Biochimica et Biophysica Acta 860 (1986) 137–148
Elsevier

BBA 73210

Active labeling of phosphatidylcholines by [1-^{14}C]docosahexaenoate in isolated photoreceptor membranes

Norma M. Giusto [a], Mónica I. de Boschero [a], Howard Sprecher [b] and Marta I. Aveldaño [a,*]

[a] *Instituto de Investigaciones Bioquímicas, Universidad Nacional el Sur-Consejo Nacional de Investigaciones Científicas y Técnicas, Gorriti 43, 8000 Bahía Blanca (Argentina), and*
[b] *Department of Physiological Chemistry, Ohio State University College of Medicine, Columbus, OH 43210 (U.S.A.)*

(Received April 4th, 1986)

Key words: Photoreceptor membrane; Rod outer segment; Docosahexaenoate incorporation; (Bovine retina)

Isolated bovine rod outer segments and photoreceptor disks actively incorporated [1-^{14}C]docosahexaenoate (22:6) into phospholipids when incubated in the presence of CoA, ATP, and Mg^{2+}. About 80% of the esterified fatty acid was in phosphatidylcholine (PC). Microsomal and mitochondrial fractions incorporated as much 22:6 as rod outer segments, but it was distributed among various phospholipids and neutral glycerides. The isolated photoreceptor membrane thus contains an acyl-CoA synthetase which activates the fatty acid and a docosahexaenoyl-CoA-lysophosphatidylcholine acyltransferase activity. The specific radioactivity of PC was higher in rod outer segments than in the other subcellular fractions. About 2/3 of the label in photoreceptor membrane PC was in its dipolyunsaturated molecular species and 1/3 in hexaenes. Dipolyunsaturated PCs showed high turnover rates of 22:6 in all three subcellular membranes, especially in mitochondria. Retinal membranes in vitro seem to take up free [^{14}C]22:6 from the medium by simple diffusion or partition into the membrane lipid. The ability of these membranes to activate and esterify [1-^{14}C]22:6 indicates that docosahexaenoate-containing molecular species of retina lipids, including those of photoreceptor membranes, are subject to acylation-deacylation reactions in situ.

Introduction

Disk membranes packed in the outer segment of rod photoreceptor cells display a continuous process of renewal, as demonstrated by autoradiographic and radiobiochemical studies [1–5]. The lipid and protein components of these membranes are assembled at the base of the rod outer segment, while disks situated at its apex are concomitantly shed and engulfed by the pigment epithelium. There is also an intracellular traffic of intact lipid and protein molecules along the rod outer segment, the molecular replacement of disk membrane lipids being faster than that of protein [2–5]. Disk membranes thus rely on other subcellular membranes, located in the inner segment of the rod, for the supply of their phospholipid constituents, which is consistent with the fact that isolated rod outer segments lack key enzymes for the de novo synthesis of phospholipids like phosphatidylcholine (PC) [6]. When ^{3}H-labeled fatty acids are injected in vivo, a peak of label transiently appears at the site of phospholipid synthesis (the base of the outer segment), but labels from fatty acids diffuse throughout the disk membranes

* To whom correspondence should be addressed.
Abbreviations: [^{14}C]22:6, 4,7,10,13,16,19-[1-^{14}C]docosahexaenoate; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PS, phosphatidylserine.

at rates that are faster than expected from membrane replacement of intact phospholipid replacement mechanisms [2]. This suggested that phospholipid molecules in disk membranes may undergo an active turnover of their fatty acyl moieties [2]. This paper is concerned with the incorporation of 4,7,10,13,16,19-[1-^{14}C]docosahexaenoic acid ([^{14}C]22 : 6) into lipids of isolated rod outer segments and disks, as well as in other retina subcellular fractions. It is shown that photoreceptor membranes contain, in situ, enzymatic mechanisms which lead to the esterification of 22 : 6 in their phospholipids, and that the main target for such reactions is phosphatidylcholine.

Photoreceptor membrane phospholipids are made up by a whole range of docosahexaenoate-containing molecular species, from the well-known 'hexaenes' (e.g., 16 : 0/22 : 6, 18 : 0/22 : 6, etc.) to various 'dipolyunsaturated' species (e.g., 20 : 4/22 : 6, 20 : 5 or 22 : 5/22 : 6, 22 : 6/22 : 6, etc.) [7,8]. The latter separate as decaenoic, undecaenoic and dodecaenoic molecular species after argentation TLC [8], since they have a total of 10, 11, and 12 double bonds per molecule. In rod outer segment phosphatidylcholine these species contain 22 : 6 and, in addition to the familiar 20- and 22-carbon polyenes, a whole range of fatty acids having 4, 5 and 6 double bonds and 24–36 carbon atoms [9]. Since PC is shown here to be the phospholipid predominantly labeled with [^{14}C]22 : 6 in photoreceptor membranes, it was of interest to investigate how this fatty acid is distributed among the various docosahexaenoate-containing molecular species of PC. It is shown that a large proportion of the [^{14}C]22 : 6 incorporated in PC is introduced into its dipolyunsaturated molecular species in all retinal membranes.

During the development of this work, the collateral observation was made that an important fraction of the free [^{14}C]22 : 6 added to the incubation media was taken up by all retina subcellular membranes studied, irrespectively of their kind, apparently by an energy-independent mechanism. These observations are presented as an appendix to this paper.

Materials and Methods

Bovine eyes were obtained from a local slaughterhouse and kept at 0–4°C in the dark for about 2 h. The retinas were gently excised, swirled in tubes containing 34% (w/w) sucrose, and briefly centrifuged [10] to separate the floating rod outer segments from the rest of the retina. The procedure was repeated and rod outer segments were isolated from the combined supernatants using the discontinuous sucrose density gradient described by Papermaster [10]. Photoreceptor disks were obtained by subjecting rod outer segments from the 1.11/1.13 and 1.13/1.15 g/ml interfaces to osmotic shock, followed by flotation on a 5% Ficoll-water interface [11]. Rhodopsin content was determined by reading the absorbance at 500 nm in aliquots treated with buffered 1% Emulphogen and using a molar extinction coefficient of 40 000. The A_{280}/A_{500} (absorbance ratios) were usually 2.3 and the phospholipid/rhodopsin molar ratios were 70–75. The membranes were pelleted, washed twice with 10 mM Tris-HCl (pH 7.4), and resuspended in 50 mM Tris-acetate buffer (pH 7.4) containing 50 mM KCl and 5 mM NaCl. When indicated, rod outer segment suspensions in this medium were sonicated for 5 min at 4°C.

