Phospholipid Species Containing Long and Very Long Polyenoic Fatty Acids Remain with Rhodopsin after Hexane Extraction of Photoreceptor Membranes

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ABSTRACT: About one-fourth the phosphatidylcholines (PCs) from bovine disk photoreceptor membranes contain very long chain (24–36 carbons) polyunsaturated (4, 5, and 6 double bonds) fatty acids of the n-3 and n-6 series (VLCPUFA). Such fatty acids, exclusively occurring in dipolyunsaturated species, are esterified to the *sn*-1 position of their glycerol backbone, docosahexaenoate being the major fatty acid at *sn*-2. Chromatographically, such PCs display a weakly polar character relative to other species, ascribable to their exceedingly large number of carbons. After hexane extraction of lyophilized disks, PC is the major component of the fraction of lipids that remains associated with rhodopsin, followed by phosphatidylserine, while a large proportion of the phosphatidylethanolamine is removed. The fatty acid composition of the hexane-removable and protein-bound lipid fractions markedly differs, the latter being enriched in lipid species containing long-chain and very long chain polyenes. This is observed for all lipid classes except free fatty acids. VLCPUFA-containing PCs are the most highly concentrated species in the rhodopsin-associated lipid fraction. The very long chain polyenes these PCs have at *sn*-1 may account for their resistance to being separated from the protein. It is hypothesized that their unusually long polyenoic fatty acids could be well suited to partially surround α-helical segments of rhodopsin.

Rhodopsin, the major protein of rod photoreceptor membranes, is surrounded by unique glycerophospholipids. The major acyl chain of all major phospholipids in bovine rod outer segments (ros)[1] is 4,7,10,13,16,19-docosahexaenoate (22:6 n-3). This long-chain polyenoic fatty acid occurs in "hexaenoic" molecular species of glycerophospholipids, where it is esterified to the *sn*-2 position of the glycerol backbone (the *sn*-1 position being occupied by a saturated fatty acid), and in dipolyunsaturated (Miljanich et al., 1979) or "supraenoic" (Aveldaño & Bazán, 1983) molecular species, where long-chain polyunsaturated fatty acids are bound to both positions of the glycerol skeleton. About one-third of the phospholipids of bovine ros are dipolyunsaturated, since these species represent 31% of the phosphatidylcholines, 20% of the phosphatidylethanolamines, and 52% of the phosphatidylserines (Aveldaño & Bazán, 1983). Recently, a second peculiarity of the lipid environment or rhodopsin has been shown: phosphatidylcholine (PC), one of the major lipids of bovine ros, contains a series of unusual fatty acids. These were found to be almost exclusive components of dipolyunsaturated PCs from bovine retinas and were identified as very long chain (24–36 carbons) polyunsaturated (4, 5, and 6 double bonds) fatty acids (VLCPUFAs) belonging to the n-3 and n-6 families of polyenes (Aveldaño, 1987; Aveldaño & Sprecher, 1987). This study contributes with further information on the chemical makeup of VLCPUFA-containing dipolyunsaturated PC in photoreceptor disks of bovine ros and describes an experimental condition in which a particularly strong kind of association between such species and rhodopsin is apparent. Treatment of photoreceptor membranes with nondenaturing solvents like hexane has been used to advantage for photochemical studies, since it is a classical observation [see Ishimoto and Wald (1946), Darszon et al. (1979), and Yoshizawa and Schichida (1982)] that it produces no deleterious effects on the spectral properties of rhodopsin and other visual pigments. Hexane extraction of lyophilized bovine ros has been reported to affect the phospholipid composition of membranes, leading to a relative enrichment in PC in the fraction of the total lipid that is not removed by the solvent (Borggreven et al., 1970; Poincelot & Abrahamson, 1970). Freeze-dried, hexane-extracted bovine ros produce, after rehydration, electron spin resonance signals indicative that the lipids which remain in such membranes are much less "fluid" than those present in the native membranes (Pontus & Delmelle, 1975), i.e., that the molecular motions of the remaining lipids are much slower, or restricted, in comparison with what is observed in the original ros. The possible connections between these two observations, namely, alterations in phospholipid composition and changes in membrane physical properties, have not been further investigated. The unusual characteristics of the fatty acids of PC in photoreceptor membranes, and the antecedent that PC apparently plays a major role in such changes, prompted me to investigate the behavior of the fatty acids of disk lipids under these conditions. Here it is shown that hexane extraction of lyophilized bovine photoreceptor membranes leads to a dramatic enrichment in phospholipid species that contain long and very long polyunsaturated acyl chains, especially of VLCPUFA-containing PCs, in the fraction of the total lipid that remains associated to rhodopsin.

[1] Abbreviations: ros, rod outer segment(s); PC, PE, and PS, phosphatidylcholine, -ethanolamine, and -serine, respectively; FFA, free fatty acid; TLC and GLC, thin-layer and gas–liquid chromatography, respectively; Tris, tris(hydroxymethyl)aminomethane; NMR and ESR, nuclear magnetic and electron spin resonance, respectively; doxyl, 4,4-dimethyloxazolidine-3-oxyl. Fatty acids are named by the following convention: number of carbon atoms:number of double bonds, with n-3 or n-6 referring to the position of the first double bond counting from the methyl end. VLCPUFA, very long chain polyunsaturated fatty acid, is used in general to denote fatty acids having more than 22 carbons and 4–6 double bonds.

Materials and Methods

Rod outer segments were isolated from bovine retinas by using a discontinuous sucrose density gradient (Papermaster, 1982), washed with 10 mM Tris–acetate, and pelleted. After subjecting these ros to osmotic shock, disks were isolated by

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flotation on a 5% Ficoll–water interface (Smith & Litman, 1982). Typically, disks obtained from membranes retained at the 1.11/1.13 g/mL and 1.13/1.15 g/mL density interfaces of Papermaster (1982) had A_{280}/A_{500} (absorbance ratios) of 2.20 ± 0.01 and 2.3 ± 0.10, respectively. Since large amounts of membranes were necessary for fatty acid compositional studies, and since their lipid and fatty acid composition was similar, disks obtained from the ros at both interfaces were combined. About 1.4 μmol of rhodopsin was usually recovered in disk membranes from 100 retinas. Disks were washed with 50 mM Tris buffer containing sodium acetate and KCl (Stubbs & Litman, 1978) and pelleted (100000*g*). Lipids were extracted from the pellets (Folch et al., 1957) and resolved into classes by two-dimensional TLC (Rouser et al., 1970). To determine the species composition (Table I) and positional distribution of fatty acids (Table II) in PC, the phospholipid was recovered from TLC plates by using the solvents described by Arvidson (1968). In some cases, the group of molecular species of PC that migrates ahead on thin-layer chromatograms—a fraction enriched in dipolyunsaturated molecular species (Miljanich et al., 1979)—was separately recovered. Molecular species of PC were separated according to unsaturation by argentation TLC of acetyldiglyceride derivatives (Aveldaño, 1987). The positional distribution of fatty acids in PC was studied by hydrolyzing the phospholipid with *Crotalus adamanteous* phospholipase A_2 until no unreacted PC remained and separating the resulting lysophosphatidylcholines (lysoPCs) and free fatty acids (FFAs) by TLC. Both were converted to methyl esters and analyzed by gas–liquid chromatography (GLC), using methyl heneicosanoate as an internal standard for quantitation. More FFA than lysoPC was obtained in the experiments (molar ratios indicated by the subindexes x and y in Table II). When these values (instead of the expected 0.5:0.5 molar ratios) were used as factors, the composition of PC was the same as that of the original phospholpid before hydrolysis. Therefore, all fatty acids from PC were recovered after enzymatic hydrolysis, chromatography, and derivatization. The uneven recovery of lysoPC and FFA may have arisen from contamination of the enzyme preparation used with lysophospholipase. [This does not invalidate the conclusion (Results) that VLCPUFAs are major constituents of the *sn*-1 position, since, in fact, part of the VLCPUFA recovered as FFA may have arisen from hydrolysis of such lysoPCs.]

In order to study the lipids extracted and not extracted by hexane, ros and disk samples having 100 nmol of rhodopsin were washed and pelleted as described. The liquid was discarded, the tubes were placed in a lyophilizer chamber at −50 °C and equilibrated at that temperature, and vacuum was applied. The dry samples were then vigorously homogenized with hexane at 10–20 °C (three successive extractions with 5 mL each). The hexane extracts were separated by centrifugation and combined. Lipids in the hexane-extracted pellets were solubilized by means of chloroform/methanol (Folch et al., 1957). Both solvents were evaporated under N_2, and lipids were resolved and quantitated by phosphorus analysis (Rouser et al., 1970). To analyze the fatty acid composition of lipids, they were converted to methyl esters. The latter were usually subjected to TLC (using hexane/ether 95:5 as developing solvent) on prewashed plates of silica gel G and analyzed by GLC as previously described (Aveldaño, 1987).

