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Synthesis of very long chain (up to 36 carbon) tetra, penta and hexaenoic fatty acids in retina

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The synthesis of very long chain (C_{24} to C_{36}) polyunsaturated (four, five and six double bonds) fatty acids (VLCPUFA) is investigated in bovine retina using [^{14}C]acetate. Saturates on the one hand (mainly palmitate), and polyenes on the other (mainly VLCPUFA), incorporate most of the label found in lipids. Phosphatidylcholine (PC) is the most highly labelled lipid class, since both types of ^{14}C-labelled fatty acids, but especially this novel series of VLCPUFA, are concentrated in this phospholipid. Radioactivity from [^{14}C]acetate is found in very long chain tetra, penta and hexaenoic fatty acids of PC. The labelling of 20:4($n-6$), 20:5($n-3$), 22:5($n-6$) and 22:6($n-3$) is much lower than that of longer polyenes of each of these series, indicating that VLCPUFA are synthesized *in situ* by successive elongations of the above polyenes, pre-existing in retina lipids. In various subcellular fractions isolated from retinas after incubations with [^{14}C]acetate (including cytosol, microsomes, mitochondria and photoreceptor membranes), the labelling of the VLCPUFA of PC is very high, even at relatively short intervals of incubation. The results suggest that not only the synthesis but also the intracellular traffic among membranes of VLCPUFA-containing species of PC are very active processes in the retina.

INTRODUCTION

The retina has long been known to be one of the vertebrate tissues with the highest content of polyunsaturated fatty acids. A large proportion of the retinal glycerophospholipids, especially those of photoreceptor membranes, is made up of dipolyunsaturated molecular species (Miljanich *et al.*, 1979; Aveldaño & Bazán, 1983). In one of the major phospholipids of retina, PC, these species have recently been reported to contain a new series of VLCPUFA. Such polyenes (tetra, penta and hexaenes) have 24 to 36 carbons and belong to the $n-3$ and $n-6$ series of fatty acids (Aveldaño, 1987, Aveldaño & Sprecher, 1987). Very long chain polyenes have also been identified in other vertebrate tissues containing polyunsaturated fatty acids. Thus, it has been reported that human vascular endothelial cells can synthesize and release 24- and 26-carbon polyenes (Rosenthal & Hill, 1984). Fatty acids with up to 30 carbons and four and five double bonds are synthesized in testis (Grogan, 1984). Increased proportions of unusually long polyenes have been found to be produced in the brain of newborns with a brain-hepato-renal (Zellweger) syndrome (Poulos *et al.*, 1986). The occurrence of very long chain polyenes add many intriguing questions to the not-yet-completely-elucidated problem of what the function of polyunsaturated fatty acids in tissues is. The studies concerning these fatty acids in retina are of particular interest. Polyenes with up to 36 carbons have been found to occur in the retina of a wide variety of vertebrate species, including fish, birds and mammals, and to be specifically concentrated in photoreceptor membrane phospholipids, especially in PC (Aveldaño, 1987). Recently it was shown that the levels of VLCPUFA in retina PC decrease with aging in rats (Rotstein *et al.*, 1987). The information about important aspects of the biochemistry and biophysics of the novel VLCPUFA-containing lipids is quite limited at present. This paper is concerned with the synthesis of these fatty acids in the bovine retina. It is shown that the VLCPUFA of PC are very actively synthesized from [^{14}C]acetate in entire retinas *in vitro*, and that in the PC of various subcellular fractions, including photoreceptor membranes, these fatty acids are labelled to a considerable extent.

MATERIALS AND METHODS

Materials

Bovine eyes were obtained from a local abattoir and transported to the laboratory in crushed ice. They were dark-adapted for 2 h and the retinas were excised on ice, under dim red light. [1-^{14}C]Acetate (sodium salt, specific activity 58.3 mCi/mmol, ethanolic solution) was obtained from New England Nuclear (Boston, MA, U.S.A.).

