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DISATURATED AND DIPOLYUNSATURATED PHOSPHOLIPIDS IN THE BOVINE RETINAL ROD OUTER SEGMENT DISK MEMBRANE

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Summary

Thin-layer chromatography was used to separate the major phospholipid headgroup classes of the rod outer segment disk membrane into subfractions which differ markedly in fatty acid composition. At least 18% of the rod outer segment phosphatidylcholine must contain two saturated fatty acids. Furthermore, two unsaturated fatty acids are found in at least 43% of the phosphatidylserine, 24% of the phosphatidylcholine, and 24% of the phosphatidylethanolamine. The unsaturated acids are predominantly polyunsaturated in all cases. A similar separation, but with less resolution, was achieved with silicic acid column chromatography.

The temperature dependence of the polarization of the fluorescence of *trans*-parinaric acid (9,11,13,15-all-*trans*-octadecatetraenoic acid) showed that the thermal behavior of aqueous dispersions of the phosphatidylcholine subfractions was consistent with their fatty acid compositions.

Introduction

The large degree of molecular heterogeneity of phospholipids in biological membranes is well known. However, the detailed combinations of two fatty acids with each other and of these pairs with particular phospholipid headgroups are poorly documented. Fractionation and characterization of these combinations might provide clues for understanding the functional role(s) of

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this molecular polydispersity and should also suggest more relevant model membrane systems for physical studies. In addition, preparative fractionation of various phospholipid species with different fatty acid compositions from natural sources could provide material for model studies [1].

In this paper we report the separation, by a one-dimensional thin-layer chromatography (TLC) method, of the major phospholipid classes of the retinal rod outer segment membrane into subfractions which differ in fatty acid composition. Fatty acid analysis reveals the presence of saturated-saturated, saturated-polyunsaturated, and polyunsaturated-polyunsaturated species of outer segment phosphatidylcholine and phosphatidylethanolamine and saturated-polyunsaturated and polyunsaturated-polyunsaturated species of outer segment phosphatidylserine. * A similar separation can be achieved, with somewhat less resolution by silicic acid column chromatography. As a further characterization of this separation, we also show that the temperature dependence of the fluorescence polarization of *trans*-parinaric acid in aqueous dispersions of outer segment phosphatidylcholine subfractions is consistent with their fatty acid compositions.

Materials and Methods

Preparation of rod outer segments. Highly purified bovine outer segments were isolated by the method of Raubach et al. [2] except that the second sucrose density gradient was centrifuged for 90 min and all solutions contained 0.1 mM EDTA and 0.15 mM $CaCl_2$ to minimize oxidative damage [3].

Lipid extraction. Under the conditions employed for column chromatography we have found that retinal forms a Schiff's base with phosphatidylethanolamine and this complexation prevents complete separation of these two components. Conversion of retinal to retinaloxime eliminates this problem. Prior to lipid extraction for column chromatography, the outer segment membrane suspension was mixed with enough 0.1 M hydroxylamine (pH 7) to give a 100-fold molar excess of hydroxylamine over retinal and then exposed to an unfiltered 300 W floodlight while the sample tube was immersed in a large beaker of cold tap water. Retinaloxime appears to be unstable, so column fractionation was commenced immediately subsequent to lipid extraction. If the lipids were to be used solely for TLC the oximation step was unnecessary and was omitted. Total outer segment lipids were extracted by a modification of the procedure of Folch et al. [4]. The chloroform and methanol contained 50 mg/l butylated hydroxytoluene. In this and in the following methods, the chloroform and methanol were redistilled and were bubbled with argon immediately before use. Whenever feasible, manipulations were performed under an argon atmosphere.

Column chromatographic fractionation of phospholipid headgroup classes. The total lipids were first applied to a diethylaminoethyl-cellulose column prepared and eluted essentially by the procedure of Rouser et al. [5]. Elution with chloroform removed butylated hydroxytoluene, cholesterol, retinal,

* Positional analyses of the fatty acids in these subfractions were not performed. Therefore, the order of fatty acid types used in naming these phospholipid species is arbitrary.

retinaloxime, vitamin E, and free fatty acids. Elution with 1 : 1 chloroform/methanol removed phosphatidylcholine and phosphatidylethanolamine and glacial acetic acid removed pure phosphatidylserine. The phosphatidylcholine-phosphatidylethanolamine mixture was reduced to dryness by rotary evaporation, redissolved in chloroform and further fractionated on a silicic acid column (approx. 1 g silicic acid per 5 mg phospholipid).