Results

Disk Phosphatidylcholine and Its Fatty Acids. The fatty acid composition of dipolyunsaturated species from bovine disk PC, as separated by argentation TLC of acetyldiglyceride derivatives, is given in Table I. Large percentages of VLCPUFAs characterized the bands containing dodecaenoic, undecaenoic, and decaenoic molecular species. All of the VLCPUFAs of disk PC were recovered in these bands, the rest of the species (hexaenes to disaturates) having the composition already reported for ros (Aveldaño & Bazán, 1983). Except for the most strongly retained fraction (band I, ≃5% of the PC species, mainly derived from didocosahexaenoyl-PC), all dipolyunsaturated species contained, on a molar basis, about half 22:6 and half other polyenes, predominantly with very long chains. In contrast to PC, dipolyunsaturated PEs contained low amounts of polyenes longer than 22 carbons, while PS was rich in dipolyunsaturates containing 24 and 26 carbon fatty acids (not shown, but see Table V). Polyenes longer than 26 carbons were only found in significant amounts in PC. C_{24}–C_{36} polyenes accounted for about 13 mol % (≃20% of the weight of fatty acids) of total disk PC (Table II). Since there is virtually one VLCPUFA per molecule, about 26% of the PCs of disk membranes contain a VLCPUFA.

Partial resolution into groups of molecular species occurs when phospholipids from bovine photoreceptor membranes are subjected to TLC on silica. The phenomenon was first described by Miljanich et al. (1979), who observed that dipolyunsaturated molecular species of ros lipids always migrated *ahead* of other species on thin-layer chromatograms. When lipids from bovine disks were separated by two-dimensional TLC, an almost clear-cut separation of PC into three groups of species was observed, mainly made up by saturates, hexaenes, and dipolyunsaturates, in that order. The group of species migrating ahead contained, in addition to docosahexaenoate, virtually all of the VLCPUFA of disk PC (Table II). The weak retention by silica distinguishes these PCs to such an extent that the same (simply chromatographic) separation can be achieved when the PC of the entire retina is subjected to this procedure. The fatty acid composition of supraenoic PCs thus isolated, whether from retina or disks, was closely similar despite the differences in total PC composition (Table II).

The distribution of fatty acids on silica follows the rules that (1) for a given unsaturation, the longer the chain, the weaker the retention and (2) for a given chain length, the higher the unsaturation, the stronger the retention (if the fatty acids belong to the same family, i.e., if the first double bond counting from the methyl end is the same). These general rules are also followed by phospholipids, and thus a dipolyunsaturated species should be more strongly retained than a hexaenoic, and this more than a saturated species if the three had the same chain lengths in their fatty acids. However, since dipolyunsaturated PCs have the largest unsaturation but also the longest hydrocarbon chains of all PCs, their fast migration on silica indicates that the number of carbons overcomes the effect of the number of double bonds in determining their chromatographic "segregation". Such behavior was also displayed by acetyldiglycerides (Table I), for it is a property exclusively conferred by the acyl chains. (See also Figure 1, B_1 and B_2, showing that 32:6 distinctly migrates ahead of 22:6.)

The quantitative composition of lysoPCs and FFAs obtained after hydrolysis of PCs from intact retina and disks, as well as of dipolyunsaturated PCs obtained from both, is compared in Table II. About one-third of the fatty acids of the *sn*-1 position of disk PC were made up by VLCPUFA, the two major saturates, 16:0 and 18:0, adding up to 60% (versus 5% and 80%, respectively, in PC from intact retina). Docosahexaenoate, docosapentaenoate, and arachidonate were predominantly located at the *sn*-2 position. Some 22:6 occurred at the *sn*-1 position and some saturates at the *sn*-2 position,

Table I: Fatty Acid Composition of Dipolyunsaturated Phosphatidylcholines from Disks[a]

	fraction				
	I	II	III	IV	V
20:4 n-6	0.4 ± 0.2	0.8 ± 0.1	5.5 ± 1.1	1.3 ± 0.1	1.9 ± 0.2
20:5 n-3	1.2 ± 0.1	0.2 ± 0.1	0.5 ± 0.2	0.5 ± 0.0	0.9 ± 0.3
22:4 n-6			0.3 ± 0.1	0.5 ± 0.0	
22:5 n-6	0.3 ± 0.1	1.0 ± 0.1	0.7 ± 0.3	0.5 ± 0.2	2.0 ± 0.1
22:5 n-3	2.0 ± 0.2	0.6 ± 0.1	1.4 ± 0.2	1.3 ± 0.2	1.2 ± 0.2
22:6 n-3 (+24:4 n-6)	88.6 ± 1.8	54.4 ± 1.1	54.4 ± 2.9	53.8 ± 1.3	51.5 ± 1.2
24:5 n-3	0.9 ± 0.2	7.6 ± 0.3	0.9 ± 0.1	1.0 ± 0.2	0.8 ± 0.2
24:6 n-3 (+26:4 n-6)	4.4 ± 0.1	0.5 ± 0.1	0.4 ± 0.0	1.1 ± 0.1	1.0 ± 0.1
26:5 n-3		2.7 ± 0.2	4.9 ± 1.0		
26:6 n-3	1.7 ± 0.2	0.5 ± 0.0			
28:4 n-6					2.0 ± 0.4
28:5 n-3			4.0 ± 1.2	0.5 ± 0.1	0.5 ± 0.1
28:6 n-3	0.3 ± 0.1	0.4			
30:4 n-6					1.7 ± 0.2
30:5 n-3			2.1 ± 0.9	3.7 ± 0.1	0.4 ± 0.1
30:6 n-3		1.5 ± 0.2			
32:4 n-6					29.3 ± 1.7
32:5 n-3				20.8 ± 3.1	0.8 ± 0.1
32:6 n-3		27.9 ± 0.9	5.2 ± 1.3		
34:4 n-6					59.8 ± 0.8
34:5 n-3				14.7 ± 0.6	
34:6 n-3		2.0 ± 0.8	19.3 ± 1.0		
36:4 n-6					0.03 ± 0.0
36:5 n-3				0.1 ± 0.0	
36:6 n-3			0.4 ± 0.2		
total VLCPUFA (mol %)[b]	7.3	43.1	37.2	41.9	41.9
total VLCPUFA (wt %)	8.2	51.0	45.5	50.7	50.7
av fatty acid[c]					
M_r	334.7	374.7	373.8	389.6	388.5
unsaturation	5.9	5.9	5.7	5.5	5.1
chain length	21.9	25.5	25.4	26.1	25.7
av species[d]	44:12	51:12	51:11	52:11	51:10

[a] Acetyldiglycerides from PC were subjected to argentation TLC and their fatty acids analyzed by GLC. Band I mainly contained didocosahexaenoyl and 24:6/22:6 species. Such "dodecaenes" separated from species like 30:6/22:6 or 32:6/22:6 (band II), and the latter in turn lagged behind species like 34:6/22:6 and 36:6/22:6 (band III). The same occurred for species having 22:6 and tetra- or pentaenoic fatty acids, and thus dipolyunsaturates with shorter polyenes tended to lag behind those enriched in longer polyenes of the same unsaturation. Even when argentation TLC separations are based on the total number of double bonds per molecule, species of the same unsaturation differring in more than two carbons tended to separate on the silica support, indicating that the large number of carbons in VLCPUFA-containing species overcomes their large number of double bonds in determining their TLC retention properties. The composition is expressed as mole percent (mean ± SD from four samples). [b] Sum of polyenes with 24–36 carbons. [c] Average values of molecular weight (M_r), unsaturation, and chain length of fatty acids in each band. [d] Average number of carbons and unsaturation of PC species in each band.

consistent with the known presence of didocosahexaenoyl and disaturated species in photoreceptor membrane PC (Miljanich et al., 1979; Aveldaño & Bazán, 1983). In the group of species migrating ahead upon TLC, nearly 80% of the fatty acids of the *sn*-1 position were VLCPUFAs, and 75% of those of the *sn*-2 position were 22:6. The data in Table II clearly demonstrate the selectivity with which VLCPUFAs occur in the *sn*-1 position of dipolyunsaturated PCs of the disk membrane.