Incubation of retinas

Retinas were incubated in a CO_2-saturated Krebs–Ringer bicarbonate buffer (118 mM-NaCl, 5 mM-KCl, 2.5 mM-$CaCl_2$, 1 mM-KH_2PO_4, 1mM-$MgSO_4$ and 25 mM-$NaHCO_3$), pH 7.0 (7 ml/retina). Glucose (2 mg per ml of medium) was added at the beginning of incubation and (dissolved in 500 μl of medium) after every 1 h of incubation. The [^{14}C]acetate was added to the media and thoroughly mixed before adding the retinas. Three retinas per sample (each weighing 300–500 mg) were used in the

Abbreviations used: PC, phosphatidylcholine; VLCPUFA, very long chain polyunsaturated fatty acids; FAME, fatty acid methyl esters; ROS, rod outer segments; fatty acids are abbreviated by the convention, number of carbon atoms: number of double bonds.

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experiments with entire retinas. When subcellular fractionation was to be performed, 12 retinas per sample were used for the incubations. These were done at 37 °C, with gentle agitation, under O_2/CO_2 (19:1). A trap containing a 30% NaOH solution was used to capture the CO_2 possibly released during incubations. After the periods indicated in the Results section, retinas and incubation media were transferred to plastic tubes and centrifuged at 17000 rev./min for 15 min. The medium was discarded and the tissue was either directly extracted with chloroform/methanol (entire retinas) or subjected to the following subcellular fractionation procedures.

Subcellular fractionation

The sedimented tissue was resuspended by vortexing in the medium described by Papermaster (1982) to obtain the rod outer segments (ROS), which were isolated using the discontinuous sucrose density gradient also described by this author. The A_{280}/A_{500} (absorbance ratio) was 2.1 ± 0.3 in the fraction here referred to as ROS. After separating these membranes, the retinas were homogenized in 0.32 M-sucrose (in 50 mM-Tris/HCl and 1 mM-EDTA, pH 7.4) and centrifuged at 2500–3000 rev./min for 15 min, collecting the supernatants. The procedure was repeated and the combined supernatants were centrifuged at 15000 *g* for 45 min. From the resulting pellets the fraction called 'mitochondria' was obtained, using the sucrose density-gradient procedure described by Dienel *et al.* (1977). From the supernatants, the fractions called 'microsomes' and 'postmicrosomal supernatant' were obtained by centrifuging at 140000 *g* for 45 min (pellets and supernatants respectively). The four fractions thus isolated (Rotstein & Aveldaño, 1987*a*) differed significantly in phospholipid composition. The major phospholipids also showed characteristic patterns of fatty acid and molecular species distribution (Rotstein & Aveldaño, 1987*b*). Photoreceptor disks isolated from ROS by the procedure described by Smith & Litman (1982) gave a similar composition (and distribution of [^{14}C]acetate among lipids) as ROS.

Lipid extraction and separation

Lipids from retinas and retina subcellular fractions were extracted and partitioned according to the procedure of Bligh & Dyer (1959). After evaporating the organic solvents under nitrogen, chloroform/methanol (2:1, v/v) was added and the partitions and washings described by Folch *et al.* (1957) were carried out to remove non-incorporated [^{14}C]acetate.

Phospholipid classes were resolved using two-dimensional t.l.c. as described by Rouser *et al.* (1970). Neutral lipids, eluted from (the top of) the above plates were separated with hexane/ether (2:3, v/v) on plates of silica gel G. For phosphorus and radioactivity quantification, lipid spots were located by exposing the plates to iodine vapours. Lipid phosphorus was quantified according to Rouser *et al.* (1970). Radioactivity was determined by liquid-scintillation counting. The lipid spots were scraped into vials containing 0.4 ml of water; 10 ml of 0.4% Omnifluor in Triton X-100/toluene (1:4) was added as scintillation cocktail.

Fatty acid separations and analysis

To study the labelling of fatty acids in lipids, these were isolated by t.l.c. as described and located under u.v. light, after spraying the plates with 0.05% dichlorofluorescein in methanol. Fatty acid methyl esters (FAME) were prepared from lipids as described by Morrison & Smith (1964) and resolved into fractions according to their unsaturation by argentation t.l.c. ($AgNO_3$-t.l.c.). The plates were prepared using silica gel G and $AgNO_3$ (4:1, w/w); chloroform/methanol (47:3, v/v) was used as solvent to resolve hexaenes, pentaenes, tetraenes and trienes. The bands at the top of the plates were eluted and dienoic, monoenoic and saturated FAME were separated after a second $AgNO_3$-t.l.c. using chloroform. Bands were located under u.v. light, after spraying the plates with 0.05% dichlorofluorescein in methanol/water (1:1, v/v). These bands were either eluted, for further studies of their individual fatty acids, or directly scraped into vials containing 0.5 ml of 2 M-NaCl to determine radioactivity. In this case, 0.4% Omnifluor in Triton X-100/toluene (3:7, v/v) was used as scintillation cocktail; the AgCl formed was allowed to settle before counting.