4,7,10,13,16,19-[1-^{14}C]Docosahexaenoic acid ([^{14}C]22 : 6), specific activity 40 Ci/mol, was prepared as described in Ref. 12. Aliquots from membrane suspensions were incubated at 37°C with [^{14}C]22 : 6 (added as the K^+ salt) and the following cofactors: 2.5 mM ATP, 10 mM $MgCl_2$, 0.1 mM CoASH, and 0.1 mM α-D-dithiothreitol, in a total volume of 0.5 ml of Tris-acetate-KCl buffer. After incubation, the membranes were diluted 20-fold with cold buffer, and immediately pelleted. All procedures were done under dim red light.

Lipids were extracted from the membrane pellets with chloroform/methanol [13], washed, and separated by two-dimensional thin-layer chromatography [14]. Since the extracts contained high levels of radioactivity from unesterified [^{14}C]22 : 6 in comparison with the label esterified in lipids, controls were prepared to subtract a certain amount of unspecifically adsorbed radioactivity present in the lipid spots (perhaps originated from trailing of labeled 22 : 6 along the plate). Aliquots containing the same amount of membranes as the experimental samples were heated in a boiling water bath for 5 min. Then [^{14}C]22 : 6 was added and incubation, as well as further procedures, were identical to those of the respective samples.

After chromatographic development, lipid spots were located with iodine vapors and scraped into vials containing 0.4 ml water. 10 ml of Triton X-100/5% Omnifluor in toluene (New England Nuclear) were then added, and the samples were subjected to liquid scintillation counting. Quantitation of lipids was done by phosphorus analysis of spots after TLC [14].

To study [^{14}C]22 : 6 incorporation into molecular species of PC, the lipid, isolated as before, was converted to acetyldiacylglycerols, which were separated by argentation TLC [8]. Chloroform/methanol/water (65 : 25 : 4, by vol.) was used to resolve dipolyunsaturated molecular species. The bands were located after spraying with dichlorofluorescein in methanol/water (1 : 1, by vol.), and scraped into vials containing 1 ml of 2 M NaCl. After mixing, 10 ml Aquasol-2 (New England Nuclear) were added, the contents were mixed, the solids settled down, and the samples counted by liquid scintillation.

For quantitation of phosphatidylcholine species, PC was isolated from unlabeled aliquots of the corresponding membrane preparations, converted to ^{3}H-labeled acetyldiacylglycerols, and treated as described above. The amount of ^{3}H in species was used to calculate the amount of species as detailed in Ref. 8.

Incorporation of [^{14}C]22 : 6 in photoreceptor membranes was compared to that of other subcellular membranes from retina. A crude mitochondrial fraction (also containing synaptosomes) and a microsomal fraction were obtained from the retinas remaining after rod outer segment separation. Retinas were homogenized in 0.32 M sucrose and centrifuged for 20 min at $3000 \times g$. The first pellet was discarded and the supernatants were centrifuged at $11\,500 \times g$ to isolate a second pellet (P_2). After separating this pellet, microsomes were obtained from the supernatants (60 min centrifugation at $100\,000 \times g$). Membranes thus prepared were subjected to identical procedures as photoreceptor membranes. In all cases, at least 95% of the membranes originally added to the incubation tubes was recovered after incubation with [^{14}C]22 : 6, dilution, and centrifugation, as determined by measuring lipid phosphorus before and after the experiments.

Results

Labeling of photoreceptor membrane lipids

The incorporation of [1-^{14}C]docosahexaenoate into lipids of isolated rod outer segments and disks was studied in the presence of ATP-Mg^{2+} and CoASH. In the absence of these cofactors, labeling of lipids was negligible. Thus, eight samples incubated for 1 h with no cofactors only yielded 0.4 ± 0.1 pmol ^{14}C-labeled phospholipids/nmol rhodopsin (not shown) against the 25.1 (Table I) obtained when cofactors were present. To facilitate comparison between rod outer segments and disks, initial incubations (Table I) used presonicated suspensions of the former, to ensure access of cofactors to their disk membranes. However, sonication was later found to be unnecessary, since non-sonicated rod outer segments incorporated similar amounts of [^{14}C]22 : 6. This indicates that the rod outer segment plasma membrane was permeable to ATP and CoA from the medium. Such permeabilization may have occurred during the isolation procedures, which in some steps include washings with somewhat hypotonic solutions (10 mM Tris-acetate [10]).

The pattern of [^{14}C]22 : 6 incorporation into photoreceptor membrane lipids was dominated by phosphatidylcholine (about 80% of the incorporated label, Table I). Phosphatidylethanolamine (PE), which was the major lipid (Table I), and phosphatidylserine (PS), incorporated much less fatty acid. This is peculiar, since in these membranes the proportions of 22 : 6-containing molecular species are higher for PE and PS than for PC [8]. In addition, PE and PS seem to be preferentially located in the outer monolayer of disk membranes [15] (the asymmetric distribution of lipids described by Litman [15] was reproduced when his procedure was applied to our disk preparations). Thus, the low level of labeling of these lipids is not caused by lack of accessibility of enzymes to cofactors and/or substrates. LysoPE and lysoPS, as well as lysoPC, were present in the membranes (Table I). The preferential labeling of PC is thus noteworthy, and suggests that the turnover of 22 : 6 in this lipid may play some special role in photoreceptor membranes.

Phosphatidate, which amounted to only 1% of the lipids, was the third phospholipid in the order

TABLE I

LABELING BY [1-^{14}C]DOCOSAHEXAENOATE AND COMPOSITION OF LIPIDS FROM BOVINE RETINA PHOTORECEPTOR MEMBRANES

Rod outer segment and disk membrane suspensions containing 15.4 and 7.2 nmol rhodopsin, respectively, were incubated for 60 min at 37°C with 6 nmol [^{14}C]22:6 in the presence of ATP-Mg^{2+} and CoASH in 0.5 ml of Tris-acetate-KCl buffer. After incubation, the samples were diluted 20-fold with cold buffer and pelleted. Lipids were extracted from the pellets and separated by two-dimensional TLC. Results are mean values ± S.D. from eight samples in each case. The average distribution of the incorporated activity (%) is given in parentheses. The phospholipid composition was determined in four samples of the respective preparations. The phospholipid/rhodopsin mole ratio was 70.9 for outer segments and 71.9 for disks.

