Docosahexaenoate-Containing Molecular Species of Glycerophospholipids from Frog Retinal Rod Outer Segments Show Different Rates of Biosynthesis and Turnover†

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Received May 27, 1988; Revised Manuscript Received August 1, 1988

ABSTRACT: We have studied the de novo synthesis and subsequent turnover of major docosahexaenoate-containing molecular species in frog rod outer segment (ROS) phospholipids following intravitreal injection of [2-^{3}H]glycerol. On selected days after injection, ROS were prepared and phospholipids extracted. Phosphatidylcholine (PC), phosphatidylethanolamine (PE), and phosphatidylserine (PS) were isolated and converted to diradylglycerols with phospholipase C. Diradylglycerols were derivatized with benzoic anhydride and resolved into diacylglycerobenzoates and ether-linked glycerobenzoates. The diacylglycerobenzoates were fractionated into molecular species by HPLC, quantitated, and counted for radioactivity. Label was incorporated into ROS phospholipids by day 1 and was followed up through the eighth day. The dipolyenoic species 22:6–22:6 from PC showed a 3–5 times higher radiospecific activity than the same species from either PE or PS. In PC, the specific activities of 16:0–22:6 and 18:0–22:6 were 3–5 times lower than the specific activity of 22:6–22:6. In contrast, for PE, the specific activities of 16:0–22:6 and 18:0–22:6 were 2–5 times higher than that of 22:6–22:6. The specific activities of 18:0–22:6 and 22:6–22:6 were similar in PS. Specific activities of the docosahexaenoate-containing species began approximating an exponential decline 6–8 days postinjection and continued through the 31st day. The rate of decline was determined by calculating the half-life of each molecular species, which was used as a measure of the turnover of the species. The species 22:6–22:6-PE and 18:0–22:6-PE showed a 2–3 times slower turnover rate than the corresponding species from either PC or PS. The turnover rates for 22:6–22:6, 18:1–22:6, and 16:0–22:6 were similar in PC but were 1.5 times faster than 18:0–22:6. In PE, 18:1–22:6 and 16:0–22:6 had similar rates but were 2–3 times faster than 22:6–22:6 and 18:0–22:6. For PS, 22:6–22:6 and 18:0–22:6 turned over at the same rate. The percent distribution of radioactivity in the molecular species of PC and PE was quite different from the relative mass distribution at day 1. However, percent dpm approached the mole percent by 31 days. In PS, percent dpm and mole percent were the same at all time points. These results indicate that the molecular species composition of PC and PE in frog retinal ROS is determined by a combination of factors, which include rate of synthesis, rate of degradation, and selective interconversions. In contrast, PS composition appears to be determined at the time of synthesis.

Glycerophospholipids of cell membranes represent a heterogeneous population of molecular species that occur in characteristic proportions. Each molecular species is defined by the chemical nature of the polar head group, the type of linkage to glycerol, and the aliphatic chains at both the *sn*-1 and *sn*-2 positions (Holub & Kuksis, 1978). Several laboratories (Miljanich et al., 1979; Miljanich & Dratz, 1982; Aveldano & Bazan, 1983), including our own (Wiegand & Anderson, 1983), have examined the molecular species of vertebrate rod outer segment (ROS)[1] membranes. These studies had demonstrated the presence of dipolyenoic molecular species in the glycerophospholipid classes of phosphatidylethanolamine (PE), phosphatidylserine (PS), and phosphatidylcholine (PC). Docosahexaenoic acid (22:6)[2] is the most abundant polyenoic fatty acid of the dipolyenoic species, which also contain tetraenoic and pentaenoic fatty acids with chain lengths of 20, 22, or 24 carbons. In addition, ROS phospholipids contain large amounts of 18:0–22:6, 16:0–22:6, and 18:1–22:6.

The outer segments of retinal rod photoreceptor cells are dynamic structures whose components are constantly being renewed. The tips of ROS are shed periodically (Young, 1971) and are phagocytized and digested by retinal pigment epithelial cells (Young & Bok, 1969). To compensate for this regular loss of membranes, newly synthesized components are incorporated into the base of the outer segments (Hall et al., 1969). ROS renewal proceeds by two mechanisms: membrane replacement and molecular replacement (Young, 1976). Integral proteins are renewed by membrane replacement. Newly synthesized proteins are transported from the site of synthesis in the inner segment to the apical region of the inner segment in vesicles which fuse with the plasma membrane (Papermaster et al., 1979). Proteins destined for the outer segment diffuse into the plasma membrane of the outer segment. As the total mass of newly added membrane increases, the plasma membrane invaginates to produce a stack of basal enfoldings, the most distal of which eventually pinch off from the plasma membrane and become rod outer segment disks (Steinberg et al., 1980). These disks are not continuous with the plasma membrane but are ordered through interactions with some cytoskeletal structures (Roof & Heuser, 1982). Integral proteins such as rhodopsin, the visual photopigment of rod

† This work was supported by NIH Grants EY04149, EY00871, and EY07001 and by a grant from Research to Prevent Blindness, Inc.

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[1] Abbreviations: ROS, rod outer segment(s); PC, phosphatidylcholine; PE, phosphatidylethanolamine; PS, phosphatidylserine; BHT, butylated hydroxytoluene; TLC, thin-layer chromatography; HPLC, high-performance liquid chromatography; GLC, gas–liquid chromatography.

