MOLECULAR SPECIES OF PHOSPHATIDYLCHOLINE AND PHOSPHATIDYLETHANOLAMINE IN DEVELOPING RAT BRAIN: QUANTITATIVE ANALYSIS AND RATES OF BIOSYNTHESIS

by

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I hereby recommend that this dissertation prepared under my direction by Claude Gerald Crawford

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be accepted as fulfilling the dissertation requirement of the degree of Doctor of Philosophy

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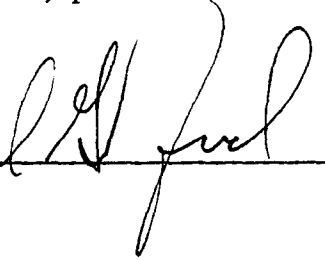
SIGNED: 

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ABSTRACT

The fatty acid composition of diacyl phosphatidylcholine and phosphatidylethanolamine from the brains of rats 3, 6, 9, 12, 15, 18, and 21 days old was determined. In phosphatidylcholine the relative amount of stearic and oleic acid increased from 25% to 33% while the relative amounts of myristic, palmitic, and palmitoleic decreased from 65% to 50% during this time period. The same pattern was seen in phosphatidylethanolamine with stearic and oleic increasing from 38% to 49% and the shorter chain acids decreasing from 17% to 13%. In both lipids the relative amounts of polyunsaturated fatty acids remained fairly constant accounting for about 10% of the total fatty acids in phosphatidylcholine and approximately 38% in phosphatidylethanolamine.

Techniques were devised to convert phosphatidylcholine and phosphatidylethanolamine to diacyl glyceride acetates without loss or degradation of polyunsaturated fatty acids, and separate the molecular species of diglyceride acetates by argentation chromatography on a single plate.

The molecular species composition of phosphatidylcholine and phosphatidylethanolamine was determined in developing brains of rats 3, 6, and 9 days old. The relative amounts of the molecular species remained fairly constant during this time period with phosphatidylcholine containing 35% saturated, 40% monoenoic, 6% dienoic, 11% tetraenoic, 2% pentaenoic, and 5% hexaenoic, with dipalmitoyl phosphatidylcholine accounting for approximately 25% of the total molecular species.

Phosphatidylethanolamine contained 1% saturated, 8% monoenoic, 3% dienoic, 40% tetraenoic, 9% pentaenoic, and 37% hexaenoic species.

The relative specific activities of the molecular species were determined after injection of 1,3, 2-[^{3}H]glycerol, and U-[^{14}C]glucose into the brains of 6-day old rats. The relative specific activities of phosphatidylcholine varied from 1 for the monoenoic species to 3 for the hexaenoic species at 2 hours, and from 1 for the saturated species to 1.4 for the hexaenoic species after 24 hours. Phosphatidylethanolamine showed much greater differences at 2 hours with the relative specific activity of the tetraenoic species being 1 and the saturated species 15. After 24 hours the relative specific activity of the tetraenoic species was still the lowest at 1 but the highest was the dienoic species which was only 2. The three substrates gave identical results in the relative specific activity measurements.

This is the first report of the fatty acid and molecular species composition of diacyl phosphatidylethanolamine from developing rat brain and the changes in that composition during the three weeks following birth. The in vivo incorporation of labeled substrates show that the newly synthesized molecular species of phosphatidylcholine and phosphatidylethanolamine are nearly identical to the total molecular species of these lipids. This indicates that the enzymes of phospholipid biosynthesis are specific in the acylation of fatty acids to glycerophosphate and that the resulting diacyl glycerols are either in separate pools or the CDP transferases are specific for certain species within a common pool.

INTRODUCTION

With the introduction of gas liquid chromatography (GLC) by James and Martin (1952) and its refinement by Lovelock (1958), the detection and quantitation of fatty acids became routine. Hydrolysis of phospholipids with phospholipases in conjunction with fatty acid analysis led to the knowledge that the fatty acids of phospholipids were not distributed randomly between the 1 and 2 position of the glycerol backbone of the molecule.[1] It was also found that a specific phospholipid obtained from different tissues or different phospholipids from the same tissue had different fatty acid compositions (Hanahan, 1960). These studies showed that in general polyunsaturated fatty acids are found predominantly at the 2 position of phospholipids while the 1 position is occupied by a saturated or monoenoic fatty acid (Hanahan, Brockerhoff, and Barron, 1960). These observations led to the concept of molecular species of lipid classes.

The most stringent definition of molecular species is one which defines the fatty acids found at each position of the lipid and in the case of phosphatidylcholine and phosphatidylethanolamine the molecular species are defined by the fatty acid at the 1 and the 2 position of the glycerol backbone. For example, one molecular species would be the species having the fatty acid 16:0 at the 1 position and 20:4 at the

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1. The position numbering is derived from Lα glycerophosphate and is shown in Appendix A. In fatty acids the first number refers to the number of carbon atoms and the second to the number of double bonds found in the fatty acid.

2 position while a molecule having 18:0 at the 1 position and 20:4 at the 2 position would be another molecular species. In general, it is a term used to define subfractions of a lipid class which contain the same number of double bonds or the same number of fatty acid carbons.

The determination of molecular species of phospholipids has been advanced with the development of argentation chromatography. This technique provides separation of compounds by the degree of unsaturation and in the case of phospholipids by the number of double bonds in the fatty acyl groups. Argentation chromatography has been combined with other analytical techniques to determine the molecular species of phospholipids from a number of sources.

Renkonen (1966a) converted phosphatidylcholine into diglyceride acetate and utilized argentation chromatography, GLC of the subfractionated diglyceride acetates, and fatty acid analysis of lipase hydrolysates to determine the complete molecular species composition of phosphatidylcholine from ox brain. Using the techniques developed by Renkonen, the molecular species compositions have been determined for phosphatidylcholine isolated from egg yolk (Kuksis and Marai, 1967), rat heart, kidney, and plasma (Kuksis et al., 1969), and rat liver (Holub and Kuksis, 1971a). Arvidson (1965, 1968a) separated intact rat liver phosphatidylcholine and phosphatidylethanolamine by argentation chromatography and further fractionated the subspecies by reversed phase thin layer chromatography (Arvidson, 1967). These detailed studies of the molecular species of the lipid classes phosphatidylcholine and phosphatidylethanolamine, and the development of these

techniques has enabled a more detailed assessment of the biosynthesis of molecular species.

Through the pioneering work of Kennedy (1953) and Kornberg and Pricer (1953) the pathways for de novo synthesis of phosphatidylcholine and phosphatidylethanolamine have been elucidated and are shown in Figure 1 (Bishop, 1971). Once these lipids are synthesized they can undergo further reactions, which are shown in Figure 2 (Eichberg, Hauser, and Karnovsky, 1969; Rossiter and Strickland, 1969).

The studies which determined the pathways for biosynthesis of phosphatidylcholine and phosphatidylethanolamine did not reveal how the fatty acid asymmetry is introduced into the lipids or how the difference in fatty acid composition between different phospholipids arises, leaving the following questions to be answered. Are the enzymes involved in de novo synthesis specific in the placement of saturated fatty acids at the 1 position and unsaturated fatty acids at the 2 position? Are the differences in the fatty acid composition between phosphatidylcholine and phosphatidylethanolamine introduced by the specificity of enzymes converting phosphatidic acid to the respective phosphoglycerides? Does the de novo synthesis of phosphatidylcholine and phosphatidylethanolamine merely provide a random population of molecules which are then tailored into molecular species by modification reactions? Alternatively, are the molecular species of phosphatidylcholine and phosphatidylethanolamine synthesized by the de novo reactions with a fatty acid composition which is nearly identical to the cells' requirements, needing only minor modification by the subsequent reactions?

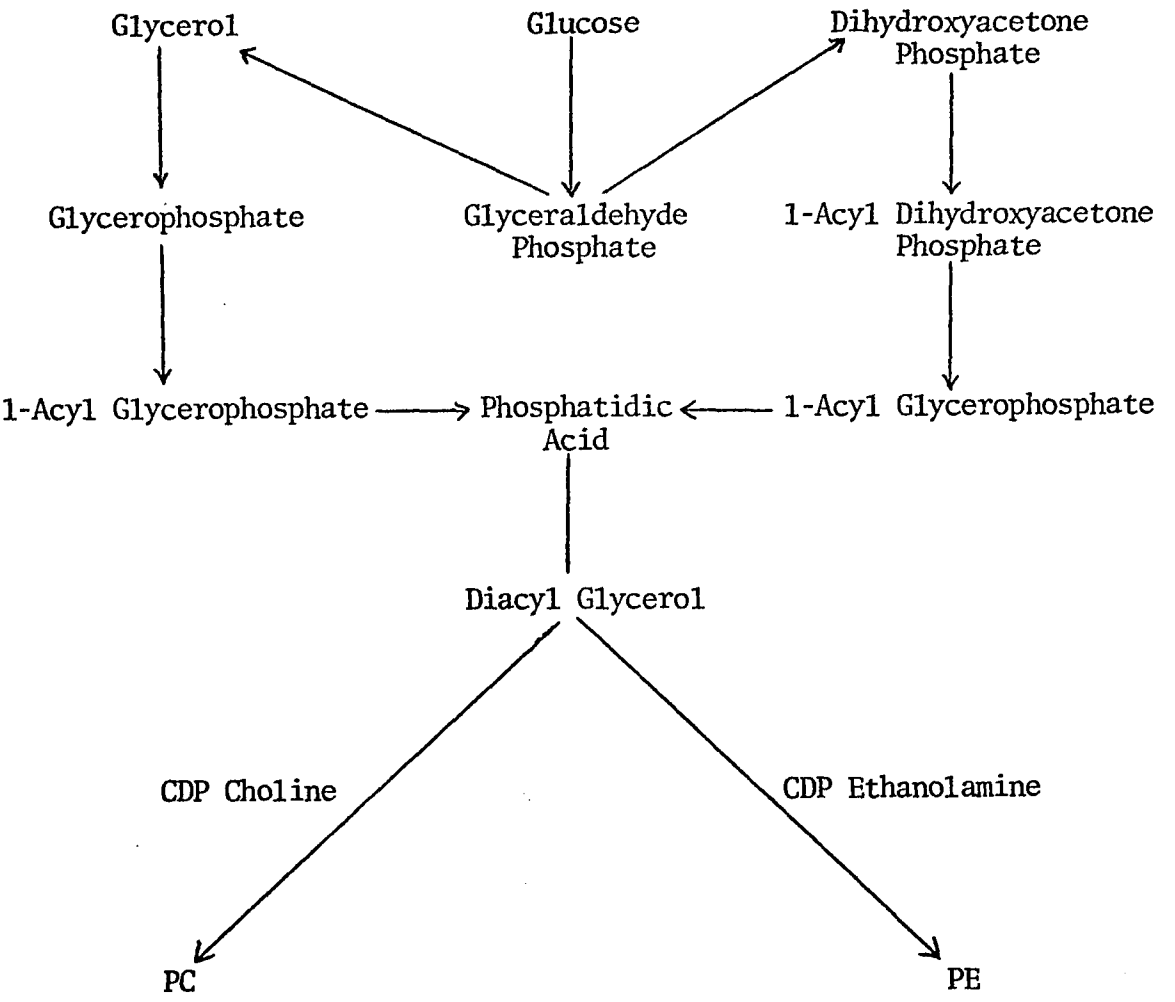
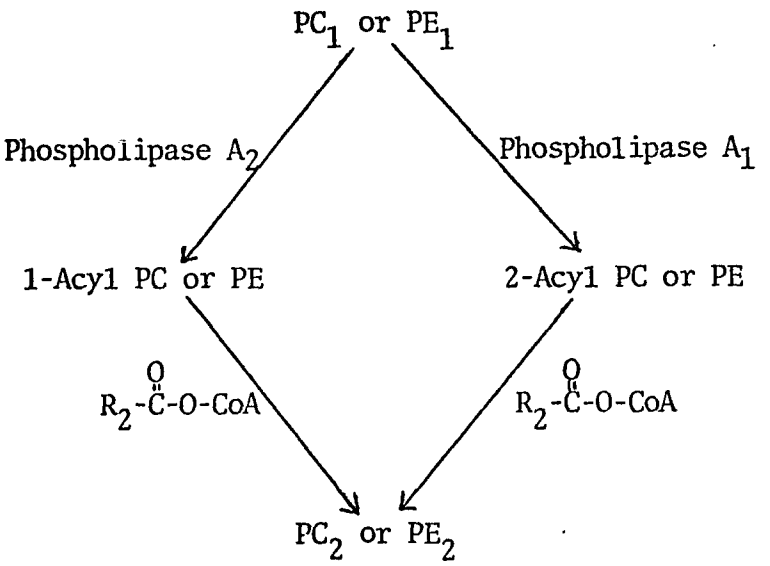


Figure 1. Pathways for the de novo synthesis of phosphatidylcholine and phosphatidylethanolamine.

Structures and abbreviations are shown in Appendix A.

A)



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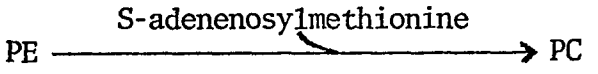

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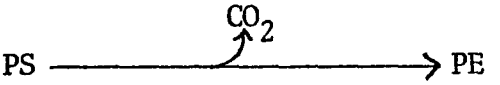


D) Base Exchange

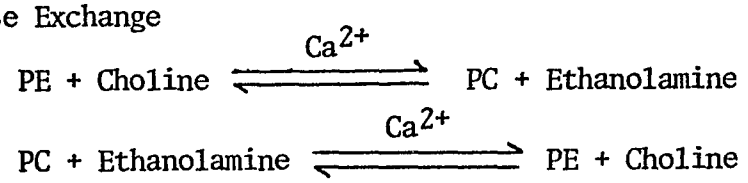


Figure 2. Modification reactions undergone by intact phospholipids. Structures and abbreviations are shown in Appendix A.

With the development of the techniques to separate lipids into molecular species it became possible to study the extent to which the reactions shown in Figures 1 and 2 are responsible for the observed fatty acid asymmetry and fatty acid specificity of phospholipids.

The biosynthesis of phosphatidic acid by the acylation of glycerophosphate has been studied both in vitro and in vivo with somewhat conflicting results as to the specificity of the enzymes involved. Using liver microsomal preparations, Lands and Hart (1964) studied the incorporation of stearate and linoleate into phosphatidic acid and found random placement of the fatty acids at the 1 and the 2 positions. They concluded that the enzymes involved in the formation of phosphatidic acid did not have the specificity required to produce the fatty acid patterns found in phospholipids. They suggested that the fatty acid patterns observed in tissue phospholipids could arise from the redistribution of fatty acids after the nitrogenous base had been attached to the molecule.