To quantify the incorporation of [^{14}C]acetate in fatty acids, the different FAME fractions separated by $AgNO_3$-t.l.c., were eluted and resolved into their individual constituents using reverse-phase h.p.l.c. An octadecylsilane column (5 μm particle size; 0.4 cm × 15 cm) at 37 °C was used. Acetonitrile (flow rate 0.5 ml/min) was the mobile phase. The FAME, detected by their absorption at 205 nm using a u.v.-visible variable detector, were collected into vials as they eluted from the column. The solvent was allowed to evaporate at room temperature and radioactivity was measured by liquid-scintillation counting (as described for samples not containing Ag^+ ions).

To obtain the specific radioactivities of fatty acids of PC (Table 2), portions of each fraction of FAME were taken for quantification of its individual components. This was done by means of g.l.c., using glass columns packed with 15% OV-275 on Chromosorb WAW 80–120 mesh (Varian, Sunnyvale, CA, U.S.A.) and temperature programs previously described (Aveldaño, 1987). For lipids other than PC, unlabelled FAME (prepared from whole retina PC) were added as carriers to the samples, in order to improve the location of the different bands on argentation t.l.c. plates (and also facilitate their h.p.l.c. detection). Elution of PC, neutral lipids and methyl esters after the described t.l.c. steps, was performed using the solvents described by Arvidson (1968) which allowed quantitative recoveries of these lipids from the silica. All procedures (except the t.l.c.) were carried out under an atmosphere of nitrogen.

RESULTS

Labelling of retina lipid classes by [^{14}C]acetate

Bovine retinas incorporated [^{14}C]acetate at a constant rate during an incubation period of 3 h (Fig. 1). The total lipid extract showed an uptake of 290 pmol of [^{14}C]acetate/μmol of total lipid phosphorus per h. Most of the incorporated radioactivity was in phospholipids. Neutral lipids showed a relatively lower labelling, except for a band which migrated in t.l.c. with the solvent front. Cholesterol and triacylglycerols incorporated little [^{14}C]acetate even after 3 h incubation (1.6 and 2.0% respectively, of the total radioactivity). The percentage of label in free fatty acids decreased with incubation time, since this was the only lipid class whose incorporation

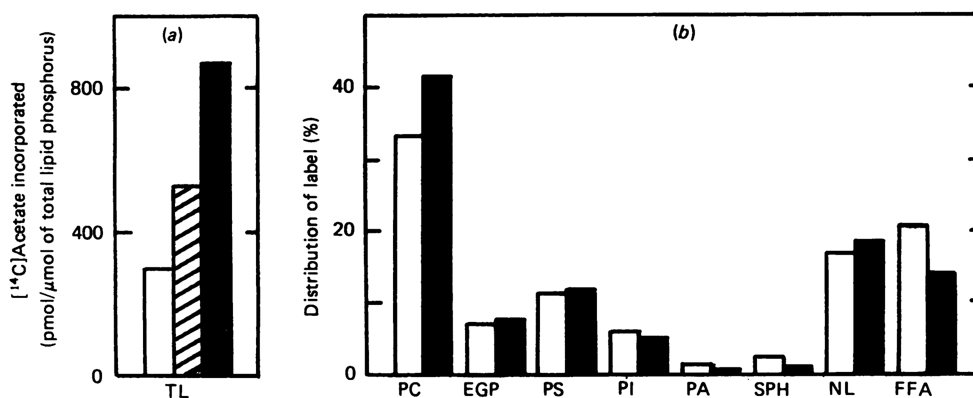

Fig. 1. Labelling of retina lipids with [^{14}C]acetate

Bovine retinas (three per sample) were incubated for 1 h (□), 2 h (▨) and 3 h (■) with 90 μCi of [^{14}C]acetate in a glucose-containing Krebs–Ringer bicarbonate buffer. Lipids were extracted with chloroform/methanol and separated by t.l.c. (*a*) Incorporation of ^{14}C from acetate in lipids (pmol of [^{14}C]acetate/μmol of total lipid phosphorus); (*b*) distribution of radioactivity among lipids (% of total incorporated label). Abbreviations: TL, total lipids; EGP, ethanolamine glycerophospholipids; PS, phosphatidylserine; PI, phosphatidylinositol; PA, phosphatidate; SPH, sphingomyelin; NL, neutral lipids; FFA, free fatty acids.