[2] The fatty acids are designated by number of carbon atoms: number of double bonds.

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outer segments, remain in the original disk in which they were incorporated until the ROS tips are shed (Hall et al., 1969). Complete renewal of ROS integral proteins in mammals occurs over a period of 9–10 days (Young, 1976). In poikilotherms, however, the renewal rate is dependent on body temperature. In frogs maintained at 25 °C, complete renewal of ROS integral proteins occurs over about a 40-day time period (Anderson et al., 1980b). Soluble and perhaps peripheral proteins are renewed by molecular replacement, in which newly synthesized, soluble materials are diffusely incorporated into the cytoplasmic compartment of the outer segments (Bok & Young, 1972). There are no published renewal rates for these ROS constituents.

Lipids may be renewed by both membrane and molecular replacement (Bibb & Young, 1974a,b). In membrane replacement, newly synthesized lipids are transported in vesicles that are incorporated as a unit into the growing plasma membrane of the outer segment. Unlike rhodopsin, however, these membrane phospholipids undergo rapid interdisk exchange, so that, shortly after incorporation of radioactive phospholipid into the basal disks, the label becomes diffusely distributed throughout the entire outer segment (Anderson et al., 1980a–d). These studies indicated that the turnover of individual phospholipid classes (PI, PS, PC, and PE) occurred faster than could be explained on the basis of renewal only by membrane replacement. Therefore, molecular replacement of phospholipids or their individual components must also occur in these membranes, perhaps through phospholipid exchange proteins, which have been identified in bovine retina (Dudley & Anderson, 1978).

The renewal of ROS integral proteins (Young, 1976) and phospholipids (Bibb & Young, 1974a,b; Fliesler & Anderson, 1983) has been described in some detail. However, the metabolism of the molecular species of ROS membrane phospholipids has not been studied. In this paper, we have utilized the chromophoric benzoate derivatives of diradylglycerols to study the synthesis and turnover of the diacylglycerol molecular species derived from PC, PE, and PS. Our study provides evidence for different rates of incorporation and turnover of the docosahexaenoate molecular species of these phospholipids in frog ROS.

Materials and Methods

Materials. Isoproterenol and *d*-tubocurarine chloride (3 mg/mL) were purchased from Sigma Chemical Co. (St. Louis, MO) and Eli Lilly Co. (Indianapolis, IN), respectively. [2-^{3}H]Glycerol (200 mCi/mmol) was obtained from ICN Radiochemicals (Irvine, CA), and the radioactive samples were counted in ACS purchased from Amersham (Arlington Heights, IL). Phospholipase C (grade I, *Bacillus cereus*) was purchased from Boehringer Mannheim (Indianapolis, IN). Benzoic anhydride and 4-(dimethylamino)pyridine were from Aldrich Chemical Co. (Milwaukee, WI). 1,2-Diolein and other 1,2-diacylglycerols from Serdary Research Laboratory (London, Ontario, Canada) were derivatized with benzoic anhydride and used as the benzoate standards. All other chemicals were reagent grade or better.

Animals. *Rana pipiens* obtained from West Jersey Biological (Wenonah, NJ) were maintained for at least 2 weeks in a controlled-temperature incubator (25 °C) on a diurnal light cycle (12 h light/12 h dark) under fluorescent illumination (Vita-Lite, Duro-Test Corp.) of 10–15 ft-c at the side of the clear plastic cage nearest the light. The cages, covered with window screen, were placed on a slant so that about half of the bottom was covered with water. Water within the cages was changed daily. Frogs were fed crickets weekly throughout the course of the study. At the beginning of the experiment, groups of frogs weighing 30–40 g were immobilized with an injection of *d*-tubocurarine chloride (9 μg/g body weight) into the dorsal lymph sac. Pupil dilation was achieved by injecting isoproterenol (1 nmol/g body weight) in the same manner. Frogs were injected intravitreally through the pars plana of both eyes with 40 μCi of [2-^{3}H]glycerol (5 μCi/μL in sterile aqueous solution), allowed to recover from immobilization, and returned to the metabolic incubator for the duration of the experiment.

Preparation of Rod Outer Segments. The method for preparing retinal rod outer segments (ROS) was a modification (Wiegand & Anderson, 1983) of the discontinuous sucrose gradient procedure originally described by Papermaster and Dreyer (1974). Briefly, frogs were decapitated and pithed, and the eyes were enucleated and hemisected. The retinas, containing some pigment epithelium plus choroid, were dissected from the eyecup and gently homogenized in 4 mL of 1.175 g/mL sucrose buffered with 7 mM Tris–acetate (pH 7.4) containing 70 mM NaCl and 0.7 mM $MgCl_2$ by five strokes of a Teflon pestle in a glass homogenization tube. Clearance between pestle and tube was 0.10–0.15 mm. The homogenate was transferred to a 17-mL Ultra-Clear centrifuge tube (Beckman, 16 × 102 mm) and overlaid sequentially with 4 mL of d = 1.155 g/mL sucrose, 5 mL of d = 1.135 g/mL sucrose, and 4 mL of d = 1.115 g/mL sucrose. These latter three sucrose solutions were buffered with 1 mM Tris–acetate (pH 7.4) containing 0.1 mM $MgCl_2$. ROS were separated by centrifugation at 82000g for 60 min at 4 °C in a Sorvall OTD-65 ultracentrifuge equipped with an AH627 rotor. The ROS layer at the d = 1.115–1.135 g/mL interface was removed and diluted with 3–4 volumes of 50 mM Tris–acetate (pH 7.4) containing 5 mM $MgCl_2$ and 0.1 mM EDTA. The ROS membranes were pelleted at 27000g for 20 min and washed once with the same buffer. All procedures including dissections were carried out under dim ambient light. Recovery of ROS membranes was greater than 90%. Polyacrylamide gel electrophoresis of the ROS fraction indicated 90–95% of the total protein to be opsin.