The occurrence in bovine disk PC of a whole series of polyenoic fatty acids with different chain lengths, degrees of unsaturation, and position of the double bonds allowed the interesting collateral observation that the preferential distribution of fatty acids between the *sn*-1 and *sn*-2 positions was closely similar to the distribution of the same fatty acids between upper and lower bands (respectively) when methyl esters from this lipid were subjected to TLC on silica (Figure 1). Thus, the upper band of methyl esters contained most of the saturates and VLCPUFA (just as the *sn*-1 position of PC, Table II) and the lower band most of the 22:6 and related polyenes (as the *sn*-2 position). The same was observed for the fatty acids of dipolyunsaturated PCs [see Aveldaño and Sprecher (1987) for their TLC separation and Table II for their positional distribution]. Thus, the longer the acyl chain of a polyene, the weaker its retention on silica and the larger its proportion in the *sn*-1 position. In general (Table II), the *sn*-1/*sn*-2 ratios were higher (1) the lower the unsaturation for fatty acids of the same chain length (18:0 > 18:1 > 18:2 > 18:3) and (2) the longer the chain for fatty acids of the same unsaturation (18:0 > 16:0 > 14:0; 32:4 n-6 > 22:4 n-6 > 20:4 n-6; 32:5 n-3 > 24:5 n-3 > 22:5 n-3; 36:6 n-3 > 32:6 n-3 ≫ 22:6 n-3). These and other well-known examples of phospholipid species suggest that, in general, when the distribution of fatty acids between the *sn*-1 and *sn*-2 positions is uneven, the more "hydrophobic" or "lipophilic" (or less "polar") of the two (in the chromatographic sense) tends to be in the *sn*-1 position.[2]

[2] Many examples of naturally occurring phospholipid species may be recalled that follow, or at least do not rule out, this apparent correlation. (1) Familiar species of lipids such as 16:0/22:6, 18:0/22:6, 18:0/20:4, etc., almost invariably have the saturate at *sn*-1 and the polyene at *sn*-2. (2) If both fatty acids have the same chain length, the more saturated is at *sn*-1 and the more unsaturated is at *sn*-2 (e.g., 18:0/18:1, 18:0/18:2, 18:0/18:3, 16:0/16:1, etc.). (3) If both acyl chains have the same unsaturation and position of the double bonds, the longer is at *sn*-1 and the shorter at *sn*-2 (as shown for major dipolyunsaturated PCs like 32:6/22:6, 34:6/22:6, etc.). (4) In dipolyunsaturated species of octopus microvillar membranes, 20:4/20:5 and 20:4/22:6 are predominant lipid species (of PE), with 20:4 at *sn*-1 and 20:5 or 22:6 at *sn*-2 (Akino & Tsuda, 1979). The former species fits into (2) and the seond is not an exception to (3) since 20:4 belongs to the n-6 and 22:6 to the n-3 series of polyenes. In all cases, methyl esters from the fatty acids at *sn*-1 will be more weakly retained on polar chromatographic supports and more strongly retained on nonpolar ones than those from the fatty acids located at *sn*-2.

Table II: Composition and Positional Distribution of Fatty Acids in Phosphatidylcholines from Retina and Photoreceptor Disks[a]

	total phosphatidylcholine							dipolyunsaturated PCs					
		retina			disks			retina			disks		
	disks (6)	*sn*-1 (A)	*sn*-2 (B)	$A_x + B_y$	*sn*-1 (A)	*sn*-2 (B)	$A_x + B_y$	*sn*-1 (A)	*sn*-2 (B)	$A_x + B_y$	*sn*-1 (A)	*sn*-2 (B)	$A_x + B_y$
14:0	0.34 ± 0.11	0.58 ± 0.16	0.65 ± 0.10	0.62	0.30 ± 0.01	0.43 ± 0.02	0.28	0.2	1.2	0.3	0.3	0.2	0.2
15:0	0.20 ± 0.02	0.53 ± 0.03	0.44 ± 0.05	0.47	0.17 ± 0.01	0.27 ± 0.01	0.23		0.3	0.2	0.2	0.2	0.2
16:0	22.33 ± 1.22	47.50 ± 0.36	32.15 ± 1.01	37.60	28.15 ± 0.17	18.45 ± 0.14	21.97	2.5	0.9	1.3	2.0	3.5	3.0
16:1	1.10 ± 0.44	0.28 ± 0.04	2.65 ± 0.32	1.81	1.01 ± 0.01	1.10 ± 0.01	1.06	0.1	0.9	0.7	0.1	0.3	0.2
17:0	0.40 ± 0.20	0.44 ± 0.05		0.15	0.79 ± 0.01	0.30 ± 0.01	0.48	0.2		0.1	0.3	0.2	0.2
18:0	15.74 ± 0.60	30.60 ± 0.81	4.04 ± 0.38	13.46	31.25 ± 0.33	6.36 ± 0.39	15.39	14.0	6.1	8.4	12.2	4.8	7.2
18:1	2.41 ± 0.20	9.92 ± 0.32	22.24 ± 0.38	17.86	2.23 ± 0.09	2.72 ± 0.08	2.54	1.5	3.0	2.5	1.4	1.9	1.8
18:2	0.53 ± 0.23	0.68 ± 0.02	1.55 ± 0.07	1.24	0.31 ± 0.03	0.76 ± 0.03	0.59	0.1	0.2	0.2	0.3	0.5	0.4
20:0	0.06 ± 0.01	0.26 ± 0.04		0.09	0.11 ± 0.01		0.04	0.2		0.2	0.1		0.1
18:3	0.19 ± 0.02		0.54 ± 0.02	0.34		0.17 ± 0.04	0.14		0.2	0.2		0.03	0.02
20:1	0.08 ± 0.02	0.90 ± 0.11		0.31	0.11 ± 0.01		0.04	0.2		0.1	0.1	0.1	0.1
20:3 n-6 (+22:0)	0.21 ± 0.01	0.24 ± 0.03	0.94 ± 0.05	0.69	0.14 ± 0.01	0.22 ± 0.02	0.19		0.5	0.4	0.1	0.1	0.1
20:4 n-6	2.23 ± 0.19	0.38 ± 0.13	7.59 ± 0.29	5.02	0.25 ± 0.02	3.32 ± 0.18	2.20	0.5	2.6	2.0	0.1	1.6	1.2
20:5 n-3	0.42 ± 0.08	0.09 ± 0.01	0.66 ± 0.16	0.43	0.12 ± 0.02	0.62 ± 0.01	0.43	0.03	0.3	0.2	0.2	0.4	0.4
22:4 n-6	0.20 ± 0.02	0.20 ± 0.01	0.47 ± 0.04	0.37	0.09 ± 0.01	0.20 ± 0.01	0.16	0.3	0.3	0.3	0.2	0.1	0.2
22:5 n-6	0.25 ± 0.04	0.09 ± 0.02	0.21 ± 0.03	0.16	0.15 ± 0.01	0.29 ± 0.01	0.23	0.7	0.9	0.8	0.1	0.3	0.2
22:5 n-3	1.32 ± 0.09	0.13 ± 0.05	1.67 ± 0.21	1.12	0.16 ± 0.00	1.96 ± 0.03	1.31	0.2	2.0	1.5	0.1	1.6	1.2
22:6 n-3 (+24:4 n-6)	38.92 ± 0.96	2.35 ± 0.14	23.47 ± 0.13	15.96	5.39 ± 0.08	57.53 ± 0.49	38.61	2.2	75.4	54.1	2.2	75.5	54.6
24:5 n-3	1.41 ± 0.06	0.40 ± 0.02	0.22 ± 0.01	0.28	1.47 ± 0.01	1.23 ± 0.02	1.32	0.7	0.9	0.8	1.5	1.5	1.5
24:6 n-3 (+26:4 n-6)	0.75 ± 0.03	0.23 ± 0.02	0.16 ± 0.06	0.18	0.76 ± 0.01	0.64 ± 0.04	0.69	0.9	0.6	0.7	0.8	0.9	0.9
26:5 n-3	0.71 ± 0.04	0.18		0.06	0.88 ± 0.02	0.34 ± 0.02	0.54	1.8	0.4	0.8	2.5	0.9	1.3
26:6 n-3 (+28:4 n-6)	0.08 ± 0.04	0.06	0.06 ± 0.01	0.06	0.23 ± 0.01		0.08	0.6	0.03	0.2	0.4	0.1	0.2
28:5 n-3	0.18 ± 0.02	0.07 ± 0.01		0.02	0.43 ± 0.01	0.04 ± 0.03	0.18	1.0	0.1	0.4	1.3	0.1	0.4
28:6 n-3	0.05 ± 0.01	0.02 ± 0.01		0.01	0.01 ± 0.01	0.01	0.01	0.3		0.1	0.3	0.02	0.1
30:4 n-6	0.04 ± 0.01	0.03		0.01	0.12 ± 0.01	0.01	0.01	0.2		0.1	0.3	0.01	0.1
30:5 n-3	0.58 ± 0.02	0.26 ± 0.01	0.01	0.02	1.43 ± 0.01	0.13 ± 0.01	0.60	3.8	0.2	1.3	4.4	0.4	1.5
30:6 n-3	0.10 ± 0.01	0.03		0.01	0.28 ± 0.01	0.14 ± 0.01	0.19	0.3		0.1	0.8	0.1	0.3
32:4 n-6	0.64 ± 0.07	0.35 ± 0.06	0.01	0.13	1.32 ± 0.02		0.48	3.5		1.0	4.5	0.3	1.5
32:5 n-3	2.79 ± 0.07	1.04 ± 0.09	0.05 ± 0.01	0.40	6.97 ± 0.06	0.58 ± 0.05	2.90	21.4	0.7	6.7	21.5	1.4	7.1
32:6 n-3	2.59 ± 0.17	0.97 ± 0.12	0.08 ± 0.02	0.39	6.29 ± 0.03	0.58 ± 0.06	2.65	15.8	1.4	5.6	17.9	1.3	6.0
34:4 n-6	0.09 ± 0.07	0.10 ± 0.01	0.01	0.04	0.22 ± 0.01	0.01	0.09	0.5		0.2	0.7	0.02	0.2
34:5 n-3	2.07 ± 0.07	0.81 ± 0.02	0.04	0.31	5.27 ± 0.06	0.39 ± 0.04	2.16	18.2	0.5	5.6	16.3	1.0	5.4
34:6 n-3	0.96 ± 0.07	0.37 ± 0.03	0.02	0.14	2.58 ± 0.06	0.15 ± 0.03	1.04	7.7	0.1	2.3	6.5	0.4	2.1
36:5 n-3 (+36:6 n-3)	0.04 ± 0.01	0.07 ± 0.04		0.02	0.11 ± 0.01		0.04	0.3		0.1	0.3		0.1
total VLCPUFA (mol %)	13.08	5.00	0.66	2.08	28.37	4.25	13.00	77.0	5.0	26.0	80.0	8.4	28.7
total VLCPUFA (wt %)	18.10	7.90	1.02	3.28	39.70	5.63	18.04	83.3	6.4	31.6	88.5	10.9	34.9
av M_r[b]	320	281	288	285	333	312	320	436	330	360	437	340	368