Using microsomal preparations or liver slices as the enzyme source Hill, Husbands, and Lands (1968) confirmed the results of Lands and Hart (1964) that the microsomal system showed no specificity in the placement of fatty acids in the synthesis of phosphatidic acid. On the other hand, when labeled glycerol was incubated with liver slices, the fatty acids of the newly synthesized phosphatidic acid, phosphatidylcholine, and phosphatidylethanolamine were not randomly placed but were incorporated in an asymmetric manner indicating that in intact tissue the acyl transferase enzymes have the specificity in fatty acid

placement to account for the asymmetry found in phosphatidylcholine and phosphatidylethanolamine.

In contrast to the results of Lands and coworkers, Possmayer et al. (1969) found that the phosphatidic acid formed in the presence of acyl-CoA and microsomal or mitochondrial preparations showed specificity in the placement of saturated and unsaturated fatty acids. These findings were confirmed and extended by Lamb and Fallon (1970) who reported the isolation of monoacyl glycerophosphate. They found that when palmitoyl-CoA was added to the incubation mixture 1-acyl glycerophosphate was the predominant product while 2-acyl glycerophosphate was the major product when oleoyl-CoA was used as the substrate.

Although Okuyama, Ebil, and Lands (1971) and Daae (1972) were unable to isolate the 1-acyl intermediate reported by Lamb and Fallon (1970), Lands and Hart (1965) had suggested that two acylating enzymes were involved in the synthesis of phosphatidic acid. When they added mercaptan binding reagents to microsomal incubation mixtures, the formation of phosphatidic acid from glycerophosphate was strongly inhibited but the conversion of 1-acyl glycerophosphate to phosphatidic acid was relatively unaffected.

The two enzyme activities inferred from the work of Lands and Hart (1965) have been isolated from microsomal preparations by Yamashita and Numa (1972), Yamashita, Hosaka, and Numa (1972) and from mitochondria by Monroy, Kelker, and Pullman (1973) and Monroy, Rola, and Pullman (1972). In both the microsomal and mitochondrial preparations the fractions catalyzing the acylation of glycerophosphate to form 1-acyl glycerophosphate preferentially utilize saturated fatty acyl-CoA while

unsaturated fatty acyl-CoA is much less effective as a substrate (Yamashita and Numa, 1972; Monroy et al., 1973). On the other hand, the acyl-CoA:1-acyl glycerophosphate acyltransferase from mitochondria preferentially utilize unsaturated fatty acyl-CoA (Monroy et al., 1972), while the microsomal preparation used either saturated or unsaturated fatty acyl-CoA equally as substrates (Yamashita et al., 1972).

Another biosynthetic route to phosphatidic acid was discovered by Hajra and Agranoff (1968a, 1968b), whereby dihydroxyacetone phosphate formed by the Embden-Meyerhof degradation of glucose is acylated to form 1-acyl dihydroxyacetone phosphate. This is then reduced to 1-acyl glycerophosphate and further acylated to form phosphatidic acid. Glyceraldehyde phosphate can also enter the pathway but it must first be converted to dihydroxyacetone phosphate (Puelo, Rao, and Reiser, 1970). Using brain microsomes as the enzyme source Hajra (1973) found that the rate of acylation of dihydroxyacetone phosphate by oleoyl-CoA was half the rate of acylation by palmitoyl or stearoyl-CoA. Okuyama and Lands (1970) had previously shown that the fatty acids of phosphatidic acid formed through the dihydroxyacetone phosphate pathway by liver slices were incorporated in an asymmetric manner with the saturated acids at the 1 position and unsaturated acids at the 2 position but that no unique class of molecular species was synthesized by this route.

Thus, the phosphatidic acid and resulting diacyl glycerol formed in tissue slices contain fatty acids which are not esterified in a random manner. On the other hand, the experiments conducted using microsomal preparations or partially purified enzymes include reports that the glycerophosphate acylating enzymes show specificity with regard to

placement of fatty acids and other reports that the enzymes are non-specific.

The in vitro studies concerning the specificity of the CDP choline and CDP ethanolamine transferases again conflict with the in vivo observations. De Kryuff, Van Golde, and Van Deenen (1970) have shown that liver microsomal preparations show no specificity toward different unsaturated species of diacyl glycerol when incubated with CDP choline or CDP ethanolamine. Weiss, Smith, and Kennedy (1958) showed that the reaction of diacyl glycerol with CDP choline to form phosphatidylcholine and CMP was reversible, and Kanoh and Ohno (1973a, 1973b) studied the products formed upon addition of CMP to microsomal preparations. Their results showed that the fatty acid composition of the diacyl glycerol formed by the back reaction was identical to the composition of the phosphatidylcholine in the microsomes before the addition of CMP indicating that the choline transferase was non-specific with regard to acyl composition.

The in vivo studies of Arvidson (1968b) and Holub and Kuksis (1971a) on the de novo synthesis of phosphatidylcholine and phosphatidylethanolamine show that newly synthesized phospholipids in the liver have fatty acid compositions which differ from each other and an asymmetric distribution of fatty acids. This indicates that there is either a compartmentation of different species of diacyl glycerol which the different CDP aminotransferases utilize or that the aminotransferases show specificity toward certain molecular species within a common pool. Although the various molecular species are not synthesized at rates which would produce the molecular species distribution found in

the liver, there is fatty acid asymmetry and molecular species specificity found in newly synthesized material, indicating that de novo synthesis is not a process which produces random species whose fatty acids must be completely altered to provide the molecular species utilized by the liver.

The reactions shown in Figure 2 could modify the molecular species of phosphatidylcholine and phosphatidylethanolamine. Phospholipase A_1 (Gatt, 1968; Webster and Cooper, 1968), and phospholipase A_2 (Webster and Cooper, 1968) activity has been found in brain and these enzymes can react with phosphatidylcholine and phosphatidylethanolamine to form monoacyl compounds which can then be reacylated with other fatty acids to form different molecular species. In the case of these reactions, the in vitro results conform to those expected from the observed asymmetry. Hill and Lands (1968) and Van Den Bosch et al. (1968) have shown that liver microsomal preparations preferentially acylate 1-acyl phosphatidates with unsaturated fatty acids while saturated fatty acids are more reactive with the 2-acyl phosphatidates.

The study by Hill, Husbands, and Lands (1968) using liver slices indicated that the polyunsaturated fatty acids were introduced into the lipids by reacylation of a deacylated phospholipid and the in vivo studies by Holub and Kuksis (1971b) have shown that the majority of arachidonic acid-containing molecular species of liver phosphatidylcholine and phosphatidylethanolamine are formed by these reactions.

The formation of phosphatidylcholine from phosphatidylethanolamine by successive methylations has been studied in liver by determining the incorporation of radioactivity into the molecular species of

phosphatidylcholine after injection of labeled ethanolamine (Arvidson, 1968a; Tinoco et al., 1970; Salerno and Beeler, 1973) or after injection of labeled Me-methionine (Tinoco et al., 1968; Salerno and Beeler, 1973). The results of these workers indicated that the arachidonic acid (20:4) and docosahexaenoic (22:6) species of phosphatidylethanolamine were preferentially methylated to form phosphatidylcholine. Bremer and Greenberg (1961) and Bjornstad and Bremer (1966) studying this reaction in vivo, found it to be negligible in adult rat brain but Chida and Arakawa (1971) found by in vitro measurements that about 5% of the phosphatidylcholine in brain could be formed by this pathway in rats up to 3 weeks old.

The conversion of phosphatidylserine to phosphatidylethanolamine, which involves the decarboxylation of serine, has been studied in brain by Mc Murray (1964) and Abdel-Latif and Abood (1966) and in liver by Dennis and Kennedy (1972) and Van Golde et al. (1974) but the specificity of the reaction with regard to molecular species has not been determined.

A calcium-stimulated, non-energy dependent exchange of free choline and ethanolamine has been reported to occur in brain tissue by Lunt and Lapetina (1970) and the solubilization of this enzymatic activity has been reported by Saito and Kanfer (1973). This reaction could involve the exchange of choline with the ethanolamine group of phosphatidylethanolamine (or vice-versa) thus modifying the molecular species of these phospholipids but to date no report on the acyl specificity of this activity has been published.

Though the in vitro experiments utilizing membrane bound enzymes and substrates which have limited solubility in aqueous solutions have proved ambiguous, it appears that in vivo the liver fatty acid composition and molecular species of phosphatidylcholine and phosphatidylethanolamine are predominantly defined by the specificity of de novo reactions. Modification reactions are present which could further define the molecular species produced by that organ but the specificities of those reactions are ill-defined.

STATEMENT OF THE PROBLEM

Preliminary investigations in this laboratory showed a significant difference between the fatty acid composition of phosphatidylcholine and phosphatidylethanolamine isolated from developing rat brain. These differences were much more striking than the differences found in the fatty acid composition of these two lipid classes from liver. Thus, it was of interest to determine the fatty acid composition of diacyl phosphatidylcholine and diacyl phosphatidylethanolamine during the period of rapid brain development from 3 to 21 days.

The developing rat brain also offers unique advantages in studying the de novo synthesis of these two phospholipids. First, there is a rapid increase in the total mass of phosphatidylcholine and phosphatidylethanolamine and experiments utilizing labeled substrates would provide accurate measurement of de novo incorporation of those substrates into lipids. The rapid increase in the mass of the lipids would also minimize the effect of turnover on those measurements. Second, the phospholipids produced by the developing rat brain are deposited in the structures of the brain whereas the liver produces lipids not only for membranes within the liver but also releases lipids into the plasma and bile. The transient nature of the exported phospholipids probably does not contribute significantly to the static phospholipid composition, but a rapid rate of synthesis of these lipids would significantly influence conclusions about the mechanism of biosynthesis of structural components of the liver. Third, from the available data the predominant

pathways for molecular species formation is through de novo synthesis and the deacylation-reacylation reactions with only minor contributions from the other modification reactions found in liver. For these reasons the molecular species composition of phosphatidylcholine and phosphatidylethanolamine and the relative specific activities of the molecular species of these lipids after in vivo incorporation of labeled substrates were determined.

EXPERIMENTAL METHODS

Materials

Methanol used for fatty acid transmethylation was redistilled and stored over molecular sieve 4-A, and spectral grade carbon disulfied was used as the solvent for gas liquid chromatography. Other solvents were reagent grade and used without purification and all solvents were mixed in proportion by volume. 2-[^{3}H]Glycerol, [^{3}H] and [^{14}C]acetic anhydride, and Aquasol liquid scintillation fluid were purchased from New England Nuclear (Boston, Mass.). 1,3-[^{3}H]Glycerol and U-[^{14}C]glucose were purchased from Amersham/Searle (Arlington Heights, Ill.). Labeled compounds were obtained from the supplier at greater than 98% purity and were used without further purification. Fatty acid standards were obtained from The Hormel Institute (Austin, Minn.). Silicic Acid CC-4 and CC-7 were purchased from Mallinkrodt (St. Louis, Mo.) and Silica Gel G from E. M. Laboratories (Elmsford, N. Y.). Whatman DE-23 was obtained from Reeve Angel (Clifton, N. J.) and B. cereus phospholipase C from Calbiochem (La Jolla, Ca.). Diethylene glycol succinate (DEGS) and SE-30 were purchased from Alltech Associates (Arlington Heights, Ill.).

Sprague-Dawley rats from a colony maintained at The University of Arizona College of Medicine, Division of Animal Resources, were used throughout this study. The mothers were maintained on Formulab Chow 5008, Ralston Purina (St. Louis, Mo.), and the pups were sacrificed immediately after being removed from the mother except in the 24 hour

glycerol incorporation study. In that case, the pups were returned to the mother for 24 hours after the isotope was injected. Brains were removed immediately upon decapitation of unanesthetized animals and frozen on Dry Ice. Extraction of brain lipids was begun within 30 min of removal and all lipids were stored in chloroform at -18° under nitrogen.

Analytical Methods

Phosphorus was determined by the method of Bartlett (1959) after digestion of the sample with 70% perchloric acid. Lipids were detected on thin layer chromatographic (TLC) plates by spraying with either 0.02% 2,7, dichlorofluorescein (DCF) in methanol or 0.1% 8 anilino-1-naphthalenesulfonic acid (ANS) in H_2O (Gitler, 1972) and for plates impregnated with silver nitrate, both sprays were used. After spraying, the lipids were visualized under 380 nm UV light. The purity of phosphatidylcholine and phosphatidylethanolamine was routinely checked by TLC on silica gel G plates developed in chloroform-methanol-water (95:35:4).

Radioactivity was determined by drying a sample in a scintillation vial under a stream of nitrogen then adding 10 ml Aquasol and counting in a Beckman LS 230 or LS 250 liquid scintillation spectrometer. At the settings used for these experiments the LS 230 had a counting efficiency of 39% for [^{3}H] and 75% for [^{14}C]. There was 8.2% spillover of the [^{14}C] counts into the [^{3}H] window and 0.75% spillover of the [^{3}H] counts into the [^{14}C] window. The LS 250 counted [^{3}H] with an efficiency of 49% and [^{14}C] at 74%. The spillover from [^{3}H] to

[^{14}C] was 1.4% and from [^{14}C] to [^{3}H] 13.8. Spillover corrections were made on all samples containing both [^{3}H] and [^{14}C].

Gas liquid chromatography (GLC) was done on a Hewlett Packard 102 equipped with a Hewlett Packard 3370A integrator. For fatty acid determinations the GLC was operated isothermally at 180° using 0.6 x 100 cm columns packed with 15% DEGS on 100-200 mesh Gas Crom P. Helium was used as the carrier gas and the flow rate was maintained at 100 ml/min with the flash heater maintained at 190° and the hydrogen flame detector at 210°. Fatty acids were identified by comparing retention times to the retention times of known standards.

GLC of diacyl glyceride acetates was performed following the procedure of Kuksis and Ludwig (1966). The GLC was operated with a temperature gradient of 2°/min from 275° to 315° using 1/4" x 2' columns packed with 2.25% SE-30 on 60-80 mesh Chromabsorb W AW-PMCS. The carrier gas flow rate was maintained at 75 ml/min with the flash heater at 310° and the hydrogen flame detector at 350°. Carbon number was determined by comparing retention times with synthetic dipalmitoyl and distearoyl glycerol acetates prepared in this laboratory by K. Yabusaki (Department of Biochemistry, College of Medicine, The University of Arizona). Carbon number refers to the number of carbon atoms of the acyl groups excluding those of the acetyl group.