Table 1. Incorporation of [^{14}C]acetate into fatty acids of the retina

Fractions of the total lipid extracts and of PC were treated with BF_3 in methanol, and the resulting FAME were recovered after t.l.c. (silica gel G; hexane:ether, 19:1, v/v). Then FAME were separated according to unsaturation by argentation t.l.c. The values represent the pmol of [^{14}C]acetate incorporated in fatty acids fractions in total lipid and in PC (expressed per μmol of total lipid phosphorus and per μmol of total PC, respectively). The values in parentheses on the right of each column represent the percentages of the total label contributed by the fractions concentrating most of the radioactivity (saturates and the most highly unsaturated polyenes, the rest not being depicted). Unidentified, denotes two tenuous bands detected between saturated and monoenoic FAME bands, observed in total lipid but not in PC (probably representing fatty aldehydes produced from the alkenyl–acyl subclass of ethanolamine glycerophospholipids). They had less than 1% of the total label both in these total extracts and in the ethanolamine glycerophospholipids shown in Fig. 3.

		[^{14}C]Acetate incorporation			
		Total lipids (pmol/μmol of total lipid phosphorus)		Phosphatidylcholine (pmol/μmol of total PC)	
FAME	Incubation time ...	1 h	3 h	1 h	3 h
Saturates		166.6 (56.1)	399.7 (46.0)	84.5 (45.5)	326.4 (53.9)
Unidentified		1.8	5.2	0.5	1.6
Monoenes		2.1	6.1	0.9	4.3
Dienes		0.3	1.8	0.3	1.2
Trienes		0.9	0.9	0.1	0.1
Tetraenes		36.8 }	113.8 }	25.7 }	58.5 }
Pentaenes		68.6 } (42.2)	259.9 } (52.3)	54.5 } (53.6)	154.1 } (44.9)
Hexaenes		19.9 }	80.8 }	19.4 }	59.0 }
Totals		297.0	869.0	185.9	605.2
Ratio 3 h/1 h saturates		2.4		3.9	
Tetra to hexaenes		3.6		2.7	

reached a plateau after 2 h incubation (not shown). The incorporation in other lipids increased constantly in the 1–3 h interval. Phosphatidylcholine was, at all incubation times, the most highly labelled lipid class (Fig. 1).

Labelling of fatty acids in total lipids and phosphatidylcholine

When methyl ester derivatives of the fatty acids from total retina lipids and from PC were separated into fractions according to their unsaturation, the most highly unsaturated polyenes (that is, tetra, penta and hexaenes), as well as the saturates, were the groups of fatty acids most actively labelled with [^{14}C]acetate (Table 1). Half the radioactivity incorporated in PC after 1 h incubation was in these three polyenoic fractions. The contribution of polyenes to the labelling of PC was larger after short than at long incubation periods while the opposite was true for saturates. This was not the case with the fatty acids of total lipids since the percentage contribution of [^{14}C]saturated fatty acids decreased and that of polyenes increased with incubation time.

When the distribution of radioactivity among indi-

Table 2. [^{14}C]Acetate incorporation in individual fatty acids of retina total lipid and PC

FAME of total lipids and of PC, resolved as described in Table 1, were separated by reverse-phase h.p.l.c. The fatty acids were quantified by g.l.c. using methyl heneicosanoate as internal standard. The specific radioactivities are expressed as pmol of [^{14}C]acetate per nmol of fatty acid. Others, sum of all the fatty acids other than those depicted. VLCPUFA, sum of fatty acids with 24–36 carbons.