[a] Phosphatidylcholine from (six samples of) disks were separated by TLC and its fatty acids analyzed by GLC. Three samples of total disk or retina PC, as well as one large sample of the fraction of PC (from disks or retina) that migrates ahead on thin-layer chromatograms, were isolated and hydrolyzed with phospholipase A_2. The resulting lysoPCs (*sn*-1) and free fatty acids (*sn*-2) were separated and also analyzed by GLC. x and y represent the fractional amount (mol) of fatty acid methyl esters recovered as lysoPCs and free fatty acids, respectively (see Materials and Methods). For total PC, $x = 0.355$ and $y = 0.645$ (retina); $x = 0.363$ and $y = 0.637$ (disks). For total PC, $x = 0.355$ and $y = 0.645$ (retina); $x = 0.363$ and $y = 0.637$ (disks). For dipolyunsaturated PCs, $x = 0.291$ and $y = 0.709$ (retina); $x = 0.285$ and $y = 0.715$ (disks). The fatty acid composition is expressed as mole percent (mean value ± SD). [b] Molecular weight of the average fatty acid.

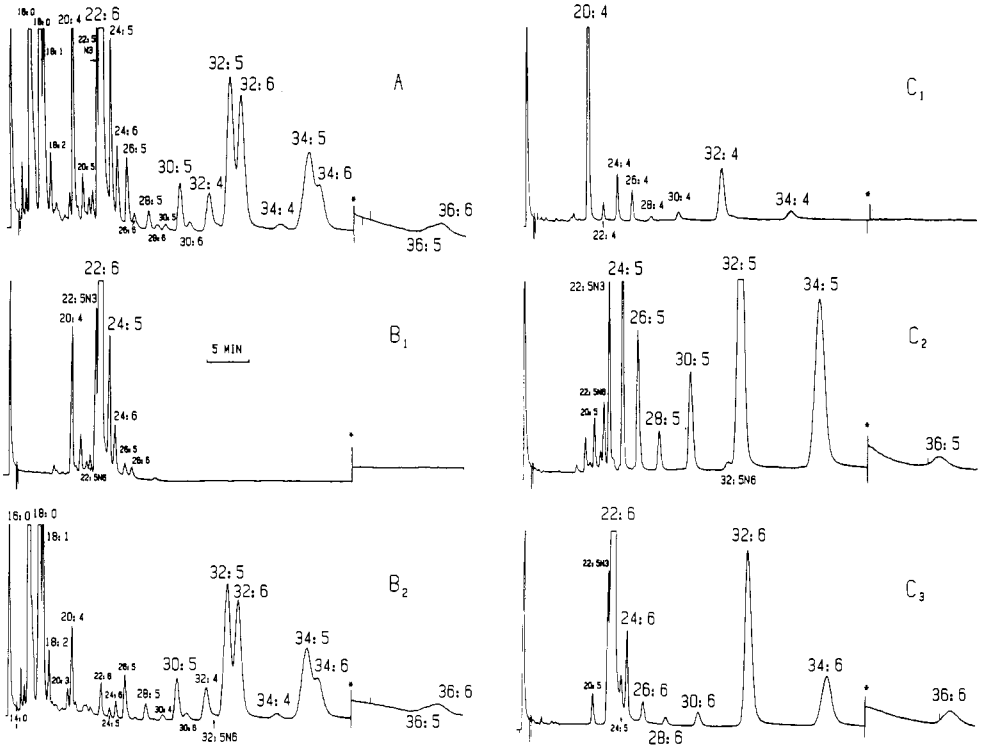

FIGURE 1: Gas–liquid chromatographic patterns of fatty acid methyl esters from disk membrane phosphatidylcholine. (*) denotes a 10-fold increase in detector sensitivity. (A) Methyl esters from total PC. (B) Separation of such methyl esters on thin layers of silica gel (solvent, hexane/ether 95:5). (B_1 and B_2) Constituents of the lower and upper bands of methyl esters, respectively. Note that VLCPUFA migrated ahead of shorter polyenes of the same unsaturation on silica. Also, VLCPUFA (and saturates) clearly separated from 22:6 and related long-chain polyenes (C_{20}–C_{24}). Such chromatographic "segregation" seems to correlate with the preferential distribution of fatty acids between the *sn*-1 and *sn*-2 positions of the glycerol backbone in disk PC (Table II). The fatty acids of isolated dipolyunsaturated species also separated on silica (Aveldaño & Sprecher, 1987) in a way that closely resembled their distribution between the *sn*-1 and *sn*-2 positions (Table II). (C) (n-6) tetraenoic (C_1), (n-3) pentaenoic (C_2), and (n-3) hexaenoic (C_3) fatty acid constituents of disk PC, as separated by argentation TLC using chloroform/methanol 95:5. Note that very long chain (n-6) tetra- and pentaenes were minor constituents of bovine disk PC in comparison with (n-3) penta- and hexaenes.

Table III: Phospholipid Removable (A) and Nonremovable (B) after Hexane Extraction of Lyophilized Bovine Disks[a]

	lipid composition (%)			fraction of each lipid in extracts		
	A	B	A + B	A	B	B/A
PC	26.1 ± 0.4	50.0 ± 0.6	38.3 ± 0.8	32.8 ± 1.9	67.2 ± 1.9	2.05
PE	60.2 ± 0.6	32.6 ± 0.4	46.1 ± 0.4	63.2 ± 2.3	36.8 ± 2.3	0.58
PS	13.7 ± 0.2	17.4 ± 0.4	15.6 ± 0.4	42.6 ± 2.2	57.4 ± 2.2	1.35
PC/PE	0.43 ± 0.01	1.53 ± 0.04	0.83 ± 0.02			
total lipid				49.2 ± 2.9	50.8 ± 2.9	1.03

[a] Disks were lyophilized and then extracted (3 times, successively) with hexane (fraction A). The lipid not extracted with hexane (B) was removed by chloroform/methanol. Total lipid phosphorus was 36.6 ± 2.2 and 38.0 ± 2.1 (mol/mol of disk rhodopsin) in A and B, respectively. Phospholipids were analyzed after TLC separations. A + B is the phospholipid composition after adding up the phosphorus in A and in B. It did not differ from that of untreated disks.

Hexane Extraction of Disk Membrane Lipid Classes and Their Fatty Acids. When lyophilized disks were extracted with hexane, about half the lipid was removed (Table III). The extracted (A) and nonextracted (B) fractions of the total lipid dramatically differed in phospholipid head group composition. Much more PC than other lipids remained in the fraction associated with rhodopsin, and much more PE than other lipids was extracted by hexane. The lipid fraction removed by the solvent (A) differed not only in phospholipid class but also in fatty acid composition from the fraction that remained associated with rhodopsin (B) (Table IV). The latter was enriched in long, and especially in very long, polyenoic fatty acids. The *amount* of 22:6 was similar in both fractions, since the amount of lipid (Table III) and the percentage of 22:6 in such lipid (Table IV) were similar. However, fraction A contained more saturates (e.g., stearate) than fraction B, and the latter contained about 3.1-fold more very long chain polyunsaturated fatty acids than fraction A. Thus, the ratio 18:0/22:6 was much higher in A than in B, and conversely the ratio VLCPUFA/22:6 was much higher in B than in A (see Table VIII). This indicates that hexane removed mainly hexaenoic molecular species, while the fraction that remained associated with rhodopsin became richer in dipolyunsaturated species of lipids. Consistently, the phospholipid class most easily removed by the solvent was PE, which is mainly made up of hexaenoic species, while the classes most strongly retained by the protein were PC and PS, the lipids constituted by the largest proportions of dipolyunsaturated species.