Lipid Extraction

Extraction of lipids was carried out by the method of Wells and Dittmer (1965), omitting the acidified chloroform-methanol extraction, and non-lipid material was removed by partitioning with a salt solution

after Folch, Lees, and Sloane-Stanley (1957). Each gram of brain was ground in 10 ml chloroform-methanol (1:1) for 3 min at 23,000 rpm in a Virtis Model 23 homogenizer. The homogenate stood 45 min in the dark then was filtered through a sintered glass funnel. The residue was reextracted with the same volume of chloroform-methanol (3:1) and after standing in the dark 45 min was filtered. The filtrates were combined and 0.2 vol 0.9% NaCl was added. After thorough mixing the sample was transferred to a separatory funnel and chilled 1 hr at -18°. The separatory funnel was centrifuged at 1000 rpm for 10 min in a centrifuge maintained at 4°. The lower phase was transferred to a round bottom flask and dried in vacuo with additions of benzene to remove H_2O. The lipid was then taken up in 25 ml chloroform/10 g brain material and stored at -18°.

DE-23 Chromatography

Phosphatidylcholine and sphingomyelin were separated from phosphatidylethanolamine and ethanolamine plasmalogen following the procedure of Rouser et al. (1969). DE-23 was prepared by washing in 3 vol 1 N HCl, rinsing to neutrality with H_2O followed by washing with 3 vol 0.1 N KOH then again rinsing to neutrality with H_2O. These steps were repeated 3 times then the DE-23 was converted to the acetate form by washing with 3 vol glacial acetic acid. The excess acid was removed by washing with methanol then dried overnight at 55° under vacuum. The dried DE-23 was stored in a tightly sealed bottle.

Columns were prepared by suspending 10 g of DE-23 in glacial acetic acid and stirring for several hours to break up aggregates. The

slurry was poured in 5 to 7 portions into a 2.5 x 30 cm column containing a glass wool plug. After each portion was poured the acetic acid was forced down to the bed head under nitrogen pressure then the bed pressed down firmly with a 2.4 cm glass rod. After the last portion was packed the column was washed with 300 ml methanol, 150 ml chloroform-methanol (1:1), and 300 ml chloroform.

A brain extract containing 100 to 200 μmoles of lipid phosphorus was applied to the column in chloroform then the lipids were eluted with 200 ml chloroform, 200 ml chloroform-methanol (12:1), 200 ml chloroform-methanol (9:1), and finally with 300 ml chloroform-methanol (4:3). A flow rate of 3 ml/min was maintained under nitrogen pressure and 15-ml fractions were collected. The elution profile of the column with this solvent system was determined by applying 100 μl samples from every other tube to a silica gel G plate then developing the plate in chloroform-methanol-water (95:35:4). Thereafter, the tubes containing phosphatidylcholine and phosphatidylethanolamine were located by applying 50 μl from every other tube to 3 x 3 cm squares scribed on a silica gel G plate and visualized after spraying with ANS. The tubes containing phosphatidylcholine and sphingomyelin were combined and dried in vacuo as were the fractions containing phosphatidylethanolamine and ethanolamine plasmalogen. Phosphatidylcholine and sphingomyelin eluted primarily in the chloroform-methanol (12:1) fraction and phosphatidylethanolamine and ethanolamine plasmalogen in the chloroform-methanol (4:3) fractions. The samples were taken up in 10 ml chloroform and stored at -18°.

Phosphatidylcholine Purification

After heat activating 10 g silicic acid CC-4 for 1 hr at 110°, it was cooled and slurried in 80 ml chloroform. The slurry was poured into a 1.5 x 30 cm column containing a glass wool plug and after washing the column with 100 ml chloroform, up to 100 μmoles of lipid phosphorus of the phosphatidylcholine plus sphingomyelin fraction from the DE-23 column was applied in chloroform. The lipids were eluted from the column with 200 ml chloroform-methanol (6:1), 400 ml chloroform-methanol (3:1) and 200 ml chloroform-methanol (1:1). A flow rate of 2 ml/min was maintained and 10 ml fractions were collected. Purified diacyl phosphatidylcholine was eluted primarily with chloroform-methanol (3:1) with traces appearing in the chloroform-methanol (1:1) fractions. The fractions containing purified phosphatidylcholine were combined, dried in vacuo, and then taken up in 10 ml chloroform and stored at -18°.

Ethanolamine Plasmalogen Hydrolysis

Ethanolamine plasmalogen was converted to 2-acyl phosphatidyl-ethanolamine by a modification of the method of Wells and Dittmer (1966). Approximately 100 μmoles of lipid phosphorus of the phosphatidylethanol-amine plus ethanolamine plasmalogen fractions from the DE-23 column were dried under nitrogen then incubated 30 min at 37° with 8.0 ml chloroform-methanol-0.025 M $HgCl_2$ (5:11:4). After cooling to room temperature, 2.0 ml isobutanol, 6.8 ml chloroform and 1.0 ml H_2O were added. This solution was mixed vigorously then centrifuged at 1800 x g. The upper phase was removed and the lower phase washed with 4.0 ml methanol-water (1:1). This was again centrifuged and the lower phase

was taken to dryness in vacuo. The phosphatidylethanolamine plus 2-acyl phosphatidylethanolamine was taken up in 10 ml chloroform and stored at -18°.

Phosphatidylethanolamine Purification

Ten grams of silicic acid CC-7 were slurried in 80 ml chloroform and poured into a 1.5 x 30 cm column containing a glass wool plug. After washing the column with 100 ml chloroform, up to 100 μmoles lipid phosphorus of the phosphatidylethanolamine plus 2-acyl phosphatidylethanolamine hydrolysate were applied to the column in chloroform and eluted with 100 ml chloroform, 300 ml chloroform-methanol (8:1), and 200 ml chloroform-methanol (4:1). A flow rate of 2 ml/min was maintained and 10 ml fractions were collected. The purified diacyl phosphatidylethanolamine was eluted in the chloroform-methanol (8:1) fractions, which were combined and taken to dryness in vacuo. The phosphatidylethanolamine was then taken up in 10 ml chloroform and stored at -18°.

Fatty Acid Methylation

The fatty acids of the purified phosphatidylcholine, phosphatidylethanolamine, diacyl glycerol, and diacyl glycerol acetates were converted to methyl esters using a modification of the method of Morgan, Hanahan, and Ekholm (1963). A solution of 5 to 10 μmoles of the purified lipid was taken to dryness under nitrogen and the residue incubated 15 min at room temperature with 1.0 ml of freshly prepared 0.5 M KOH in methanol. The reaction mixture was neutralized with 0.4 ml glacial acetic acid then one ml H_2O was added and the mixture was transferred to a separatory funnel containing 4.0 ml H_2O and 20 ml petroleum ether.

The petroleum ether phase was washed 3 times with 4.0 ml H_2O then dried 30 min over Na_2SO_4. The petroleum ether phase was taken to dryness in vacuo and the methyl esters of the fatty acids were taken up in 1.0 to 5.0 µl CS_2 and 0.2 to 0.5 µl injected into the GLC.

Hydrolysis of Phospholipids With Phospholipase C

Ten to 20 µmoles of purified phosphatidylcholine or phosphatidylethanolamine were dried under nitrogen then taken up in 2.0 ml diethyl ether and 2.0 ml 0.02 M N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES) pH 7.3. To this was added 0.1 ml *B. cereus* phospholipase C solution and 4.0 µl of 1 M $CaCl_2$. The enzyme solution contained approximately 80 IU activity in 1.0 ml 0.02 M HEPES pH 7.3 and 1 mM $ZnCl_2$. The reaction mixture was incubated at 37° for 1 to 2 hours, with frequent mixing and was then extracted twice with 8.0 ml petroleum ether.

Acetylation of Diacyl Glycerols

The crude diacyl glycerols from the ether phase of the phospholipase C hydrolysis were taken to dryness under nitrogen and the residue dissolved in 0.25 ml anhydrous pyridine and stirred 1 to 2 hours with 0.1 ml acetic anhydride. When [^{3}H] or [^{14}C] labeled acetic anhydride was used the acetic anhydride was diluted to 0.2 to 5 µCi/µmole with anhydrous benzene and unlabeled acetic anhydride. A 10- to 100-fold excess of acetic anhydride was reacted with the crude diacyl glycerol mixture. To remove the excess acetic anhydride the reaction mixture was chilled in ice, then 1.0 ml methanol was added and the mixture was allowed to warm to room temperature. After addition of 1.0 ml H_2O the

reaction mixture was extracted twice with 8.0 ml petroleum ether. The combined petroleum ether phases were taken to dryness under nitrogen, taken up in 100 µl chloroform and applied to a 20 x 20 x 0.05 cm silica gel G plate and developed in petroleum ether-diethyl ether (4:1). The plate was sprayed with 0.1% ANS and the lipids visualized with UV light. The band of 1,2 diacyl glycerol acetate was scraped into a screw cap tube and the lipids extracted 3 times with 10 ml chloroform-methanol-NH_4OH-water (50:39:1:10) (Arvidson, 1968a). Ten ml H_2O was added to the extract and the upper phase containing the ANS was discarded. The lower phase was taken to dryness in vacuo and the purified 1,2 diacyl glycerol acetates were taken up in chloroform and stored at -18°.

Argentation Chromatography

Separation of the diacyl glycerol acetates into molecular species was carried out using a modification of the procedures described by Renkonen (1964, 1966b). Fifty grams of silica gel G were slurried into 100 ml H_2O containing 5.0 g $AgNO_3$. The mixture was degassed and spread on 20 x 20 x 0.025 cm plates. After air drying 30 min in the dark the plates were dried for 1.5 hrs at 120°. The cooled plates were stored for up to two weeks in the dark over desiccant. Five to 10 µmoles of diacyl glycerol acetate were applied in 50 µl chloroform about 1 cm from the bottom of the plate then the plate was developed 10 cm in chloroform-methanol (97:3). The plate was dried under vacuum 15 min then developed the full length in benzene-chloroform (9:1). After drying under vacuum the plate was sprayed with 0.02% DCF then 0.1% ANS and the bands visualized with UV light. The bands were scraped into screw

cap tubes and the lipids eluted 3 times with 10 ml chloroform-methanol-NH_4OH-water (50:39:1:10). Ten ml H_2O was added and the upper phase containing the ANS, DCF, and silver ions was discarded and the lower phase taken to dryness in vacuo. The samples were taken up in chloroform and stored at -18°.

Injection of Labeled Substrates

Ten µl of sterile H_2O containing 1 µCi/µl of labeled substrate (1 µCi/µmole) was injected over a 15-second period into unanesthetized 6-day old rats just beneath the meninges at the suture of the frontal and parietal bones. At the times indicated the animals were sacrificed and the brain lipids immediately extracted.

Determination of Molecular Species Composition

Diacyl glycerols, derived from purified phosphatidylcholine and phosphatidylethanolamine were acetylated with [^{3}H] or [^{14}C] labeled acetic anhydride and separated into molecular species by argentation chromatography. The amount of radioactivity in each band was determined and the relative mole percentage of each molecular species was calculated from the total radioactivity recovered.

Preliminary experiments indicated that there were only small amounts of saturated, monoenoic, and dienoic species in phosphatidylethanolamine while there was a much larger amount of those species in phosphatidylcholine. The opposite was indicated for the polyenoic species with phosphatidylcholine containing much smaller amounts of tetraenoic, pentaenoic, and hexaenoic species than phosphatidylethanolamine. Therefore, to aid in the visualization of minor bands unlabeled diacyl

glycerol acetates derived from one phospholipid were added to the labeled diacyl glycerol acetates derived from the other phospholipid before separating the molecular species by argentation chromatography.

Incorporation of Labeled Substrates Into Fatty Acids

An aliquot of the purified phosphatidylcholine or phosphatidylethanolamine extracted from animals which had been injected with labeled substrate was dried under nitrogen and the amount of radioactivity in the intact molecule determined. Another aliquot was treated with methanolic KOH (see Fatty Acid Methylation) and the amount of radioactivity in the fatty acid methyl esters was determined. The radioactivity of the latter was divided by the radioactivity of the original sample to determine the relative degree of incorporation of the substrate into the fatty acids.

Hydrogenation of Diacyl Glycerol Acetates

Purified diglyceride acetates were fractionated by argentation chromatography and the eluted lipids were transferred in hexane to a hydrogenation flask. A small amount of platinum oxide was added and the samples were hydrogenated overnight under 50 psi hydrogen with continuous shaking. Upon completion of hydrogenation the sample was removed from the flask and taken to dryness under a stream of nitrogen. The samples were taken up in CS_2 and analyzed by gas liquid chromatography.

Relative Specific Activity Determination

Phosphatidylcholine and phosphatidylethanolamine were purified from animals which had been injected with 1,3 or 2-[^{3}H] labeled glycerol, hydrolyzed with phospholipase C, and acetylated with [^{14}C] labeled acetic anhydride. Unlabeled material was added as described above and the purified 1,2 diglyceride acetates were separated by argentation chromatography. The [^{14}C] counts from each band were used to determine the relative mole percentage of each molecular species and the relative specific activities for each molecular species were determined by dividing the [^{3}H] counts from each band by the [^{14}C] counts in that band.

To determine the specific activity of the glycerol portion of the molecule lipids obtained from rats injected with [^{14}C] labeled glucose required removal of the fatty acids, which had also incorporated label. Therefore, the purified phosphatidylcholine and phosphatidylethanolamine were hydrolyzed with phospholipase C and acetylated with [^{3}H] labeled acetic anhydride. The purified diacyl glycerol acetates were separated by argentation chromatography and the bands from one plate were counted directly to determine the [^{3}H] counts and thus the relative mole percentage of each band.

The diglyceride acetates were eluted from the bands of a second plate and treated with methanolic KOH (see Fatty Acid Methylation). The aqueous phase, which contained the glycerol portion of the diglyceride acetate, was counted and the [^{14}C] counts from the glycerol moiety were then divided by the [^{3}H] counts found in the corresponding band from the

first plate to determine the relative specific activity of the glycerol portion of the molecule.

RESULTS

Methodology

Purification and Recovery of Lipids

To insure the validity of the results of this study it was necessary to quantitatively, or nearly quantitatively, recover purified diacyl phosphatidylcholine and phosphatidylethanolamine and determine that the diacyl glycerol acetates derived from these lipids were identical in fatty acid composition to the original material.

Figure 3 outlines the scheme used to obtain purified diacyl phosphatidylcholine and phosphatidylethanolamine. To determine the recovery of these lipids the amount of diacyl phosphatidylcholine and phosphatidylethanolamine was determined in the total brain extract by the method of Wells and Dittmer (1966). This involves mild alkaline hydrolysis of the total brain lipids, chromatography of the water soluble phosphate diesters and quantitation of the glycerol phosphorylcholine and glycerol phosphorylethanolamine. Diacyl phosphatidylcholine and phosphatidylethanolamine from the total brain extract were then purified and the amount recovered was compared to the amount present in the total brain extract.

