THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 267, No. 15, Issue of May 25, pp. 10362-10369, 1992
Printed in U.S.A.
© 1992 by The American Society for Biochemistry and Molecular Biology, Inc.

Individual Molecular Species of Phosphatidylcholine and Phosphatidylethanolamine in Myelin Turn Over at Different Rates*

(Received for publication, December 17, 1991)

Andrea H. Ousley and Pierre Morell‡

From the Department of Biochemistry and Biophysics and the Brain and Development Research Center, University of North Carolina, Chapel Hill, North Carolina 27599-7250

Phosphatidylcholine (PC) and phosphatidylethanolamine (PE) of the myelin membrane exhibit heterogeneity with respect to metabolic turnover rate (Miller, S. L., Benjamins, J. A., and Morell, P. (1977) *J. Biol. Chem.* 252, 4025–4037). To test the hypothesis that this is due to differential turnover of individual molecular species (which differ in acyl chain composition), we have examined the relative turnover of individual molecular species of myelin PC and PE. Phospholipids were labeled by injection of [2-^{3}H]glycerol into the brains of young rats. Myelin was isolated at 1, 15, and 30 days post-injection, lipids were extracted, and phospholipid classes were separated by thin-layer chromatography. The PC and PE fractions were hydrolyzed with phospholipase C, and the resulting diacylglycerols were dinitrobenzoylated and fractionated by reverse-phase high performance liquid chromatography. The distribution of radioactivity among individual molecular species was determined. The labeled molecular species of myelin PC were 16:0-16:0, 16:0-18:0, 16:0-18:1, and 18:0-18:1, with most of the label present in 16:0-18:1 and 18:0-18:1. Changes in distribution of label with time after injection indicated that 16:0-18:1 turned over more rapidly than 18:0-18:1. The labeled molecular species of myelin PE were 18:0-20:4, 18:1-18:1, 16:0-18:1, 18:0-18:2, and 18:0-18:1. As with myelin PC, 16:0-18:1 (and 18:1-18:1) turned over more rapidly than 18:0-18:1. The relative turnover of individual molecular species of PC in the microsomal fraction from forebrain was also examined. The molecular species profile was different from myelin PC, but again, 16:0-18:1 turned over more rapidly than the other molecular species. Thus, within the same membrane, individual molecular species of a phospholipid class are metabolized at different rates. Comparison of our results with previous studies of turnover of molecular classes of phospholipids indicates that in addition to polar head group composition (Miller *et al.*, 1977), fatty acid composition is very important in determining the metabolic fate of a phospholipid.

Plasma membrane lipids in mammalian cells are subject to continuous degradation and replacement. Individual phospholipid classes have been shown to turn over at different rates in plasma membrane (Lee *et al.*, 1973) and in myelin from rat brain (Smith and Eng, 1965; Smith, 1967). Furthermore, the turnover of an individual phospholipid class can itself be multiphasic, *e.g.* phosphatidylcholine (PC)[1] and phosphatidylethanolamine (PE) of the myelin membrane from rat brain exhibit more than one turnover rate (Miller *et al.*, 1977). We now report a test of the hypothesis that multiphasic turnover of a phospholipid class in a plasma membrane is due to individual molecular species turning over at different rates.

Myelin, a specialized extension of the oligodendroglial cell plasma membrane, is a uniquely favorable system for investigation of the metabolic turnover of plasma membrane phospholipids *in vivo*. Myelin is derived from a single cell type and can be obtained in high yield and very high purity. Rapid accumulation of this membrane is concentrated in a defined postnatal period. Thus, when young animals are injected with radiolabeled precursors, lipids in myelin are well labeled, and this is due primarily to net synthesis rather than to metabolic turnover. Phospholipids synthesized in the endoplasmic reticulum of the oligodendroglial cell body and pulse-labeled by precursor injection are assembled into the myelin membrane within minutes (Benjamins *et al.*, 1976). The myelin phospholipids are subsequently turned over at a rate slower than in most other membranes (reviewed in Benjamins and Smith, 1984). Thus, in myelin relative to other plasma membranes, there is less overlap of biosynthetic and catabolic events, simplifying interpretation of experimental results. Another unique advantage of this system is that in the oligodendroglial cell there is a great deal of myelin; more than 99% of the cell may consist of this membranous material. Thus, it can be assumed that loss of radioactivity from myelin is temporally closely related to a catabolic event; there is no compartment large enough to account for long term storage of material removed from myelin.

EXPERIMENTAL PROCEDURES

Materials—[2-^{3}H]Glycerol (15 Ci/mmol), [^{3}H]acetic acid, sodium salt (5 Ci/mmol), and [1,3-^{14}C]glycerol (55 mCi/mmol) were from American Radiolabeled Chemicals, Inc. (St. Louis, MO). Phospholipase C, type III from *Bacillus cereus*, 3,5-dinitrobenzoyl chloride, 4-dimethylaminopyridine, butylated hydroxytoluene, 1,2-dilauroyl-*rac*-glycerol, and 1,3-dipentadecanoin were from Sigma. Standards for fatty acid methyl esters were from Sigma or Nu-Chek Prep, Inc. (Elysian, MN). Standards for diacylglycerol molecular species (18:2-18:2, 18:0-20:4, 18:1-18:1, 16:0-18:1, 16:0-16:0, and 18:0-18:1) were prepared from defined PC standards purchased from Sigma, with the exception of 16:0-20:4 and of 16:0-18:0, for which synthetic PC standards were obtained from Avanti Polar Lipids, Inc. (Birmingham, AL). Silylation grade pyridine was from Pierce Chemical Co. High performance liquid chromatography (HPLC) grade acetonitrile, *n*-

* This work was supported by United States Health Service Grant NS11615. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ To whom correspondence and reprint requests should be addressed: Brain and Development Research Center, CB 7250, University of North Carolina, Chapel Hill, NC 27599. Tel.: 919-966-2128; Fax: 919-966-1844.

[1] The abbreviations used are: PC, phosphatidylcholine; HPLC, high performance liquid chromatography; PE, phosphatidylethanolamine.

This is an Open Access article under the CC BY license.

hexane, and 2-propranol, and silica gel G (500 μm), LK6, and LHP-K thin layer chromatography (TLC) plates were from Fisher. Silica gel G plates (500 μm) impregnated with 15% $AgNO_3$ were from Analtech. The Ultrasphere C18 HPLC column (5 μm, 25 cm × 4.6 mm) was from Alltech Associates, Inc. (Deerfield, IL). The SP-2331 (45 m × 0.25 mm) and Nukol (30 m × 0.25 mm) capillary columns were from Supelco, Inc. (Bellefont, PA). Male Sprague-Dawley rats were obtained from Charles River Breeding Laboratories (Raleigh, NC). All other materials were reagent-grade.