Thin-layer chromatography. TLC of outer segment total lipid extract was performed on 5 cm by 20 cm plates coated with a 0.25 mm thick layer of silica gel H (EM Laboratories, Inc.). For identification of the various lipid species, authentic standards were cochromatographed with a few μg of outer segment lipids in chloroform/methanol/water (65 : 25 : 5, v/v) and 50 mg/l butylated hydroxytoluene. Standards used included bovine brain sphingomyelin (Applied Sciences Laboratories); lysophosphatidylcholine and lysophosphatidylethanolamine (Miles Laboratories); soybean phosphatidylinositol, dimyristoyl phosphatidylcholine, dipalmitoyl phosphatidylcholine, distearoyl phosphatidylcholine, dipalmitoylethanolamine, *N,N*-dimethyldipalmitoyl phosphatidylcholine, vitamin E, cholesterol, retinal, retinol, triolein and oleic acid (Sigma Chemical Co.); and highly purified bovine brain phosphatidylserine (a generous gift from Dr. D. Papahadjopoulos, University of California, San Francisco). 1-Palmitoyl-2-docosahexaenoyl phosphatidylcholine and didocosahexaenoyl phosphatidylcholine, synthesized as described below, were also used as standards. Chromatograms were visualized by either exposure to I_2 vapor, charring with 9 M H_2SO_4, or with 0.2% ninhydrin in ethanol.

The same TLC system was also used to isolate phospholipid subfractions for fatty acid and phosphorus analyses. Approx. 1.0—1.5 mg of outer segment total lipid in approx. 75 μl chloroform was applied to the TLC plate as a thin band 3 cm long and was developed in an argon-saturated jar.

Phosphorus and fatty acid analysis of the subfractions. The plate was dried quickly in a stream of argon and sprayed with 0.4% dichlorofluorescein in methanol under argon pressure. Lipid bands were visualized under ultraviolet light, marked with a pencil and quickly scraped with a razor blade into 10-ml screw-capped tubes containing 5 μg butylated hydroxytoluene and 14.7 μg heneicosanoic acid (21 : 0) (Supelco, Inc.) in 100 μl chloroform. Fatty acid methyl esters were formed under argon by a modification of the method of Morrison and Smith [6] and centrifuged at 3000 × *g* for 10 min to remove suspended silica gel. The fatty acid methyl esters (in the upper layer) were analyzed by gas-liquid chromatography (GLC) using a 6 ft glass column containing 10% SP-2330 on 100/120 Supelcoport (Supelco, Inc.) which was temperature programmed from 130 to 250°C at 4°C/min. Identification of peaks was made by comparison with reference methyl ester mixtures (Supelco, Inc.). The integrated area (obtained with an Autolab model 6300 digital integrator) of each peak was divided by the molecular weight of the corresponding methyl ester to yield relative molar concentrations. The lower phases from the transmethylation procedure were heated at 110°C for several hours until dry and subjected to phosphorus analysis by the method of Chen et al. [7] modified for smaller sample sizes.

Synthesis of palmitoyl-docosahexaenoyl phosphatidylcholine. 1-Palmitoyl-2-docosahexaenoyl phosphatidylcholine was synthesized by the method of

Evans and Tinoco [8]. TLC of the synthesized material showed two closely migrating bands. GLC analysis demonstrated that the main band, with a slightly smaller R_F, contained a 1 : 1 mol : mol ratio of palmitic and docosahexaenoic acids and the minor band, with a larger R_F, contained all docosahexaenoic acid. Phosphorus analysis showed this minor band to represent approx. 4 mol% of the total phospholipid. This component is didocosahexaenoyl phosphatidylcholine.

Polarization of fluorescence of trans-Parinaric acid. The temperature dependence of the polarization of the fluorescence of *trans*-parinaric acid (9,11,13,15-all-*trans*-octadecatetraenoic acid) in phospholipid dispersions was measured using a Hitachi-Perkin-Elmer MPF-2A spectrofluorometer as described previously [9].