Lipid	Rod outer segments	Disks
pmol[^{14}C]22:6/nmol rhodopsin		
PC	20.18 ± 1.62 (80.4)	15.60 ± 1.90 (78.1)
PE	3.20 ± 0.43 (12.7)	2.44 ± 0.65 (12.2)
PS	0.52 ± 0.03 (2.1)	0.17 ± 0.02 (0.9)
PI	0.13 ± 0.01 (0.5)	0.09 ± 0.02 (0.5)
Phosphatidate	0.96 ± 0.09 (3.8)	1.30 ± 0.15 (6.5)
LysoPC	0.06 ± 0.02 (0.2)	0.30 ± 0.10 (1.5)
LysoPE	0.05 ± 0.01 (0.2)	0.07 ± 0.15 (0.4)
Total phospholipid	25.10 ± 1.49	19.97 ± 2.72
Free fatty acid	153.4 ± 10.2	309.2 ± 14.5
Phospholipid composition (%)		
PC	35.8 ± 0.6	35.9 ± 0.3
PE	44.5 ± 0.6	42.8 ± 0.3
PS	15.3 ± 1.3	14.9 ± 0.3
PI	0.9 ± 0.01	1.3 ± 0.3
Phosphatidate	0.9 ± 0.13	1.0 ± 0.2
LysoPC	0.4 ± 0.04	0.7 ± 0.06
LysoPE	0.4 ± 0.02	1.4 ± 0.08
LysoPS	0.7 ± 0.02	0.7 ± 0.19
Sphingomyelin	0.4 ± 0.12	0.5 ± 0.04
Diphosphatidylglycerol	0.2 ± 0.01	0.2 ± 0.04
Others (origin, unknown, etc,)	0.3 ± 0.03	0.6 ± 0.04

of 22:6 incorporation. Phosphatidylinositol, present in similar amounts, incorporated 10-fold less [^{14}C]22:6 than phosphatidate. LysoPC from disks contained more label than PI, phosphatidic acid and PS, and more than rod outer segment lysoPC (Table I). The content of lysoPC was apparently small, but significantly higher in disks than in rod outer segments, and the specific radioactivity of lysoPC was similar in both (299 ± 103 and 224 ± 60 pmol [^{14}C]22:6/μmol lipid phosphorus), despite the fact that disks incorporated 20% less label.

The amount of [^{14}C]22:6 incorporated in lipids was linearly related to the amount of membranes (and hence to the amount of protein and lipid phosphorus) in a relatively small range of membrane concentrations, after which further increases in the amount of membranes did not lead to proportional increases in labeled phospholipids, as shown in Fig. 1A for PC. This is probably related to the fact that for a constant amount of fatty acid in the system, the actual concentration of free [^{14}C]22:6 in the membranes decreases as the amount of membranes increases, as shown in the appendix section.

Esterification of [^{14}C]22:6 in photoreceptor membrane lipids is an energy-dependent process, since it requires ATP for the activation of the fatty acid, as mentioned before. In the presence of ATP-Mg^{2+} and CoASH, lipid labeling by [^{14}C]22:6 was linearly dependent on the incubation time in the interval 0–60 min, as shown in Fig. 1B for PC. To test whether the label incorporated decreased after removing the cofactors from the medium, membranes incubated for 30 min with [^{14}C]22:6 and cofactors were diluted 20-fold, pelleted, and reincubated in plain buffer (no added [^{14}C]22:6 or cofactors). The label in phospholipids significantly decreased (see PC in Fig. 1B and in Table II). There was no apparent redistribution of [^{14}C]22:6 among phospholipid classes, since the percentage distribution of esterified label remained constant in the interval 0–60 min incubation under these conditions (not shown). The starting concentrations of free [^{14}C]22:6 in membranes also decreased (being recovered in media), for the ratio esterified/unesterified [^{14}C]22:6 in membranes was also approximately constant. However, when membranes were similarly incubated, but in the presence of cofactors, (1) label from free [^{14}C]22:6 decreased as before, but (2) the label in phospholipids was maintained as shown in Table II for PC, which resulted in significantly higher ratios of esterified versus free [^{14}C]22:6 in the presence than in the absence of cofactors. The fate of the radioactivity lost by PC could not be detected as an increase in labeled

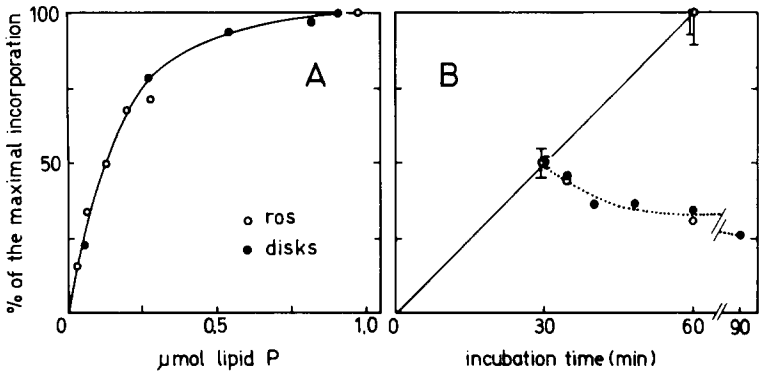

Fig. 1. Labeling of photoreceptor membrane phosphatidylcholine by [1-^{14}C]docosahexaenoate as a function of amount of membranes (A) and incubation time (B). In A, various aliquots from a rod outer segment (ros), or disk, preparation were incubated for 60 min at 37°C and at pH 7.4, with [^{14}C]22:6, ATP-Mg^{2+}, and CoASH. In B, equal amounts of rod outer segments (or disks) were incubated for 30 or 60 min under the same conditions as in A. Results are expressed as percent of the maximum incorporation attained in each experiment. Also in B, the decrease in PC label in membranes incubated with no cofactors is shwon. Membranes which had been incubated for 30 min with [^{14}C]22:6 and cofactors were diluted with fresh buffer, pelleted, and reincubated at 37°C in plain buffer for additional periods up to 60 min. (dotted line).

free 22:6 due to the exceedingly large amount of [^{14}C]22:6 already present in membranes (Table II). Similarly, it was also undetectable as an increase in free 22:6 by gas-liquid chromatographic (GLC) quantitations of free fatty acids in membranes. The 'endogenous' level of free 22:6 in disks was 0.039 mol/mol lipid phosphorus, i.e., about 2700 pmol/nmol rhodopsin. Disks incubated for 60 min at 37°C (with or without cofactors) did not display significant changes in the levels of free fatty acids with respect to unincubated membranes, as measured by GLC. Therefore, apparent phospholipid breakdown by phospholipases A did not occur under the present conditions (even though such enzyme activities are probably present, since rod outer segments incubated under similar conditions in Ca^{2+}-containing buffers show a significant increase in free fatty acids easily measurable by GLC [16]). Even when we were unable to detect lipid deacylation under the present experimental (and methodological) conditions, the significant decrease in PC label in the absence of cofactors (Fig. 1B, Table II) is likely to reflect an unbalance in the equilibrium between deacylation and reacylation reactions (see Fig. 2), the acylation step being obviously dependent on the presence of cofactors.