In Vivo Labeling of Brain Lipids with Radioactive Precursors—Rats 18 days of age were given intracranial injections of 250–500 μCi of [2-^{3}H]glycerol or 1.0 mCi of [^{3}H]acetic acid in 10 μl of 0.9% NaCl to label forebrain lipids (Wiggins *et al.*, 1976). Myelin lipids in brainstem were labeled by intracisternal injections of 500 μCi of [2-^{3}H]glycerol into 14-day-old rats. For the experiments where turnover of lipids in different myelin subfractions was examined, animals were given 62 μCi of [1,3-^{14}C]glycerol at 20 days of age, followed by injection of 320 μCi of [2-^{3}H]glycerol 33 days later. For the second injection, animals were anesthetized, an incision was made along the sagittal suture to the level of the cranium, and a dental drill was used to make an injection access hole over the right hemisphere (Miller and Morell, 1978).

Preparation of Tissue Subcellular Fractions—Myelin was isolated from rat forebrain and brainstem homogenates on discontinuous sucrose gradients by a modification of the method of Norton and Poduslo (1973a, 1973b). The forebrain or brainstem was homogenized in 10 ml of cold 0.32 M sucrose using a motor-driven Potter-Elvehjem Teflon/glass homogenizer, and the homogenate was layered over 19 ml of 0.85 M sucrose. Crude myelin was collected at the interface between 0.32 and 0.85 M sucrose following centrifugation in a Beckman SW-27 rotor at 69,800 × *g* for 35 min. Material collected from the interface was diluted with ice-cold water and pelleted at 69,800 × *g* for 20 min. The pellet was resuspended in ice-cold water, homogenized using a Dounce homogenizer, and kept on ice for 30 min. The osmotically shocked myelin was collected by centrifugation at 13,300 × *g* for 10 min using a Sorvall SS-34 rotor and then recycled through the discontinuous sucrose gradient step as above. The purified myelin was collected from the gradient, diluted with water, collected by centrifugation at 39,000 × *g* for 15 min using a Sorvall SS-34 rotor, and then washed once under the same conditions.

Myelin subfractions were isolated by resuspending in 0.32 M sucrose myelin collected from the osmotic shock step and layering over a 3-step discontinuous sucrose gradient consisting of 9 ml of 0.50 M sucrose, 13 ml of 0.63 M sucrose, and 9 ml of 0.72 M sucrose (Benjamins *et al.*, 1976). After centrifugation at 69,800 × *g* for 35 min using a Beckman SW-27 rotor, material at the 0.50–0.63 M interface (subfraction B), the 0.63–0.72 M interface (subfraction C), and the pellet (subfraction D) were collected. The isolated subfractions were resuspended in water and centrifuged at 39,000 × *g* for 15 min using a Sorvall SS-34 rotor.

Preparation of microsomes involved centrifugation of forebrain homogenates at 13,300 × *g* for 15 min using a Sorvall SS-34 rotor. The supernatant was then centrifuged at 80,800 × *g* for 65 min using a Beckman type 40 rotor, and the pellet was collected.

Extraction and Separation of Lipids—Lipids were extracted from isolated myelin, myelin subfractions, microsomes, and brain homogenate by the procedure of Bligh and Dyer (1959). Aliquots of tissue fraction samples were suspended in 2 ml of water, and 7.5 ml of chloroform/methanol (1:2) was added. The mixture was then partitioned into organic and aqueous phases by the addition of 2.4 ml of chloroform and 2.0 ml of water. Following agitation on a Vortex mixer, the phases were separated by centrifugation and the organic phase was removed and dried under nitrogen. Lipid classes were separated by two-dimensional TLC on silica gel G plates. The TLC plates were developed in the first dimension with chloroform/methanol/ammonium hydroxide (65:25:4), exposed to HCl vapors for 10 min (this treatment destroys the acid-sensitive plasmalogen), and developed in the second dimension with chloroform/methanol/acetone/acetic acid/water (75:15:30:15:7.5; Horrocks and Sun, 1972). This TLC method does not separate 1-alkyl,2-acyl from diacyl phospholipids. However, [2-^{3}H]glycerol does not significantly label 1-alkyl,2-acyl phospholipids (or plasmalogens), because dihydroxyacetone phosphate, the obligatory intermediate in their biosynthesis, is not labeled.

Lipids were visualized by brief exposure to iodine vapor and identified by comparing R_F values with those of known lipid standards. The iodine was allowed to evaporate, and appropriate regions of the TLC plates were scraped into vials for liquid scintillation counting or into test tubes for further analysis. PC and PE were eluted from the silica by extracting three times with 4 ml of chloroform/methanol/water (7:7:1) (Miller *et al.*, 1977).

Hydrolysis of Phospholipids—PC and PE were hydrolyzed to diacylglycerol by treatment with phospholipase C in the presence of H_3BO_3 (Yeung and Kuksis, 1974; Kito *et al.*, 1985). H_3BO_3 was present to reduce 2,3-acyl migration, which causes conversion of 1,2- to 1,3-diacylglycerol. Briefly, 10 μg of butylated hydroxytoluene was added to 0.5 mg of phospholipid and dispersed by sonication in 1.2 ml of 0.1 M Tris-HCl (pH 7.4) containing 10 mM $CaCl_2$ and 30 mM H_3BO_3. Three units of phospholipase C were added to the dispersed lipid in 30 μl of 0.1 M Tris-HCl (pH 7.4) containing 10 mM $CaCl_2$. Two ml of water-saturated diethyl ether were added, and the reaction mixture was shaken for 3 h at room temperature. The ether layer was then removed and the aqueous phase washed three times with 2 ml of water-saturated ether. The combined ether phases were dried under nitrogen and stored at −20 °C. An aliquot of the ether phase was spotted on LHP-K TLC plates and developed in heptane/isopropyl ether/acetic acid (60:40:4) (Breckenridge and Kuksis, 1968) to check for incomplete hydrolysis, acyl migration to 1,3-diacylglycerol, or degradation to monoacylglycerol and free fatty acid. The phospholipase C reaction products were visualized by spraying with 10% $CuSO_4 \cdot 5H_2O$ in 8% H_3PO_4 and charring for 10 min at 180 °C (Ganser *et al.*, 1988).

Derivatization of Diacylglycerols—The diacylglycerols derived from individual phospholipid classes and diacylglycerol standards were dinitrobenzoylated (Kito *et al.*, 1985). Briefly, 3,5-dinitrobenzoyl chloride, 4-dimethylaminopyridine, and the diacylglycerol samples were dried in a desiccator for 30 min prior to use. Derivatization with 50 mg of 3,5-dinitrobenzoyl chloride was carried out in 1 ml of silylation grade pyridine also containing 2 mg of 4-dimethylaminopyridine (added to reduce acyl migration during the reaction (Batley *et al.*, 1980; Hassner *et al.*, 1978)) in sealed test tubes for 30–60 min at 60 °C. One ml of water was added to stop the reaction, and the samples were heated for 10 min at 60 °C. After addition of 4 ml of 0.1 N HCl and 6 ml of *n*-hexane, the mixture was gently agitated overnight at 4 °C. The solvent layer was removed and dried under nitrogen, and the residue was dissolved in acetonitrile.