As indicated by the fatty acid compositions in Tables IV–VI, *all* phospholipids contributed to the observed enrichment in long-chain and very long chain polyenes. However, PC contributed with the largest proportion and with the longest acyl chains. The fraction of PC remaining with rhodopsin was 2.5-fold richer in VLCPUFA than the PCs that were removed (Table IV). Since the amount of PC in the protein-associated fraction was in addition 2.05-fold larger, it follows that this

Table IV: Fatty Acid Composition of Total Lipid and Phosphatidylcholine Fractions Removed by Hexane (A) and Associated with Rhodopsin (B) after Hexane Extraction of Lyophilized Bovine Disks[a]

	total lipid			phosphatidylcholine		
	A	B	total[b]	A	B	total[b]
14:0	0.14 ± 0.05	0.34 ± 0.04	0.23	0.31	0.29 ± 0.05	0.29
15:0	0.10	0.13	0.11	0.16	0.16	0.16
16:0	13.50 ± 0.15	14.58 ± 0.14	14.04	27.10 ± 0.99	20.44 ± 0.79	22.62
16:1	0.71 ± 0.01	1.21 ± 0.01	0.96	1.09 ± 0.01	0.89 ± 0.03	0.96
17:0	0.56	0.53	0.54	0.64	0.51	0.55
18:0	28.35 ± 0.39	16.28 ± 0.23	22.23	24.94 ± 0.19	13.53 ± 0.33	17.27
18:1	2.84 ± 0.13	3.40 ± 0.35	3.13	2.65 ± 0.02	2.50 ± 0.08	2.55
18:2	0.42	0.71 ± 0.03	0.57	0.36 ± 0.01	0.67 ± 0.05	0.57
20:0	0.10	0.06	0.08	0.03		0.01
18:3 + 20:1	0.04	0.14	0.09	0.02	0.16	0.12
20:3 n-9	0.03	0.02	0.03	0.01	0.09	0.06
20:3 n-6	0.14 ± 0.01	0.38 ± 0.03	0.26	0.14	0.22 ± 0.03	0.20
20:4 n-6	3.11 ± 0.03	1.89 ± 0.04	2.49	1.53 ± 0.01	2.06 ± 0.10	1.88
20:5 n-3	0.38 ± 0.07	0.37 ± 0.02	0.38	0.32	0.52 ± 0.02	0.45
22:4 n-6	0.36 ± 0.01	0.57 ± 0.03	0.46	0.13	0.17 ± 0.03	0.15
22:5 n-6	0.29	0.36	0.32	0.16	0.25 ± 0.03	0.22
22:5 n-3	1.54 ± 0.03	2.07 ± 0.05	1.81	1.31 ± 0.03	1.48 ± 0.02	1.42
22:6 n-3	43.73 ± 0.09	45.65 ± 0.46	44.70	33.08 ± 0.30	40.81 ± 0.13	38.27
24:5 n-3	2.10 ± 0.08	4.19 ± 0.03	3.16	1.00 ± 0.03	1.67 ± 0.01	1.45
24:6 n-3	0.53	1.11 ± 0.02	0.82	0.38 ± 0.03	0.78 ± 0.01	0.64
26:5 n-3		0.63 ± 0.05	0.32	0.41 ± 0.02	0.67 ± 0.05	0.58
26:6 n-3		0.14	0.07	0.02	0.13 ± 0.04	0.01
28:5 n-3	0.01	0.09	0.08	0.06	0.24 ± 0.02	0.18
28:6 n-3		0.02	0.01	0.01	0.06	0.04
30:4 n-6		0.03	0.02		0.05	0.03
30:5 n-3	0.06	0.35 ± 0.01	0.21	0.24 ± 0.01	0.75 ± 0.03	0.58
30:6 n-3		0.05	0.03	0.02	0.12	0.09
32:4 n-6	0.06	0.29 ± 0.01	0.18	0.27 ± 0.01	0.64 ± 0.01	0.52
32:5 n-3	0.33 ± 0.02	1.47 ± 0.06	0.91	1.33 ± 0.04	3.45 ± 0.22	2.75
32:6 n-3	0.16 ± 0.02	1.24 ± 0.08	0.72	0.79 ± 0.10	3.15 ± 0.10	2.38
34:4 n-6	0.01	0.03	0.03	0.03	0.06	0.05
34:5 n-3	0.23 ± 0.01	1.11 ± 0.07	0.67	0.98 ± 0.01	2.54 ± 0.06	2.03
34:6 n-3	0.13 ± 0.01	0.43 ± 0.02	0.28	0.49 ± 0.01	1.01 ± 0.03	0.84
36:5 + 36:6 n-3		0.01	0.01		0.02	0.01
total VLCPUFA	3.6 ± 0.1	11.2 ± 0.2	7.5	6.0 ± 0.3	14.8 ± 0.3	12.3

[a] The fatty acid composition of lipids from the experiment described in Table III was analyzed by GLC. Results are expressed as mole percent and are mean values ± SDs from three disk preparations (SDs smaller than 0.005 are not depicted). [b] The composition of the total lipid (or PC) in disks was obtained after adding up the amounts (in moles) of each fatty acid present in each extract and recalculating percentages. It did not differ significantly from the composition observed in untreated membranes (see Table II).

Table V: Fatty Acid Composition of Phosphatidylethanolamine and Phosphatidylserine Fractions Removed by Hexane (A) and Associated with Rhodopsin (B) after Hexane Extraction of Lyophilized Disks[a]

	phosphatidylethanolamine			phosphatidylserine		
	A	B	total	A	B	total
14:0	0.07 ± 0.01	0.06 ± 0.01	0.06	0.12 ± 0.03	0.10 ± 0.01	0.11
15:0	0.04 ± 0.03	0.04 ± 0.01	0.04	0.06 ± 0.01	0.04 ± 0.01	0.05
16:0	13.10 ± 0.16	8.90 ± 0.24	11.77	1.10 ± 0.10	0.86 ± 0.13	0.96
16:1	0.35 ± 0.01	0.49 ± 0.01	0.40	0.11	0.13	0.15
17:0	0.70 ± 0.02	0.55 ± 0.01	0.64	0.06	0.03	0.06
18:0	32.32 ± 0.88	18.25 ± 0.27	26.51	30.67 ± 0.52	8.94 ± 0.04	17.93
18:1	3.00 ± 0.10	6.18 ± 0.13	4.17	0.71 ± 0.08	0.75 ± 0.02	0.73
18:2	0.42 ± 0.04	1.37 ± 0.01	0.76	0.04	0.06	0.05
20:0	0.04		0.03	0.06	0.02	0.04
18:3 n-6	0.03	0.35 ± 0.01	0.14	0.01	0.02	0.01
18:3 n-3 (+20:1)	0.04	0.18	0.09			
20:3 n-9		0.21	0.07	0.02	0.01	0.01
20:3 n-6	0.20	0.80 ± 0.01	0.43	0.04	0.07	0.06
20:4 n-6	1.19 ± 0.02	1.95 ± 0.01	1.47	1.09 ± 0.02	0.94 ± 0.40	1.00
20:5 n-3	0.13 ± 0.01	0.34 ± 0.03	0.21	0.20 ± 0.05	0.27 ± 0.04	0.24
22:4 n-6	0.18 ± 0.02	0.48 ± 0.04	0.29	1.12 ± 0.11	2.06 ± 0.02	1.66
22:5 n-6	0.26 ± 0.01	0.44 ± 0.03	0.32	0.35 ± 0.02	0.59 ± 0.08	0.49
22:5 n-3	0.99 ± 0.02	1.63 ± 0.06	1.23	2.73 ± 0.01	5.51 ± 0.07	4.33
22:6 n-3	47.16 ± 0.70	55.51 ± 0.51	50.31	51.69 ± 0.26	58.82 ± 0.54	55.78
24:5 n-3	0.89 ± 0.06	1.98 ± 0.04	1.29	8.10 ± 0.16	17.52 ± 0.22	13.56
24:6 n-3	0.35 ± 0.07	0.56 ± 0.01	0.43	1.44 ± 0.03	3.26 ± 0.08	2.48
26:5 n-3				0.19 ± 0.03	0.36 ± 0.05	0.29
26:6 n-3				0.06 ± 0.02	0.18 ± 0.03	0.14
total VLCPUFA	1.2 ± 0.13	2.5 ± 0.03	1.7	9.8 ± 0.18	21.2 ± 0.38	16.5

[a] Details as in Table IV.

Table VI: Major Fatty Acids of Phosphatidate, Phosphatidylinositol, and Diacylglycerol Fractions Extracted (A) and Nonextracted (B) by Hexane from Lyophilized Bovine Disks[a]

	phosphatidate (PA)			phosphatidylinositol (PI)			diacylglycerol
	A	B	total	A	B	total	A
16:0	19.4 ± 1.3	10.6 ± 0.7	14.6	18.8	9.1	15.5	18.6
18:0	25.0 ± 2.1	13.1 ± 0.6	18.5	32.0	22.0	28.6	20.5
18:1	21.1 ± 5.3	7.1 ± 0.3	13.4	5.6	8.0	6.4	3.4
20:4 n-6	4.4 ± 1.8	2.8 ± 0.2	3.3	31.3	28.6	30.4	39.1
20:5 n-3	1.1 ± 0.4	1.2 ± 0.1	1.2	2.4	2.5	4.0	4.0
22:5 n-3	1.0 ± 0.2	3.7 ± 0.8	2.5	0.2	2.6	1.0	1.7
22:6 n-3	24.3 ± 1.1	47.5 ± 0.8	37.1	9.0	22.1	13.2	11.7
24:5 n-3	3.1 ± 0.6	11.3 ± 1.2	7.6	0.6	1.3	0.8	0.8
24:6 n-3	0.9 ± 0.2	2.7 ± 0.4	1.9	0.1	0.2	0.1	0.2

[a] Details as in Table IV. Hexane extracted an average of 45% of the total PA, 66% of the total PI, and 97% of the diacylglycerols. Only the depicted fatty acids (which represent higher than 90% of the fatty acid weight on gas–liquid chromatograms) were considered for calculations.

fraction (B) was 5-fold richer in VLCPUFA-containing PCs than fraction A. Similarly, lipid fraction B contained 1.3-fold more PS (Table III), and the PSs of this fraction were 2.2-fold richer in VLCPUFA than those of fraction A (Table V), giving a 2.9-fold enrichment of VLCPUFA-containing PSs in the rhodopsin-associated lipid fraction.