Table 1 shows the results of four separate determinations on the amount of diacyl phosphatidylcholine and diacyl phosphatidylethanolamine present in 100 μmoles lipid phosphorus of a total brain extract from 6 and 21-day old rats. Two aliquots containing 100 μmoles lipid

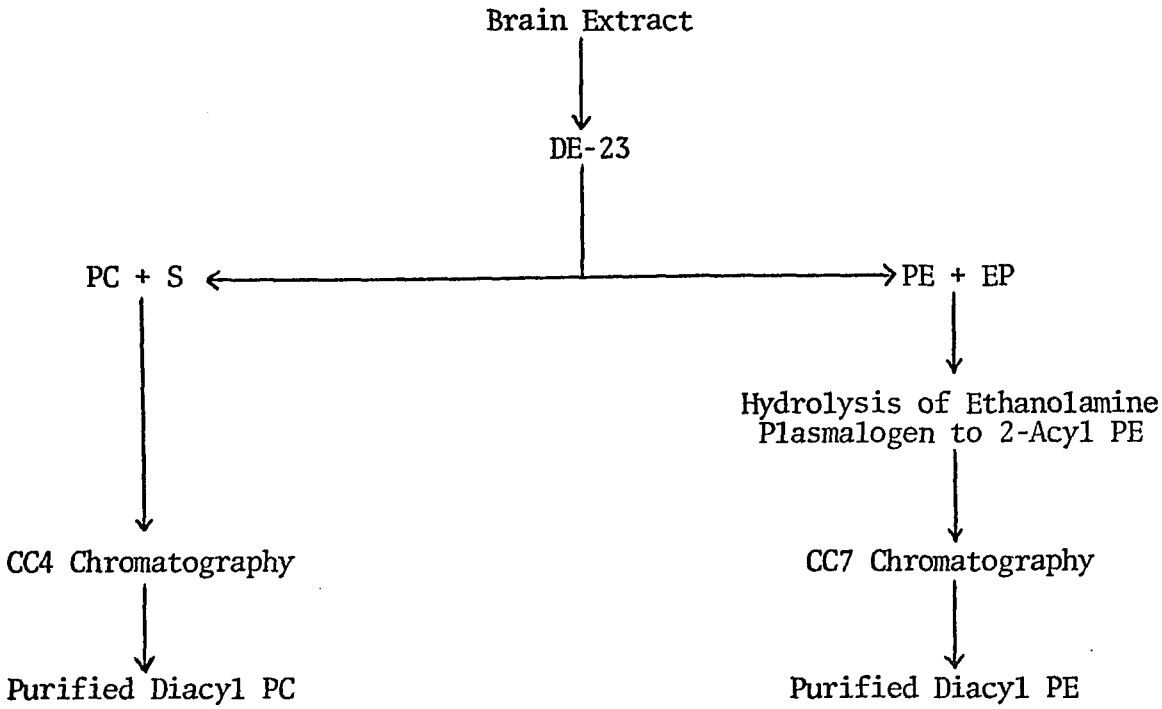


Figure 3. Purification scheme used to obtain diacyl phosphatidylcholine and diacyl phosphatidylethanolamine.

Structures and abbreviations are shown in Appendix A.

Table 1. Recovery of diacyl phosphatidylcholine (PC) and diacyl phosphatidylethanolamine (PE) after purification from total rat brain extract.

Rats	PC	PE
6 Day		
μmoles/100 μmoles lipid phosphorus	50.7	18.0
μmoles purified	44.5	16.7
% recovered	87.8%	92.8%
21 Day		
μmoles/100 μmoles lipid phosphorus	39.0	16.8
μmoles purified	35.2	14.7
% recovered	90.2%	87.5%

phosphorus from each age were then purified as outlined in Figure 3, and the amounts of diacyl phosphatidylcholine and phosphatidylethanolamine were determined. Approximately 90% of the phosphatidylcholine and over 85% of the phosphatidylethanolamine were recovered using this purification scheme. Vinyl ether titration (Wells and Dittmer, 1966) indicated less than 1% plasmalogen contamination and over 98% of the phosphorus was recovered in the aqueous phase after mild alkaline hydrolysis. These results are consistent with the assumption that the lipids recovered are diacyl phospholipids.

Conversion of Phospholipids to Diglyceride Acetates

For separation of the molecular species by argentation chromatography and isotope incorporation studies phosphatidylcholine and phosphatidylethanolamine were converted to their diglyceride acetate derivatives. Argentation chromatography separates compounds on the basis of degree of unsaturation by interaction of the unsaturated portions of the molecule with the silver ions impregnated in the gel. Phospholipids can thus be separated on the basis of the number of double bonds in the acyl groups. Although Arvidson (1968a) has obtained good resolution of rat liver phospholipids, more complete resolution can be obtained by converting the phospholipid to a less polar form. The phospholipids can be converted to diacyl glycerol by the action of phospholipase C and separated in that form by argentation chromatography (Van Golde and Van Deenen, 1966) but the acetylated derivative provides several advantages.

First, by acetylating the diacyl glycerols, the acyl group at the 2 position cannot migrate to the 3 position. Since the R_f of the 1,3 diacyl species on argentation TLC is different than the R_f of the 1,2 diacyl species (Kuksis and Marai, 1967) the presence of 1,3 diacyl glyceride could give ambiguous results. Second, small amounts of material can be accurately quantitated when the diglycerol acetates are labeled with radioactive acetate and third, the diglyceride acetates can be analyzed directly by GLC. Figure 4 shows the scheme for converting the phospholipids to diglycerol acetates and purification of the 1,2 diglyceride acetates. To insure the validity of the results it was necessary to show that the fatty acid composition of the diacyl glycerol acetates was identical to the parent compound.

Initially crude phospholipase C was obtained from the growth media of cultures of B. cereus as outlined by Otnaess et al. (1972) and was shown to hydrolyze phosphatidylcholine and phosphatidylethanolamine. When commercial preparations became available complete hydrolysis of 10 to 15 μmoles of phosphatidylcholine was accomplished by reacting the phospholipids with 10 IU of the phospholipase for 60 min at 37°. When the reaction mixture was chromatographed on silica gel G using diethyl ether-petroleum ether (4:1) as the developing solvent a small amount of 1,3 diacyl glycerol but no unreacted phosphatidylcholine was found. However, in the case of phosphatidylethanolamine a trace of phosphatidylethanolamine was found at the origin and prolonged hydrolysis did not seem to reduce that small amount of unhydrolyzed material but did increase somewhat the amount of 1,3 diacyl glycerol.

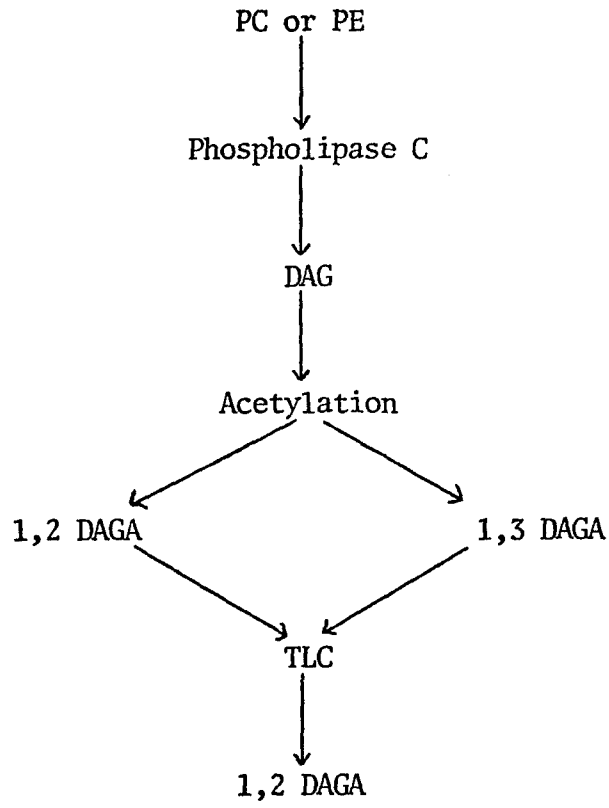


Figure 4. Scheme used to obtain purified 1,2 diacyl glyceride acetates from phosphatidylcholine or phosphatidylethanolamine.

Structures and abbreviations are shown in Appendix A.

Originally the diacyl glycerols were acetylated at 80° by the method of Kuksis et al. (1969) but it was found that up to 25% of the 22:4, 22:5, and 22:6 fatty acids were lost when the fatty acid composition of the 1,2 diglyceride acetate was compared to the fatty acid composition of the original phospholipid. Table 2 shows the fatty acid composition of the original sample of phosphatidylethanolamine, the fatty acid composition of the purified 1,2 diacyl glycerol after hydrolysis and the fatty acid composition of the purified 1,2 and 1,3 diglyceride acetates after acetylation at 80°. Thus, there was no preferential loss during hydrolysis, but the loss occurred during the acetylation step due to the formation of 1,3 diglyceride acetate.

At higher temperatures there was considerable migration of polyunsaturated fatty acids from the 2 to the 3 position but the migration was negligible when the reaction was carried out at room temperature. Table 3 shows the fatty acid composition of the major fatty acids of phosphatidylcholine and phosphatidylethanolamine from 6-day old rats and the fatty acid composition of the 1,2 diacyl glycerol acetates obtained by acetylating the diacyl glycerols at room temperature as outlined in Experimental Methods. Thus, even though there may have been some loss of material during hydrolysis and acetylation the recovered material was identical in fatty acid composition to the original lipid.

Argentation Chromatography

The diglyceride acetates were further analyzed as shown in Figure 5. To facilitate the determination of relative mole percentage and relative specific activity of each species it was desirable to be

Table 2. Fatty acid composition in mole % of phosphatidylethanolamine (PE), 1,2 diacyl glycerol (DAG) after hydrolysis with phospholipase C, and 1,2, and 1,3, diacyl glyceride acetates (DAGA) after acetylation at 80°.

Structures and abbreviations are shown in Appendix A.

Fatty Acid	PC	1,2 DAG	1,2 DAGA	1,3 DAGA
16:0	12.4	12.3	13.1	11.4
16:1	1.2	1.2	1.4	4.1
18:0	26.0	26.3	27.9	3.9
18:1	8.8	8.9	9.8	4.7
18:2	1.1	1.1	1.3	1.5
20:4	20.1	20.0	22.8	12.5
22:4	4.8	4.8	3.8	14.0
22:5	2.5	2.4	1.8	3.6
22:6	19.5	19.3	14.2	29.5

Table 3. Fatty acid composition in mole % of phosphatidylcholine (PC) and phosphatidylethanolamine (PE) and the resulting 1,2 diacyl glyceride acetates (DAGA) after acetylation at room temperature.

Structures and abbreviations are shown in Appendix A.

Fatty Acid	PC	1,2 DAGA	PE	1,2 DAGA
14:0	4.2	3.9	1.4	0.9
16:0	51.1	51.6	14.5	14.5
16:1	7.1	7.0	1.8	1.6
18:0	5.2	5.4	24.7	25.0
18:1	18.1	18.6	9.5	9.9
18:2	1.9	1.8	1.1	1.0
20:4	6.3	6.3	19.0	19.9
22:4	0.9	0.8	4.7	4.7
22:5	-	-	3.0	2.9
22:6	2.8	2.7	18.1	18.2

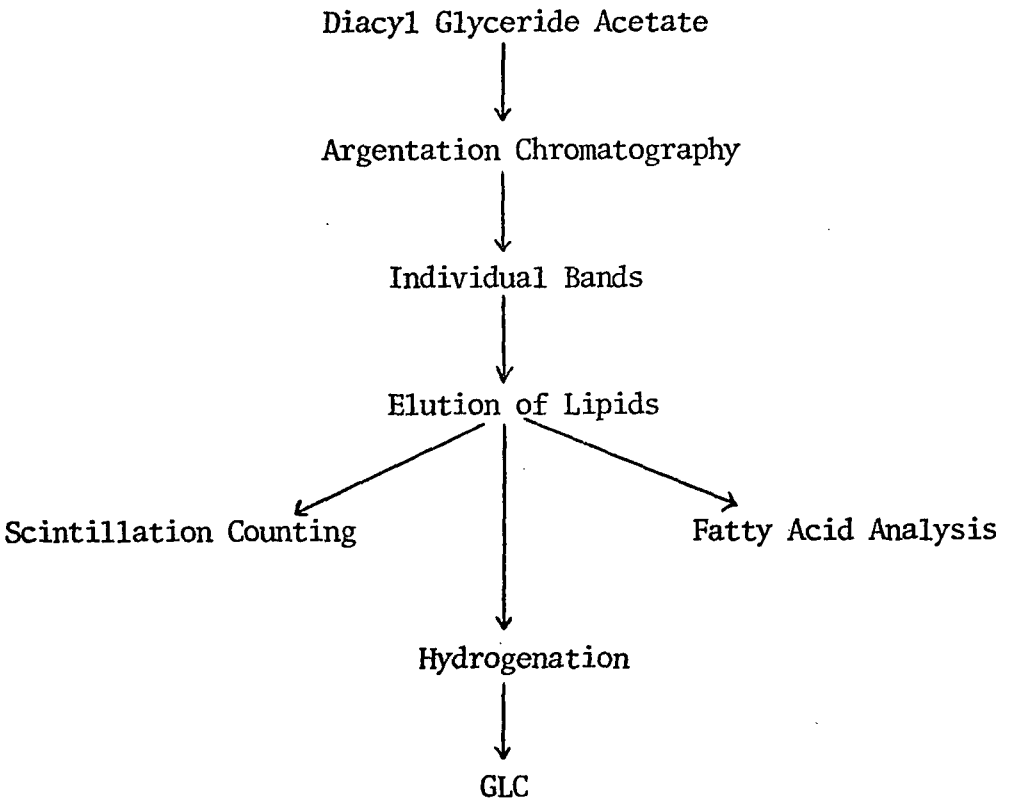


Figure 5. Scheme used to analyze diacyl glyceride acetates derived from phosphatidylcholine and phosphatidylethanolamine.

Structures and abbreviations are shown in Appendix A.

able to separate the diglyceride acetates into their subfractions on a single plate. The published procedures of Kuksis and Marai (1967) or Renkonen (1966a) for the separation of diglyceride acetates by argentation chromatography provided unsatisfactory resolution and/or were not reproducible. Conditions were therefore determined which provided reproducible resolution of the diglyceride acetates derived from rat brain phosphatidylcholine and phosphatidylethanolamine.

Increasing the quantity of silver impregnated in the gel affects the resolution of the species by increasing the interaction between the double bonds and the silver ions thereby retarding migration. Kuksis and Marai (1967) used plates impregnated with 20% silver nitrate for the resolution of diglyceride acetates derived from egg yolk phosphatidylcholine, but this concentration of silver proved to be too high for resolution of the relatively large amounts of polyunsaturated species found in brain lipids. Plates were impregnated with 5, 7.5 and 10% silver and it was found that good resolution of the polyunsaturated species could be obtained on plates containing 5% silver while the higher concentrations of silver markedly reduced their resolution.