Separation of Diacylglycerol Derivatives by Reverse-phase High Performance Liquid Chromatography and Identification of Molecular Species—The derivatized diacylglycerols obtained from individual phospholipid classes were loaded onto an Ultrasphere C18 column (Kito *et al.*, 1985), and individual molecular species were eluted with acetonitrile, 2-propanol (85:15) using an isocratic gradient at a flow rate of 1 ml/min at room temperature. The amount loaded for an individual HPLC run varied from 200 to 600 μg since it depended on an initial estimate of how many preparative TLC plates were required to obtain sufficient labeled material for accurate analysis. Eluate was monitored at 254 nm, 1-ml fractions were collected, and radioactivity was quantified by liquid scintillation spectrometry. HPLC traces in the following figures are indexed to an early eluting peak of UV-absorbing side reaction products formed during the derivatization reaction.

Identification of molecular species was made by comparing peak retention times with those of known standards. To confirm the assignments made and to establish identity of molecular species for which no standards were available, the fractions associated with each HPLC peak were collected and subjected to alkaline methanolysis at room temperature for 60 min following the addition of 2 ml of chloroform and 1 ml of 0.2 N methanolic NaOH. After neutralizing with 0.6 ml of 0.33 M acetic acid, the lower phase, which contains the fatty acid methyl esters, was isolated (Miller *et al.*, 1977). An aliquot of the lower phase was spotted on LK6 plates, and the plates were developed in heptane/isopropyl ether/acetic acid (60:40:4) to check for completeness of reaction. The reaction products were visualized by acid charring as noted above. The remaining extract was dried under nitrogen and dissolved in chloroform or *n*-hexane to a final concentration of 1–5 ng of methyl ester/μl. The fatty acid methyl esters were subjected to gas chromatography on a 30-m × 0.25-mm capillary column coated with Nukol stationary phase. Other samples were also subjected to gas chromatography-mass spectroscopy. In this case, the methyl esters were chromatographed on a SP-2331 capillary column (45 m × 0.25 mm) coated with a 0.25-μm film, and the output was analyzed using a VG 70-250 SEQ Tandem Hybrid MS/MS system operated in "mass spectroscopy" only mode. Production of ions was by electron impact, and assignments were made based on retention time and the presence of the appropriate parent molecular ion.

Separation of Fatty Acid Methyl Esters—In some experiments,

HPLC fractions corresponding to UV-absorbing peaks were collected and subjected to alkaline methanolysis. The resulting methyl esters were separated by TLC with chloroform on 15% $AgNO_3$ plates. The plates were dried and exposed to iodine vapors (this treatment reduces subsequent background charring). The section containing fatty acid methyl ester standards was sprayed with acid and charred as noted above. Regions of the TLC plates containing samples (protected by glass during acid charring) were collected for determination of radioactivity.

RESULTS

Experimental Design—The present study involved intracranial injections of [2-^{3}H]glycerol. This protocol is suitable for pulse labeling the glycerol backbone of brain lipids. Label in the precursor pool drops rapidly, and thus, incorporation of radioactivity into phospholipids is quantitatively significant only during the first few hours following precursor injection. After incorporation into phospholipids, reutilization of this label is minimal because glycerol 3-phosphate released from degraded phospholipid is rapidly equilibrated with dihydroxyacetone phosphate, and this results in loss of ^{3}H to water (Benjamins and McKhann, 1973; Miller *et al.*, 1977).

Forebrains or brainstems were labeled *in vivo* with [2-^{3}H] glycerol at an age when myelin is being deposited at a rapid rate. So that both brain regions would be labeled during an equivalent developmental stage with respect to myelination, forebrain was labeled at 18 days of age, and brainstem (which is phylogenetically older than forebrain and myelinates earlier) was labeled at 14 days of age. Myelin was isolated from forebrain or brainstem at 1, 15, and 30 days postinjection. Individual classes of myelin phospholipid were separated by two-dimensional TLC and hydrolyzed to diacylglycerol by treatment with phospholipase C. The diacylglycerols derived from PC and PE were derivatized with 3,5-dinitrobenzoyl chloride, and different molecular species were separated by reverse-phase HPLC as described under "Experimental Procedures."

Time points for analysis of turnover were chosen based on previous work suggesting the presence in myelin of at least two metabolic pools of each phospholipid class, with turnover times on the order of days and weeks, respectively (Miller *et al.*, 1977). At 1 day postinjection, both the "fast" and "slow" pools of myelin phospholipid are labeled. By 15 or 30 days, much of the label in the fast pool has disappeared and remaining label corresponds predominantly to the slow pool. If individual molecular species of myelin PC or PE turn over at the same rate, then one would expect to see no change in the distribution of ^{3}H radioactivity among individual molecular species with time following precursor injection. Conversely, if molecular species turn over at different rates, the percentage of total label in the molecular species that turn over at a faster rate should decrease with time, relative to that in species that turn over at a slower rate. With respect to data presentation, comparison of the absolute amounts of radioactivity in particular molecular species in different animals is not meaningful because of the large injection variability typical of intracranial injections. The data were normalized by calculation of distribution of radioactivity as presented in the bottom panel of relevant figures.

Identification of Diacylglycerol Molecular Species Derived from Individual Phospholipid Classes—The molecular species detected in individual HPLC peaks are shown in Table I. For myelin lipids, assignments were made based on peak retention time and gas chromatography or gas chromatography-mass spectroscopy analysis as described under "Experimental Procedures." Most molecular species were easily identified since there was a 1:1 proportion of only two fatty acids. When more than one molecular species was present in an HPLC peak, the assignment was made based on the relative ratios of the fatty acids present. The order of elution for molecular species, which is dependent upon hydrophobicity, was consistent with that previously reported by other investigators (Kito *et al.*, 1985; Lee and Hajra, 1991). For microsomal PC, the assignments were made only by comparison of peak retention times with those of known standards.

TABLE I

Identification of diacylglycerol molecular species derived from phosphatidylcholine and phosphatidylethanolamine

Myelin PC, myelin PE, or microsomal PC was hydrolyzed to diacylglycerol. The resulting diacylglycerol was then dinitrobenzoylated and fractionated by reverse-phase HPLC as described under "Experimental Procedures." Molecular species detected in individual HPLC peaks are shown below. Assignments were made assuming that the more unsaturated fatty acid is present in the *sn*-2 position. The peak numbers correspond to those used in Figs. 1-6.

Peak number	Molecular species		
	Myelin PC	Myelin PE	Microsomal PC
1	18:0-20:4 (25%) 16:0-18:1 (1,3)[a] (75%)	18:0-20:4	ND
2	ND[b]	18:1-18:1	ND
3	16:0-18:1	16:0-18:1 (75%) 18:0-18:2 (25%)	16:0-18:1
4	16:0-16:0	ND	16:0-16:0
5	18:0-18:1 (1,3)	18:0-18:1 (1,3)	ND
6	18:0-18:1	18:0-18:1	18:0-18:1
7	16:0-18:0	16:0-18:0	16:0-18:0

[a] (1,3) indicates the 1,3-isomer of diacylglycerol; others are 1,2-isomers.

[b] ND, not detected.