TABLE II

LABELING OF PHOTORECEPTOR MEMBRANE PHOSPHATIDYLCHOLINE AFTER REMOVAL OF [^{14}C]22:6 FROM THE MEDIUM

Samples of rod outer segments containing 10 nmol rhodopsin were preincubated with 6 nmol [1-^{14}C]22:6 and cofactors for 30 min. The media were diluted 20-fold with cold buffer, the membranes were pelleted, and the supernatants discarded. Some of the pellets were directly extracted with chloroform/methanol (a). The rest were incubated for 5 or 30 additional minutes with fresh buffer containing (+) or lacking (−) cofactors. FFA, free fatty acid.

Time after prelabeling (min)	Cofactors	pmol[^{14}C]22:6/nmol rhodopsin		PC/FFA (pmol ratio × 10^3)
		PC	FFA	
0 [a]	+	7.1 ± 0.8	219 ± 5.4	32.4 ± 0.4
5	+	8.3 ± 0.7	200 ± 9.0	42.0 ± 2.5
5	−	6.4 ± 0.7	196 ± 5.4	32.5 ± 3.6
30	+	7.2 ± 0.3	117 ± 5.1	61.1 ± 0.8
30	−	4.2 ± 0.1	125 ± 5.7	33.8 ± 1.3

Comparison with other subcellular membranes

The distribution of [^{14}C]22 : 6 among lipids of photoreceptor membranes as well as their specific radioactivities are compared with those of other subcellular membranes in Table III. All of them incorporated similar levels of 22 : 6, both on the bases of protein and lipid phosphorus content. Phosphatidylcholine concentrated most of the label in phospholipids, and phosphatidate had the highest specific radioactivity in all three membrane fractions. However, both the distribution of label and the specific activities showed marked quantitative differences among subcellular fractions. Neutral lipids, which were not labeled in rod outer segments and disks, concentrated about 1/3 of the [^{14}C]22 : 6 incorporated in microsomes and in the P_2 fraction. Phosphatidylserine and phosphatidate also showed significantly higher incorporations in the latter. Labeling of phosphatidylinositol was highest in microsomes and lowest in disks. Phosphatidylethanolamine and -serine attained the highest specific radioactivities in the P_2 fraction. The results in Table III are indicative of differences and similarities in the activity of acyltransferases in each membrane since, at least for the major phospholipids, important cofactors that would be necessary for de novo synthesis or other lipid interconversions are not present.

The comparison with other subcellular membranes was originally designed to test whether phospholipid labeling in photoreceptor membranes arose from contamination with fragments from other retinal membranes, especially mitochondria and synaptosomes. For this reason rather crude fractions were prepared from the same retinas, and were washed and incubated under the same conditions as rod outer segments. Interestingly, highly significant differences in the distribution of the fatty acid among lipids were observed in the three fractions. These dissimilarities, and especially the fact that the total level of incorporation in all fractions was similar, make the possibility of cross-contamination unlikely as an explanation for photoreceptor membrane lipid labeling. For instance, if contamination with microsomes were significant, some label should have

TABLE III

LABELING OF LIPIDS FROM RETINA SUBCELLULAR MEMBRANES BY [1-^{14}C]DOCOSAHEXAENOATE

Subcellular membranes isolated from retinas were incubated and analyzed as detailed in Table I for photoreceptor membranes. Protein content was determined from aliquots in parallel of the respective membrane preparations.

	Membrane			
	rod outer segments (11)	disks (8)	P_2 fraction (3)	microsomes (3)
Distribution of [^{14}C]22 : 6 (%)				
Phosphatidylcholine	82.0 ± 2.5	80.8 ± 2.0	42.4 ± 2.0	36.5 ± 2.9
Phosphatidylethanolamine	12.8 ± 2.3	11.7 ± 2.2	9.9 ± 1.2	1.5 ± 0.8
Phosphatidylserine	2.0 ± 0.2	0.9 ± 0.1	6.1 ± 0.3	3.7 ± 0.1
Phosphatidylinositol	0.5 ± 0.1	0.5 ± 0.1	1.7 ± 0.6	6.7 ± 0.03
Phosphatidate	3.2 ± 0.7	6.6 ± 1.0	11.5 ± 1.0	15.6 ± 0.5
Diacylglycerol	–	–	5.9 ± 0.4	1.6 ± 0.8
Triacylglycerol	–	–	25.5 ± 2.1	34.1 ± 1.7
Total incorporated				
(pmol [^{14}C]22 : 6/mg protein)	473 ± 25	347 ± 31	348 ± 10	308 ± 1
(pmol [^{14}C]22 : 6/μmol lipid P)	405 ± 27	296 ± 44	383 ± 11	267 ± 28
Specific radioactivities (pmol [^{14}C]22 : 6/μmol lipid)				
Phosphatidylcholine	739 ± 53	602 ± 82	384 ± 26	239 ± 19
Phosphatidylethanolamine	89 ± 9	76 ± 17	148 ± 19	22 ± 11
Phosphatidylserine	41 ± 3	16 ± 4	246 ± 9	150 ± 8
Phosphatidylinositol	116 ± 19	111 ± 20	147 ± 52	382 ± 0
Phosphatidate	1148 ± 109	1764 ± 315	5194 ± 486	4810 ± 146

been recovered as triacylglycerols in rod outer segments, which was not the case. If label in rod outer segment PC came from cross-contamination, its specific radioactivity should be much lower than in the other fractions, not higher as found. It is concluded that enzymes controlling the turnover of 22:6 are present in all subcellular fractions from retina. They are active towards various lipid substrates, including major membrane phospholipids, and key intermediates like phosphatidate.