Results

Fig. 1 shows a thin-layer chromatogram of outer segment total lipids co-chromatographed with four authentic standards. The phosphatidylserine region is separated into two spots, PS-1 and PS-2; the phosphatidylcholine region into five spots, PC-1 through PC-5; and the phosphatidylethanolamine region into three spots, PE-1, PE-2, and PE-3. Several pieces of evidence rule out significant contributions to any of these resolved spots by minor phospholipids. Treatment of an identical plate with ninhydrin showed that all spots attributed to phosphatidylserine and phosphatidylethanolamine contained primary amino groups. Authentic dipalmitoyl phosphatidylcholine, dipalmitoyl phosphatidylethanolamine and bovine brain phosphatidylserine co-migrated with PE-3, PC-5 and PS-2, respectively. Upon exposure to 9 M H_2SO_4 and heat, PC-5, PC-4 and PE-3 charred only weakly, in contrast to the dense black charring of the other outer segment phospholipid spots. Both authentic dipalmitoyl phosphatidylcholine and dipalmitoyl phosphatidylethanolamine also exhibited weak charring which is characteristic of very saturated compounds. No spot attributable to highly saturated phosphatidylserine was detected. Synthetic palmitoyl-docosahexaenoyl phosphatidylcholine containing a small amount of didocosahexaenoyl phosphatidylcholine co-migrated with PC-3 and PC-1, respectively. Bovine brain sphingomyelin and lysophosphatidylcholine standards (not shown) identified two very minor spots with R_F values smaller than the phosphatidylserine spots. Another spot, below these, was ninhydrin positive and may be lysophosphatidylserine. All three of these minor spots are not visible in Fig. 1 and can only be detected on plates with a heavy sample load. The phosphatidylinositol standard (not shown) co-migrated with PC-5, and lysophosphatidylethanolamine (also not shown) co-migrated with PS-1 and PS-2. Resolution of phosphatidylinositol and lysophosphatidylethanolamine, each into single spots, was obtained with a solvent system containing chloroform/methanol/acetic acid/water, v/v) (65 : 50 : 5 : 3, v/v). Phosphorus analysis showed that phosphatidylinositol constitutes less than 2% and lysophosphatidylethanolamine less than 0.2% of the total lipid phosphorus. These contributions to PC-5 and to PS-1 and PS-2 were ignored. The various neutral lipids were identified with the appropriate standards.