Peaks 1 and 5 were identified as the 1,3-isomers of peaks 3 and 6 (see Table I). The 1,3-isomers were eluted before their corresponding 1,2-isomers, as expected. Formation of the 1,3-isomers was an artifact of the phospholipase C or derivatization reaction (Kito *et al.*, 1985). In most experiments, 2,3-acyl migration resulted in significant production of 1,3-isomers (8–15% of the total derivatized diacylglycerol).

Individual Molecular Species of Myelin Phosphatidylcholine are Turned Over at Different Rates—Brainstems were labeled *in vivo* by injection of [2-^{3}H]glycerol, and molecular species of diacylglycerol derived from myelin PC were fractionated by reverse-phase HPLC. The profile of UV-absorbing HPLC peaks, shown in Fig. 1 (*panel A*), demonstrates that myelin PC is composed of few molecular species, mainly 16:0-18:1, 16:0-16:0, 18:0-18:1, and 16:0-18:0 (this profile did not change appreciably during the time course of the experiment). Other molecular species, presumably containing long chain fatty acids, were detectable by UV at low levels, but these were not significantly labeled in our experiments. The mass distribution of molecular species was consistent with published reports of the fatty acid composition in myelin PC (Skrbic and Cumings, 1970).

Representative traces of radioactivity in HPLC peaks are shown for 1 and 30 days post-injection (Fig. 1, *panels B* and *C*). The labeled molecular species were 16:0-18:1 (*peak 3*), 16:0-16:0 (*peak 4*), 18:0-18:1 (*peak 6*), and 16:0-18:0 (*peak 7*), with most of the label (60–70%) in peaks 3 and 6. Data were averaged from multiple experiments (Fig. 1, *panel D*). The fraction of label in 16:0–18:1 (*peak 3*) decreased from 45 to 24% with time. Concomitantly, the fraction of label in 18:0-18:1 (*peak 6*) increased from 24 to 45%, indicating that 16:0-18:1 turned over more rapidly than 18:0-18:1 (this corresponds to a 4-5-fold difference in turnover rate; see "Discussion").

Comparison of the HPLC trace of UV-absorbing peaks (Fig. 1, *panel A*) with a trace of radioactivity in HPLC peaks at 1 day postinjection (Fig. 1, *panel B*) indicated that individual

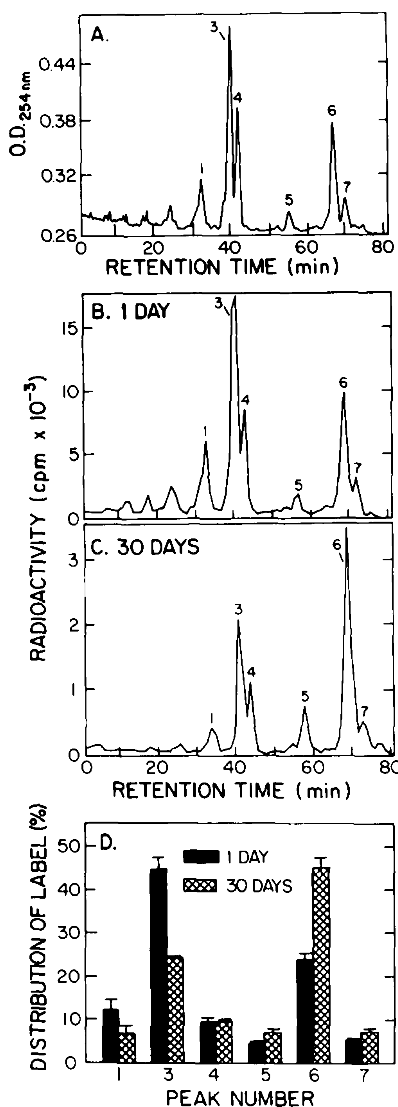

FIG. 1. **Distribution of radioactivity among individual molecular species of brainstem myelin PC.** Brainstems of 14-day-old rats were labeled by intracisternal injection of 500 μCi of [2-^{3}H] glycerol. Myelin was isolated at 1 and 30 days postinjection. Lipids were extracted, and PC was isolated by TLC and hydrolyzed by phospholipase C to diacylglycerol. Derivatized diacylglycerol species were resolved by reverse-phase HPLC. The mass distribution of molecular species is shown in *panel A*. Distribution of the radioactivity in HPLC peaks is shown for 1 and 30 days postinjection (*panels B* and *C*). In *panel D*, the mean percentage of radioactivity in HPLC peaks is shown for 1 day (*solid bars*) and 30 days (*hatched bars*) postinjection. Data are averages ± S.E. for brainstem myelin samples from five to six animals. Peak identification numbers refer to those given in Table I.

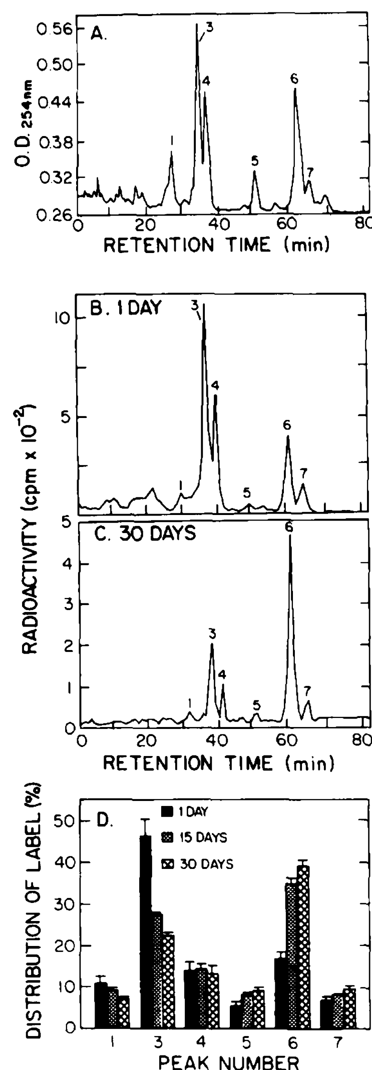

FIG. 2. **Distribution of radioactivity among individual molecular species of forebrain myelin PC.** Forebrains of 18-day-old rats were labeled by injection of 250–500 μCi of [2-^{3}H]glycerol. Myelin was isolated at 1, 15, and 30 days postinjection. Lipids were extracted, and PC was isolated by TLC and hydrolyzed to diacylglycerol. Derivatized diacylglycerol species were resolved by reverse-phase HPLC. The mass distribution of molecular species is shown in *panel A*. Distribution of the radioactivity in HPLC peaks is shown for 1 and 30 days postinjection (*panels B* and *C*). In *panel D*, the mean percentage of radioactivity in HPLC peaks is shown for 1 (*solid bars*), 15 (*stippled bars*), and 30 days (*hatched bars*) postinjection. Data are the averages ± S.E. for forebrain myelin samples from six to eight animals. Peak identification numbers refer to those in Table I.

molecular species were labeled to approximately the same relative specific radioactivity by 1 day. This was as expected since myelin lipids were labeled during a developmental period, which corresponds to the maximal rate of myelin accumulation. Because incorporation of label into myelin lipids was due to net synthesis, the amount of radioactivity incorporated into individual molecular species was proportional to the mass distribution.