Phosphatidate and phosphatidylinositol (less than 3% of the total lipid phosphorus from disks) displayed the same behavior as the major phospholipids, and thus the fraction of each remaining with rhodopsin was richer in longer and more highly unsaturated fatty acids than the fraction removed by hexane (Table VI). On the basis of the amounts of lipids recovered as methyl esters, there was about twice as much diacylglycerol as phosphatidylinositol in disks, and their fatty acid compositions were in addition quite similar. This suggests the interesting possibility [also suggested by ^{14}C-labeled polyenoic fatty acid incorporation experiments; see Rotstein and Aveldaño (1987)] that a considerable proportion of the former may have risen from phosphoinositides during the process of disk membrane isolation. Diacylglycerols were almost completely removed from lyophilized membranes by hexane (Table VI), as was also the case with cholesterol (not shown).

The free fatty acids (FFAs) present in disk membranes did not behave as the mentioned neutral lipids with respect to their extractability by the solvent, since only about 40% of the total FFA was removed (Table VII). In addition, they behaved in a way that was exactly the opposite as that of phospholipids with respect to the type of fatty acids involved: the fraction of FFA removed by hexane was richer in longer and more unsaturated fatty acids than the protein-associated fraction, as emphasized by the 7-fold larger saturated/unsaturated FFA molar ratio in the latter. Even though on a weight basis FFAs were relatively minor constituents of the disk lipid, on a mole basis their levels were quite high (about 5 mol/mol of rhodopsin, i.e., larger than phospholipids like phosphatidylinositol and others). Their metabolic role and origin in the membrane are not known. In contrast to the high unsaturation of phospholipids, there were about 2.7-fold more saturated than unsaturated fatty acids in the FFA pool. Free docosahexaenoate accounted for only 11% of the FFA, and in addition, saturates like 24:0, which were negligible components of glycerophospholipids, occurred as free fatty acids.

A similar hexane extraction experiment carried out in rod outer segments (not shown) gave similar qualitative and quantitative results as those described for disks. In Table VIII the ratios between the fatty acids mainly involved are shown to differ markedly and significantly for the major lipid classes present in fractions A and B. The columns on the right of Table VIII contain the relevant quantitative characteristics of the average of all the acyl chains of such lipids, calculated from their composition. It is apparent that the lipids of fraction B, especially PC and PS, contain heavier, longer, and more unsaturated fatty acids than those of A.

Table VII: Composition of Free Fatty Acid Fractions Extracted (A) and Nonextracted (B) by Hexane from Lyophilized Bovine Disks[a]

	A	B	total[b]
14:0	1.5 ± 0.1	1.9 ± 0.1	1.7
15:0	1.3 ± 0.1	1.3 ± 0.1	1.3
16:0	25.0 ± 1.4	44.3 ± 0.3	36.5
16:1	2.7 ± 0.1	1.9 ± 0.1	2.2
17:0	1.0	2.2 ± 0.1	1.7
18:0	20.6 ± 1.1	34.1 ± 0.7	28.6
18:1	9.2 ± 0.4	4.9 ± 0.2	6.6
19:0	0.3	0.4	0.4
18:2	1.4 ± 0.1	0.8	1.1
20:0	0.2	0.4	0.3
18:3 + 20:1	0.2	0.1	0.1
21:0		0.2	0.1
20:3 n-6	0.6 ± 0.1		0.2
22:0		0.6	0.4
20:4 n-6	5.5 ± 1.0	0.5 ± 0.1	2.5
23:0	0.4	0.5	0.4
20:5 n-3	0.5 ± 0.1		0.2
24:0	0.8 ± 0.1	1.1 ± 0.1	1.0
22:4 n-6	0.7 ± 0.1		0.3
25:0		0.2	0.1
22:5 n-6	0.2		0.1
22:5 n-3	1.9 ± 0.1	0.1	0.9
22:6 n-3	22.0 ± 0.6	3.6 ± 0.5	11.1
26:0	0.3	0.5	0.4
24:5 n-3	3.2 ± 0.1	0.3 ± 0.1	1.4
24:6 n-3	0.5 ± 0.1		0.2
28:0		0.2	0.1
S/NS[c]	1.1	7.3	2.7

[a] Details as in Table IV. SDs smaller than 0.05 are not depicted [b] The composition of the total did not differ from that of untreated disks. In these preparations there was 5.2 ± 0.5 mol free fatty acid/mol of rhodopsin. Hexane extracted 41 ± 3% of the total free fatty acids. [c] S/NS, ratio between the sum of saturated and that of unsaturated FFAs.

Discussion

The presented results provide further chemical characterization of the dipolyunsaturated species of PC in bovine disk photoreceptor membranes. Such PCs are shown to be endowed with unusually long polyenes, mainly of the n-3 series, which are predominantly esterified to the *sn*-1 position of the glycerol backbone, 22:6 n-3 being the major acyl chain at *sn*-2. The selective lipid class extraction with hexane and other nondenaturing solvents has been repeatedly and consistently observed in photoreceptor membranes, whether invertebrate microvillii (Mason & Fager, 1974; Tsuda & Akino, 1981) or vertebrate rod outer segments (Borggreven et al., 1970; Poincelot & Abrahamson, 1970), but evidence that the hydrophobic acyl

Table VIII: Characteristics of Fatty Acids in Hexane-Removable (A) and Protein-Associated (B) Fractions of Lipids from Freeze-Dried Rod Outer Segments and Disks[a]

		ratios × 10			av fatty acid[b]		
		18:0/22:6	VLCPUFA/22:6	S/NS	M_r	length	unsaturation
total lipid	A	6.2 ± 0.3 (6.5)	1.1 ± 0.1 (0.8)	7.2 ± 0.3 (7.5)	305 ± 3 (302)	19.8 (19.8)	3.0 (3.1)
	B	3.3 ± 0.3 (3.6)	2.7 ± 0.1 (2.5)	4.4 ± 0.3 (4.7)	318 ± 4 (315)	20.8 (20.5)	3.7 (3.6)
PC	A	7.2 ± 0.9 (7.5)	2.4 ± 0.3 (1.8)	10.5 ± 0.7 (12.2)	303 ± 4 (300)	19.6 (19.5)	2.5 (2.5)
	B	4.0 ± 0.8 (3.3)	4.5 ± 0.5 (3.6)	7.6 ± 0.6 (5.5)	320 ± 3 (320)	20.8 (21.3)	3.3 (3.5)
PE	A	6.4 ± 0.1 (6.6)	0.2 ± 0.0 (0.3)	8.0 ± 1.1 (9.3)	305 ± 2 (302)	19.8 (19.9)	3.1 (3.1)
	B	4.0 ± 0.8 (3.3)	0.6 ± 0.0 (0.5)	4.7 ± 0.9 (3.9)	308 ± 2 (308)	20.1 (20.9)	3.6 (3.8)
PS	A	7.5 ± 0.9 (5.9)	1.9 ± 0.1 (1.9)	5.8 ± 0.7 (4.7)	317 ± 2 (315)	20.5 (20.8)	3.5 (3.9)
	B	2.2 ± 0.3 (1.4)	4.2 ± 0.7 (3.6)	1.5 ± 0.2 (1.1)	329 ± 4 (329)	22.0 (22.0)	4.9 (5.1)

[a] Four samples of rod outer segments were treated with hexane as described for disks and analyzed by GLC. The depicted values were calculated from the fatty acid composition of lipids (not shown). The values for disks, calculated from the data in Tables IV and V, are given for comparison in parentheses. The ratios between the depicted fatty acids were multiplied by 10. S/NS = the ratio between the sum of all saturated and the sum of all unsaturated fatty acids. [b] Values of molecular weight (M_r), length, and unsaturation of the "average" fatty acid of each lipid in A and B.

chains of lipids might play a role in this separation was lacking. Here it is shown that the fraction of the total lipid which remains associated to rhodopsin after hexane extraction contains phospholipids which in all cases have (1) heavier, (2) longer, and (3) more highly unsaturated acyl chains than the fraction easily removed. The fatty acid composition of both fractions indicates that dipolyunsaturated species are "preferred" over other species, including hexaenes, under these conditions. Since the fatty acid at *sn*-2 (22:6 n-3) is the same for hexaenes and dipolyunsaturates, the "preference" of rhodopsin for the latter may be related to the presence of long and very long acyl chains in the *sn*-1 position of dipolyunsaturated phospholipids. Much more PC than PS, and in turn more PS than PE, is held by the protein, which is consistent with the fact that PC has longer polyenoic acyl chains than PS, and this in turn longer polyenes than PE.