Tables I—III show the fatty content and phosphorus content of the TLC sub-

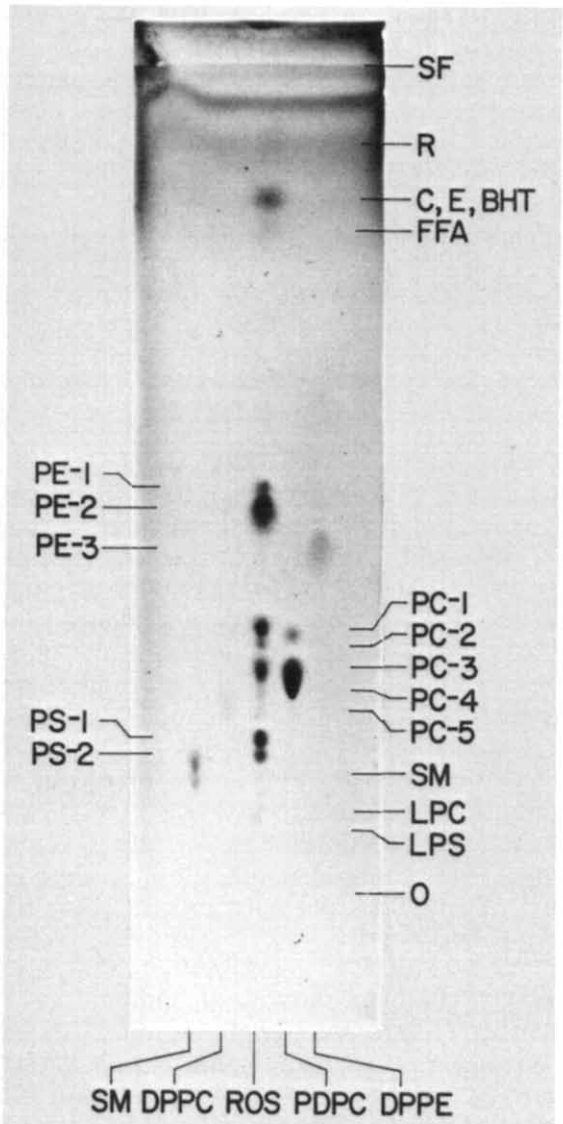

Fig. 1. A chromatogram of four authentic phospholipid standards and total lipid extract of rod outer segment disk membranes. A few μg of each sample were eluted with chloroform/methanol/water, (65 : 25 : 5, v/v). The chromatogram was visualized by charring at 150°C after spraying with 9 M H_2SO_4. From left to right are: bovine brain sphingomyelin (SM), dipalmitoyl phosphatidylcholine (DPPC), total rod outer segment lipids (ROS), palmitoyl-docosahexaenoyl phosphatidylcholine (PDPC) (containing approx. 4% didocosahexaenoyl phosphatidylcholine, DDPC), and dipalmitoyl phosphatidylethanolamine (DPPE). Other standards used for identification are listed in Materials and Methods. Labels on the left and right of the chromatogram refer to components of the outer segment lipid extract. They are from bottom to top: the origin (O), lysophosphatidylserine (LPS), lysophosphatidylcholine (LPC), sphingomyelin (SM), phosphatidylserine (PS-2 and PS-1), phosphatidylcholine (PC-5 through PC-1), phosphatidylethanolamine (PE-3 through PE-1), free fatty acids (FFA), cholesterol (C), vitamin E (E), butylated hydroxytoluene (BHT), retinal (R), and the solvent fron (SF). Outer segment lysophosphatidylserine, lysophosphatidylcholine and sphingomyelin are not visible in this chromatogram, but are clearly visible in chromatograms heavily loaded with sample.

TABLE I

FATTY ACID COMPOSITION OF ROD OUTER SEGMENT PHOSPHATIDYLSERINE SUBFRACTIONS *

Values are mol percentages and are given as the mean ± S.D. of three independent determinations.

	PS-1	PS-2
Phosphorus (%):	46.0 ± 5.4	54.0 ± 5.4
Saturated fatty acids		
16 : 0	2.0 ± 1.5	5.3 ± 0.6
18 : 0	5.7 ± 2.6	41.3 ± 2.2
other	tr	0.1 ± 0.1
Mean total ± S.D.	7.8 ± 3.9	46.7 ± 2.0
Unsaturated fatty acids		
18 : 1	0.7 ± 0.6	3.4 ± 1.0
18 : 2	0.1 ± 0.2	tr
20 : 4	0.7 ± 1.0	8.8 ± 1.7
22 : 4	3.6 ± 0.6	0.6 ± 0.5
22 : 5ω6	0.5 ± 0.5	0.2 ± 0.2
22 : 5ω3	5.0 ± 0.3	1.3 ± 0.4
22 : 6	56.1 ± 5.3	35.6 ± 1.6
24 : 4	6.2 ± 0.3	0.3 ± 0.3
24 : 5	17.3 ± 2.8	3.1 ± 3.8
other	1.9 ± 1.0	tr
Mean total ± S.D.	92.2 ± 3.9	53.3 ± 2.0

* See Fig. 3 for percentage of total phosphorus in each headgroup class.
tr, trace amount.

TABLE II

FATTY ACID COMPOSITION OF ROD OUTER SEGMENT PHOSPHATIDYLCHOLINE SUBFRACTIONS *

Values are mol percentages and are given as the mean ± S.D. of three independent determinations.

	PC-1 + PC-2	PC-3	PC-4 + PC-5
Phosphorus (%):	26.1 ± 2.1	43.5 ± 2.0	29.3 ± 2.3
Saturated fatty acids			
16 : 0	5.0 ± 1.5	19.0 ± 1.8	69.9 ± 4.0
18 : 0	2.0 ± 1.0	28.9 ± 0.9	8.6 ± 0.5
other	0.2 ± 0.4	0.5 ± 0.5	2.7 ± 0.5
Mean total ± S.D.	7.3 ± 1.0	48.5 ± 1.8	81.2 ± 3.5
Unsaturated fatty acids			
18 : 1	1.7 ± 0.4	2.9 ± 0.5	12.5 ± 1.5
18 : 2	0.2 ± 0.2	0.3 ± 0.2	1.0 ± 0.2
20 : 4	1.6 ± 0.9	4.9 ± 0.5	0.6 ± 0.2
22 : 4	0.1 ± 0.2	1.1 ± 1.4	tr
22 : 5ω6	0.3 ± 0.4	0.6 ± 0.5	tr
22 : 5ω3	1.0 ± 0.4	2.1 ± 0.3	tr
22 : 6	84.1 ± 1.8	38.2 ± 3.3	2.4 ± 0.5
24 : 4	0.7 ± 0.9	0.8 ± 0.7	0.2 ± 0.2
24 : 5	1.4 ± 0.6	0.4 ± 0.4	2.1 ± 1.8
other	1.4 ± 0.6	0.4 ± 0.4	2.1 ± 1.8
Mean total ± S.D.	93.1 ± 2.4	51.7 ± 2.0	18.7 ± 3.6

* See Fig. 3 for percentage of total phosphorus in each headgroup class.
tr, trace amount.

