min</th>
<th></th>
</tr>
</thead>
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<tr>
<td></td>
<td>% dist.</td>
<td>sd</td>
<td>% dist.</td>
<td>sd</td>
<td>% dist.</td>
<td>sd</td>
</tr>
<tr>
<td>16:0 (ol)</td>
<td>54.8</td>
<td>4.7</td>
<td>51.0</td>
<td>5.8</td>
<td>33.8</td>
<td>1.3</td>
</tr>
<tr>
<td>PakOH</td>
<td>6.5</td>
<td>1.5</td>
<td>8.7</td>
<td>3.7</td>
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<td>3.5</td>
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<tr>
<td>PakEtn + PIsEtn</td>
<td>21.5</td>
<td>1.1</td>
<td>24.3</td>
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<td>37.2</td>
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<tr>
<td>PakCho + PIsCho</td>
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<td>16.0</td>
<td>2.9</td>
<td>20.5</td>
<td>0.9</td>
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Table 4.3: Distribution of counts found in the brain membrane fractions following the intravenous infusion of [1,1-\(^3\)H]hexadecanol. Values represent the means ± sd in units of percent distribution (n = 4). Abbreviations are: hexadecanol (16:0 (ol)), 1-O-alkyl-2-acyl-sn-glycero-3-phosphate (PakOH), ethanolamine glycerophospholipid (EtnGpl), and choline glycerophospholipid (ChoGpl).
Brain dispersion
(50 mM Tris, pH 7.5 + 0.32 M sucrose)

1,500 RPM,
270 x g,
10 min.

Pellet
(Cell debris,
discard)

Supernate

9,000 RPM,
12,000 x g,
20 min.

Pellet

1. Resuspend in 0.32 M sucrose
2. Layer onto 0.8 M sucrose
3. 19,000 RPM, 40,000 x g,
   60 min.

Supernate

40,000 RPM,
100,000 x g,
60 min.

Suspension
(myelin)

1. Dilute with H₂O
2. 19,000 RPM, 40,000 x g,
   60 min.

Cytosol
(discard)

Pellet
(myelin)

Pellet
(synaptosome +
mitochondria)

Pellet
(plasma +
microsomal
membranes)

Figure 4.1: Outline of the isolation procedure for the myelin, synaptosomal plus mitochondrial, and plasma plus microsomal membrane fractions from the rat brain.
Figure 4.3. Autoradiographs of brains at 30 minutes following the start of the infusion of [1,1-^3^H]hexadecanol.
Figure 4.3. Autoradiographs of brains at 60 minutes following the start of the infusion of [1.1-\textsuperscript{3}H]hexadecanol.
Figure 4.4. Autoradiographs of brains at 240 minutes following the start of the infusion of [1,1-^3H]hexadecanol.
CHAPTER 5

CONCLUSIONS

In this dissertation, I have outlined the pathways responsible for biosynthesis de novo of ether phospholipid in the brain. These pathways make ether phospholipids using a substrate-product relationship involving a series of sequential reactions. This substrate-product relationship was exploited in the development of a pulse-chase fatty alcohol model used to quantitate the biosynthetic rate and turnover of ether phospholipid synthesis in vivo. This model maintains steady-state levels of all brain ether phospholipids and produces a brain PakOH pool, which has a constant specific activity between 20 and 120 minutes. These conditions allow the application of steady-state tracer kinetic analysis (Zilversmit et al., 1942) to measure the formation and turnover of brain PakEtn, PakCho, PlsEtn, and PlsCho. The use of the tritium-labeled [1,1-\(^{3}\)H]hexadecanol permits measurement of ether phospholipid synthesis only, because the tritium atoms are lost during the oxidation of [1,1-\(^{3}\)H]hexadecanol to palmitic acid. Furthermore, there is stoichiometric retention of label following the desaturation step involved in plasmalogen formation, allowing for the direct measure of plasmalogen biosynthesis. Application of tracer kinetics to this model permits the determination of the rate and turnover of brain
ether phospholipids *in vivo*. The primary importance of this work is that the application of tracer kinetic analysis to brain ether phospholipid biosynthesis *de novo* allows assessment of the biological importance of these molecules within the central nervous system. Also, development of an animal model to study ether phospholipid biosynthesis *in vivo* provides a means to efficiently test drugs and therapeutic strategies that are targeted to specific pathways involved in ether phospholipid metabolism.