	[^{14}C]Acetate incorporation				
		Phosphatidylcholine			
	Total lipid (%)	(%)		Specific radioactivity (pmol of [^{14}C]acetate per nmol of fatty acid)	
Fatty acid / Incubation time ...	3 h	1 h	3 h	1 h	3 h
14:0	4.9	6.8	3.5	1.10	2.04
16:0	32.4	29.8	40.0	0.09	0.43
18:0	8.9	9.3	10.8	0.05	0.20
18:1	0.3	0.3	0.3	0.003	0.01
20:4($n-6$)	0.2	1.5	0.1	0.02	0.004
20:5($n-3$)	0.03	0.04	0.1	0.013	0.07
22:4($n-6$)	7.0	5.8	4.4	0.80	2.11
22:5($n-3$)	4.1	3.0	3.3	0.22	0.86
22:5($n-6$)	0.3	0.1	0.1	0.02	0.05
22:6($n-3$)	0.2	0.2	0.1	0.001	0.003
24:4($n-6$)	4.7	4.6	3.5	1.78	0.47
24:5($n-3$)	18.2	14.4	12.7	1.90	5.93
24:5($n-6$)	0.1	3.9	0.9	26.20	10.52
24:6($n-3$)	4.0	3.4	2.7	0.75	2.10
26:4($n-6$)	0.6	0.6	0.6	1.24	4.02
26:5($n-3$)	2.3	2.4	1.9	–	–
26:6($n-3$)	1.3	1.6	1.1	1.28	3.00
28:4($n-6$)	0.1	0.2	0.1	–	–
28:5($n-3$)	0.5	0.7	0.5	1.86	5.16
28:6($n-3$)	0.3	0.5	0.3	2.09	4.53
30:4($n-6$)	1.3	0.3	0.3	0.43	1.60
30:5($n-3$)	0.6	2.8	1.7	3.74	7.77
30:6($n-3$)	0.2	0.6	0.7	5.09	22.11
32:4($n-6$)	0.2	0.8	0.6	0.42	1.20
32:5($n-3$)	2.5	1.8	3.4	0.58	3.82
32:6($n-3$)	2.6	2.9	3.9	1.09	5.10
34:4($n-6$)	0.4	0.1	0.1	0.25	0.78
34:5($n-3$)	1.0	0.7	1.3	0.27	1.69
34:6($n-3$)	0.5	0.5	0.8	0.45	2.39
36:5($n-3$)	0.02	–	0.01	–	0.70
36:6($n-3$)	0.03	0.01	0.03	0.14	2.38
Others	0.6	0.3	0.5	0.02	0.11
VLCPUFA	41.5	42.8	37.1	1.40	4.24
Total $n-6$ polyenes	14.3	16.4	10.6	0.22	0.45
Total $n-3$ polyenes	38.4	35.5	34.4	0.16	0.55

vidual fatty acids was determined, VLCPUFA were found to be responsible for the high labelling observed in the polyenoic fractions (Table 2). Thus, about 40 % of the radioactive fatty acids of both PC and total lipids were VLCPUFA. After 3 h incubation, the total lipids had more [^{14}C]24:5 and [^{14}C]24:6($n-3$) than PC, which in turn showed a considerably higher proportion of labelled fatty acids of longer chains. The percentage of total radioactive VLCPUFA in PC tended to decrease slightly between 1 and 3 h incubation, mainly due to the fact that saturates (especially palmitate) increased more than polyenes in this period. Among the latter, the contribution of ^{14}C-labelled 24- and 26-carbon fatty acids decreased while that of longer fatty acids remained relatively constant (11.9 and 13.7 % of the total label of PC fatty acids, after 1 and 3 h incubation, respectively).

The specific radioactivities of both individual and total VLCPUFA of PC were remarkably high (Table 2). Both the percentage of labelling and the specific radioactivities of 20:4($n-6$), 20:5($n-3$), 22:5($n-6$) and 22:6($n-3$) were much lower than those of longer polyenes. In general, the $n-3$ polyenes were more actively labelled than those of the $n-6$ series. Their specific radioactivities did not differ markedly, however, since the latter were