TABLE III

FATTY ACID COMPOSITION OF ROD OUTER SEGMENT PHOSPHATIDYLETHANOLAMINE SUBFRACTIONS *

Values are mol percentages and are given as the mean ± S.D. of four independent determinations for PE-1 and PE-2, and two independent determinations for PE-3.

	PE-1	PE-2	PE-3
Phosphorus (%):	10.6 ± 1.4	87.6 ± 1.7	2.5 ± 0.1
Saturated fatty acids			
16 : 0	2.8 ± 1.7	12.3 ± 0.4	47.0 ± 1.6
18 : 0	2.7 ± 1.5	27.7 ± 0.8	22.1 ± 4.1
other	0.1 ± 0.1	1.5 ± 1.3	0.2 ± 0.2
Mean total ± S.D.	5.6 ± 3.1	41.5 ± 1.5	69.3 ± 5.6
Unsaturated fatty acids			
18 : 1	1.0 ± 0.9	3.0 ± 0.2	12.9 ± 1.3
18 : 2	0.2 ± 0.2	0.6 ± 0.1	0.9 ± 1.3
20 : 4	1.8 ± 0.2	2.8 ± 0.2	0.6 ± 0.8
22 : 4	1.1 ± 0.8	0.6 ± 0.2	6.5 ± 5.6
22 : 5ω6	1.0 ± 0.8	0.6 ± 0.3	tr
22 : 5ω3	2.0 ± 0.6	1.1 ± 0.2	tr
22 : 6	80.3 ± 4.5	48.7 ± 2.3	6.2 ± 2.5
24 : 4	1.3 ± 1.5	tr	3.3 ± 4.6
24 : 5	3.7 ± 1.6	0.3 ± 0.2	tr
other	1.8 ± 2.4	0.2 ± 0.3	0.6 ± 0.8
Mean total ± S.D.	94.4 ± 3.2	58.5 ± 1.4	31.0 ± 5.6

* See Fig. 3 for percentage of total phosphorus in each headgroup class.
tr, trace amount.

fractions of outer segment phosphatidylserine, phosphatidylcholine and phosphatidylethanolamine, respectively. The total combined phosphorus content of all of the minor bands not included in the tables is approx. 4% of the total and is ignored in the following analyses. The phosphatidylserine fraction is approximately evenly divided between PS-1 and PS-2. PS-2 is comprised of approximately half saturated acids and half unsaturated acids, while PS-1 contains over 90% polyunsaturated acids. Subfraction PC-5 (Table II) represents approx. 20% of the total phosphatidylcholine phosphorus and contains about 90% saturated fatty acids. PC-4, which possesses about 10% of the phosphatidylcholine phosphorus, contains approx. 50% 16 : 0, 13% 18 : 0 and 28% 18 : 1. PC-5 and PC-4 differ mainly in their content of 18 : 1 and are summed in Table II. PC-3, accounting for greater than 40% of the phosphatidylcholine, is evenly divided between saturated and unsaturated species. The smallest phosphatidylcholine band, PC-2, contains nearly all 22 : 6 as does the much larger band, PC-1. Why these two bands, which appear to be nearly identical chemically, are resolved is not clear. PC-2 and PC-1 are summed in Table II. PE-3 (Table III), which is a small fraction of the total phosphatidylethanolamine is predominantly saturated. PE-2, containing approx. 90% of the phosphatidylethanolamine, possesses about 40% saturated fatty acids and about 60% unsaturated fatty acids. Finally, PE-1, 10% of the phosphatidylethanolamine, contains nearly all polyunsaturated acids. Quantitative analysis of the fatty acids by means of the

TABLE IV

FATTY ACID COMPOSITION OF SUBFRACTIONS OF ROD OUTER SEGMENT PHOSPHATIDYLCHOLINE FROM SILICIC ACID COLUMN CHROMATOGRAPHY USED FOR FLUORESCENCE POLARIZATION MEASUREMENTS

Values given are mol%.