Forebrains were labeled *in vivo* by injection of [2-^{3}H]glycerol, and molecular species of diacylglycerol derived from myelin PC were fractionated by reverse-phase HPLC. The profile of UV-absorbing peaks, shown in Fig. 2 (*panel A*), was similar to that for brainstem myelin PC. Changes in the percentage distribution of label between 1 and 15 or 30 days postinjection (Fig. 2, *panel D*) indicated that, again, 16:0-18:1 (*peak 3*) turned over more rapidly than 18:0-18:1 (*peak 6*). Most of the change in percentage distribution of label observed between 1 and 30 days postinjection had occurred by 15 days postinjection. Therefore, even for a single molecular species of myelin PC, there appeared to be more than one metabolic pool, *e.g.*, one pool of 16:0-18:1 PC turned over at a faster rate than 18:0-18:1, whereas another pool (containing most of the label still remaining by 15 days postinjection) turned over at a slower rate than that pool.

Individual Molecular Species of Myelin Phosphatidylethanolamine Are Turned Over at Different Rates—The profile of UV-absorbing peaks for brainstem myelin PE, shown in Fig. 3 (*panel A*), indicates that myelin PE is composed mainly of

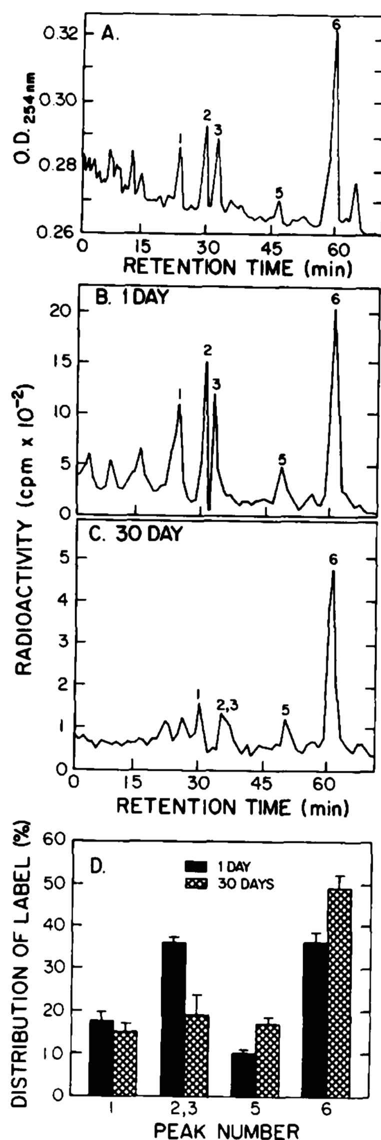

FIG. 3. **Distribution of radioactivity among individual molecular species of brainstem myelin PE.** The mass distribution of molecular species is shown in *panel A*. Distribution of the ^{3}H radioactivity in HPLC peaks is shown for 1 and 30 days (*panels B* and *C*) postinjection. In *panel D*, the mean percentage of radioactivity in HPLC peaks is shown for 1 (*solid bars*) and 30 days (*hatched bars*) postinjection. Data are the averages ± S.E. for brainstem myelin samples from three to four animals. Peak identification numbers refer to those given in Table I.

18:0-20:4, 18:1-18:1, 16:0-18:1, 18:0-18:2, and 18:0-18:1. The distribution profile of individual molecular species is similar to that recently reported for rat brain PE (Lee and Hajra, 1991).

Traces of radioactivity in HPLC peaks are shown for 1 and 30 days postinjection (Fig. 3, *panels B* and *C*). The labeled species were 18:0-20:4 (*peak 1*), 18:1-18:1 (*peak 2*), 16:0-18:1 and 18:0-18:2 (*peak 3*), and 18:0-18:1 (*peak 6*), with much of the label (35–50%) present in peak 6. Data averaged for multiple experiments are shown in *panel D*. These data indicate that 18:1-18:1 (*peak 2*) and 16:0-18:1 (the primary component of *peak 3*) turned over more rapidly than 18:0-18:1 (*peak 6*). Because peaks 2 and 3 were sometimes poorly resolved, radioactivity in these two peaks was combined for *panel D*.

The profile of UV-absorbing peaks for forebrain myelin PE was similar to that for brainstem (Fig. 4, *panel A*). Changes in the percent distribution of label indicated that, again, 18:1-18:1 (*peak 2*) and 16:0-18:1 (*peak 3*) turned over more rapidly than 18:0-18:1 (*peak 6*) (Fig. 4, *panel D*).

Previous studies indicated that myelin PE turns over more rapidly than PC (Miller *et al.*, 1977). An independent confirmation of this result was obtained by comparing the ratio of ^{3}H radioactivity in myelin PC to that in PE at 1 and 30 days postinjection. The ratio of ^{3}H radioactivity in PE to that in PC was 1:2 and 1:4 at 1 and 30 days, respectively, indicating that radioactivity was lost more rapidly from PE than from PC. This suggested that an experiment over a shorter time frame would be useful for study of PE metabolism. Thus, myelin PE from forebrain was isolated at 15 rather than 30 days postinjection. Comparison of Fig. 3 (*panel D*) and Fig. 4 (*panel D*) revealed that most of the rapid turnover of 18:1-18:1 (*peak 2*) and 16:0-18:1 (*peak 3*) observed in brainstem

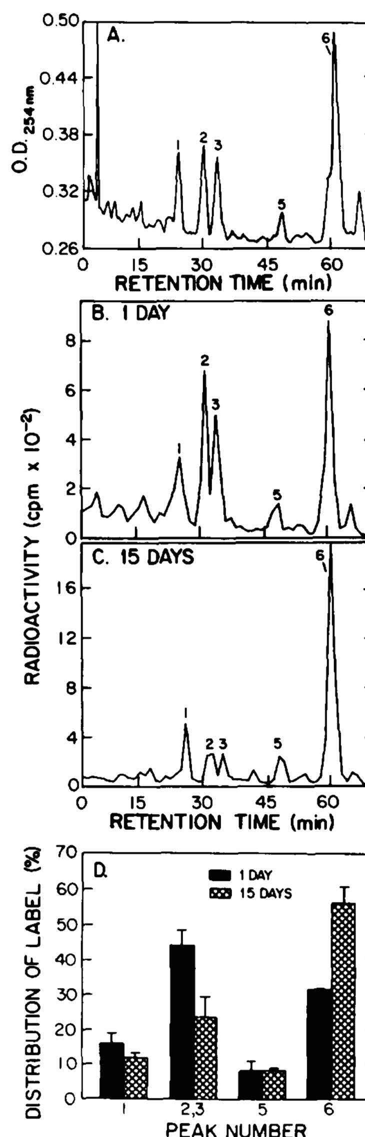

FIG. 4. **Distribution of radioactivity among individual molecular species of forebrain myelin PE.** The mass distribution of molecular species is shown in *panel A*. Distribution of the ^{3}H radioactivity in HPLC peaks is shown for 1 and 15 days (*panels B* and *C*) postinjection. The mean percentage of radioactivity in HPLC peaks is shown for 1 (*solid bars*) and 15 days (*hatched bars*) postinjection. Data are averages ± S.E. for forebrain myelin samples from three animals. Peak identification numbers refer to those given in Table I.

between 1 and 30 days postinjection had occurred by 15 days postinjection in forebrain. By analogy to the results for PC, this may indicate that more than one metabolic pool of 16:0-18:1 PE or 18:1-18:1 PE is present in myelin.