Extraction of Lipids and Separation into Phospholipid Classes. Lipids from ROS were extracted according to the procedure described by Bligh and Dyer (1959). The lipid extract was dried under argon at room temperature and made to a known volume with chloroform. Aliquots were removed for lipid phosphorus assay (Rouser et al., 1970). An aliquot of the phosphorus solution was subsequently taken for determination of radioactivity as described by Anderson et al. (1980d).

The remaining lipid extract of 200–300 μg of phospholipid was resolved into phospholipid classes by two-dimensional TLC on silica gel HR (Anderson et al., 1969). Individual phospholipids were visualized by spraying the chromatoplate with aqueous 0.01% rhodamine 6G. Regions on the chromatoplate corresponding to PC, PE, and PS were scraped from the plate, and BHT (250 μg) was added to the scrapings. The phospholipid classes were then extracted from the gel scrapings with two washings of 5 mL of chloroform–methanol (1:1 v/v), followed by one wash with 5 mL of chloroform–methanol–water (65:45:12 by volume) and once more with 5 mL of chloroform–methanol (1:1 v/v). The combined washes were subsequently evaporated under an inert atmosphere of argon on a vacuum rotoevaporator.

Preparation of Benzoate Derivatives. The individual phospholipid classes were hydrolyzed with phospholipase C by a modification of the method of Takamura et al. (1986).

The purified phospholipid class was dispersed by bath sonication in 1.25 mL of 10 mM Tris-HCl (pH 7.5) containing 30 mM H_3BO_3 and 10 mM $CaCl_2$. Sixty units of phospholipase C and 2 mL of diethyl ether were added to the mixture and vortexed. Complete hydrolysis was achieved by maintaining the suspension overnight at room temperature under constant agitation by magnetic stirring. Hydrolysis at higher temperatures was avoided, since polyunsaturated fatty acids are susceptible to oxidative degradation. The ether layer containing the released 1,2-diradylglycerols (DGs) was removed, and the aqueous phase was extracted twice with diethyl ether. The combined ether extracts were dried under argon.

Benzoate derivatives of the DGs were prepared immediately by a modification of the procedure described by Blank et al. (1984). The DGs were dissolved in 0.25 mL of benzene containing 8 mg of benzoic anhydride and 3 mg of 4-(dimethylamino)pyridine and allowed to stand at room temperature for 2 h. The samples were placed in an ice bath, and 1 mL of concentrated NH_4OH was added slowly. The benzoate derivatives were extracted 3 times with 2 mL of hexane. The diradylglycerobenzoates were separated into subclasses (alkenylacyl, alkylacyl, and diacyl) by TLC on silica gel HR in a solvent system of toluene–hexane–diethyl ether (50:45:5 by volume). After solvent development, the chromatoplate was sprayed with 0.05% 2′,7′-dichlorofluorescein in 75% aqueous methanol, and the diradylglycerobenzoate subclasses were visualized by UV light. The diacylglycerobenzoate (DGBz) fraction was scraped from the plate into 2 mL of ethanol and mixed vigorously; 2 mL of water was added and the suspension extracted 3 times with 2 mL of hexane. The solvent was evaporated under argon, the DGBzs were redissolved in a known volume of hexane, and an aliquot was taken for determination of radioactivity.

HPLC of Diacylglycerobenzoates. Separation of the DGBz molecular species was accomplished with a HPLC system comprised of a Kontron Model T-414 pump, a Valco Model SV-7 injector, a Spherisorb ODS II column (5 μm, 25 cm × 4.6 mm i.d.), and a Kratos Spectraflow 773 absorbance detector. DGBzs were diluted with the elution solvent acetonitrile–2-propanol (70:30 v/v), and molecular species were separated by isocratic elution at a flow rate of 0.5 mL/min. Molecular species were detected at 230 nm, and the UV detector signal was fed into a Spectra-Physics 4270 computing integrator. Peaks corresponding to the eluted species were collected in scintillation vials, the solvent was evaporated, and each vial was counted for radioactivity. Identification of the molecular species of the DGBz in each collected peak was confirmed by HPLC analysis of DGBz standards and by GLC analysis of the fatty acid methyl esters of each peak (Wiegand & Anderson, 1982).

Molecular Species Quantitation by HPLC. Molar amounts of molecular species eluting from the HPLC column were quantitated on the basis of the linear relationship between area units obtained from the computing integrator and the mass of 1,2-dioleylbenzoate standards injected on the column. An appropriate mass of purified 1,2-dioleylbenzoate was dissolved in acetonitrile–2-propanol (70:30 v/v) and the absorbance determined at 230 nm. The concentration of the benzoate standard was calculated by using the molar absorptivity value, 1.317×10^4 (Blank et al., 1984). The concentration of the 1,2-dioleylbenzoate standard was also determined by GLC analysis of the oleyl esters in the presence of an internal standard (heneicosanoic acid, 21:0).