Fraction	PC-1′	PC-2′	PC-3′	PC-4′
Phosphorus (%):	20.8	50.1	19.6	9.6
Saturated fatty acids	26.6	34.1	57.2	76.6
Monounsaturated fatty acids	7.5	5.5	12.6	13.3
Polyunsaturated fatty acids	65.3	60.2	30.3	9.9

internal standard heneicosanoic acid (21 : 0) yields values very similar to those obtained by phosphorus analysis.

The summed fatty acid and phosphorus contents of PS-1 and PS-2, PC-1 through PC-5, and PE-1, PE-2 and PE-3 are very close to those of outer segment total phosphatidylserine, -ethanolamine and -choline from DEAE-cellulose and silicic acid column chromatography (data not shown). TLC, GLC and phosphorus analyses of fractions from silicic acid column chromatography of these phospholipids show that a subfractionation similar to that obtained with TLC is achieved, but with somewhat less resolution. Table IV shows the fatty acid composition and phosphorus content of the four subfractions of outer segment total phosphatidylcholine from a typical silicic acid column elution.

The fluorescence polarization of *trans*-parinaric acid versus temperature in

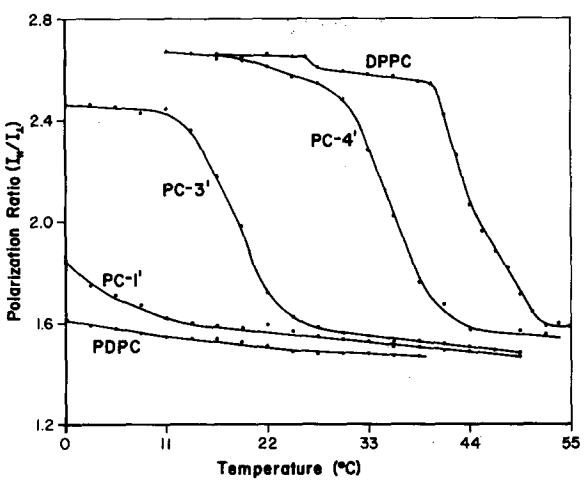

Fig. 2. The temperature dependence of the polarization of fluorescence of *trans*-parinaric acid in phosphatidylcholine subfractions. The polarization of fluorescence ($I_\parallel/I_\perp$) refers to the ratio of intensities emitted parallel and perpendicular to a vertically polarized exciting beam and has been corrected for instrumental anisotropy. Phospholipid subfractions (PC-1′, PC-2′ and PC-4′) from rod outer segment phosphatidylcholine were obtained as described in Materials and Methods. Details of these fluorescence procedures will be presented elsewhere [17] but are similar to those described by Sklar et al. [9]. Data for dipalmitoyl phosphatidylcholine (DPPC) and palmitoyl-docosahexaenoyl phosphatidylcholine (PDPC) are shown for comparison.

aqueous dispersions of column fractions PC-1′, PC-3′ and PC-4′ is presented in Fig. 2. The same measurements for dipalmitoyl phosphatidylcholine and our synthetic palmitoyl-docosahexaenoyl phosphatidylcholine are included for comparison. An increase in the critical temperature (where the polarization ratio changes steeply) is observed with increasing degree of average saturation of the subfraction.

Discussion

One of the initial objectives of the work presented here was to develop a preparative procedure for the isolation of phospholipid headgroup classes from retinal rod outer segment membranes for study by physical methods. Fluorescence polarization of parinaric acid probes in bilayers made from eluted fractions of pure outer segment phosphatidylserine, phosphatidylcholine and phosphatidylethanolamine from column chromatography revealed large differences in the physical properties of subfractions within these three headgroup classes (Fig. 2 shows representative data for outer segment phosphatidylcholine). These observations led to efforts to characterize the nature of the molecular heterogeneity responsible for these variations in physical properties. Employing silica gel TLC and GLC we found that the major phospholipids of the outer segment can be resolved into distinct subfractions which differ substantially in fatty acid composition. The data from these investigations are shown in Tables I—III. Since the physical properties of phospholipids depend strongly on the degree of saturation of their constituent fatty acids, the total mol percentages of saturated and unsaturated acids in each subfraction are also presented in Tables I—III.