Distribution of [^{14}C]22:6 among docosahexaenoate-containing phosphatidylcholines

The distribution of [^{14}C]22:6 among phosphatidylcholines (Table IV) significantly differed among retinal membranes, especially between rod outer segments and microsomes. More than half the radioactivity, against 32%, was in dipolyunsaturated PCs in the former and the latter, respectively. Among dipolyunsaturated species, the fraction at the origin of the argentation TLC plates (I), which mainly contains didocosahexaenoyl species, was the most heavily labeled in photoreceptor membranes. This fraction showed the highest specific radioactivity in all subcellular membranes, while the lowest corresponded to hexaenoic species of PC. The turnover of 22:6 in the dipolyunsaturated species which contain other polyenes in addition to 22:6 (mainly very long-chain hexaenes, pentaenes and tetraenes, in fractions II, III and IV, respectively [9]) was about 10- and 3-fold higher in the P_2 fraction and microsomes, respectively, than in photoreceptor membranes, as indicated by their specific radioactivities in Table IV. This is interesting because dipolyunsaturated molecule species, including those containing very-long-chain polyenoic fatty acids, are specific constituents [9] of photoreceptor membranes, and are probably synthesized in intracellular membranes of the rod inner segments. How-

TABLE IV

DISTRIBUTION OF [1-^{14}C]DOCOSAHEXAENOATE AMONG PHOSPHATIDYLCHOLINES FROM RETINAL MEMBRANES

Phosphatidylcholine from membranes incubated for 60 min with [^{14}C]22:6, ATP-Mg^{2+}, and CoASH was isolated by TLC and converted to acetyldiacylglycerols. The latter were resolved into fractions of similar unsaturation by argentation TLC. Quantitation of species in each band was done in unlabeled aliquots of the respective membrane preparations after conversion of PC to ^{3}H-labeled acetyldiacylglycerols, which were separated and counted as were the [^{14}C]22:6-labeled derivatives. [a] The term 'supraenes' is used to designate species with more than six double bonds per molecule. Band I mainly contains didocosahexaenoyl species (90% 22:6). Bands II, III and IV contain 50% 22:6 and 50% hexa-, penta- and tetraenoic fatty acids, respectively (mainly with very long carbon chains [9]). Band V, nona- to heptaenes (mostly heptaenes, 18:1/22:6). Incubations were done in duplicate (disks, mitochondria, microsomes) or triplicate (rod outer segments), for which mean values ± S.D. are given. Due to the very small amounts of supraenoic species in P_2 and microsomes, fractions II–IV were collected together, and fraction V together with hexaenes. To allow for comparison between membranes, the sum of radioactivity in these species is also provided for photoreceptor membranes ([b]). [c], is the sum of radioactivity in these species divided by the sum of their mass.

Species	Distribution of [^{14}C]22:6 (%)				Specific radioactivity (pmol [^{14}C]22:6/nmol species)			
	rod outer segments		disks		rod outer segments		disks	
Supraenes [a] I	38.6 ± 5.6		47.8		5.0		3.2	
II	9.2 ± 0.5		8.4		0.8		0.6	
III	3.9 ± 0.7	16.8 [b]	4.1	15.6 [b]	0.3	0.62 [c]	0.3	0.46 [c]
IV	3.7 ± 0.7		3.1		1.2		0.8	
V	10.6 ± 0.2		11.2		3.0		2.6	
Hexaenes	34.0 ± 4.2	44.6 [b]	25.4	36.6 [b]	0.8	0.92 [c]	0.5	0.62 [c]
	P_2 fraction		microsomes		P_2 fraction		microsomes	
Supraenes I	29.0		16.0		5.3		4.8	
II to IV	23.1		16.3		5.5		1.8	
V + hexaenes	47.9		67.7		1.1		1.0	

ever, since microsomes and mitochondria were obtained from entire retinas, only a small fraction of these membranes derive from (the inner segments of) photoreceptor cells, for it is noteworthy that high specific radioactivities are still manifested in these species of PC, despite the considerable dilution with mitochondrial and microsomal membranes from many other cell types.

Discussion

The results in this paper show that photoreceptor membrane lipids, especially phosphatidylcholine, undergo an active turnover of their docosahexaenoyl moieties. The reactions leading to 22 : 6 incorporation into PC are independent from the lipid biosynthetic processes that occur in the inner segment of rods, since (1) they take place in isolated rod outer segments and disks, and (2) occur in the absence of cofactors that would be necessary for de novo synthesis and/or alternative lipid interconversions. As long as the fatty acid is only esterified in the presence of ATP-Mg^{2+} and CoA, is not utilized in membranes previously denatured by heat, and esterification depends on incubation time and amount of protein, the process is enzymatic. The enzymes involved are necessarily (1) a membrane-bound acyl-CoA synthetase which produces docosahexaenoyl-CoA, and (2) a docosahexaenoyl-CoA-lysoPC acyltransferase (reactions 1 and 2, Fig. 2).

In general, the process of lipid acyl moiety turnover may be envisaged as a cyclic event in which a third enzyme must intervene to provide an acceptor lyso compound (Fig. 2). This third enzyme may be a phospholipase A (as in the Lands' cycle [17], or an acyltransferase such as the activity catalyzing the CoA-mediated, ATP-independent breakdown of phospholipids demonstrated in rat liver microsomes [18] and in lymphocytes [19]. Both will produce lysophospholipids, but only phospholipases A will produce in addition free fatty acids. In the presence of ATP and CoA, the latter may be rapidly converted to acyl-CoA esters and recombined with lysophospholipids. The two possible ways of acyl-CoA ester synthesis (reactions 3 and 4, Fig. 2) are not necessarily exclusive of one another. In intact cells the acylation capacity is always greater than the degradation of phospholipids by phospholipases A, which is biologically sensible, since otherwise the cycle of fatty acyl moiety turnover would result in a futile expenditure of high-energy phosphates [20].