Another factor contributing to the interaction of the unsaturated bonds with silver ions is the polarity of the developing solvent. The more polar solvents reduce interaction between the double bonds while less polar solvents permit stronger interactions. Renkonen (1971) has described a solvent system of benzene-chloroform (9:1) which separates diglyceride acetates into saturated, monoenoic, dienoic, and polyenoic species while a more polar solvent, chloroform-methanol (97:3), separates the polyenoic species into trienes, tetraenes,

pentaenes, and hexaenes. By developing the plates impregnated with 5% silver in chloroform-methanol (97:3) to varying heights, allowing the solvent to dry then developing the full height in benzene-chloroform (9:1), it was found that excellent separation of all species was achieved by developing the plate one-half the distance to the top in chloroform-methanol (97:3), removing that solvent from the plate under vacuum, then developing the full height in benzene-chloroform (9:1). It was also noted that the solvents could not be used for more than 4 plates/100 ml of developing solvent. If more than 4 plates were developed the resolution of the bands was very poor and not reproducible.

There was considerable difficulty in obtaining reproducible separation until it was noted that conditions for drying the plates were critical. It was found that uniform drying at constant temperature was necessary and that drying 1.5 hrs at 120° in a clean forced air oven and storage in a desiccated chamber gave excellent resolution and reproducible results.

Arvidson (1968a) has shown that the solvent system and technique used to elute lipids from the gel did so quantitatively and in this study the recovery of radioactivity applied to silver plates was greater than 85%. Therefore, the techniques used in this study provide a nearly quantitative recovery of diacyl phosphatidylcholine and phosphatidylethanolamine from rat brain and the enzymic and chemical modifications of these lipids do not alter their fatty acid composition.

Analytical Data

Fatty Acid Composition of Brain Diacyl Phosphatidylcholine and Diacyl Phosphatidylethanolamine From Rats During Maturation

Tables 4 and 5 show the fatty acid composition of rat brain phosphatidylcholine and phosphatidylethanolamine during development from 3 to 21 days. The values shown in the tables are the average of at least four separate determinations and the standard deviations were less than 1% for the major fatty acids and less than 0.2% for the minor constituents. In Figure 6, the data from Tables 4 and 5 for the major fatty acids is presented graphically. Although there are no dramatic changes during this time there are some gradual changes in proportions of some of the fatty acids. In phosphatidylcholine the fatty acids containing 14 and 16 carbon atoms account for 64% of the total fatty acids at 3 days but decrease to 52% of the total by the twenty-first day, while stearic and oleic acid increase from 25% on day 3 to 33% on day 21. The relative amounts of the polyunsaturated fatty acids remain fairly constant during this time period.

The fatty acids of phosphatidylethanolamine show the same trend with the 14 and 16 carbon fatty acids accounting for 18% on the third day and only 13% on the twenty-first day, while stearic and oleic acid increase from 38% to 49% during the same time. Unlike phosphatidylcholine, polyunsaturated fatty acids in phosphatidylethanolamine show variation during the first 21 days. Arachidonic acid (20:4) shows a slight increase the first 6 days then remains fairly constant until the fifteenth day and then declines. The relative amount of 22:4 remains

Table 4. Fatty acid composition in mole % of diacyl phosphatidylcholine from developing rat brain at 3, 6, 9, 12, 15, 18, and 21 days after birth.

	Days						
Fatty Acid	3	6	9	12	15	18	21
12:0	0.3	0.1	tr	tr	tr	tr	tr
14:0	4.2	4.0	3.9	3.2	2.6	1.6	1.7
14:1	0.4	0.4	0.3	0.5	0.3	0.4	.4
16:0	50.5	51.3	53.8	51.7	51.0	50.2	46.7
16:1	9.0	7.7	6.5	5.2	4.8	2.9	3.1
18:0	5.1	5.2	6.0	6.5	7.2	9.5	10.1
18:1	19.8	18.0	18.4	18.7	20.7	22.7	23.0
18:2	1.5	1.9	1.9	2.3	1.9	1.7	2.0
18:3ω6	0.2	0.8	0.2	0.4	0.1	0.2	0.5
18:3ω3	0.4	0.8	0.4	0.8	0.4	0.8	1.8
20:3	0.3	0.7	0.4	0.6	0.3	0.6	0.9
20:4	5.6	6.1	6.0	7.0	7.4	6.1	7.0
22:4*	0.4	0.8	0.4	0.6	0.7	0.6	0.8
22:5	tr	tr	tr	tr	tr	tr	tr
22:6	2.1	2.0	1.7	2.3	2.4	2.3	2.6

* Tentative identification. Values are the average of at least 4 separate determinations.

Table 5. Fatty acid composition in mole % of diacyl phosphatidyl-ethanolamine from developing rat brain at 3, 6, 9, 12, 15, 18, and 21 days after birth.

	Days						
Fatty Acid	3	6	9	12	15	18	21
12:0	0.4	0.5	tr	tr	tr	tr	tr
14:0	0.9	1.2	0.9	1.0	1.0	1.1	1.0
14:1	tr	tr	tr	tr	tr	tr	tr
16:0	14.8	14.5	14.5	13.9	12.8	10.6	11.2
16:1	1.8	1.8	1.6	0.9	0.8	0.7	0.5
18:0	27.1	28.5	29.2	32.5	32.5	31.5	33.9
18:1	10.9	10.6	10.6	11.0	11.5	14.4	15.1
18:2	1.0	1.1	1.3	0.7	1.1	1.4	0.8
18:3ω6	tr	tr	tr	tr	tr	tr	tr
18:3ω3	0.3	0.3	0.4	0.2	0.5	1.7	0.9
20:3	0.5	0.4	0.6	0.4	0.5	1.1	0.4
20:4	18.2	19.5	20.0	19.2	18.6	16.4	15.7
22:4*	4.4	4.1	4.1	4.0	4.2	4.6	4.2
22:5	2.8	1.7	1.4	1.1	1.1	1.1	0.9
22:6	16.8	15.6	15.2	15.0	15.0	15.1	15.0

* Tentative identification. Values are the average of at least 4 separate determinations.

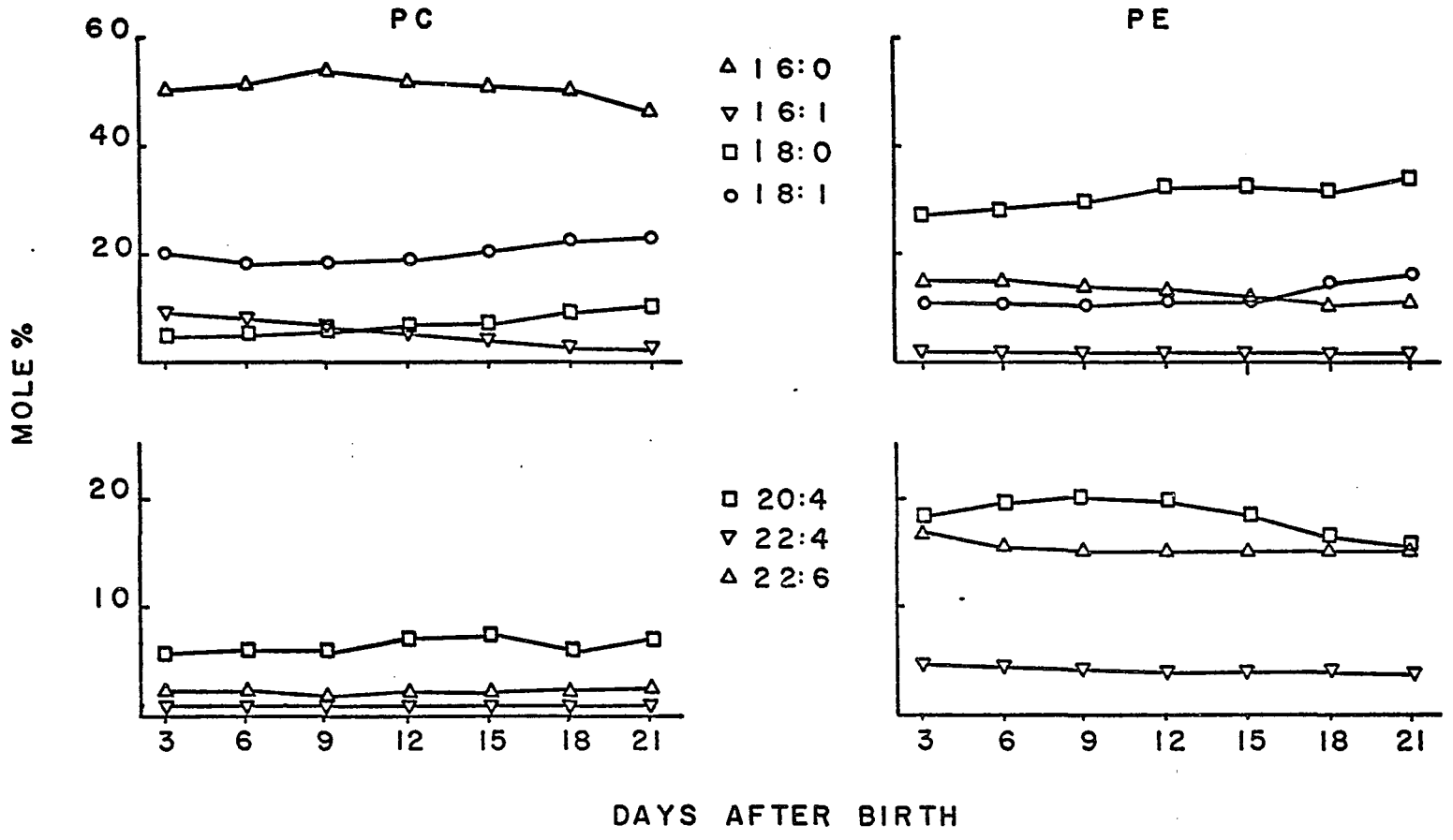


Figure 6. Composition of the major fatty acids of phosphatidylcholine and phosphatidylethanolamine from the brain of rats 3, 6, 9, 12, 15, 18, and 21-days old.

constant throughout the 21 days while 22:5 decreases in the first 12 days. Docosahexanoic (22:6) decreases slightly the first 6 days then remains constant. Small amounts of lauric acid were detected in both phosphatidylcholine and phosphatidylethanolamine on the third and sixth day but could not be detected even in trace amounts after that. In Figure 7, the data from Tables 4 and 5 for the major fatty acids of phosphatidylcholine and phosphatidylethanolamine from 6-day old rats are presented. This shows graphically the marked difference in fatty acid composition between the two lipids.

Molecular Species Determination

The molecular species of phosphatidylcholine and phosphatidylethanolamine and relative molar percentage of each species from 3, 6, and 9-day old animals are shown in Table 6, and the data for 6-day old rats is presented graphically in Figure 8. The band number indicates the number of double bonds predominantly found in the molecular species of that band. There are some variations of the molecular species during this time period but as in the case of the fatty acid composition there are no dramatic changes.

Fatty acids of phospholipids are not distributed randomly and the polyunsaturated species are found predominantly at the 2 position while the less saturated fatty acids are generally located at the 1 position. This is reflected in the molecular species of phosphatidylcholine and phosphatidylethanolamine since the fatty acids arachidonic (20:4) through docosahexanoic (22:6) comprise over 40% of the fatty acids of unfractionated phosphatidylethanolamine, and the polyunsaturated

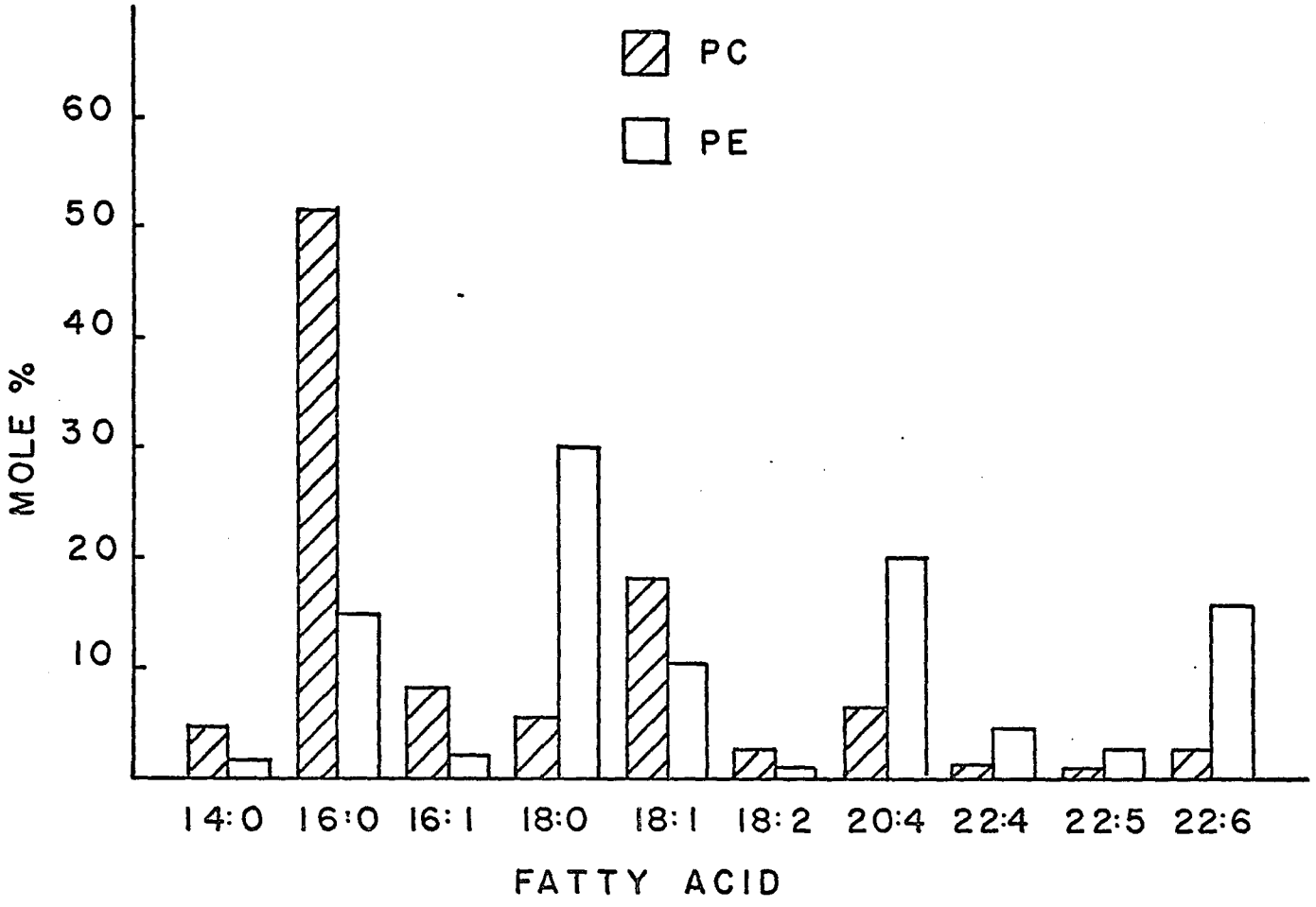


Figure 7. Fatty acid composition of the major fatty acids of phosphatidylcholine and phosphatidyl-ethanolamine from the brain of rats 6-days old.