Known amounts of 1,2-dioleylbenzoate (0.5–20 nmol) were injected on the column, and the integrator response was determined at 230 nm. The integrator area units were proportional to the amounts of dioleylbenzoate injected on the column. In addition, the integrator area units were found to be inversely proportional to and exact multiples of the absorbance unit limits preset on the detector. A regression line was fitted for the data ($r^2 = 0.999$) and the resulting slope (integrator area units per nanomole) calculated and used to quantify the molecular species collected for radioactivity determination.

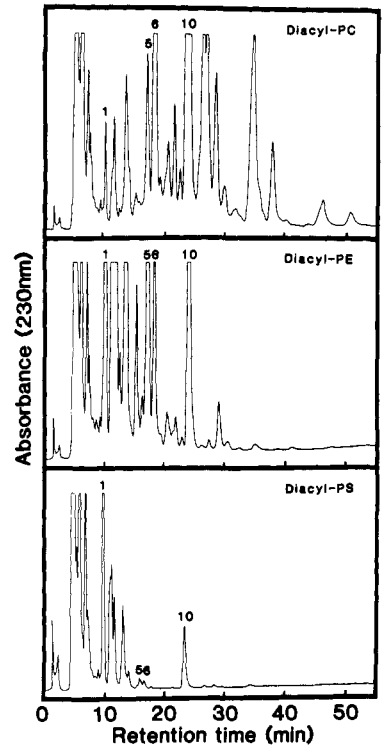

FIGURE 1: HPLC elution profile of the diacylglycerobenzoate species derived from phosphatidylcholine (diacyl-PC), phosphatidylethanolamine (diacyl-PE), and phosphatidylserine (diacyl-PS). Benzoate derivatives, obtained as described under Materials and Methods, were injected onto a reverse-phase HPLC column. The integrator response was determined at 230 nm. Peaks were identified as described in the text. Peak 1, 22:6–22:6; peak 5, 18:1–22:6; peak 6, 16:0–22:6; and peak 10, 18:0–22:6.

RESULTS

Molecular Species Composition of ROS Glycerophospholipids. ROS phospholipids were converted to benzoate derivatives and separated by TLC into diacylglycerobenozates and ether-linked glycerobenzoates. Ether-linked phosphoglycerols account for less than 1 mol% of the total molecular species of frog ROS (Anderson & Risk, 1974), and their benzoate derivatives were found to contain little or no radioactivity. Therefore, no attempt was made to quantitate them in the present study. Figure 1 shows a typical HPLC elution profile of the DGBz species derived from PC, PE, and PS. Reverse-phase HPLC had resolved the DGBz with respect to number of double bonds and length of the carbon chain. In general, the more unsaturated species elute with the shortest retention times, while the more saturated species elute with the longest retention times.

GLC analysis of the fatty acid methyl esters of peak 1 for the three glycerophospholipid classes (Figure 1) showed that 22:6 contributed over 95 mol % of the total fatty acids, allowing identification of peak 1 as 22:6–22:6. Similar analysis of peak 10 showed 18:0 and 22:6 to be 48 and 45 mol %, respectively, establishing 18:0–22:6 as the molecular species

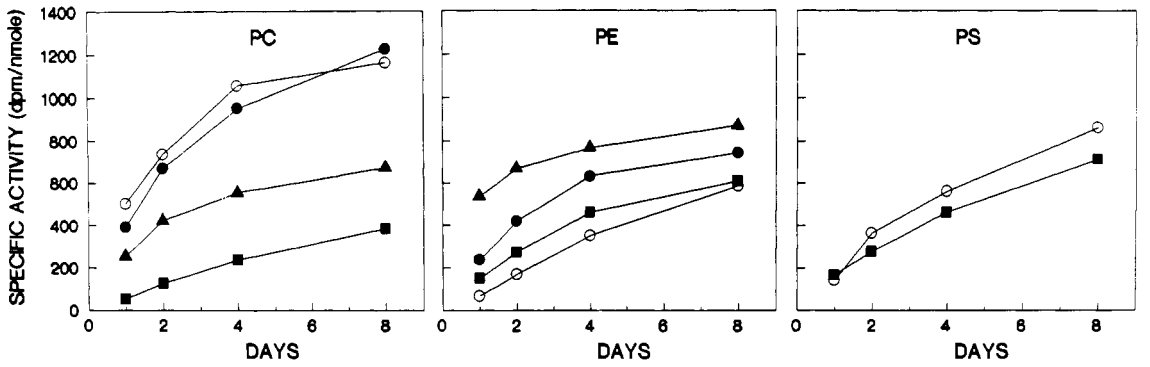

FIGURE 2: Time course of incorporation of [2-^{3}H]glycerol into docosahexaenoate-containing species of phosphatidylcholine (PC), phosphatidylethanolamine (PE), and phosphatidylserine (PS). Diacylglycerobenzoates of each phospholipid class were fractionated into molecular species by HPLC, quantitated, collected, and counted for radioactivity as described under Materials and Methods and in the text. Specific activities (dpm per nanomole) of the docosahexaenoate-containing species were determined. The number of samples per time point varied from 4 to 11 individual determinations. (○) 22:6–22:6; (●) 18:1–22:6; (▲) 16:0–22:6; (■) 18:0–22:6.