Whatever the source of acyl CoA(s) and lyso-compounds, lysophosphatide acyltransferase(s) must play a central role (1) in the maintenance of lipid (and hence of membrane) integrity, (2) in the maintenance of the characteristic acyl group composition of lipids, and (3) in mechanisms of fatty acid replacements to synthesize new molecular species of lipids through inter- or intra-molecular rearrangements of their acyl chains. By selecting specific CoA esters from the pool of acyl-CoA(s), acyltransferases may be involved in the control of physicochemical properties of membranes that depend on lipid acyl moiety structural features such as chain length and unsaturation. The densely packed disks of photoreceptor cells, especially those located near the apex of the rod outer segments, are quite far from the site of synthesis to rely on the resynthesis and transport of new molecules for rapid replacement, let us say, of a 'damaged' (e.g., hydrolyzed, peroxidized) phospholipid molecule. In addition, local modifications in the physical properties (e.g., fluidity) of membranes, brought about by transient changes in their immediate environment (e.g., changes in pH, Ca^{2+} movements, etc), may be compensated by in situ rearrangements of lipid acyl chains. Acyltransferases involved in polyunsaturated fatty acid turnover may play important roles in these mechanisms.

Studies on the specificities of liver acyltransferases have indicated that arachidonate and related long-chain polyenes may be esterified mainly through the 1-acyl-*sn*-glycerol-3-phosphocholine acyltransferase system (reaction 2, Fig. 2), whereas saturated, monoenoic and dienoic fatty acids may be incorporated mainly by glycerophosphate and 1-acyl-*sn*-glycerol-3-phosphate acyltransferases (reactions 5 and 6, Fig. 2) [24]. Those enzyme activities have been separated, and their acyl donor specificities have indicated that the acylation that gives rise to phosphatidate, and the deacylation-reacylation cycle operating after de novo synthesis of lipids are respectively the most important steps in controlling the non-random distribution of fatty

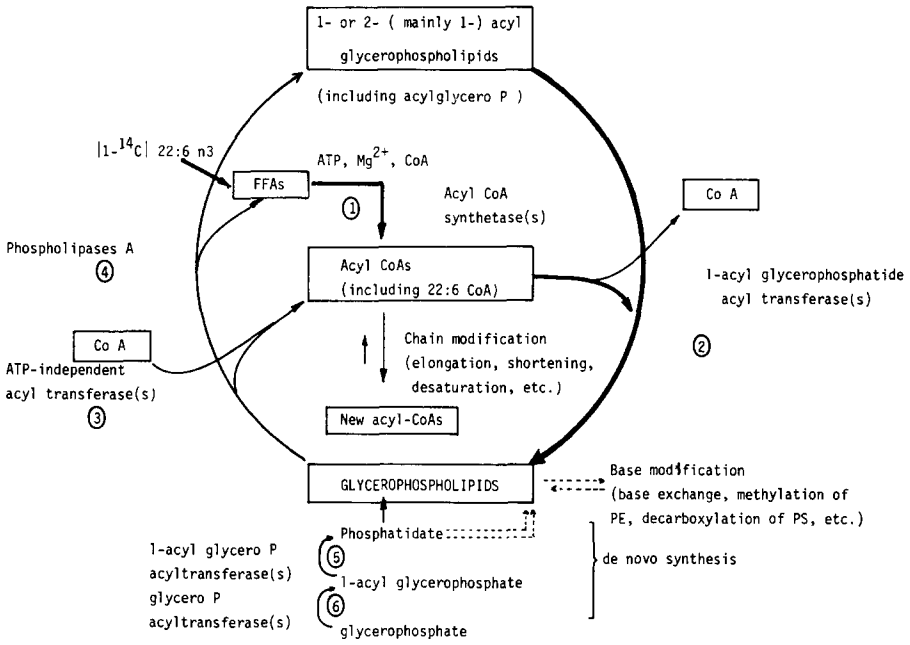

Fig. 2. Role of acyltransferases in the turnover of fatty acyl moieties of lipids in membranes. The heavy arrows indicate the pathways probably followed by [^{14}C]22 : 6 in the present experiments. The encircled numbers refer to enzymatic reactions discussed in the text.

acids between the *sn*-1 and *sn*-2 positions of naturally occurring glycerophospholipids [22,23]. These enzymes have not been studied in retina, and lipid labeling by [^{14}C]22 : 6 has not yet been studied in liver. Docosahexaenoate is predominantly located at the *sn*-2 position of the glycerol backbone in the various molecular species of lipids which contain this fatty acid in photoreceptor membranes (including hexaenoic and dipolyunsaturated molecular species of PC other than didocosahexaenoyl-PC, which has 22 : 6 in both positions) (unpublished results). The results presented here indicate that all these species of PC are subject to a continuous turnover of their docosahexaneoyl moieties in situ, independently of de novo synthetic mechanisms, which is consistent with the way polyunsaturated fatty acids are introduced into membrane lipids in general. In membranes potentially able to carry out de novo synthesis of lipids, like those of microsomes and mitochondria, however, phosphatidate is also actively labeled with [^{14}C]22 : 6 (Table III). This may be consistent with the interpretation [24] that 22 : 6 is mainly introduced early on in the de novo biosynthetic sequence, i.e., during the synthesis of phosphatidate. However, under the present conditions, label in phosphatidate is unlikely to arise from acylation of glycerophosphate (the first step in the de novo synthetic pathway), since this water-soluble precursor was probably lost during membrane isolation and washing. Unless this phosphatidate arose from degradation of other labeled phospholipids, the present results indicate that various lipid substrates including phosphatidate are potentially subject to undergo deacylation-reacylation reactions in retinal membranes, and hence that 22 : 6 may be introduced in multiple steps of glycerophospholipid metabolism.

Appendix

Uptake of free [1-^{14}C]docosahexaenoate in retinal membranes

During incubations of rod outer segments with [^{14}C]22 : 6, it was observed that a significant proportion of the free fatty acid in the incubation system was taken up by the membranes. However, the apparently contradictory effect that the smaller

the amount of membranes used for the incubations, the larger the concentration of free [^{14}C]22 : 6 attained in the membranes was noted as shown in Table I for rod outer segments and disks incubated under identical conditions. This prompted us to analyze in closer detail how free [^{14}C]22 : 6 was taken up, which led to various observations that may be useful to understand one of the possible mechanisms by which free fatty acids are incorporated in membranes, and perhaps in cells. The total amount of 22 : 6 taken up by rod outer segments incubated for 5, 10, 20, 30, or 60 min was similar (S.D. ± 10% approx., not shown). This indicates that 22 : 6 uptake is very rapid, since it is virtually completed in a few minutes, in contrast to its esterification (Fig. 1B). Free 22 : 6 uptake occurred to similar extents in the absence or presence of cofactors, was similar for the three kinds of subcellular fractions studied, and even occurred in controls where the native structural organization of membranes was destroyed by heating (Table V). This indicates that free 22 : 6 is taken up by an energy-independent, non-enzymatic process, and that the fatty acid partitions into the membranes by simple diffusion into the lipid matrix. The (lipid in the) membranes had a high affinity for the free acid, since even when small membrane/medium volume ratios were used, a large proportion of the added [^{14}C]22 : 6 (about one third, Table V) was recovered in the membranes.