Using this model, the time-dependent transfer of labeled [1,1-\(^{3}\)H]hexadecanol between stable brain ether phospholipid pools is used to calculate the transfer coefficients of tracer between brain ether phospholipid compartments. These transfer coefficients are then used to calculate the biosynthetic rate *de novo*, turnover, and half-lives of brain ether phospholipids *in vivo*. Whole brain analysis showed that at 2.0 hours, 29.1 ± 1.5, 32.6 ± 2.3, and 31.4 ± 1.0 percent of total labeled brain phospholipid was found in the PakOH, EtnGpl and ChoGpl fractions, respectively. The EtnGpl and ChoGpl fractions contained only trace amounts of radioactivity in the diacyl subclass, indicating that the oxidation of [1,1-\(^{3}\)H]hexadecanol to palmitic acid resulted in the complete loss of the tritium atoms.

The rates of synthesis and turnover *in vivo* of brain ether phospholipids containing hexadecanol were calculated using tracer kinetics. The rates for labeled PakCho, PakEtn, and PlsEtn were 33.6, 63.3, and 1,157.4 nmol x gram\(^{-1}\) x hour\(^{-1}\), respectively. However, depending on the size of the active PlsEtn pool, the calculated rates for PlsCho were between 2,861.6 and 533.6 nmol x gram\(^{-1}\) x hour\(^{-1}\). Although these rates are considerably different, they are both fast enough to suggest the involvement of PlsCho in dynamic processes such as signal transduction. The half-life of total brain PlsEtn is 12.3 hours and
the half-life of PlsCho is between 0.1 and 1.1 hours, respectively. The rates of formation of both PakEtn and PakCho are in good agreement with published values using microsomal preparations (Radominska-Pyrek and Horrocks, 1972, Radominska-Pyrek et al., 1977). The rates of synthesis and turnover of PlsEtn and PlsCho are an order of magnitude greater than those values observed using microsomal assays (Ansell and Metcalfe, 1971, Radominska-Pyrek and Horrocks, 1972, Radominska-Pyrek et al., 1977) and efflux calculations (Horrocks, 1972, Miller et al., 1977, Porcellati et al., 1983). On the other hand, the flux and turnover of PlsEtn is supported from steady-state experiments in which intracerebral injection of [1-^3H]glycerol was performed (Masuzawa et al., 1984).

Autoradiographic experiments show that [1,1-^3H]hexadecanol was localized in distinct brain regions in the adult awake rat. These studies, combined with the fractionation studies, show that approximately 97 percent of the biosynthesis \textit{de novo} of the brain ether phospholipids was accounted for by synthesis in gray matter regions. Very little incorporation was found in the white matter or myelin membrane fractions. This suggests that in the adult rat, myelin membrane maintenance is principally supported through recycling mechanisms and not ether phospholipid biosynthesis \textit{de novo}. Alternatively, movement of PlsEtn and PlsCho into myelin, from the gray matter, is a slow process that was not observed under my experimental conditions. These studies support the hypothesis that there is heterogeneity in the biosynthesis \textit{de novo} of ether phospholipids in the brain.

In summary, I have measured the \textit{de novo} rate and turnover of brain ether phospholipid synthesis in the awake adult rat brain \textit{in vivo}. Utilization of an intravenous
infusion of [1,1-^3^H]hexadecanol and tracer kinetics allowed specific assessment of all brain ethanolamine and choline ether phospholipids, including the complete analysis of plasmalogen formation throughout the duration of the experiment. The rates of plasmalogen synthesis and turnover reported here suggest that plasmalogens may have a greater function within the central nervous system than previously thought. Furthermore, the rapid turnover of PlsEtn and PlsCho suggests that these lipids are actively involved in dynamic neurological functions, perhaps receptor-mediated signal transduction. Application of this model to in vivo ether phospholipid biosynthesis offers an opportunity to more fully characterize the role of these lipids in brain function, and provides a means to evaluate their participation in signal transduction, neuroplasticity, and brain injury, as well as other diseases.
BIBLIOGRAPHY


sn-glycerol ethanolaminephosphotransferase from microsomal fraction of rat brain. 