Table I: Distribution of Docosahexaenoate Molecular Species in Diacylphospholipids of Frog Retinal Rod Outer Segments[a]

		mol %		
peak[b]	species[c]	PC (48.6 ± 2.5)[d]	PE (39.4 ± 1.0)	PS (10.7 ± 1.4)
1	22:6–22:6	3.8 ± 0.9	27.2 ± 4.9	42.9 ± 7.4
5	18:1–22:6	3.3 ± 0.2	10.2 ± 1.2	0.8 ± 0.3
6	16:0–22:6	20.9 ± 3.9	5.7 ± 0.5	0.4 ± 0.2
10	18:0–22:6	39.1 ± 3.8	13.1 ± 0.8	11.5 ± 1.7
	others	32.9 ± 3.4	43.8 ± 2.5	44.4 ± 4.5

[a] Molecular species of the phospholipid classes were analyzed by HPLC as described under Materials and Methods. Values represent the average of eight individual rod outer segment preparations and are reported as mole percent of the total phospholipid class. [b] Peak number corresponds to the peak labeled in the HPLC elution profile of Figure 1. [c] The first number denotes the carbon chain length and the second the number of double bonds in the aliphatic chain. The sequence of numbers in the symbols is not meant to designate the positional distribution of the aliphatic chains. [d] Value in parentheses for each phospholipid class is reported as mole percent of the total phospholipid fraction.

of this peak. Peaks 5 and 6 were likewise identified as 18:1–22:6 and 16:0–22:6, respectively. Species eluting between peaks 1 and 5 account for the remainder of the dipolyenoic species. These dipolyenoic species contain 22:6 and 20-, 22-, or 24-carbon polyenoic acids having 4, 5, and/or 6 double bonds. Acyl chain analysis of the other peaks revealed multiplicity of coeluting molecular species. Only those four molecular species containing 22:6 that could be obtained in pure form from the HPLC column were used for determination of specific radioactivity.

Quantitation of four docosahexaenoate-containing species of diacyl-PC, -PE, and -PS is shown in Table I. The species distributions were markedly different for each phospholipid class. Diacyl-PC species were considerably more saturated than the species of diacyl-PE or diacyl-PS. The saturated docosahexaenoate species, 16:0–22:6 and 18:0–22:6, comprised almost 60 mol % of the species of diacyl-PC, compared to less than 4 mol % for 22:6–22:6 and slightly greater than 3 mol % for the monoenoic species 18:1–22:6. Tetraenoic, dienoic, monoenoic, and saturated species comprise at least 20 mol % of the diacyl-PC species (data not shown). Diacyl-PE contains a much higher proportion of the dipolyenoic species than diacyl-PC. The major molecular species of diacyl-PE is 22:6–22:6, comprising 27.2 mol %. The saturated docosahexaenoate species, 16:0–22:6 and 18:0–22:6, represent 5.7 and 13.1 mol %, respectively, of the diacyl-PE species, while the monoenoic docosahexaenoate species, 18:1–22:6, comprises 10.2 mol %. Compared to diacyl-PC or diacyl-PE, diacyl-PS contains the highest percentage of dipolyenoic species, accounting for over 80% of the PS species (data not shown). The major species 22:6–22:6 and the species 18:0–22:6 comprise 42.9 and 11.5 mol %, respectively, of the total molecular species in PS. The values for species of PC and PE agree with those we have previously published for frog ROS (Wiegand & Anderson, 1983), which were determined by argentation chromatography and methyl ester analysis of diacylglycerol acetate derivatives of these phospholipids.

Incorporation of [2-^{3}H]Glycerol into Glycerophospholipids. DGBzs of each ROS phospholipid class were fractionated into molecular species by HPLC, quantitated, collected, and counted for radioactivity. The specific activity (dpm per nanomole) of each species was determined. Figure 2 shows the time course of incorporation of [2-^{3}H]glycerol into docosahexaenoate-containing species of diacyl-PC, -PE, and -PS. Specific activities of these species plotted against days postinjection reveal striking differences in the rates of incorporation for species within individual phospholipid classes. In general, reduction in the rate of incorporation for most species occurred after the fourth day postinjection. During days 1 through 4, the diacyl-PC species 22:6–22:6 and 18:1–22:6 exhibited specific activities 3–5 times greater than those of 16:0–22:6-PC or 18:0–22:6-PC. In contrast, the specific activity of the 22:6–22:6-PE was 2–5 times less than that of 16:0–22:6-PE and 18:1–22:6-PE. The major saturated docosahexaenoate species of diacyl-PS incorporating a significant amount of [2-^{3}H]glycerol was 18:0–22:6. Specific activities of 18:0–22:6-PS and 22:6–22:6-PS were similar up through the eighth day postinjection.

There were appreciable differences among the three phospholipid classes in specific activities of species containing the same fatty acids. The specific activities for 22:6–22:6 were higher in diacyl-PC than in diacyl-PE or -PS. The species 22:6–22:6-PS had higher specific activities than 22:6–22:6-PE. On the other hand, the specific activities of 18:0–22:6-PC were 2–3 times lower than the corresponding species of PE or PS. However, there appeared to be no differences in the specific activities of 18:0–22:6-PE and 18:0–22:6-PS. The specific activities of 16:0–22:6-PE were about twice that of 16:0–22:6-PC, while the 18:1–22:6 species had a higher specific activity in PC than in PE. The small amounts of 18:1–22:6 and 16:0–22:6 in PS precluded an accurate mass determination, so their specific activities were not included in Figure 2.