Phospholipids are commonly found with one saturated and one unsaturated fatty acid. If the mol percentage of saturated or unsaturated fatty acids in a subfraction is less than 50%, the maximum mol percentage of phospholipid that contains one saturated and one unsaturated chain is equal to twice the mol percentage of the lesser fatty acid type. The remaining percentage of phospholipid is then the minimum percentage, in that particular subfraction, which possesses two fatty acids of the major type, i.e. saturated-saturated or unsaturated-unsaturated species. In Tables I—III, it is seen that PS-1, PS-2, PC-3, PC-1 + PC-2, PE-1 and PE-2 all contain significant amounts of unsaturated-unsaturated phospholipids. The minimum percentages of diunsaturated species from each of these subfractions are multiplied by the mol fraction each represents within its headgroup class. Those with the same headgroup are then summed to yield the total minimum percentage of unsaturated-unsaturated phospholipids per headgroup class. These values are given in Fig. 3 along with the minimum mol percentage saturated-saturated species of phosphatidylcholine and phosphatidylethanolamine calculated from the fatty acid composition of PC-4 + PC-5 and PE-3. Total outer segment phosphatidylserine must contain at least 40% species with two unsaturated fatty acids while the remainder must be saturated-unsaturated phosphatidylserine. The unsaturated acids in both species are preponderantly polyunsaturated.

The data require that at least 18% of the outer segment total phosphatidylcholine be saturated-saturated, and that this be largely dipalmitoyl phos-

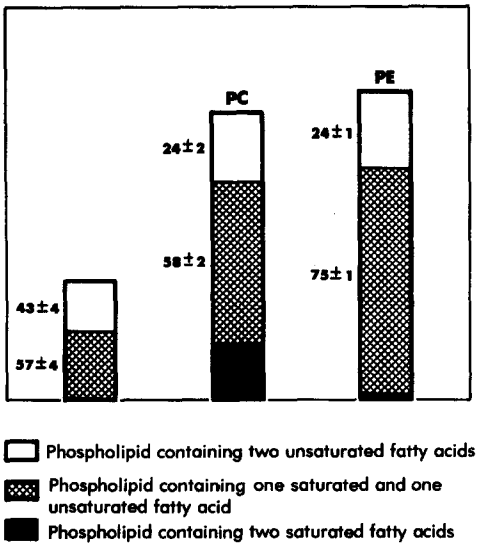

Fig. 3. Mol percentages of disaturated, saturated-unsaturated and diunsaturated species of major retinal rod outer segment phosphatidylserine (PS), phosphatidylethanolamine (PE) and phosphatidylcholine (PC). The values given are the mean ± S.D. for at least three determinations. Phosphatidylserine represents 16 ± 1 mol% of the total of the three phospholipid headgroup classes; phosphatidylethanolamine represents 39 ± 2 mol%; and phosphatidylethanolamine represents 42 ± 2 mol%.

phatidylcholine. Phosphatidylcholine must also contain approximately one-quarter unsaturated-unsaturated species, most of which are dipolyunsaturated.

Outer segment phosphatidylethanolamine contains a small amount of saturated-saturated component and a substantial amount of unsaturated-unsaturated component (also largely dipolyunsaturated). These account for at least 1% and 24% of total phosphatidylethanolamine, respectively. In both the unsaturated-unsaturated and remaining saturated-unsaturated species, the unsaturation is predominantly polyunsaturation.

Prior work suggests that slightly different mobilities of the subfractions of each phospholipid class may be ascribed to differences in the total number of fatty acid carbons per phospholipid [10]. That is, a larger number of carbons confers a slightly greater hydrophobicity to the phospholipid which in turn results in higher solubility in the mobile phase. This explanation is consistent with the R_F values of dimyristoyl phosphatidylcholine, dipalmitoyl phosphatidylcholine, and distearoyl phosphatidylcholine which we have observed to increase according to increasing total fatty acid chain length (data not shown). The degree of unsaturation appears to have much less influence on mobility in this TLC system. Each resolved subfraction is thus actually composed of phospholipids with pairs of fatty acids with similar total numbers of carbons. The fatty acid compositions in Tables I—III bear this out in large measure.

The predominant saturated fatty acids in the outer segment are the relatively short chained acids palmitic (16 : 0) and stearic (18 : 0). The major unsaturated acid is the relatively long chained docosahexaenoic acid. Most of the other unsaturated acids also have long chains. The large difference in length

between these two types of fatty acids makes it possible to conclude that the minimum mol percentages of phospholipids with either two unsaturated or two saturated fatty acids in Fig. 3 are also not very far from the maximum mol percentages for these species. For example, if phosphatidylserine were to contain more than the minimum calculated percentage of unsaturated-unsaturated species there would also have to be some saturated-saturated components present. However, no spot that would correspond to phosphatidylserine with two short chains was detected. Therefore, the minimum value of 42.5% unsaturated-unsaturated species very closely approximates the true value. Since phosphatidylethanolamine contains only a very small amount of saturated-saturated component a similar argument holds for it also. The presence of substantial amounts of all three possible combinations of fatty acid types make a determination of the actual levels of these components less straightforward for phosphatidylcholine.

