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

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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 [2-3H]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 16:0-18:1. 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 16:0-18:1. 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

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

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hexane, and 2-propanol, 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% AgNO₃ were from Analtech. The Ultrasphere C₁₈ HPLC column (5 μm, 25 cm × 4.6 mm) was from Alltech Associates, Inc. (Deerfield, IL). The SP-2535 (45 µm) silica gel G plates (500 µm) were from Supelco, Inc. (Bellefonte, 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 18-20 g were given intraperitoneal injections of 2-3H]glycerol or 1.0 ml of [3H]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 [2-3H]glycerol into 14-day-old rats. For the experiments where turn-over of lipids in different myelin subfractions was examined, animals were given 62 µCi of [3H]glycerol at 20 days of age, followed by injection of 320 µCi of [2-3H]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 Podulco (1973a, 1973b). The forebrain or brainstem was homogenized in 10 ml of sucrose using a 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 recylced through the discontinuous sucrose gradient step as above. The purified myelin was collected from the gradient, diluted with water, homogenized 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 (Benjamin 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 69,800 × g for 15 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.

**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 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 Dicylglycerol Derivatives by Reverse-phase High Performance Liquid Chromatography and Identification of Molecular Species**—The derivatized dicylglycerols obtained from individual phospholipid classes and dicylglycerol standards were resolved using a C₁₈ HPLC column (5 µm, 25 cm × 0.46 cm) coated with a 0.25-µm film, and the output 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.

**Derivatization of Dicylglycerols**—The dicylglycerols derived from individual phospholipid classes and dicylglycerol standards were derivatized by a modification of the method of Poduslo (1973a, 1973b). Briefly, 4,4-dimethylamino pyridine, and the dicylglycerol 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., 1986; Hauser et al., 1989) 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 Dicylglycerol Derivatives by Reverse-phase High Performance Liquid Chromatography and Identification of Molecular Species**—The derivatized dicylglycerols 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 collected 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.

**Derivatization 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 methanolation at room temperature for 60 min following the addition of 2 ml of chloroform and 1 ml of 0.2 M 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 incomplete hydrolysis, acyl migration to 1,3-dicylglycerol, or degradation to monoacylglycerol and free fatty acid. The phospholipase C reaction products were visualized by spraying with 10% CuSO₄/5% H₂O in 8% H₃PO₄, and charring for 10 min at 180 °C (Gänsler et al., 1988).

**Derivatization of Dicylglycerols**—The dicylglycerols derived from individual phospholipid classes and dicylglycerol standards were subjected to alkaline methanolysis (Kito et al., 1985). Briefly, 4,4-dimethylamino pyridine, 4-dimethylaminopyridine, and the dicylglycerol 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., 1986; Hauser et al., 1989) 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 Dicylglycerol Derivatives by Reverse-phase High Performance Liquid Chromatography and Identification of Molecular Species**—The derivatized dicylglycerols 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 collected 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.
HPLC fractions corresponding to UV-absorbing peaks were collected and subjected to alkaline methanalysis. The resulting methyl esters were separated by TLC with chloroform on 15% AgNO₃ 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 [2-3H]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 3H to water (Benjamins and McKhann, 1973; Miller et al., 1977).

Forebrains or brainstems were labeled in vivo with [2-3H] 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 myelinales 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 3H 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 experimental 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.

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 Phosphatidyicholine are Turned Over at Different Rates—Brainstems were labeled in vivo by injection of [2-3H]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

| Peak number | Myelin PC | Myelin PE | Microsomal PC |
|-------------|-----------|-----------|---------------|
| 1           | 18:0-20:4 (25%) | 18:0-20:4 | ND            |
| 16:0-18:1 (1,3)* (75%) | 18:0-18:1 | ND          |
| 16:0-18:1 (ND) | 16:0-18:1 | ND          |
| 16:0-18:1 (ND) | 16:0-18:1 | ND          |
| 16:0-18:1 (ND) | 16:0-18:1 | ND          |

*(1,3) indicates the 1,3-isomer of diacylglycerol; others are 1,2-isomers.
ND, not detected.
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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 [2-3H]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 [2-3H]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 [2-3H]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
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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...
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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 [3H]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 molecular species was consistent with published reports of fatty acid composition in brain microsomal PC (Skrbic and Cumings, 1970).

**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 5), 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.

**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 intracranial injection of [2-3H]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.

**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 [2-3H]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.
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).

**Differential Turnover of 16:0-18:1 and 18:0-18:1 Accounts for Much of the Heterogeneity in Turnover of Myelin Phosphatidylincholine**—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,

$$\log\left[\frac{N_y}{N_x}\right]_{10^{t+\Delta t}} - \log\left[\frac{N_y}{N_x}\right]_{10^t} = \frac{1}{t_y} - \frac{1}{t_x} \Delta t$$

where, $[N_y/N_x] = \text{the ratio of radioactivity in molecular species x to that in y at time t}$, $\Delta t = \text{time interval during which turnover was examined}$, in days, $t_x = \text{half-life of molecular species x}$, in days, $t_y = \text{half-life of molecular species y}$, 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 predominately 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 decylation catalyzed by phospholipase A$_2$, or A$_x$ 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 PynPC (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.

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