Turnover of Molecular Species. Specific activities of the molecular species of PC, PE, and PS began approximating an exponential decline 6–8 days postinjection (Figure 3). The rate of this decline is represented by the half-life of each

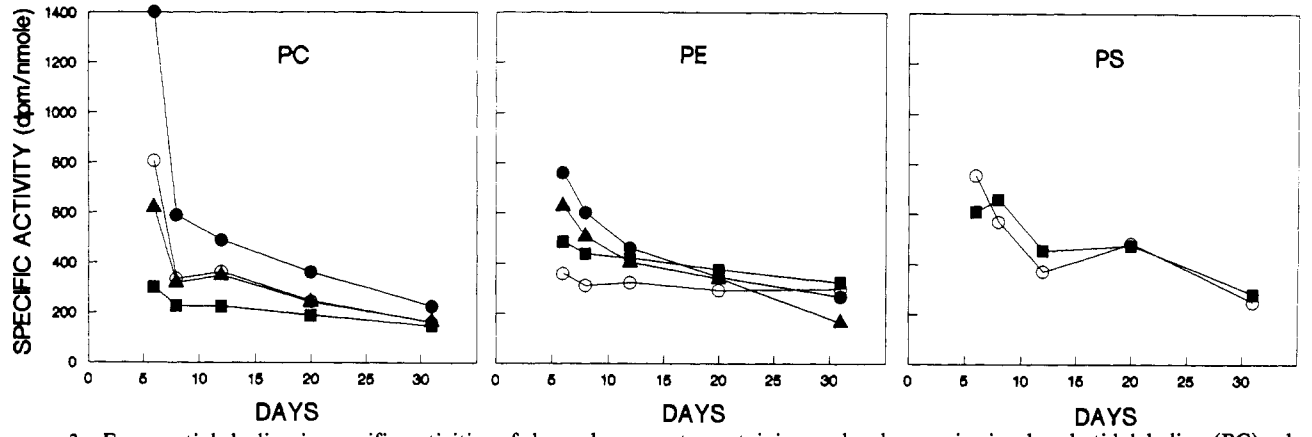

FIGURE 3: Exponential decline in specific activities of docosahexaenoate-containing molecular species in phosphatidylcholine (PC), phosphatidylethanolamine (PE), and phosphatidylserine (PS). The protocol was as described under Figure 2. The number of independent samples varied from 6 to 11 per time point. (○) 22:6–22:6; (●) 18:1–22:6; (▲) 16:0–22:6; (■) 18:0–22:6.

Table II: Calculated Half-Life of Docosahexaenoate Molecular Species in Diacylphospholipids of Frog Retinal Rod Outer Segments

		half-life (days)[a]		
peak	species	PC	PE	PS
1	22:6–22:6	12.3	32.7	14.5
5	18:1–22:6	10.2	13.1	*b*
6	16:0–22:6	12.0	17.0	*b*
10	18:0–22:6	17.8	30.6	18.7

[a] Half-life (days) = log 2.0/m, where m = the slope of the regression line, calculated from the linear regression analysis of log specific activity versus time for 6, 8, 10, 12, 20, and 31 days postinjection. [b] The small amount of these species precluded accurate mass determination.

molecular species (Table II), calculated from a linear regression analysis of log specific activity versus time for 6, 8, 10, 12, 20, and 31 days postinjection. In PC, the species 22:6–22:6, 18:1–22:6, and 16:0–22:6, which have half-lives of 10–12 days, turned over at similar rates but were 1.5 times faster than 18:0–22:6. For PE, 18:1–22:6 and 16:0–22:6 (half-lives of 13–17 days) had similar turnover rates, which were 2–3 times faster than 22:6–22:6 and 18:0–22:6. In PS, turnover of 22:6–22:6 and 18:0–22:6 had half-lives of 15–19 days. The diacyl-PE species 22:6–22:6 and 18:0–22:6 showed a 2–3 times slower turnover rate than the corresponding species in PC and PS, while 18:1–22:6 and 16:0–22:6 species had similar half-lives in PE and PC.

The differences in synthesis and turnover rates were reflected in the relative distributions of radioactivities of these species within the three phospholipid classes when compared to their relative mass distributions (Table III). Variations in the distribution of label, indicating differences in the rates of incorporation, were already apparent on day 1. Higher values for percent dpm compared to mole percent reflect the perferential incorporation of label into that molecular species. For example, in PC, 22:6–22:6 had 16% of the total label at day 1, although its mass accounted for only 5% of the PC molecules. Likewise, 16:0–22:6 had 47% of the label in 28% of the molecular mass. The species 18:0–22:6-PC, which makes up 61% of the mass, had only 23% of the label. In PE, 22:6–22:6, which comprises 40% of the lipid mass, had only 15% of the label. Other, less dramatic, differences were observed between the percent dpm and mole percent distributions at day 1. With the onset of the decline in specific activities on the sixth to eighth day, the incorporated label began to show some redistribution among the species, while, as expected, the relative mass of each molecular species remained the same. By day 31, the distribution of label in each species approximated the steady-state level of its mass distribution (Table III). The only exceptions were 18:0–22:6-PE, 22:6–22:6-PS, and 18:0–22:6-PS. These three species had maintained a constant distribution of label over the entire time interval.