Table 6. Molecular species found in phosphatidylcholine and phosphatidylethanolamine from developing rat brain at 3, 6, and 9 days after birth.

Band number refers to the number of double bonds predominantly found in that band. Values are the average of at least 4 separate determinations.

	Band	3 Day Mole %	6 Day Mole %	9 Day Mole %
Phosphatidylcholine	0	32.3	36.1	34.1
	1	42.6	40.8	39.9
	2	6.2	5.8	6.1
	4	11.2	10.3	12.8
	5	2.3	2.2	1.6
	6	5.5	4.8	5.4
Phosphatidylethanolamine	0	1.4	1.1	1.0
	1	8.6	8.6	7.2
	2	2.9	3.8	3.9
	4	38.5	40.9	43.4
	5	9.7	8.9	7.4
	6	38.9	36.5	37.1

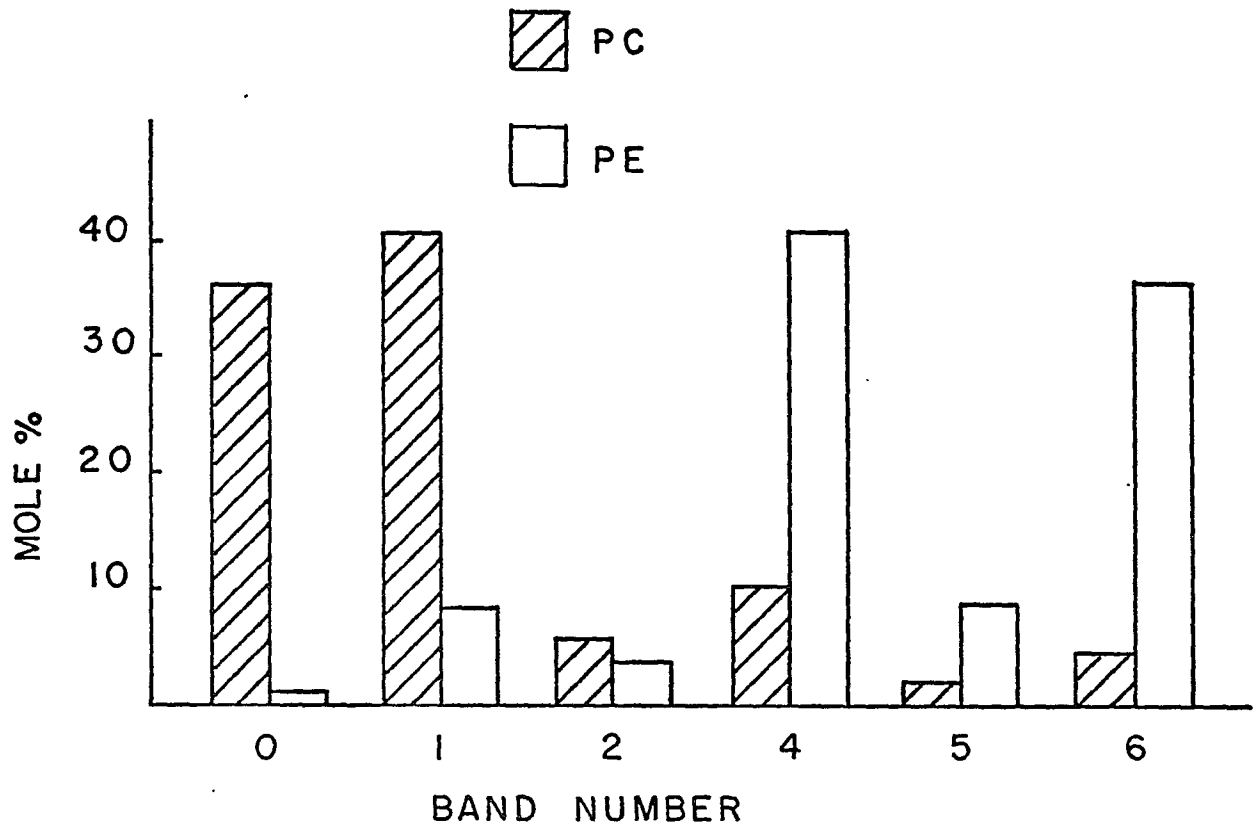


Figure 8. Molecular species composition of phosphatidylcholine and phosphatidylethanolamine from the brain of rats 6-days old.

molecular species account for about 85% of the total molecular species. This indicates that most of the polyunsaturated fatty acids are paired with a less saturated fatty acid in the diacyl molecule.

In the case of phosphatidylcholine the polyunsaturated fatty acids account for approximately 9% of the fatty acids in unfractionated lipid and the polyenoic molecular species account for approximately 19% of the total species. Therefore, most of the polyunsaturated fatty acids are paired with a less saturated acid and the majority of the molecular species of phosphatidylcholine are fully saturated and monoenoic species.

Fatty Acid Composition of the Molecular Species and Recalculated Values

Tables 7, 9 (p. 51), and 11 (p. 53) show the fatty acid composition of the 6 bands of phosphatidylcholine and phosphatidylethanolamine from 3, 6, and 9-day old rat brain and Tables 8, 10, and 12 show the recalculated fatty acid composition of the major fatty acids.

While the relative amount of each molecular species reflects the amount of saturated and unsaturated fatty acids found in the unfractionated lipid, the fatty acid composition of the individual molecular species shows a distribution of fatty acids which is not necessarily similar to that found in the unfractionated phospholipids. For example, the ratio of 16:0 to 18:0 in unfractionated phosphatidylcholine from 6-day old rats is 9.9 yet the ratio of the same fatty acids in the molecular species range from 2.6 to 12.9. The ratio of 16:0 to 18:0 in unfractionated phosphatidylethanolamine of the same age is 0.5 but the ratio of those acids in the molecular species range from 0.3 to 1.5.

Table 7. Fatty acid composition in mole % of the molecular species of phosphatidylcholine and phosphatidylethanolamine from developing rat brain at 3 days after birth.

	Band	Mole %*	14:0	16:0	16:1	18:0	18:1	18:2	20:4	22:4	22:5	22:6
Phosphatidyl-choline	0	32.3	9.5	84.3		6.2						
	1	42.6	2.2	43.1	14.2	4.4	36.1					
	2	6.2	3.5	22.6	18.2	5.3	31.5	18.9				
	4	11.2	2.0	30.2	1.6	12.8	6.9		42.7	3.8		
	5	2.3	5.9	23.5	11.4	7.4	17.9		16.2	4.4	13.3	
	6	5.5	3.2	30.4	4.4	12.1	8.6		4.4			36.8
Phosphatidyl-ethanolamine	0	1.4	21.6	58.7		19.7						
	1	8.6	4.3	29.0	8.5	18.3	39.9					
	2	2.9	6.9	15.9	9.8	18.6	40.1	8.7				
	4	38.5	1.4	8.3		37.7	4.4		41.8	6.4		
	5	9.7	2.3	13.8	2.8	17.6	19.3		16.7	7.1	18.7	1.8
	6	38.9	1.7	19.4	1.7	23.3	7.3		2.6	1.7		42.3

* From Table 6.

Table 8. Recalculated values for the major fatty acids found in (A) phosphatidylcholine and (B) phosphatidylethanolamine from the molecular species found in 3-day old rats.

Values were obtained by multiplying the percentage of the fatty acid found in that band by the mole percentage that band represents.

(A) Phosphatidylcholine

Band	Mole %*	16:0	Recalc	16:1	Recalc	18:0	Recalc	18:1	Recalc	20:4	Recalc
0	32.3	84.3	27.2			6.2	2.0				
1	42.6	43.1	18.3	14.2	6.0	4.4	1.9	36.1	15.4		
2	6.2	22.6	1.4	18.2	1.1	5.3	0.3	31.5	1.9		
4	11.2	30.2	3.4	1.6	0.2	12.8	1.4	6.9	0.8	42.7	4.8
5	2.3	23.5	0.5	11.4	0.3	7.4	0.2	17.9	0.4	16.2	0.4
6	5.5	30.4	1.7	4.4	0.2	12.1	0.7	8.6	0.5	4.4	0.2
			52.5 (50.5)†		7.8 (9.0)†		6.5 (5.1)†		19.0 (19.8)†		5.4 (5.6)†

(B) Phosphatidylethanolamine

Band	Mole %*	16:0	Recalc	18:0	Recalc	18:1	Recalc	20:4	Recalc	22:6	Recalc
0	1.4	58.7	0.8	19.7	0.3						
1	8.6	29.0	2.5	18.3	1.6	39.9	3.4				
2	2.9	15.9	0.5	18.6	0.5	40.1	1.2				
4	38.5	8.3	3.2	33.8	13.0	3.9	1.5	41.8	16.1		
5	9.7	13.8	1.3	17.6	1.7	19.3	1.9	16.7	1.6	1.8	0.2
6	38.9	19.4	7.6	23.3	9.1	7.3	2.8	2.6	1.0	42.3	16.5
			15.9 (14.8)†		26.2 (27.1)†		10.8 (10.9)†		18.7 (18.2)†		16.7 (16.8)†

* From Table 6.
† Values found in the unfractionated material (Tables 4 and 5).

Table 9. Fatty acid composition in mole % of the molecular species of phosphatidylcholine and phosphatidylethanolamine from developing rat brain at 6 days after birth.

	Band	Mole %*	14:0	16:0	16:1	18:0	18:1	18:2	20:4	22:4	22:5	22:6
Phosphatidyl-choline	0	36.1	12.4	81.3		6.3						
	1	40.8	2.8	43.3	13.9	3.7	36.2					
	2	5.8	6.8	25.0	14.3	6.2	28.9	18.8				
	4	10.3	1.8	35.7		13.8	2.3		46.4			
	5	2.2	4.4	20.9	6.6	4.7	22.1		18.3	6.5	16.4	
	6	4.8	3.0	31.1	2.4	8.6	6.3		3.6			45.0
Phosphatidyl-ethanolamine	0	1.1	24.6	45.1		30.2						
	1	8.6	5.3	29.3	9.6	17.2	38.5					
	2	3.8	7.6	13.7	9.5	10.5	45.5	13.2				
	4	40.9	1.3	9.6	1.5	34.1	1.9		37.6	14.0		
	5	8.9	2.6	11.7	2.9	13.7	19.5		19.4	9.6	20.5	
	6	36.5	1.4	17.7	1.9	20.9	6.5		3.7	2.5	42.7	

* From Table 6.

Table 10. Recalculated values for the major fatty acids found in (A) phosphatidylcholine and (B) phosphatidylethanolamine from the molecular species found in 6-day old rats.

Values were obtained by multiplying the percentage of fatty acid found in that band by the mole percentage that band represents.

(A) Phosphatidylcholine

Band	Mole %*	16:0	Recalc	16:1	Recalc	18:0	Recalc	18:1	Recalc	20:4	Recalc
0	36.1	81.3	29.3			6.3	2.3				
1	40.8	43.3	17.7	13.9	5.7	3.7	1.5	36.2	14.8		
2	5.8	25.0	1.4	14.3	0.8	6.2	0.4	28.9	1.7		
4	10.3	35.7	3.7			13.8	1.4	2.3	0.2	46.4	4.8
5	2.2	20.9	0.5	6.6	0.1	4.7	0.1	22.1	0.5	18.3	0.4
6	4.8	31.1	1.5	2.4	0.1	8.6	0.4	6.3	0.3	3.6	0.2
			54.1 (51.3)†		6.7 (7.7)†		6.1 (5.2)†		17.5 (18.0)†		5.4 (6.1)†

(B) Phosphatidylethanolamine

Band	Mole %*	16:0	Recalc	18:0	Recalc	18:1	Recalc	20:4	Recalc	22:6	Recalc
0	1.1	45.1	0.5	30.2	0.3						
1	8.6	29.3	2.5	17.2	1.5	38.5	3.3				
2	3.8	13.7	0.5	10.5	0.4	45.5	1.7				
4	40.9	9.6	3.9	34.1	14.0	1.9	0.8	37.6	15.4		
5	8.9	11.7	1.0	13.7	1.2	19.5	1.7	19.4	1.7		
6	36.5	17.7	6.5	20.9	7.6	6.5	2.4	3.7	1.4	42.7	15.6
			14.9 (14.5)†		25.0 (28.5)†		9.9 (10.6)†		18.5 (19.5)†		15.6 (15.6)†

* From Table 9.
† Values found in unfractionated material (Tables 4 and 5).

Table 11. Fatty acid composition in mole % of the molecular species of phosphatidylcholine and phosphatidylethanolamine from developing rat brain at 9 days after birth.

	Band	Mole %*	14:0	16:0	16:1	18:0	18:1	18:2	20:4	22:4	22:5	22:6
Phosphatidyl-choline	0	34.1	10.2	83.9		5.9						
	1	39.9	2.0	43.5	12.9	3.7	37.9					
	2	6.1	5.3	24.5	13.7	5.1	30.3	21.1				
	4	12.8	2.9	35.3		14.8	4.6		39.4	3.0		
	5	1.6	5.5	19.0	6.0	5.6	24.0		31.8	6.2	2.0	
	6	5.4	4.1	34.8		9.1	10.6		8.0			33.4
Phosphatidyl-ethanolamine	0	1.0	18.7	53.6		27.7						
	1	7.2	4.1	27.2	8.7	20.5	39.5					
	2	3.9	2.8	10.4	7.6	9.4	57.0	12.8				
	4	43.4	0.8	8.8	0.6	39.8	2.5		40.9	6.6		
	5	7.4	1.6	10.5	3.0	15.5	23.6		23.8	9.0	13.0	
	6	37.1	0.9	17.3	1.1	24.1	7.3		3.0	2.3	1.9	42.0

* From Table 6.

Table 12. Recalculated values for the major fatty acids found in (A) phosphatidylcholine and (B) phosphatidylethanolamine from the molecular species found in 9-day old rats.

Values were obtained by multiplying the percentage of fatty acid found in that band by the mole percentage that band represents.