Subfraction PC-4 + PC-5 could contain phospholipids which paired 16 : 1, 18 : 1 and 18 : 2 acids. These would be unsaturated-unsaturated yet would also possess short enough chains to have mobilities characteristic of PC-4 and PC-5. This arrangement would serve to increase the amount of both saturated-saturated and unsaturated-unsaturated species, each by a maximum of 4.5%. In a like manner 18 : 1 and 18 : 2 could be paired with 22 : 4, 22 : 5 and 22 : 6 acids and 20 : 4 paired with itself to give phospholipids which were diunsaturated but which also had mobilities which would place them in PC-3. This could increase the saturated-saturated and unsaturated-unsaturated content of each phosphatidylcholine species by as much as 4.5%. It is unlikely that both of these combinatorial possibilities could exist simultaneously and to their maximum degree. Both would require significant amounts of phosphatidylcholine with two 18-carbon fatty acids (36 total fatty acid carbons) which would have given a detectable spot midway between PC-4 (33.5 average number of carbons per phospholipid) and PC-3 (38.7 average number of carbons per phospholipid). Therefore, outer segment phosphatidylcholine could contain at most 27.4% disaturated species and 33.7% diunsaturated species, but the true values very probably lie much closer to the minimum 18.0% and 24.3%, as given in Fig. 3.

Anderson and Sperling [11] have reported positional localization of fatty acids in outer segment phospholipids. Their fatty acid contents are somewhat different than the results presented here (largely due to less polyunsaturated fatty acids in their reported composition). Anderson and Sperling's results demonstrate that the saturated-unsaturated phospholipids have saturated fatty acids in the one position.

As stated above, silicic acid column chromatography also subfractionates the phospholipids of the outer segments in a manner similar to but with somewhat less resolution than TLC. The fatty acid compositions of column subfractions (PC-1′ through PC-4′) from a typical silicic acid column separation are shown in Table IV. It is clear from the table that some proportion of subfraction PC-1′ must be didocosahexaenoyl phosphatidylcholine and that some portion of PC-4′ must be dipalmitoyl phosphatidylcholine. The fluorescence polarization of *trans*-parinaric acid in dispersions of these column subfractions (Fig. 2) shows that their thermal properties differ markedly. Based on a comparison of

these data with those of mixtures of synthetic palmitoyl-docosahexaenoyl phosphatidylcholine and dipalmitoyl phosphatidylcholine [17], we conclude that PC-1′ PC-2′ and PC-4′ contain approx. 3% or less, 20% and 60% dipalmitoyl phosphatidylcholine, respectively. The fluorescence of all four pooled subfractions is very similar to PC-2′, in reasonable agreement with the estimate of 18% disaturated component shown in Fig. 3.

Several methods are available to characterize the molecular heterogeneity of membrane phospholipids [10,12]. While these methods may provide a more detailed picture of the fatty acid distribution, they also require more manipulations, including chromatographic steps, than the simple TLC procedure described here. Minimal manipulation favors preservation of easily oxidized polyunsaturated fatty acids and determination of the true fatty acid distribution (see ref. 13, for example).

Electroretinograms of rats possessing disk membranes with altered fatty acid composition [14] and kinetic studies of the conversion of rhodopsin photointermediates [15] both suggest that phospholipids containing specific fatty acids may be required for outer segment membrane function. In addition, Tinoco et al. [16] have demonstrated that rats deprived of linolenic acid (18 : 3ω3), the metabolic precursor for docosahexaenoic acid (22 : 6ω3), retain normal saturated : unsaturated : polyunsaturated ratios in their retinal tissues by replacing, on a nearly mol-for-mol basis, docosahexaenoic acid with docosapentaenoic acid (22 : 5ω6). Presumably the normal complements of disaturated, saturated-unsaturated and diunsaturated phospholipids in the disk membranes of these animals are preserved.

Whether the disaturated or diunsaturated phospholipid species that we have shown to be present actively participate in the steps of visual transduction or are simply structural elements defining the physical character of the lipid environment surrounding rhodopsin remains to be determined.

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