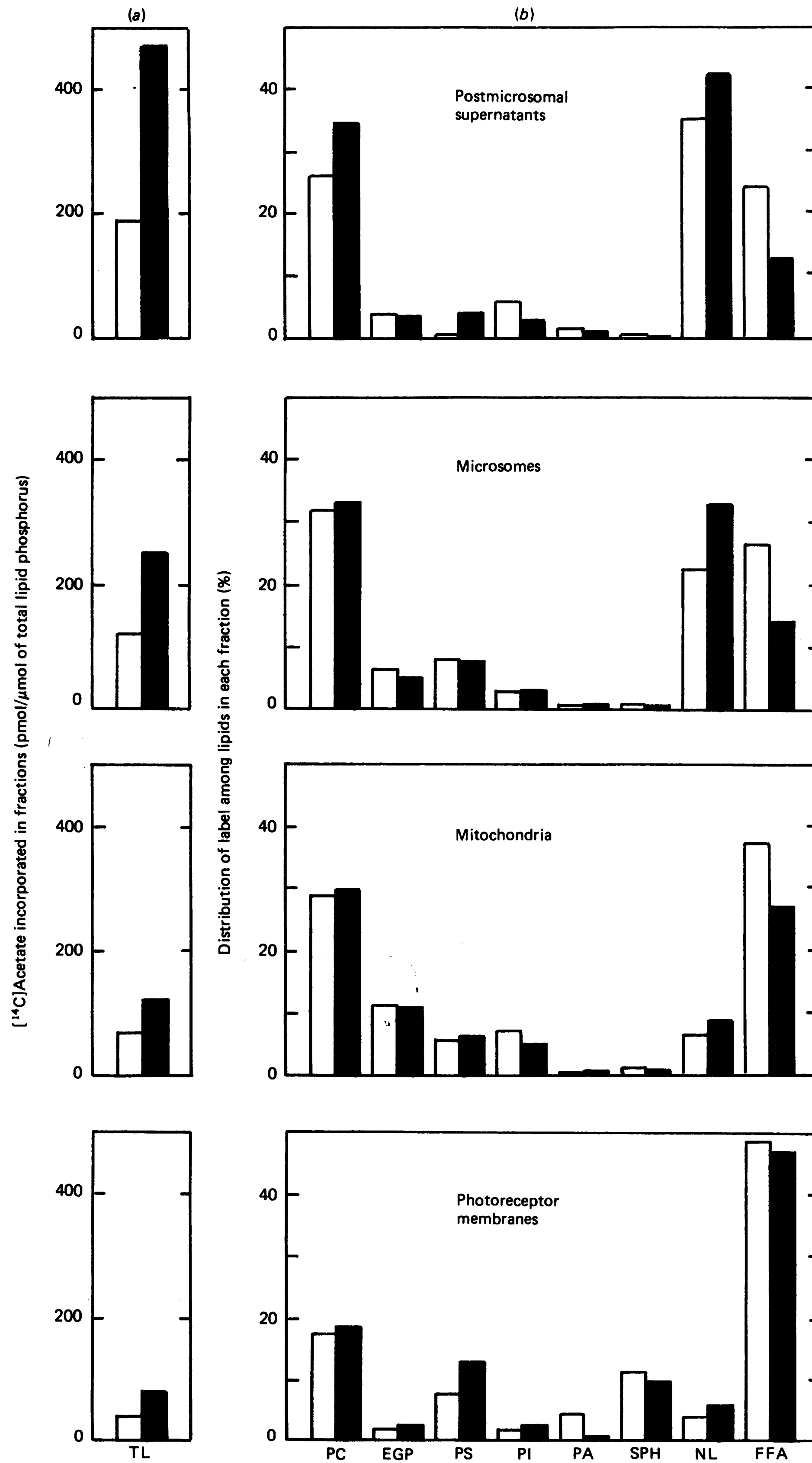

Fig. 2. Labelling of lipids of retina subcellular fractions with [^{14}C]acetate

Retinas (12 per sample) were incubated for 1 (□) and 3 h (■) with 100 μCi of [^{14}C]acetate and then subjected to subcellular fractionation. Lipids were extracted and resolved as in Fig. 1. (*a*) Incorporation of ^{14}C from acetate in lipids (pmol of [^{14}C]acetate/μmol of total lipid phosphorus); (*b*) distribution of radioactivity among lipids (% of the total incorporated label). Abbreviations as in Fig. 1.

Table 3. Incorporation of [^{14}C]acetate into fatty acids of PC from retina subcellular fractions

PC was isolated from subcellular fractions obtained after the indicated periods of incubation of retinas with [^{14}C]acetate (Fig. 2). FAME were prepared from PC and resolved by argentation t.l.c. The incorporation is expressed as pmol of [^{14}C]acetate/μmol of total PC. Other details as in Table 1.