Discussion

In the present study, we have examined the incorporation and turnover in ROS disk membranes of newly synthesized phospholipid molecular species. These species are particularly interesting because they contain one or two molecules of the long-chain, polyunsaturated fatty acid docosahexaenoic acid, which is the major fatty acid in these membrane phospholipids. Following injection of labeled glycerol, phospholipids synthesized in the inner segment were rapidly incorporated into

Table III: Comparison of dpm % and mol % of Docosahexaenoate Molecular Species in Diacylphospholipids of Frog Retinal Rod Outer Segments

		day									
		1 (n = 4)[a]		4 (n = 5)		8 (n = 11)		12 (n = 6)		31 (n = 6)	
class	species	dpm %[b]	mol %[c]	dpm %	mol %	dpm %	mol %	dpm %	mol %	dpm %	mol %
PC	22:6–22:6	15.6	5.0	9.3	3.5	7.9	4.3	5.4	4.1	4.7	2.8
	18:1–22:6	14.3	5.6	11.2	5.1	9.8	5.0	9.2	5.0	8.0	4.6
	16:0–22:6	47.4	28.4	44.4	31.8	40.7	34.8	39.6	31.9	29.3	27.5
	18:0–22:6	22.7	61.0	35.1	59.6	41.6	55.8	45.8	58.9	57.9	65.2
PE	22:6–22:6	15.3	40.4	30.0	40.9	36.6	43.2	33.0	46.1	38.4	44.1
	18:1–22:6	29.3	21.6	26.0	19.7	23.7	20.3	24.9	19.4	21.4	21.6
	16:0–22:6	32.3	10.6	18.7	11.9	14.5	10.8	13.4	11.1	11.5	10.4
	18:0–22:6	23.1	27.4	25.3	27.4	25.2	25.7	28.6	23.3	28.6	23.9
PS	22:6–22:6	69.8	71.8	79.3	75.5	77.8	75.1	77.4	80.2	65.2	76.3
	18:0–22:6	30.2	28.2	20.7	24.5	22.2	24.9	22.6	19.8	34.8	23.7

[a] Values in parentheses denote the number of independent determinations per time point. [b] Normalized for four species in PC and PE and two species in PS. [c] Normalized for four species in PC and PE and two species in PS.

the outer segment. The specific radioactivity increased over a 6–8-day period and then began approximating an exponential decline.

Initially (Figure 2), there were marked differences between the specific activities of the different molecular species of PC and PE. Assuming that these phospholipids arose from the same glycerol 3-phosphate pool through a diacylglycerol intermediate, these results indicate that the molecular species of PE and PC are not synthesized in the same proportions in which they occur in the outer segments. In addition, each molecular species of PC and PE appears to turn over independently and at different rates (Figure 3 and Table II). Indeed, the same molecular species 22:6–22:6 has a different half-life in PE and PC, indicating a selectivity in the turnover of certain molecular species in these phospholipid classes. Such selectivity has been reported by Robinson et al. (1986) in rat erythrocytes. Their studies demonstrated a selective incorporation of arachidonic acid (20:4) into the most highly unsaturated molecular species in all phospholipid classes examined. They also showed the presence of unique populations of highly unsaturated phospholipids that exhibit very rapid turnover.

At the early time points, the docosahexaenoate species in PC and PE had different specific radioactivities within each phospholipid class and among the same species in different phospholipid classes. However, they all subsequently achieved more or less the same specific radioactivities. At 31 days postinjection, the percent distributions of the radioactivities (Table III) approximated the steady-state mass compositions of the molecular species. This indicates acyl exchange within the same phospholipid class by which the radioactivity in the glycerol "backbone" becomes redistributed among the various molecular species. Such acyl exchange activities in ROS have been reported by Zimmerman and Keys (1988). However, these acyl exchange reactions do not mean that these polyunsaturated fatty acids are incorporated into the phospholipids after the lipid has been delivered to the ROS. Indeed, our results clearly show that this acid is added prior to the incorporation of the phospholipid into the ROS. Evidence for this comes from the following observation. The species 22:6–22:6-PC in ROS has the highest specific radioactivity of any PC molecular species, including 16:0–18:1-PC and 16:0–16:0-PC. The latter two species are quantifiable in PC, and their specific activities were measured in each experiment but were not reported. If 22:6–22:6-PC were synthesized in ROS by acyl exchange with any other molecular species of PC, then it would have been impossible for it to achieve a higher specific activity than its precursor. Whether 22:6 is added during de novo synthesis of PA in the inner segment or after the formation of the product phospholipid is not known.

The steady-state composition of the molecular species of PC and PE in ROS is determined by a variety of factors, which include rates of biosynthesis, rates of turnover, and rates of interconversion. Our use of glycerol as a precursor does not allow us to examine this latter possibility, but previous studies (Anderson et al., 1980a,b) have demonstrated PS decarboxylation to PE and PE transmethylation to PC. Although the cellular sites of these interconversions were not determined, substantial labeling of PE occurs in frog ROS following injection of labeled serine, and substantial labeling of PC occurs following injection of labeled ethanolamine. In addition to these unidirectional interconversions, base exchange enzymes have been reported in retina (Mizuno, 1976; Anderson & Kelleher, 1981).