Metabolic Turnover May Involve Both Catabolism and Remodeling—A shift in the radioactivity distribution between two molecular species of a phospholipid could result either from more rapid complete catabolism of one molecular species or from conversion of one molecular species to another by remodeling (change in acyl chain composition by deacylation-reacylation reactions). We tested the hypothesis that metabolism of the two predominant PC species in myelin involved complete catabolism, rather than remodeling of 16:0-18:1 PC to 18:0-18:1 PC. Forebrains were labeled *in vivo* by injection of the fatty acid precursor [^{3}H]acetate, and myelin was isolated at 1 and 15 days postinjection. Molecular species of diacylglycerol derived from myelin PC were fractionated by reverse-phase HPLC, and fractions corresponding to peaks that contained 16:0-18:1 or 18:0-18:1 were collected and subjected to alkaline methanolysis. Radioactivity in individual methyl esters was determined after methyl esters of 16:0 or 18:0 fatty acids were separated from 18:1 methyl esters by TLC. Supportive evidence for our hypothesis would have been that the ratio of radioactivity in the *sn*-1 to *sn*-2 fatty acid positions of these two PC species did not change with time. This ratio for 16:0-18:1 was 2.38 and 1.12 at 1 and 15 days, respectively, and for 18:0-18:1 it was 3.17 and 1.47 at 1 and 15 days, respectively. A second independent experiment gave similar results (values were within 10% of those given above). Thus, our hypothesis was disproved by the observed 50% decrease with time in the *sn*-1 to *sn*-2 ratio for each PC species. The simplest interpretation of our result is that 15 days following injection, half of the radioactive stearate (18:0) expected to be present in 18:0-18:1 PC had been lost by an exchange reaction. A similar interpretation would apply to the result for 16:0-18:1 PC. Thus, there may be a major although unquantified contribution of an *sn*-1 deacylation-reacylation cycle to remodel 16:0-18:1 PC.

Individual Molecular Species of Microsomal Phospholipid Are Turned Over at Different Rates—The profile of UV-absorbing peaks for microsomal PC (Fig. 5, *panel A*) was very different from that for myelin PC, as myelin PC was more enriched in 16:0-18:1 and 18:0-18:1. The mass distribution of molecular species was consistent with published reports of the fatty acid composition in brain microsomal PC (Skrbic and Cumings, 1970).

Representative traces of radioactivity in HPLC peaks are shown for 1 and 30 days postinjection (Fig. 5, *panels B* and *C*). The labeled species were 16:0-18:1 (*peak 3*), 16:0-16:0 (*peak 4*), 18:0-18:1 (*peak 6*), and 16:0-18:0 (*peak 7*), with most of the label (75–95%) present in peaks 3 and 4. Changes in the percentage distribution of label in PC molecular species with time (Fig. 5, *panel D*) indicated that 16:0-18:1 turned over more rapidly than the other molecular species.

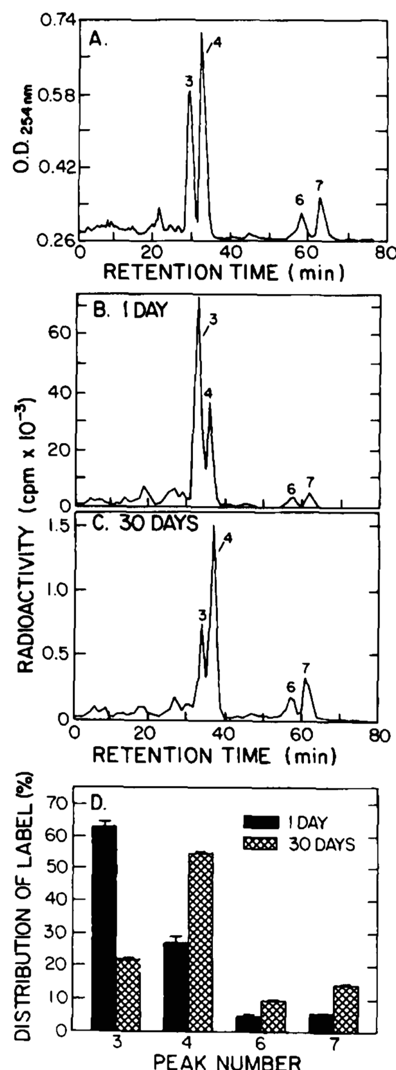

FIG. 5. **Distribution of radioactivity among individual molecular species of microsomal PC from forebrain.** Forebrains of 18-day-old rats were labeled by intracranial injection of [2-^{3}H]glycerol. Microsomes were isolated at 1 and 30 days postinjection. Lipids were extracted, and PC was isolated by TLC and hydrolyzed to diacylglycerol. Derivatized diacylglycerol species were resolved by reverse-phase HPLC. The mass distribution of molecular species is shown in *panel A*. Distribution of the radioactivity in HPLC peaks is shown for 1 and 30 days (*panels B* and *C*) postinjection. In *panel D*, the mean percentage of radioactivity in HPLC peaks is shown for 1 (*solid bars*) and 30 days (*hatched bars*) postinjection. Data are the averages ± S.E. for microsomal samples from three different animals.

Rapid Turnover of Phospholipid Is Not Specific to a Subfraction of Myelin—Myelin subfractions (A–D) can be prepared on sucrose gradients by virtue of heterogeneity in particle size and density. It is assumed that the different subfractions originate from different regions of the myelin sheath, possibly representing different contributions of compact myelin and noncompact domains such as the lateral, inner, and outer loops (reviewed in Norton and Cammer, 1984). These subfractions also have meaning in terms of precursor-product relationships in assembly of myelin (Benjamins *et al.*, 1976). Could the observed differences in metabolic turnover of molecular species of a given phospholipid class be related to this heterogeneity of myelin? To test this, forebrain lipids were labeled by injection of [1,3-^{14}C]glycerol at 20 days of age, followed by injection of [2-^{3}H]glycerol 33 days later, as described under "Experimental Procedures." Animals were sacrificed at 1 day after the ^{3}H injection, and the ^{3}H/^{14}C ratio in PC of myelin subfractions was determined. Only subfractions B and C contained enough material for HPLC analysis. Normalized to the ^{3}H/^{14}C ratio in PC from the total (nonfractionated) myelin sample (*i.e.* ^{3}H/^{14}C in total myelin PC = 1), the ^{3}H/^{14}C ratios in PC were (mean ± S.E., n = 6) 0.55 ± 0.06 (subfraction B) and 0.97 ± 0.08 (subfraction C). This difference in isotope ratio is as expected if subfraction C is derived from a domain of the myelin sheath that is closer to the entry point for newly synthesized phospholipids than is subfraction B. After 1 day, ^{3}H-labeled PC will have entered subfraction C but will not yet have equilibrated with PC in subfraction B.