The effect of hexane extraction on disk membrane FFA is an interesting exception which supports the view that the selective removal of phospholipid species is not merely a question of preferential "solubility" of such species in the solvent, but of preferential associations between the species not extracted and rhodopsin. The 3-fold enrichment in phospholipid species bearing VLCPUFA, and especially the 5-fold enrichment in phosphatidylcholines with such fatty acids, is consistent with this interpretation. (1) The dipolyunsaturated species of PC with 24–36-carbon acids at *sn*-1 and 22:6 at *sn*-2 have the largest hydrocarbon tail/polar head group ratios among disk PCs; (2) these species are very strongly retained upon reverse-phase liquid chromatography using octadecylsilane as support and a polar solvent like acetonitrile or methanol (Aveldaño, 1987) and are the most weakly retained species upon adsorption TLC using silica as support [Miljanich et al. (1979) and present results]. Therefore, if solubility in the solvent was the only physical parameter involved, one would expect these species be removed in preference to PCs with shorter acyl chains, since among the species of PC, dipolyunsaturates are the ones with the most strongly lipophilic (or less polar) character and hence should be the ones more strongly interacting with a nonpolar solvent with hexane, which was not the case. For some reason these PCs have a higher affinity for rhodopsin than for hexane and, what is even more interesting, for the lipid which *is* removed by the solvent after an exhaustive extraction. Apparently there are physical forces and/or sterical hindrances that prevent these PCs from being separated from the protein. Since there are PCs that are removed and, conversely, there are PEs that remain, the fatty acids rather than the polar head group apparently play a major role in such selective lipid class "partition". Any lipid that remains bound to a protein in the presence of a large molar excess of an organic solvent clearly has a higher affinity for that protein than the lipids readily displaced.

The lateral distribution of lipids in biological membranes is still a rather speculative matter. In most cases, we do not have direct proof of the specificity of interactions (if any) between integral membrane proteins and chemically defined groups of lipids in their native hydrophobic environments, nor as to the influence of such interactions on protein structure and function. We are caught up in the dilemma that slow chemical procedures sometimes suggest selective associations that cannot be observed in membranes in the native state, whereas fast physical procedures like ESR and NMR detect them in the native state but cannot provide information on the chemical characteristics of the lipids involved, since they "sense" bulk physical properties. Thus, an environment of phospholipids whose head group motions are restricted by the presence of rhodopsin is observed in bovine ros at 20 °C by ^{31}P NMR (Albert & Yeagle, 1983). Classical ESR studies have also revealed that rhodopsin restricts the motional freedom of a fraction of the total population of lipids present in native photoreceptor membranes: angular motions of the chains and rotation along the long molecular axis of some 30–40% of the lipids of these membranes are severely restricted by the presence of rhodopsin [Marsh and Watts (1982) and references cited therein]. Lipid molecular motions and lateral exchanges are obviously too fast to attempt an assignment of the lipids and fatty acids involved by chemical analysis of membranes in the native state (it is like trying to take a picture of fast-moving subjects by using a slow film). Lyophilization is one mild, reversible upon rehydration, nondenaturing way of obliging the membrane "subjects" to stay quiet. In the dry state, the lipids are prevented from undergoing the rapid rotational and lateral motions they display in fully hydrated membranes and may be expected to remain in relatively fixed positions in the neighborhood of integral membrane proteins. A chemical "picture" could then be taken, like here, with a nondenaturing solvent. The present observations on the behavior of phospholipids and fatty acids in relation to rhodopsin suggest the possibility that, among phospholipid classes, PC could be an important component of the fraction of the total lipid that is motionally restricted by rhodopsin (detected at the head group level by NMR). It could also be speculated that the long polyenoic acyl chains photoreceptor membrane lipids—especially PC—have at *sn*-1 could be relevant among the fatty acids whose motions are relatively restricted by the protein (detected at the acyl chain level by ESR).

Lyophilized, hexane-extracted, and then rehydrated bovine ros produce ESR spectra characteristic of strongly immobilized

spin-labels, with an outer hyperfine splitting much larger than in native membranes (Pontus & Delmelle, 1975). In fact, the order parameter, as sensed by 5-doxylstearate, is larger in membranes thus treated and taken to 37 °C (0.756) than in liposomes of dipalmitoyl-PC (whose T_c is 41 °C) taken to 28 °C (0.729). The enrichment in dipolyunsaturated species of lipids bearing long and very long acyl chains after hexane extraction of ros and disks (present results) may thus correlate with the enrichment in the motionally restricted lipid component observed by Pontus and Delmelle. Such compositional change is probably not deleterious to rhodopsin structure, and the change in the ESR spectrum is not due to protein aggregation, since it has long been known that the integrity and basic photochemical features of rhodopsin are preserved in photoreceptor membranes exhaustively extracted with mild, weak solvents like ether or petroleum ether (Ishimoto & Wald, 1946). Membranes "with or without lyophilization and extraction of lipids and retinal derivatives with petroleum ether" may be used indistinctly as a source of rhodopsin for photochemical studies (Yoshizawa & Schichida, 1982). Rhodopsin can even be transferred as a lipid–protein complex directly from bovine ros into apolar environments like ether or hexane, conserving its characteristic spectral properties and regenerability after bleaching whether in the solvents themselves or after subsequent rehydration and solvent evaporation (Darszon et al., 1979).

The unusual structural features of an important proportion of the PCs present in bovine photoreceptor membranes may provide clues to understand, or at least interpret, the involvement of this phospholipid in producing many highly individualistic physical signals observed along the extensive work already done in these membranes. For example, the temperature dependence of the polarization of fluorescence of *trans*-parinaric acid in ros PC subfractions separated by TLC (Miljanich et al., 1979) indicates that 16:0/22:6-PC shows no structural reorganization in the range 0–40 °C, giving a pattern typical of the probe in phospholipid fluid phases. Interestingly, dispersions of the fraction of PC that migrates ahead on TLC chromatograms—shown here to contain VLCPUFA and 22:6—have a higher transition temperature than the former [as indicated by the behavior shown in Figure 2 of Miljanich et al. (1979)]. The phenomenon was not explained at that moment, but the present results showing the special characteristics of such PCs may help interpret it. They may also help explain ^{1}H NMR signals from photoreceptor membrane lipids that were qualified as "unusual" or "surprising", such as the occurrence of two distinct terminal CH_3 resonances, the fact that the resolved area of the doubly allylic methylenes (CH=CH—CH_2*—CH=CH) is so low and so similar to that of the $(CH_2)_n$ protons, or the specific production (exclusively by ros membranes and by liposomes prepared from ros lipids) of a broad component that reversibly increases with decreasing temperature, apparently originating in phosphatidylcholine (Brown et al., 1977).

Another previous observation where dipolyunsaturated PCs may have been involved is that the relative area ratios of choline and allylic carbon resonances in ^{13}C NMR spectra of lipid vesicles prepared from disks are the same as in native membranes, but in the latter the relative area ratio of the CH_2 signal is half of that observed in the vesicle spectrum (Zumbulyadis & O'brien, 1979). The broadening of the CH_2 signal in membranes was interpreted by these authors as rhodopsin immobilizing more strongly the saturated acyl chains at *sn*-1 than the polyunsaturated acyl chains at *sn*-2 of disk phospholipids. Even though such an interpretation was later objected to by Brown et al. (1982), the results in this paper add a new component to be considered: the very long polyunsaturated acyl chains of dipolyunsaturated PCs, which have a long $(CH_2)_n$ segment in addition to several methylene-interrupted cis double bonds (Aveldaño & Sprecher, 1987). The preference of rhodopsin for the acyl chains at *sn*-1 was suggested by Zumbulyadis and O'Brien to be due to the ability of saturated fatty acids to conform to the geometrical requirements of the protein surface. This possibility sounds even more attractive for VLCPUFA-containing PCs, whose *sn*-1 fatty acids are twice as long and probably much more "coiled" than an average saturate.

Somewhat more direct evidence from previous work that rhodopsin and PC may sustain specific interactions in disk membranes is in the work of Sklar et al. (1979), who observed that the phase separations occurring in the lipid isolated from rod outer segments considerably differred, and in some cases did not take place at all, in native ros membranes. In the latter (i.e., when all membrane components including rhodopsin were interacting), the most conspicuous alteration was on the phase behavior of phosphatidylcholine. While the transitions typical of PS and PE occurred at somewhat lower temperatures and were broader in ros membranes than in ros lipid (but still occurred), the transition characteristic of PC was completely suppressed in ros membranes. The role of rhodopsin itself in bringing about that effect was given no detailed discussion at that moment, but this strongly suggests that rhodopsin interferes with the way in which ordered domains of laterally separated lipid classes form as temperature decreases and that such "interference" is stronger for PC than for other lipid classes of the photoreceptor membrane.