(A) Phosphatidylcholine

Band	Mole %*	16:0	Recalc	16:1	Recalc	18:0	Recalc	18:1	Recalc	20:4	Recalc
0	34.1	83.9	28.6			5.9	2.0				
1	39.9	43.5	17.4	12.9	5.1	3.7	1.5	37.9	15.1		
2	6.1	24.5	1.5	13.7	0.8	5.1	0.3	30.3	1.9		
4	12.8	35.3	4.5			14.8	1.9	4.6	0.6	39.4	5.1
5	1.6	19.0	0.3	6.0	0.1	0.6	0.1	24.0	0.5	31.8	0.5
6	5.4	34.8	1.9			9.1	0.5	10.6	0.6	8.0	0.4
			54.2 (53.8)†		6.1 (6.5)†		6.3 (6.0)†		18.6 (18.4)†		6.0 (6.0)†

(B) Phosphatidylethanolamine

Band	Mole %*	16:0	Recalc	18:0	Recalc	18:1	Recalc	20.4	Recalc	22:6	Recalc
0	1.0	53.6	0.6	27.7	0.3						
1	7.2	27.2	1.9	20.5	1.5	39.5	2.8				
2	3.9	10.4	0.4	9.4	0.4	57.0	2.2				
4	43.4	8.8	3.8	39.8	17.3	2.5	1.1	40.9	17.7		
5	7.4	10.5	0.8	15.5	1.2	23.6	1.8	23.8	1.8		
6	37.1	17.3	6.4	24.1	8.9	7.3	2.7	3.0	1.1	42.0	15.6
			13.9 (14.5)†		29.6 (29.2)†		10.6 (10.6)†		20.6 (20.0)†		15.6 (15.0)†

* From Table 6.

† Values found in unfractionated material (Tables 4 and 5).

The recalculated values of the major fatty acids of phosphatidylcholine and phosphatidylethanolamine are obtained by multiplying the relative amount of fatty acid found in a given molecular species by the relative amount of that molecular species. These values again show differences in distribution of fatty acids between phosphatidylcholine and phosphatidylethanolamine. For example, while 90% of the 16:0 and 70% of the 18:0 of phosphatidylcholine are found in the saturated, monoenoic, and dienoic species only 25% of the 16:0 and less than 10% of the 18:0 of phosphatidylethanolamine are found in these same species.

Mass Distribution in Each Molecular Species

Diglyceride acetates from each molecular species were hydrogenated and analyzed by GLC to determine the mass distribution of each molecular species. If the samples were not hydrogenated prior to analysis there was considerable broadening and overlapping of the peaks making identification difficult while the hydrogenated samples separated as sharp individual peaks. The relative mole percentage of the hydrogenated samples is presented in Table 13.

From Table 9 it can be seen that only 6% of the fatty acid found in the fully saturated molecular species of phosphatidylcholine is stearic acid, 12% is myristic, and 80% is palmitic; therefore, the majority of 32 carbon species must be dipalmitate. By multiplying the amount of 32 carbon species found in the saturated band (72.4%) by the relative amount of that band (36.1%) it can be seen that over 25% of the total molecular species of rat brain phosphatidylcholine is dipalmitoyl phosphatidylcholine. Also of interest is that both

Table 13. Mass distribution by carbon number of the molecular species of phosphatidylcholine and phosphatidylethanolamine from developing rat brain at 6 days after birth.

	Band	Mole %*	% 28	% 30	% 32	% 34	% 36	% 38	% 40	% 42	% 44
Phosphatidyl-choline	0	36.1	1.8	15.5	72.4	10.4					
	1	40.8		2.0	25.1	65.8	7.0				
	2	5.8		3.3	14.9	48.4	33.4				
	4	10.3				3.3	55.1	36.7	4.9		
	5	2.2					19.6	46.1	29.1	5.2	
	6	4.8					5.9	60.6	28.7	4.8	
Phosphatidyl-ethanolamine	0	1.1		16.1	28.7	55.2					
	1	8.6		10.1	10.5	79.4					
	2	3.8		4.5	20.2	70.2	5.0				
	4	40.9				1.4	16.0	72.5	8.6	1.5	
	5	8.9					6.8	58.1	32.0	3.1	
	6	36.5					0.8	37.0	54.8	5.3	2.0

* From Table 6.

phosphatidylcholine and phosphatidylethanolamine have molecular species which contain 42 fatty acid carbons and phosphatidylethanolamine even has a small amount of a species which contains 44 fatty acid carbons.

Incorporation of Labeled Substrates Into Fatty Acids

The amount of label incorporated from glycerol and glucose into the fatty acids is shown in Table 14. Very little glycerol was incorporated into fatty acids even after 24 hours, but the majority of counts in phosphatidylcholine and phosphatidylethanolamine were found in the fatty acids when labeled glucose was used as the substrate in phospholipid synthesis.

Relative Specific Activities of the Molecular Species

The relative specific activities of the molecualr species of brain phosphatidylcholine and phosphatidylethanolamine were determined after 2 hours using 2-[^{3}H]glycerol, 1,3-[^{3}H]glycerol, and U-[^{14}C]-glucose and after 24 hours using 2-[^{3}H]glycerol as the substrates. Tables 15 and 16 show that the specific activities of the individual molecular species are the same when glycerol or glucose are used as the substrate and that by 24 hours the relative specific activity is near one for all molecular species. The specific activities of all molecular species of phosphatidylcholine at 2 hours are similar with only a 3-fold difference between them. In phosphatidylethanolamine the small pool of disaturated species shows the highest specific activity at 2 hours while the species containing arachidonic acid is the lowest.

Table 14. Incorporation of 1,3-[^{3}H]glycerol, 2-[^{3}H]glycerol, and U-[^{14}C]glucose into the fatty acids of phosphatidylcholine and phosphatidylethanolamine from developing rat brain at 6 days after birth.

Substrate/Time	Lipid	CPM/ μmole Lipid	CPM in FA/μmole Lipid	% in FA
2-[^{3}H]Glycerol	PC	7.3×10^{4}	1.6×10^{3}	2.2
2 hours	PE	1.0×10^{5}	2.3×10^{3}	2.3
1,3-[^{3}H]Glycerol	PC	6.7×10^{4}	2.5×10^{3}	3.7
2 hours	PE	9.1×10^{4}	2.4×10^{3}	2.6
2-[^{3}H]Glycerol	PC	3.4×10^{3}	1.4×10^{2}	4.1
24 hours	PE	2.9×10^{3}	1.0×10^{2}	3.4
U-[^{14}C]Glucose	PC	2.9×10^{4}	1.9×10^{4}	65.5
2 hours	PE	2.1×10^{4}	1.1×10^{4}	52.4

Table 15. Relative specific activities of phosphatidylcholine as determined by the incorporation of labeled substrates into the glycerol moiety of the phospholipid in developing rat brain 6 days after birth.

Values are the average of at least 4 separate determinations except for the values for glucose incorporation which are the results of a single determination. Mole % was determined by the incorporation of labeled acetate.

Phosphatidylcholine	Band	Rel SA	Mole %
2-[^{3}H]Glycerol, 2 Hours	0	1.01	35.3
	1	1.00	40.7
	2	1.73	6.1
	4	1.22	11.0
	5	1.55	1.7
	6	3.06	5.2
1,3-[^{3}H]Glycerol, 2 Hours	0	1.01	35.3
	1	1.00	41.0
	2	1.68	6.1
	4	1.20	10.5
	5	1.40	1.7
	6	2.67	5.3
2-[^{3}H]Glycerol, 24 Hours	0	1.00	35.4
	1	1.25	39.0
	2	1.34	6.3
	4	1.40	11.5
	5	1.40	1.5
	6	1.18	6.2
U-[^{14}C]Glucose, 2 Hours	0	1.00	36.6
	1	1.02	40.4
	2	1.77	6.0
	4	1.84	9.8
	5	1.54	2.0
	6	2.22	5.3

Table 16. Relative specific activities of phosphatidylethanolamine as determined by the incorporation of labeled substrates into the glycerol moiety of the phospholipid in developing rat brain 6 days after birth.

Values are the average of at least 4 separate determinations except for the values for glucose incorporation which are the results of a single determination. Mole % was determined by the incorporation of labeled acetate.

Phosphatidylethanolamine	Band	Rel SA	Mole %
2-[^{3}H]Glycerol, 2 Hours	0	15.11	1.3
	1	4.88	7.7
	2	4.92	3.3
	4	1.00	41.3
	5	3.14	8.1
	6	6.39	38.4
1,3-[^{3}H]Glycerol, 2 Hours	0	15.80	1.3
	1	5.33	7.5
	2	4.67	3.5
	4	1.00	41.1
	5	2.82	7.6
	6	6.31	39.0
2-[^{3}H]Glycerol, 24 Hours	0	1.46	1.6
	1	1.59	8.2
	2	2.01	3.7
	4	1.00	40.8
	5	1.40	6.0
	6	1.38	39.7
U-[^{14}C]Glucose, 2 Hours	0	19.50	1.1
	1	4.57	7.6
	2	3.71	3.4
	4	1.00	40.7
	5	2.46	8.8
	6	5.07	38.5

DISCUSSION

Although there are slight differences in the relative amounts of fatty acids of diacyl phosphatidylcholine found in this study when compared to the results of Marshall et al. (1966), Skrbic and Cumings (1970), or Alling and Karlsson (1973), the same pattern is seen during the maturation of the rat brain. There is a decline in the relative amounts of 14:0, 16:0, and 16:1 with a concomitant rise in the relative amounts of 18:0 and 18:1 while the relative amounts of polyunsaturated fatty acids remain fairly constant.

The fatty acid composition of purified rat brain diacyl phosphatidylethanolamine has not been previously reported. Alling and Karlsson (1973) determined the fatty acid composition of a mixture of phosphatidylethanolamine and ethanolamine plasmalogen. On a basis of brain lipid values determined by Wells and Dittmer (1966), the contribution of ethanolamine plasmalogen to that mixture increases from 30% in newborn to 55% at maturity. Since Alling and Karlsson have not purified the diacyl phosphatidylethanolamine from the ethanolamine plasmalogen, the data presented here cannot be directly compared with theirs.

The data presented in this study show that the changes in the relative amounts of saturated and monoenoic fatty acids of diacyl phosphatidylethanolamine follow the same trends as the corresponding fatty acids of phosphatidylcholine but the relative amounts of the

polyunsaturated fatty acids, 20:4 and 22:6 decline slightly from birth to 21 days.

Of interest is the comparison of the fatty acid composition of phosphatidylcholine and phosphatidylethanolamine found in liver to the fatty acid composition of those phospholipids from the brain. The molar percentages of the major fatty acids of 21-day old rat brain and adult rat liver are presented in Table 17. This shows the striking difference between the fatty acid composition of brain phosphatidylcholine and phosphatidylethanolamine and also the difference between the fatty acid composition of the same phospholipids from different tissue. For example, there is twice as much 18:1 as 18:0 in brain phosphatidylcholine but the opposite is true for brain phosphatidylethanolamine and while 18:2 accounts for 12% and 6% respectively of the fatty acids of liver phosphatidylcholine and phosphatidylethanolamine the same lipids from brain contain less than 2% 18:2.

Again there are striking differences between the molecular species of phosphatidylcholine and phosphatidylethanolamine found in liver and brain. The molecular species of adult liver and 6-day old rat brain are given in Table 18, which again emphasizes the differences between the two phospholipids from the same and different tissues. In brain the monoenoic and dienoic species of phosphatidylcholine account for over 85% of the total molecular species but account for only 10% of brain phosphatidylethanolamine or liver phosphatidylcholine.

From the data presented in Tables 4 and 5 on the fatty acid composition and Table 6 on the molecular species of brain phosphatidylcholine and phosphatidylethanolamine, the differences between these two

Table 17. Mole percentage of the major fatty acids of the brain of rats 21 days after birth and adult liver.

Fatty Acid	Brain		Liver*	
	PC%	PE%	PC%	PE%
16:0	47	11	31	23
16:1	3	tr	1	tr
18:0	10	34	22	31
18:1	23	15	7	5
18:2	2	1	12	6
20:4	7	16	21	15
22:6	3	15	5	14

* Taken from Arvidson (1968a) and the average of male and female values.

Table 18. Mole percentage of each molecular species of phospholipids from the brain of rats 6 days after birth and adult rat liver.

Band	Brain		Liver*	
	PC%	PE%	PC%	PE%
0	36	1	1	tr
1	41	9	9	1
2	6	4	28	13
4	10	41	41	51
5	2	9	7	6
6	5	37	11	26

* Taken from Holub and Kuksis (1971a).

lipids are demonstrated. Even with the large differences in the overall fatty acid composition, the fatty acid composition of the molecular species shown in Tables 7, 9, and 11 indicate that there is a preferential pairing of 18:0 with 20:4. The ratio of 16:0 to 18:0 in phosphatidylcholine from 6-day old rats is 10:1 (Table 4) yet the ratio of the same acids in the molecular species containing 4 double bonds is 3:1 (Table 9). For phosphatidylethanolamine from the same age the ratio of 16:0 to 18:0 is 1:2 (Table 5) and increases to 1:3 in the molecular species containing four double bonds (Table 9). This preferential pairing of stearic acid with arachidonic acid has been observed in rat liver by Arvidson (1968b).

In the determination of the fatty acid composition of phosphatidylcholine and phosphatidylethanolamine there were only traces of fatty acids longer than 22 carbon atoms. When the individual species were hydrogenated and analyzed on the GLC as intact diacyl glycerol acetates (Table 13), appreciable amounts of diglyceride acetates were found which contained 42 carbon atoms and even a small amount of a molecular species in phosphatidylethanolamine which contained 44 fatty acyl carbon atoms. Since virtually no fatty acids longer than 22 carbon atoms were found in the unfractionated lipids there must be some species containing both 20 and 22 carbon fatty acids and a species containing two 22 carbon fatty acids.

The high amount of dipalmitoyl phosphatidylcholine is of interest because appreciable quantities of this molecular species have been found in only three tissues. Clements, Brown, and Johnson (1958) found that lung fluid had the property of lowering the surface tension

at an air-water interface and Brown (1964) showed that this property was conveyed by a lipoprotein containing dipalmitoyl phosphatidylcholine. Van Golde, Tomasi, and Van Deenen (1967) and Marai and Kuksis (1969) found dipalmitoyl phosphatidylcholine in erythrocytes and Montfoort, Van Golde, and Van Deenen (1971) found the species in lung and brain. O'Brien and Geison (1971) have isolated phosphatidylcholine from rat brain myelin, nerve endings, mitochondria, and 15,000 x g supernatant fraction and determined the molecular species of each fraction by GLC of the trimethylsilyl derivatives of phosphatidylcholine. The species containing 32 acyl carbon atoms comprise 30% of the molecular species in the nerve endings and supernatant fractions, 20% in the mitochondria, and only 10% in myelin. Although their study provides no information concerning the role of dipalmitoyl phosphatidylcholine in the brain it is interesting to note that the tissues in which appreciable amounts of this species is found are involved in oxygen uptake, oxygen transport, and high oxygen utilization.