However, both for subfractions B and C, the ratio of ^{3}H to ^{14}C in peak 3, relative to that in peak 6, was 4.6 (Fig. 6), consistent with our other results that indicate that 16:0-18:1 turns over more rapidly than 18:0-18:1. This result also demonstrates that rapid turnover of 16:0-18:1, relative to that of 18:0-18:1, is not compartmentalized with respect to the two major myelin subfractions.

DISCUSSION

We have demonstrated that, in a relatively homogeneous membrane fraction, individual molecular species of a phospholipid class are metabolized at different rates. We are not aware of any previous report dealing with such an analysis of a membrane phospholipid. Differential turnover of individual molecular species accounts for much of the multiphasic turnover observed for phospholipids in myelin (see next section). There does not appear to be heterogeneity of metabolic turnover with respect to subfractions of myelin or with respect to brain region, at least at the level of brainstem *versus* forebrain. Thus, in myelin, much of the heterogeneity in turnover of structural phospholipids is accounted for by small differences in fatty acid composition rather than by differences in polar head group composition. We note that multiphasic turnover of individual phospholipid classes has also been observed in neoplastic mast cells (Pasternak and Bergeron, 1970), MOPC 41 myeloma cells (Cohen and Phillips, 1980), microsomal membranes from rat liver (Omura *et al.*, 1967), and various subcellular fractions from brain (Pasquini *et al.*, 1973). Thus, our observation may have general significance with respect to membrane phospholipid turnover.

The only analogous report of which we are aware is that in rat hepatocytes 16:0-22:6 PE is catabolized at a faster rate than are other molecular species, *e.g.* the turnover of 16:0-22:6 was 3-fold faster than the turnover of 18:0-22:6 (Samborski *et al*, 1990).

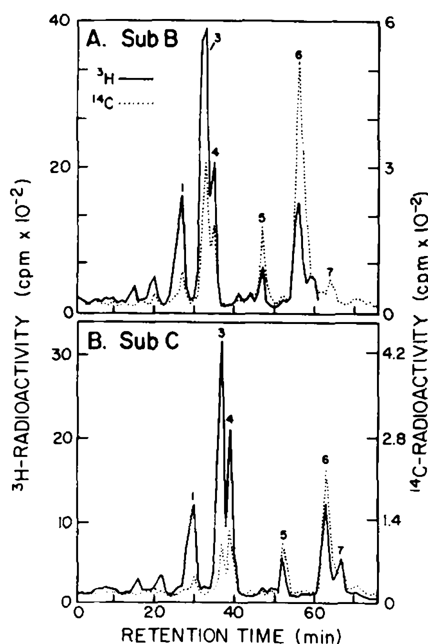

FIG. 6. **Distribution of ^{3}H and ^{14}C radioactivity among individual molecular species of myelin subfractions.** Forebrains of 20-day-old rats were labeled by injection of 62 μCi of [1,3-^{14}C]glycerol followed by injection of 320 μCi of [2-^{3}H]glycerol 33 days later. Forebrain myelin was isolated at 1 day after the second injection and then fractionated on discontinuous sucrose gradients into myelin subfractions. Traces of ^{3}H and ^{14}C radioactivity in HPLC peaks are shown for subfraction B (*panel A*) and C (*panel B*). Data are for pooled samples from six animals.

Differential Turnover of 16:0-18:1 and 18:0-18:1 Accounts for Much of the Heterogeneity in Turnover of Myelin Phosphatidylcholine—In an earlier study, forebrains were labeled by intracranial injections of [2-^{3}H]glycerol into 17-day-old rats, and the decay of radioactivity in individual classes of myelin phospholipid (PC and PE) was followed from 2 to 80 days after injection (Miller *et al.*, 1977). Myelin PC and PE showed decreases in ^{3}H radioactivity with time, which were approximated as biphasic decay curves. Half-lives of 10 and 6.5 days were calculated for PC and PE, respectively, for the first 15 days after injection, whereas half-lives of 25 days were calculated for the turnover of these two phospholipids between 15 and 80 days postinjection. We recalculated half-lives for the fast pools of PC and PE, assuming that there are two pools of PC and PE that turn over independently. When radioactivity in the slowly turning over component is first subtracted from the total radioactivity (Horrocks *et al.*, 1976), half-lives of 6 and 3 days were obtained for the fast pools of PC and PE, respectively. If we assume that the most stable species of myelin PC, 18:0-18:1 (peak 6), was turned over with a half-life of 25 days (the value previously reported for the slow phase of forebrain myelin PC turnover), then the half-life for other molecular species can be calculated from the following equation,

$$\log[N_x/N_y]_{15\,\text{days}} - \log[N_x/N_y]_{1\,\text{day}} = [(-0.693/2.3)(1/t^{1/2}_{x} - 1/t^{1/2}_{y}]\Delta t$$

where, $[N_x/N_y]_t$ = the ratio of radioactivity in molecular species x to that in y at time t, Δt = time interval during which turnover was examined, in days, $t^{1/2}_{x}$ = half-life of molecular species x, in days, $t^{1/2}_{y}$ = half-life of molecular species y, in days. Of most interest, in forebrain myelin PC, the ratio of radioactivity in 16:0-18:1 (peak 3) to that in 18:0-18:1 (peak 6) was 2.77, 0.74, and 0.59 at 1, 15, and 30 days postinjection, respectively. Assuming that 18:0-18:1 turns over with a half-life of 25 days, a half-life of 5.7 days can be calculated for the turnover of 16:0-18:1 between 1 and 15 days postinjection. Therefore, differential turnover of the two predominantly labeled molecular species can account for much of the "biphasic" turnover originally observed for myelin PC (Miller *et al.*, 1977). This calculation assumes first-order decay kinetics and is in error to an extent related to the above noted heterogeneity in turnover of even an individual molecular species.

Possible Mechanisms for Differential Turnover of Individual Molecular Species of a Phospholipid Class—Phospholipid degradation in mammalian cells involves deacylation catalyzed by phospholipase A_1 or A_2 followed by action of a lysophospholipase of the appropriate specificity. Preferential catabolism of certain molecular species may reflect *in vivo* substrate specificities of the phospholipase and lysophospholipase enzymes involved in phospholipid degradation. Results from studies that address this hypothesis are conflicting (Pind *et al.*, 1985; Holub, 1982; Lewis *et al.*, 1990). Proposed models, based on recently obtained crystal structures for phospholipase A_2 enzymes, suggest that the phospholipid molecule undergoing hydrolysis is drawn partly out of the membrane surface into the catalytic site through a hydrophobic channel (Scott *et al.*, 1990; White *et al.*, 1990; Verger *et al.*, 1973). Thus, the possibility exists that phospholipase A enzymes may exhibit substrate specificity, since the model predicts that the enzyme interacts partially with the acyl chains.