Unlike what occurs in PC and PE, the steady-state composition of two major molecular species of PS in ROS is apparently determined by their rate of synthesis. The specific activities of 22:6–22:6-PS and 18:0–22:6-PS (combined total greater than 50 mol % of total PS) were the same during their incorporation into the outer segment, and their turnover rate was the same. The percent dpm in these two species was identical with their steady-state composition at all time points examined. Thus, it appears that the immediate precursor of PS contains the exact proportion of the two major docosahexaenoate-containing species that will be present in the steady state and that these docosahexaenoyl species are synthesized de novo in the proportions in which they will occur in the outer segment. This indicates that the PS present in outer segments probably does not undergo molecular remodeling. The immediate precursor of PS is not known, although it is commonly accepted that PS is synthesized by base exchange reactions with PE and PC. If this is indeed the origin of PS in ROS, one would expect the donor phospholipid molecular species to have a higher specific radioactivity than the products. Since the specific radioactivities of the two species (18:0–22:6 and 22:6–22:6) in PS are higher than the corresponding species in PE, then the PE in ROS cannot be the precursor of PS. Also, the relative low specific activity of 18:0–22:6-PC eliminates it as a possible precursor of 18:0–22:6-PS. It is possible that the base exchange reactions that form PS took place in the inner segment. Bovine retinal microsomes have been shown to have relatively high activities of calcium-sensitive, ATP-independent base exchange activities (Anderson & Kelleher, 1981). Another possible source of the PS is direct formation from phosphatidic acid, reported by Pullarkat et al. (1981) in rat brain microsomes.

The results of our study indicate that the three major phospholipid classes of ROS membranes contain separate populations of molecular species with different rates of biosynthesis, incorporation, and turnover. At day 1, the specific activities of the docosahexaenoate-containing molecular species in ROS showed marked differences. By day 31, however, they all had achieved similar specific activities, and the percent distribution of radioactivity had approximated the steady-state mass composition of the molecular species. This would indicate extensive modifications of the molecular species in the ROS through acyl exchange reactions. However, as discussed above, these reactions would not be responsible for the initial incorporation of docosahexaenoic acid into phospholipids destined for delivery to the ROS. A problem that remains to be addressed is the stage of lipid biosynthesis during which the polyunsaturated fatty acids are incorporated into the phospholipids. This could happen during de novo synthesis of PA or after PC, PE, and PS have been formed. Further investigations of the metabolism of these molecular species in specific retinal membrane fractions at earlier time points could provide answers to this question.

Registry No. 22:6–22:6PC, 117180-33-3; 22:6–22:6PE, 117179-04-1; 22:6–22:6PS, 117179-06-3; 18:1–22:6PC, 117179-09-6; 18:1–22:6PE, 117180-34-4; 18:1–22:6PS, 117179-10-9; 16:0–22:6PC, 114818-74-5; 16:0–22:6PE, 117179-11-0; 16:0–22:6PS, 117179-12-1; 18:0–22:6PC, 117179-07-4; 18:0–22:6PE, 117179-08-5; 18:0–22:6PS, 107793-81-7.

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Investigation into the Nature of Substrate Binding to the Dipyrromethane Cofactor of *Escherichia coli* Porphobilinogen Deaminase

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Received May 6, 1988; Revised Manuscript Received July 14, 1988

ABSTRACT: The formation of the dipyrromethane cofactor of *Escherichia coli* porphobilinogen deaminase was shown to depend on the presence of 5-aminolevulinic acid. A *hemA*$^-$ mutant formed inactive deaminase when grown in the absence of 5-aminolevulinic acid since this strain was unable to biosynthesize the dipyrromethane cofactor. The mutant formed normal levels of deaminase, however, when grown in the presence of 5-aminolevulinic acid. Porphobilinogen, the substrate, interacts with the free α-position of the dipyrromethane cofactor to give stable enzyme–intermediate complexes. Experiments with regiospecifically labeled intermediate complexes have shown that, in the absence of further substrate molecules, the complexes are interconvertible by the exchange of the terminal pyrrole ring of each complex. The formation of enzyme–intermediate complexes is accompanied by the exposure of a cysteine residue, suggesting that substantial conformational changes occur on binding substrate. Specific labeling of the dipyrromethane cofactor by growth of the *E. coli* in the presence of 5-amino[5-^{14}C]levulinic acid has confirmed that the cofactor is not subject to catalytic turnover. Experiments with the α-substituted substrate analogue α-bromoporphobilinogen have provided further evidence that the cofactor is responsible for the covalent binding of the substrate at the catalytic site. On the basis of these cummulative findings, it has been possible to construct a mechanistic scheme for the deaminase reaction involving a single catalytic site which is able to catalyze the addition or removal of either NH_3 or H_2O. The role of the cofactor both as a primer and as a means for regulating the number of substrates bound in each catalytic cycle is discussed.

Porphobilinogen deaminase (hydroxymethylbilane synthase, EC 4.3.1.8) catalyzes the formation of the (hydroxymethyl)bilane preuroporphyrinogen from four molecules of porphobilinogen (Burton et al., 1979; Battersby et al., 1979a). Preuroporphyrinogen is further transformed into uroporphyrinogen III (Jordan et al., 1979) and then into all other tetrapyrroles (Scheme I). Porphobilinogen deaminases have been purified and characterized from a large number of sources

0006-2960/88/0427-9020$01.50/0