The significance of the differences in the fatty acid and molecular species composition found in the brain and liver and the differences between the fatty acid composition of phosphatidylcholine and phosphatidylethanolamine from the same tissue will have to await the elucidation of the role of these lipids in the structures in which they are found.

The de novo pathway for the synthesis of brain phosphatidylcholine and phosphatidylethanolamine has been shown to be the same as that found in liver (Ansell, 1972; Baker and Thompson, 1972). Diacyl glycerol derived from phosphatidic acid reacts with CDP choline or CDP

ethanolamine to form phosphatidylcholine or phosphatidylethanolamine. With evidence that the phosphatidic acid can be formed either by the acylation of glycerophosphate or dihydroxyacetone phosphate (Hajra and Agranoff, 1968a, 1968b), there is the possibility that a particular molecular species of phosphatidic acid could be formed by a given pathway. To insure that the phosphatidic pool is representatively labeled, a substrate must be chosen whereby the results would indicate if there is a specific labeling pattern derived from either pathway. The use of 1,3 and 2-[^{3}H] labeled glycerol as a substrate for de novo synthesis studies fulfills this requirement. If glycerophosphate is converted to dihydroxyacetone phosphate before acylation the tritium at the 2 position of 2-[^{3}H]glycerol is lost; thus the phosphatidylcholine and phosphatidylethanolamine labeled by this substrate could only be derived from phosphatidic acid formed by the acylation of glycerophosphate. Presumably the 1,3-[^{3}H]glycerol could be utilized by either pathway without losing the tritium; thus, the lipids labeled by this substrate could be derived from phosphatidic acid formed by either pathway.

The relative specific activities of the molecular species of phosphatidylcholine and phosphatidylethanolamine (Table 15), as determined by the incorporation of 1,3 or 2-[^{3}H]glycerol are identical; thus there appear to be no unique molecular species of phosphatidic acid formed by either of the alternate pathways in brain. This has also been demonstrated by Okuyama and Lands (1970) in the synthesis of lipids in the liver. The relative specific activities found when glucose is used as the labeled substrate confirm the results of the experiment utilizing 1,3-[^{3}H]glycerol. Presumably phosphatidic acid derived from

glucose could be formed by either pathway also and again the relative specific activities of the molecular species are the same as when 2-[^{3}H]glycerol is the labeled substrate.

The relative specific activities determined at 2 hours indicate that the molecular species of phosphatidylcholine may be derived primarily by de novo synthesis with little rearrangement necessary to acquire the final molecular configuration. The relative specific activities of the species differ by only a factor of 3 at 2 hours, and by 24 hours the relative specific activity of all species is nearly one. This is in good agreement with the data obtained by O'Brien and Geison (1974) with the exception that they found a 40 carbon species which maintains a comparatively high relative specific activity from 0.5 to 24 hours. From the data in Table 13 it can be calculated that a 40 carbon species in phosphatidylcholine would account for less than 2% of the total molecular species of phosphatidylcholine; therefore this species is of minor significance quantitatively.

The relative specific activities of phosphatidylethanolamine show that the species containing arachidonic acid has the lowest specific activity. At 2 hours the specific activity of the arachidonic acid-containing species is by far the lowest, but by 24 hours the relative specific activities of all the molecular species differ by only a factor of 2. If one omits the relative specific activity of the arachidonic acid-containing species and recalculates the data for 2 and 24 hours there is only a five-fold difference in relative specific activities at 2 hours and less than 0.5-fold difference at 24 hours. This indicates that with the exception of the species containing arachidonic

acid the majority of the phosphatidylethanolamine molecular species are synthesized de novo with little rearrangement of the fatty acids necessary to achieve the final configuration.

The data shown in Tables 15 and 16 were normalized by setting the lowest specific activity of a molecular species of phosphatidylcholine or phosphatidylethanolamine equal to 1 and calculating the other specific activities relative to that species. Table 19 shows the average of the $[^{3}H]/[^{14}C]$ ratios for the 2 hour incorporation experiments utilizing 1,3 and 2-$[^{3}H]$glycerol as the substrate and acetylating the diacyl glycerol derivatives with $[^{14}C]$acetic anhydride. The specific activities between phosphatidylcholine and phosphatidylethanolamine can now be compared, both with each other and with data obtained by others on rat brain lipid synthesis.

Phosphatidylcholine contains more 16 carbon fatty acids than phosphatidylethanolamine, while the opposite is true for the 18 carbon fatty acids. The molecular species also are very different, with monoenes and dienes accounting for 47% of the total molecular species of phosphatidylcholine and only 13% of the total in phosphatidylethanolamine (Table 6), yet Table 19 shows that the relative specific activities of the monoenes and dienes are nearly identical. Since phosphatidylcholine contains a much larger amount of these species than phosphatidylethanolamine yet their relative specific activities are nearly identical, there must be either separate pools of diacyl glycerol with molecular species corresponding to the molecular species of the two phospholipids, and transferases which express no specificity toward species within the

Table 19. Average of the $[^3H]/[^{14}C]$ ratios from the 2 hour 1,3 and 2-$[^3H]$glycerol incorporation studies.

Band	PC $[^3H]/[^{14}C]$	PE $[^3H]/[^{14}C]$
0	5.11	19.10
1	5.18	5.97
2	8.78	6.96
4	6.21	1.23
5	7.35	3.80
6	15.58	7.40

pool, or considerable specificity of the transferases toward certain molecular species of diacyl glycerides which are in a common pool.

Another indication for transferase specificity or compartmentation comes from consideration of the known fatty acid composition of brain diacyl glycerol. The fatty acid composition of diacyl glycerol isolated either from adult mouse brain (Sun, 1970) or adult rat brain (Keough, MacDonald, and Thompson, 1972) has been determined and the nearly identical values show the composition to be approximately 16:0 19%, 18:0 30%, 18:1 12%, 20:4 30%, and 22:6 3%. It is apparent that considerable selectivity must be exercised in the utilization of this pool in order to account for the fatty acid composition of phosphatidylcholine, again suggesting either separate pools of diglyceride or specificity of the transferases toward certain species within a common pool.

The relative specific activities of diacyl glycerol species synthesized in 10-day old rat brain after 2-[^{3}H]glycerol injection have been determined by O'Brien and Geison (1974). At 2 hours the specific activity of the 38 carbon species was the lowest while the 32, 34, and 36 carbon species showed higher and comparable specific activities. If the diacyl glycerol is in a common pool, the specific activities of the phospholipids derived from them would be expected to be the same. The data obtained in this study indicate that this is the case since the specific activity of the molecular species of phosphatidylcholine, except the species containing 22:6, and the species of phosphatidylethanolamine, except the disaturated and 20:4 species, are all similar. The low specific activity of the arachidonic acid-containing species

of phosphatidylethanolamine corresponds to the low specific activity of the 38 carbon diglyceride precursor observed by O'Brien and Geison thus the apparent low rate of initial synthesis of this species of the phospholipid could be accounted for by the low rate of synthesis of the precursor.

Although the specific activities of most of the molecular species of phosphatidylcholine and phosphatidylethanolamine compare with the specific activities of the precursor diglycerides, indicating that a common pool of diglyceride is available from which selection occurs, the fact that total brain diglyceride contains 30% arachidonic (20:4) acid coupled with the observation that this diglyceride species is synthesized at a much lower rate than the other species, indicates that the total pool of diglyceride is not available for phospholipid synthesis.

The results of the fatty acid composition studies on total brain phosphatidylcholine and phosphatidylethanolamine, along with the results of the molecular species determination and the fatty acid composition of those molecular species, emphasize the difference between the two phospholipids. The in vivo studies on their synthesis indicate that both the differences between phosphatidylcholine and phosphatidylethanolamine fatty acid composition and the difference in the molecular species within each lipid class result during de novo synthesis with apparent little need for subsequent modification. Further understanding of the mechanism whereby this specificity arises will necessitate studying the difficult question of compartmentation of some or all aspects of the

biosynthetic pathways and the re-evaluation of the in vivo studies on the specificity of the CDP choline and CDP ethanolamine transferases.

The enzymes involved in lipid biosynthesis have proven perplexing to study in vitro. Most of the enzymes are membrane bound in a strongly hydrophobic environment and many of the reactants and most of the products have limited and varied solubility in aqueous solutions. The specificity of these reactions has been shown to vary depending upon the method of extracting the enzymes (Yamashita and Numa, 1972), enzyme concentration (Lands and Hart, 1965), pH of the medium (Lamb and Fallon, 1970), concentration of glycerophosphate (Okuyama and Lands, 1972), and the concentration of monomeric or micellar forms of acyl-CoA (Zahler and Cleland, 1969). The in vivo studies show that the lipids are synthesized with specific fatty acid configuration and composition and until the micro-environment of the substrates and enzymes is known and can be reproduced, the validity of in vitro studies will be questionable.

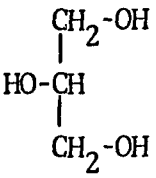
The question of compartmentation of lipid precursors in brain tissue would be even more difficult to answer. The precursors needed to resolve this question, such as specific molecular species of diacyl glycerol cannot be introduced to the tissue in such a way that they would equilibrate with the endogenous pools. Therefore, compartmentation cannot be determined by the incorporation of labeled substrates.

Isolation of cell types and fractionation of subcellular particles is a lengthy procedure during which time phospholipid exchange among the particles (Miller and Dawson, 1972), would change their lipid composition. The determination of the species present after isolation

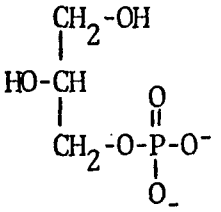
would not represent that originally in the particle and any compartmentation observed would be an artifact of the isolation procedure.

APPENDIX A

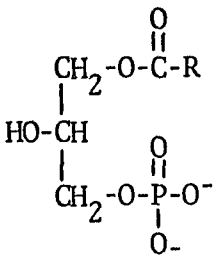
STRUCTURES AND ABBREVIATIONS OF LIPIDS DESCRIBED IN THE TEXT[1]



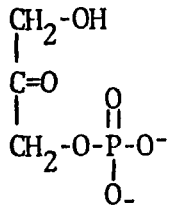
Glycerol



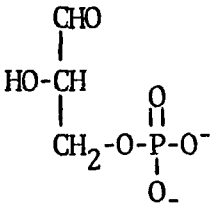
Glycerophosphate (GP)



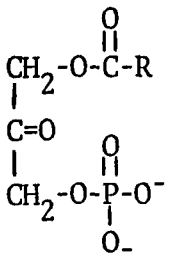
1-Acyl Glycerophosphate (1-Acyl GP)



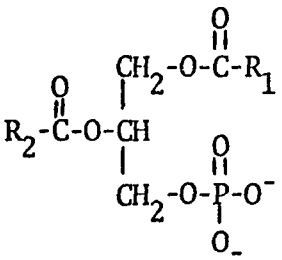
Dihydroxyacetone phosphate (DHAP)



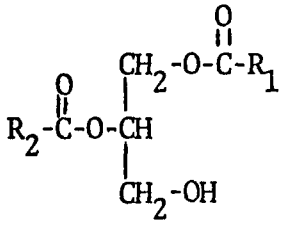
Glyceraldehyde phosphate (GAP)



1-Acyl Dihydroxyacetone phosphate (1-Acyl DHAP)

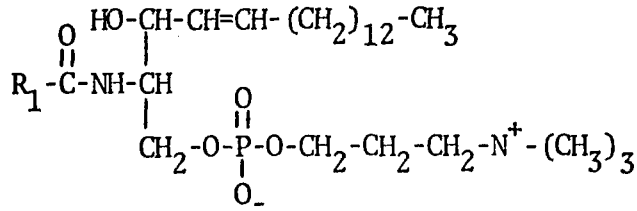


Phosphatidic Acid (PA)

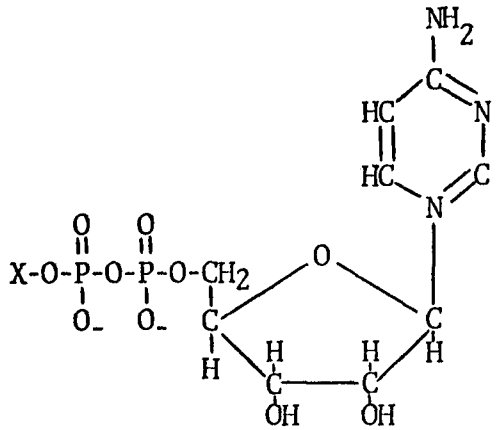


Diacyl Glycerol (DAG)

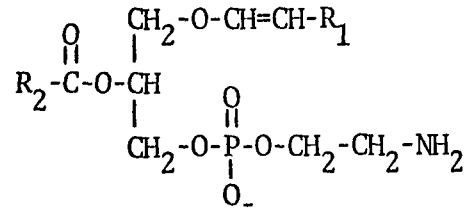
1. R denotes fatty acids.



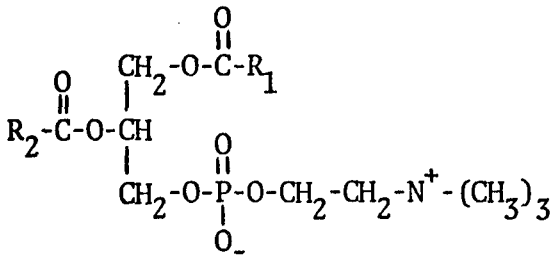
Sphingomyelin
(S)



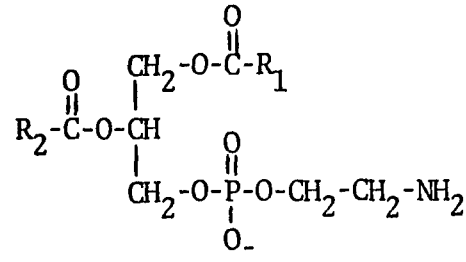
CDP Choline (X = Choline)
CDP Ethanolamine (X = Ethanolamine)



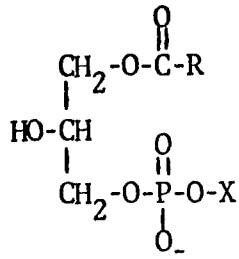
Ethanolamine Plasmalogen
(EP)



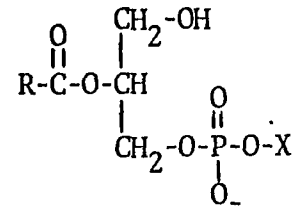
Phosphatidylcholine
(PC)



Phosphatidylethanolamine
(PE)



1-Acyl PC (X = Choline)
1-Acyl PE (X = Ethanolamine)



2-Acyl PC (X = Choline)
2-Acyl PE (X = Ethanolamine)

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