The quantitative relationship between the amount of labeled free [^{14}C]22 : 6 in membranes and the mount of membranes was obtained indirectly as follows: when the concentration of free 22 : 6 in membranes (y, expressed as pmol [^{14}C]22 : 6/μmol lipid P) was represented against the amount of membranes (x, expressed as nmol lipid P, or mg protein), a negative hyperbolic relation of the form $y = f(1/x)$ was obtained. When $1/y$ was represented against x, a straight line resulted from the data in Table V (linear correlation coefficient 0.999) where $1/y = 0.04 + 0.00043$ nmol lipid P in the membrane (Fig. 3A). Therefore, the amount of labeled 22 : 6 in membranes bore the following relationship with the amount of lipid P:

$$\text{pmol}[^{14}\text{C}]22:6 = \text{nmol P}/(0.04 + 0.00043 \text{ nmol P}) \quad (1)$$

It may be calculated that a 2-fold increase in the amount of membranes in the range 500–1000 nmol lipid P only leads to a 7% increase in the amount of 22 : 6 taken up (1960 pmol for 500 nmol and 2100 pmol for 1000 nmol P), which fits with the experimental data in Table V. If Eqn. 1 is applied to a wider range of lipid P concentrations it is apparent that a 10-fold increase of membranes in the interval 100–1000 nmol P leads to a higher increase in the pmol of 22 : 6 incorporated than if the same increase were feasible in the range 1000–10000 nmol P (1200–2100 pmol (≅ 75%), and 2100–2300 pmol (≅ 8%), respectively). The curve in Fig. 3B suggests that there is a maximum amount of 22 : 6 the membrane can take up when the total amount of 22 : 6 in the system is kept constant. The difference between the total amount

TABLE V

PARTITION OF FREE [^{14}C]DOCOSAHEXAENOATE INTO RETINAL MEMBRANE LIPIDS

The incorporation of [^{14}C]22 : 6 into various membrane preparations used in experiments described in this paper (6 nmol 22 : 6, 0.5 ml medium, presence of cofactors) is compared with similar aliquots from the same preparations which had been heated at 100 °C for 5 min before the period of incubation in the same conditions (controls).

	Controls			Membranes		
	nmol lipid P	pmol[^{14}C]22 : 6	pmol/μmolP	nmol lipid P	pmol[^{14}C]22 : 6	pmol/μmol P
Rod outer segments, 60 min	1004 ± 31 (4)	2582 ± 296	2572 ± 295	966 ± 54 (8)	2088 ± 138	2162 ± 143
60 min	782 (2)	2374	3036	760 ± 35 (3)	2074 ± 169	2729 ± 222
30 min	666 (1)	2531	3800	682 ± 40 (3)	2070 ± 107	3038 ± 75
Disks, 60 min	481 ± 26 (3)	2106 ± 141	4415 ± 296	433 ± 18 (8)	1865 ± 87	4307 ± 202
P_2 fraction, 60 min	334 (2)	1793	5368	393 ± 18 (3)	1873 ± 68	4767 ± 172
Microsomes, 60 min	657 (2)	1960	2983	766 ± 40 (3)	2072 ± 99	2705 ± 129

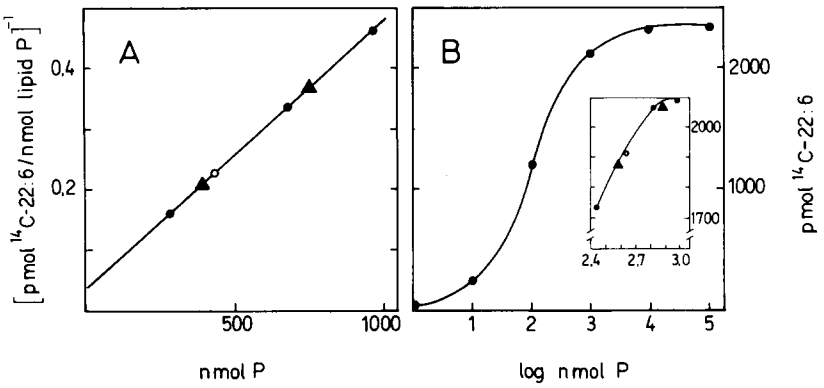

Fig. 3. Functions relating the concentration (A) and the amount (B) of free [^{14}C]22:6 attained in membranes and the amount of membranes (expressed as nmol lipid P). Membranes were incubated 60 min at 37°C and at pH 7.4 in the presence of 12 μM [^{14}C]22:6. In A, the inverses of pmol [^{14}C]22:6/nmol lipid P were calculated from Table V (plus one value from Fig. 1) and plotted against nmol lipid P. In B, the resulting function (Eqn. 1 described in the text) was applied to extend the relationship between amount of 22:6 taken up and amount of membranes to a hypothetically wider range of membrane concentrations. Experimental data from Table V are given in the inset. ●, rod outer segment; ○, disks; ▲, P_2 fraction; △, microsomes.

added and this maximum should be the amount of free 22:6 in the medium in equilibrium with the membranes, in this case 6.0–2.3 ≅ 3.7 nmol/0.5 ml medium = 7.4 μM. Obviously, higher concentrations of 22:6 in the medium should result in increased concentrations in the membranes.

The concentrations of free [^{14}C]22:6 attained in membranes after 1 h incubation ranged from 0.002 to 0.005 mol/mol phospholipid in the present experiments (Table V). It is important to point out here that this is still a trace amount, and is likely to be less disturbing to membranes than other (unavoidable) factors like the time taken for membrane isolation, low temperatures during the process, presence of extraneous buffers or ionic media, etc. For instance, in isolated disks, the endogenous amount of free 22:6 was between 8- and 20-fold higher than this, namely 0.039 ± 0.001 mol/mol lipid P, as mentioned in the text. When membranes 'preloaded' with free [^{14}C]22:6 were incubated in media lacking [^{14}C]22:6, a fraction of the free fatty acid originally present in membranes tended to equilibrate with (i.e., diffuse to) the medium. Thus, 10% and 43% was lost after 5 and 30 min incubation respectively (Table II), regardless of the presence or absence of cofactors.