We have still not established how familiar and ubiquitous glycerophospholipid species like 18:0/20:4 or 16:0/22:6 pack in the solid state, in the gel state, and in the presence of other lipids and proteins as in membranes, when we are challenged by the intriguing question of how phospholipid species like the PCs shown here—e.g., 32:6/22:6, having two acyl chains so highly unsaturated and so uneven in length—"accommodate" in the thickness of the photoreceptor membrane. Lipids isolated from disks do not form a bilayer at temperatures above 15 °C in the presence of divalent cations, while in photoreceptor membranes they do, irrespective of temperature and calcium ion concentrations (De Grip et al., 1979; Mollenwanger & De Grip, 1984). This and the observations described in the preceding paragraph indicate that rhodopsin imposes a special kind of arrangement to lipids in disk membranes which considerably differs from that in bilayers made from such lipids. The "motionally restricted" fraction of the total lipid in photoreceptor membranes was interpreted (Marsh & Watts, 1982) to be spatially disordered at the protein–lipid interface (from the lipid, not necessarily from the protein, "point of view").[3] These considerations, *plus* the large number of methylene-interrupted cis double bonds very long chain polyenes have toward the methyl end, suggest that we should depart from the idea that (all) fatty acids of disk membranes are oriented in a way strictly parallel to the bilayer normal.

The degrees of motional freedom in naturally occurring polyunsaturated acyl chains are restricted by the presence of cis double bonds. Thus, 12 of the carbon atoms of hexaenoic fatty acids like 22:6 or 32:6 are prevented from undergoing rotations, since they are "fixed" by the double bonds. Rotations

[3] Perhaps instead of proteins perturbing or decreasing the "normal" mobility of lipids in bilayers, we should describe the same phenomenon by saying that lipid mobility (and disorder) increases in the bilayers we prepare from membrane lipids due to the absence of proteins.

around the doubly allylic methylenes are possible but are likely to be restricted in biological membranes due to intramolecular hindrances (the rigid cis —C=C— bonds plus all the allowable contact radii of atoms bound to such carbons) and to intermolecular restrictions (imposed by neighbor molecules in the bilayer like proteins and other lipid acyl chains). One among the many hypothetically possible ways to visualize the arrangement of lipids with long, large polyenes at *sn*-1 in the thickness of the photoreceptor membrane could be that such fatty acids formed open, approximately helical structures, favored by the presence of so many regularly spaced double bonds toward the methyl end plus several $(CH_2)_n$ units toward the carboxyl end (n = 2–16 between 22:6 and 36:6 *n*-3). Such open helical conformations would permit polyenoic fatty acids of phospholipids (particularly those with very long chains) to partially intertwine into the grooves of intramembranes α-helical segments of rhodopsin, closely interacting with hydrophobic amino acid residues facing the bilayer. This possibility is supported by an extrapolation from previous work by Deese et al. (1981). They observed that rhodopsin has little effect on the T_c of ^{2}H-labeled 16:0/16:1-PC but considerably broadens that of 16:0/22:6-PC. Studying the ^{2}H NMR spectra, they interpreted that the second species has a longer exchange lifetime on and off the surface of the protein than the former. Whether such retardation is due to the higher unsaturation, the longer chain, or both length and unsaturation of 22:6, it is licit to predict that dipolyunsaturated PCs like 32:6/22:6-PC will have an even longer exchange lifetime. The *sn*-1 fatty acids, with their many double bonds located deep down in the chain (i.e., close to the middle of the bilayer), increase the probabilities of lipid–protein interactions, since these are possible at two levels of depth in the membrane. The resistance of dipolyunsaturated, VLCPUFA-containing PCs (and PSs) to being separated from rhodopsin after hexane extraction is consistent with the possibility that some degree of lipid acyl chain–protein interdigitation might take place with these species more than with other species of the same lipid(s). The above ideas by no means imply that in the native membranes at physiological temperatures such species would be permanently bound to rhodopsin (i.e., that they would form an especially tight "annulus" around it), but that in a relative sense their motions (lateral, rotational, segmental) have reasons to be more restricted than those of smaller, shorter, or less unsaturated lipid species.

It is interestingly coincidental that, as polyenoic fatty acids have various restrictions to chain rotations imposed by their double bonds, the major hydrophobic chain of purple membrane lipids [3(*R*),7(*R*),11(*R*),15-tetramethylhexadecyl, Kates et al. (1967)] also presents various points of restriction to free chain rotation, imposed in this case by four bulky methyl groups. Methyl groups, as double bonds, besides introducing "kinks" in lipid hydrophobic chains, obviously prevent some of their possible segmental motions to occur and hence make such chains more rigid, and at the same time bulkier, than straight saturated chains. Compositional, electron diffraction, and ESR data led Jost et al. (1978) to conclude that almost all the lipid in the purple membrane is immobilized by bacteriorhodopsin, with only very limited pools showing the molecular motions of a fluid bilayer. Therefore, no "bilayer", in the traditional sense, exists in the purple membrane. This may also be the case, to various extents, with other membrane systems including photoreceptor disks, since it is unlikely that with only two shells of lipids surrounding each rhodopsin molecule (Watts et al., 1979) there is in fact a true bilayer, i.e., that there are many lipids which do not "feel" in one way or another the influence of the protein. Helical segments of integral proteins like rhodopsin and bacteriorhodopsin may "force" many of the neighbor hydrophobic lipid chains to adopt, however transiently, a number of conformations that are not attainable in bilayers of the pure lipid (helical conformations could be one of them). Conversely, phospholipids are known to *induce* extensive segments of α-helical segments in polypeptidic chains of proteins. Wu et al. (1982) suggested that lipids may be responsible for the presence of various α-helical segments in bacteriorhodopsin, which, according to its amino acid sequence, should have to occur predominantly in the β-form. It is therefore a question that remains open whether lipids with specific structural details in their hydrophobic chains are biologically synthesized out of necessity to conform to the geometrical requirements of integral membrane proteins or if they are thus made to determine the definitive functional conformation of such proteins.

Conclusions

In comparison with the overwhelming advances made in the last decade in the knowledge of rhodopsin and other proteins of photoreceptor membranes, that of their lipid constituents has somehow lagged behind. In general, such lipids are diffusely thought of as a highly fluid matrix where rhodopsin is allowed to freely "float". However, increased unsaturation above a certain number of double bonds does not necessarily translate into increased "fluidity", neither in the sense of the potentiality of lipid molecules to undergo (segmental, rotational, lateral) motions (Stubbs et al., 1981) nor in the sense of their ability to get into ordered lipid domains, as shown by their transition temperatures (Coolbear et al., 1983; Deese et al., 1981). At physiological temperatures, a fluid lipid bilayer could be attained by lipids having much simpler and less unsaturated fatty acids than those made in photoreceptor cells (so "expensive" metabolically). Moreover, not all the lipids in these membranes are in a fluid (highly mobile) state, as shown by many experiments using NMR and ESR techniques. Reconstitution experiments indicate that fluidity of the lipid bilayer is a necessary though not sufficient condition for rhodopsin functionality (O'Brien, 1982). The results presented here disclose additional peculiarities of the chemical characteristics and behavior of the phospholipids that are naturally required for rhodopsin function. Finding ways to uncover the specific reasons for such requirement offers a stimulating challenge for future work.

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Liposomes as Model for Taste Cells: Receptor Sites for Bitter Substances Including N–C=S Substances and Mechanism of Membrane Potential Changes†

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ABSTRACT: Various bitter substances were found to depolarize liposomes. The results obtained are as follows: (1) Changes in the membrane potential of azolectin liposomes in response to various bitter substances were monitored by measuring changes in the fluorescence intensity of 3,3′-dipropylthiocarbocyanine iodide [diS-C_3(5)]. All the bitter substances examined increased the fluorescence intensity of the liposome–dye suspension, which indicates that the substances depolarize the liposomes. There existed a good correlation between the minimum concentrations of the bitter substances to depolarize the liposomes and the taste thresholds in humans. (2) The effects of changed lipid composition of liposomes on the responses to various bitter substances vary greatly among bitter substances, suggesting that the receptor sites for bitter substances are multiple. The responses to N—C=S substances and sucrose octaacetate especially greatly depended on the lipid composition; these compounds depolarized only liposomes having certain lipid composition, while no or hyperpolarizing responses to these compounds were observed in other liposomes examined. This suggested that the difference in "taster" and "nontaster" for these substances can be explained in terms of difference in the lipid composition of taste receptor membranes. (3) It was confirmed that the membrane potential of the planar lipid bilayer is changed in response to bitter substances. The membrane potential changes in the planar lipid bilayer as well as in liposomes in response to the bitter substances occurred under the condition that there is no ion gradient across the membranes. These results suggested that the membrane potential changes in response to bitter substances stem from the phase boundary potential changes induced by adsorption of the substances on the hydrophobic region of the membranes.

Among various taste stimuli, substances that elicit bitter taste are most abundant. The structures of bitter substances are extremely diverse, and it is difficult to find a chemical structure common to bitter substances. An attempt to isolate the receptor protein for bitter substances was made (Dastoli et al., 1968), but it was pointed out that the protein isolated from the porcine tongue epithelium was not a true receptor molecule for bitter substances (Price, 1969; Koyama & Kurihara, 1971).

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