Another possibility is that lipid transfer proteins, found ubiquitously in the cytosolic fraction of eukaryotic cells (reviewed in Wirtz, 1982), are involved in the retrieval of phos-

pholipids from cellular membranes prior to their degradation. Although it has been previously suggested that transfer proteins may play a role in membrane biogenesis (Dawson, 1966), the physiological role of these proteins is still unclear. Transfer proteins could mediate both the movement of newly synthesized phospholipids from the endoplasmic reticulum to other subcellular membranes and subsequent retrieval from those membranes. Preferential transfer of certain molecular species would result in more rapid turnover of those species. In this context, we note that a role for PC transfer protein has been suggested in recycling of pulmonary surfactant (which has a high 16:0-16:0 PC content) between the alveolar surface and lamellar bodies of lung. *In vitro,* PC transfer protein from lung transfers 16:0-16:0 PC at a rate 1.5-fold higher than 18:1-18:1 or 16:0-20:4 PC (Funkhouser and Read, 1985). In a recent study, the binding affinity of PC transfer protein from bovine liver for different molecular species of PC relative to that for Pyr_8PC (16:0-[1-*pyrenyl*]8:0) was determined (Kasurinen *et al.,* 1990). The binding affinity of PC transfer protein for molecular species containing 16:0 in the *sn*-1 position is higher than that for those containing 18:0 at the *sn*-1 position. For example, the relative affinity for 16:0-18:1 is 2.5-fold higher than for 18:0-18:1, a specificity very much as expected from our *in vivo* data.

Acknowledgments—We thank Dr. S. H. Zeisel for the use of his gas chromatography system and Kerry-Ann daCosta for instruction in its use for fatty acid methyl ester analysis. We thank Dean Marbury from the Department of Environmental Sciences and Engineering at this institution for performing the gas chromatography-mass spectroscopy analysis. We also gratefully acknowledge Ming Hua He for excellent technical assistance. We thank an anonymous reviewer for a particularly insightful analysis of the originally submitted manuscript and acknowledge his or her contributions to the interpretation of the results.

REFERENCES

Batley, M., Packer, N. H., and Redmond, J. W. (1980) *J. Chromatogr.* **198,** 520–525

Benjamins, J. A., and McKhann, G. M. (1973) *J. Neurochem.* **20,** 1111–1120

Benjamins, J. A., and Smith, M. E. (1984) in *Myelin* (Morell, P., ed) 2nd Ed., pp. 225–258, Plenum Press, New York

Benjamins, J. A., Miller, S. L., and Morell, P. (1976) *J. Neurochem.* **27,** 565–570

Bligh, E. G., and Dyer, W. J. (1959) *Can. J. Biochem. Physiol.* **37,** 911–917

Breckenridge, W. C., and Kuksis, A. (1968) *Lipids* **3,** 291–300

Cohen, B. G., and Phillips, A. H. (1980) *J. Biol. Chem.* **255,** 3075–3079

Dawson, R. M. C. (1966) in *Essays in Biochemistry* (Campbell, P. N., and Greville, G. D., eds) Vol. 2, pp. 69–115, Academic Press, London

Funkhouser, J. D., and Read, R. J. (1985) *Chem. Phys. Lipids* **38,** 17–27

Ganser A. L., Kerner, A., Brown, B., Davisson, M. T., and Kirschner, D. A. (1988) *Dev. Neurosci.* **10,** 99–122

Hassner, A., Krepski, L. R., and Alexanian, V. (1978) *Tetrahedron* **34,** 2069–2076

Holub, B. (1982) *Biochim. Biophys. Acta* **711,** 305–310

Horrocks, L. A., and Sun, G. Y. (1972) in *Research Methods in Neurochemistry* (Marks, N., and Rodnight, R., eds) Vol. 1, pp. 223–231, Plenum Press, New York

Horrocks, L. A., Toews, A. D., Thompson, D. K., and Chin, J. Y. (1976) in *Function and Metabolism of Phospholipids in the Central and Peripheral Nervous Systems* (Porcellati, G., Amaducci, L., and Galli, C., eds) pp. 37–54, Plenum Press, New York

Kasurinen, J., van Paridon, P. A., Wirtz, K. W. A., and Somerharju, P. (1990) *Biochemistry* **29,** 8548–8554

Kito, M., Takamura, H., Narita, H., and Urade, R. (1985) *J. Biochem. (Tokyo)* **98,** 327–331

Lee, C., and Hajra, A. K. (1991) *J. Neurochem.* **56,** 370–379

Lee, T., Stephens, N., Moehl, A., and Snyder, F. (1973) *Biochim. Biophys. Acta* **291,** 86–92

Lewis, K. A., Bian, J., Sweeney, A., and Roberts, M. F. (1990) *Biochemistry* **29,** 9962–9970

Miller, S. L., and Morell, P. (1978) *J. Neurochem.* **31,** 771–777

Miller, S. L., Benjamins, J. A., and Morell, P. (1977) *J. Biol. Chem.* **252,** 4025–4037

Norton, W. T., and Cammer, W. (1984) in *Myelin* (Morell, P., ed) 2nd Ed., pp. 147–195, Plenum Press, New York

Norton, W. T., and Poduslo, S. E. (1973a) *J. Neurochem.* **21,** 749–757

Norton, W. T., and Poduslo, S. E. (1973b) *J. Neurochem.* **21,** 759–773

Omura, T., Siekevitz, P., and Palade, G. E. (1967) *J. Biol. Chem.* **242,** 2389–2396

Pasquini, J. M., Krawiec, L., and Soto, E. F. (1973) *J. Neurochem.* **21,** 647–653

Pasternak, C. A., and Bergeron, J. J. M. (1970) *Biochem. J.* **119,** 473–480

Pind, S., Kuksis, A., Myher, J. J., and Marai, L. (1985) *Can. J. Biochem. Cell Biol.* **63,** 137–144

Samborski, R. W., Ridgway, N. D., and Vance, D. E. (1990) *J. Biol. Chem.* **265,** 18322–18329

Scott, D. L., White, S. P., Otwinowski, Z., Yuan, W., Gelb, M. H., and Sigler, P. B. (1990) *Science* **250,** 1541–1546

Skrbic, T. R., and Cumings, J. N. (1970) *J. Neurochem.* **17,** 85–90

Smith, M. E. (1967) *Adv. Lipid Res.* **5,** 241–278

Smith, M. E., and Eng, L. (1965) *J. Am. Oil Chem. Soc.* **42,** 1013–1018

Verger, R., Mieras, M. C. E., and de Haas, G. H. (1973) *J. Biol. Chem.* **248,** 4023–4034

White, S. P., Scott, D. L., Otwinski, Z., Gelb, M. H., and Sigler, P. B. (1990) *Science* **250,** 1560–1566

Wiggins, R. C., Miller, S. L., Benjamins, J. A., Krigman, R. K., and Morell, P. (1976) *Brain Res.* **107,** 257–273

Wirtz, K. W. A. (1982) in *Lipid-Protein Interactions* (Jost, P. C., and Griffith, O. H., eds) Vol. 1, pp. 151–231, John Wiley & Sons, Inc., New York

Yeung, S. K. F., and Kuksis, A. (1974) *Can. J. Biochem.* **52,** 830–837