Studies dealing with the kinetics of free fatty acid uptake by animal cells have provided evidence for two modes of fatty acid transport across cell membranes: a saturable, energy-independent component is observable at low fatty acid concentrations (1–10 μM) and simple diffusion at higher substrate concentrations [25]. The present results indicate that, using 12 μM concentrations of 22:6, a high proportion of the added fatty acid is rapidly adsorbed onto (or partitions into) the hydrophobic matrix of several intracellular membranes (irrespective of their kind) in an energy-independent process. This agrees with the proposal [25] that the striking capacity of cells to accumulate free fatty acids from incubation media may result from the association of substrates to lipophilic intracellular structures rather than to specific binding to the relatively scanty cytosolic fatty acid carrier proteins. The curve in Fig. 3B, relating the amount of free 22:6 taken up and the amount of membranes at constant fatty acid concentration, follows the same pattern as that of the function relating the amount of a solute in the stationary phase with the amount of stationary phase in chromatographic processes. Thus, the fraction of molecules in the stationary phase ($k'/1+k'$) plus the fraction of molecules in the mobile phase ($1/1+k'$) is equal to 1.0 (k' being the 'capacity factor', defined as the ratio, amount of a solute in the stationary/amount in the mobile phases). Since the volume of medium (0.5 ml) and the amount of 22:6 in the system (6000 pmol)

TABLE VI

FRACTIONAL DISTRIBUTION OF [^{14}C]22:6 BETWEEN MEMBRANES AND MEDIA

k' was obtained by dividing the amount of [^{14}C]22:6 in membranes (calculated using Eqn. 1) by the amount of [^{14}C]22:6 in media (6000 minus this amount) as explained in the text.

nmol P	k'	$1/1+k'$	$k'/1+k'$
1	0.004	0.996	0.004
10	0.039	0.962	0.038
10^2	0.251	0.799	0.201
10^3	0.549	0.646	0.354
10^4	0.623	0.616	0.384
10^5	0.63	0.613	0.686
10^6	0.63	0.616	0.686

were kept constant, using Eqn. 1 to calculate the amount of 22:6 in membranes (Fig. 3B) and 6000 minus this result to obtain the amount in media, it follows that the fractional distribution of molecules between the 'stationary' (membranes) and 'mobile' (medium) phases may be assimilated to a partition process (see Table VI).

The amount of a fatty acid that is 'solubilized' by membranes will thus depend: (1) on the volumes of both phases for a constant amount of fatty acid in the system, and (2) on the amount of fatty acid added to the incubation system for a constant membrane/medium volume ratio. If phase volumes are kept constant and the same amount of different fatty acids (solutes) are added, it is then predictable that the concentration of each solute in the membrane will differ according to the type of fatty acid (each will have its own k', since the 'stationary phase' will display different selectivities (ratios between k's) towards various solutes. The final concentration of substrates in the immediate environment (the lipid matrix) of membrane-bound enzymes which handle fatty acids (rather than the total amount added to the incubation system) must be adjusted when comparing the kinetic behavior of such enzymes towards various substrates.

References

1 Bibb, C. and Young, R.W. (1974) J. Cell Biol. 61, 327–343
2 Bibb, C. and Young, R.W. (1974) J. Cell Biol. 62, 378–389
3 Anderson, R.E., Maude, M.B., Kelleher, P.A., Maida, T.M. and Basinger, S.F. (1980) Biochim. Biophys. Acta 620, 212–226
4 Anderson, R.E., Kelleher, P.A. and Maude, M.B. (1980) Biochim. Biophys. Acta 620, 227–235
5 Anderson, R.E., Maude, M.B. and Kelleher, P.A. (1980) Biochim. Biophys. Acta 620, 236–246
6 Swartz, J.G. and Mitchell, J.E. (1970) J. Lipid Res. 11, 544–547
7 Miljanich, G.P., Sklar, L.A., White, D.L. and Dratz, E.A. (1979) Biochim. Biophys. Acta 552, 249–306
8 Aveldaño, M.I. and Bazán, N.G. (1983) J. Lipid Res. 24, 620–627
9 Aveldaño, M.I. (1986) J. Biol. Chem., in the press
10 Papermaster, D. (1982) Methods Enzymol. 81, 48–52
11 Smith, G.H. and Litman, B.J. (1982) Methods Enzymol. 81, 57–61
12 Aveldaño, M.I. and Sprecher, H. (1983) J. Biol. Chem. 258, 9339–9343
13 Folch, J., Lees, M. and Sloane-Stanley, G.H. (1957) J. Biol. Chem. 226, 497–509
14 Rouser, G., Fleischer, S. and Yamamoto, A. (1970) Lipids 5, 494–497
15 Litman, B. (1982) Methods Enzymol. 81, 309–315
16 Aveldaño, M.I., Giusto, N.M. and Bazán, N.G. (1981) Progr. Lipid Res. 20, 52–57
17 Lands, W.E.M. (1965) Annu. Rev. Biochem. 34, 313–346
18 Irvine, R.F. and Dawson, R.M.C. (1979) Biochem. Biophys. Res. Commun. 91, 1399–1405
19 Trotter, J., Flesh, I., Scmidt, B. and Ferber, E. (1982) J. Biol. Chem. 257, 1816–1823
20 Corbin, D.R. and Sun, G.Y. (1978) J. Neurochem. 30, 77–82
21 Lands, W.E.M., Inoue, M., Sugiura, Y. and Okuyama, H. (1982) J. Biol. Chem. 24, 14968–14972
22 Yamashita, S., Hosaka, K. and Numa, S. (1973) Eur. J. Biochem. 38, 25–31
23 Yamashita, S., Nayaka, N., Miki, Y. and Numa, S. (1975) Proc. Natl. Acad. Sci. USA 72, 600–603
24 Bazán, H.E.P., Sprecher, H. and Bazán, N.G. (1984) Biochim. Biophys. Acta 796, 11–19
25 Kohlwein, S.P. and Paltauf, F. (1983) Biochim. Biophys. Acta 310–317