	[^{14}C]Acetate incorporation (pmol/μmol of total PC) in:							
	Postmicrosomal supernatants		Microsomes		Mitochondria		Photoreceptor membranes	
FAME Incubation time ...	1 h	3 h	1 h	3 h	1 h	3 h	1 h	3 h
Saturates	54.5 (71.8)	223.1 (79.2)	50.1 (69.5)	117.1 (71.0)	28.3 (60.2)	81.8 (69.9)	10.7 (31.8)	27.6 (49.5)
Monoenes	1.3	3.4	0.9	2.5	1.5	2.2	0.1	0.2
Dienes + trienes	1.7	5.6	1.2	3.3	0.7	1.6	0.7	1.1
Tetraenes	3.5	8.7	3.1	6.6	2.4	4.7	2.6	2.8
Pentaenes	10.7 }(24.3)	29.6 }(17.6)	12.8 }(27.6)	26.4 }(25.4)	8.8 }(35.2)	17.9 }(26.7)	15.4 }(65.8)	19.6 }(48.0)
Hexaenes	4.2	11.3	4.0	8.9	5.4	8.7	4.2	4.3
Total PC	75.7	281.7	72.1	164.8	47.0	116.9	33.7	55.6
3 h/1 h saturates		4.1		2.3		2.9		2.6
Tetra to hexaenes		2.7		2.1		1.9		1.2

present in considerably smaller amounts than the former in bovine retina PC. An interesting difference with metabolic implications (see the Discussion section) was found between the labelling of 22:5($n-3$) and that of 22:5($n-6$), the latter showing much lower specific radioactivity than the former. The results in Table 2 clearly show that the synthesis of the VLCPUFA of PC was very active, their specific radioactivities exceeding those of palmitate and other saturated fatty acids.

Lipid labelling in retina subcellular fractions

After 1 and 3 h incubations of retinas, the post-microsomal supernatant was the most highly labelled subcellular fraction, followed by microsomes, mitochondria and photoreceptor membranes (Fig. 2). The total [^{14}C]acetate incorporated increased with time in all fractions, displaying in each a particular distribution pattern. In all cases, 1) the total label incorporated in phospholipids was higher than that in total neutral lipids and in free fatty acids, and 2) among phospholipids, PC concentrated most of the label. Neutral lipids had more radioactivity in cytosol and microsomes than in mitochondria and photoreceptor membranes. The incorporation of acetate into cholesterol was very low in all the fractions studied (4.4, 2.4, 1.4 and 1.1 % of the total acetate incorporated after 3 h incubation in cytosol, microsomes, mitochondria and photoreceptor membranes respectively). In the subcellular fractions studied ^{14}C-labelled free fatty acids contributed with large percentages to the label in the total lipid extracts. This was particularly noticeable in mitochondria and especially in ROS. The labelling of sphingomyelin was surprisingly higher in ROS than in the other fractions. However, analysis of FAME prepared from ROS sphingomyelin showed that the radioactivity was not in the fatty acid moiety, unlike all the other phospholipids.

Labelling of PC fatty acids in retina subcellular fractions

The distribution of the fatty acid formed from [^{14}C]-acetate in the PC from the subcellular fractions studied (Table 3) showed that saturates were considerably more labelled in cytosol and microsomes than in mitochondria and photoreceptor membranes. Interestingly, the amounts of [^{14}C]polyenes present in ROS PC after 1 h incubation were similar to those of the other fractions (microsomes, mitochondria and cytosol) in spite of the much lower total quantities of [^{14}C] incorporated. Moreover, while the amount of radioactivity incorporated in ROS PC polyenes was similar after 1 and 3 h incubation, that in the other fractions increased considerably in this interval, as shown by the 3 h/1 h ratios in Table 3. These ratios also show that the labelling of PC saturates in this period increased a larger number of times than that of polyenes in all fractions. Among the polyenes of PC, pentaenes had the highest incorporation in all fractions, followed by hexaenes and tetraenes, with very similar values.

Table 4 shows the distribution of label among individual fatty acids of PC. Fatty acids with more than 22 carbons actively incorporated [^{14}C]acetate in all fractions, in proportions that increased in the following order: cytosol, microsomes, mitochondria, ROS. In the ROS fraction, VLCPUFA accounted for almost half the total radioactive fatty acids of PC, and in microsomes

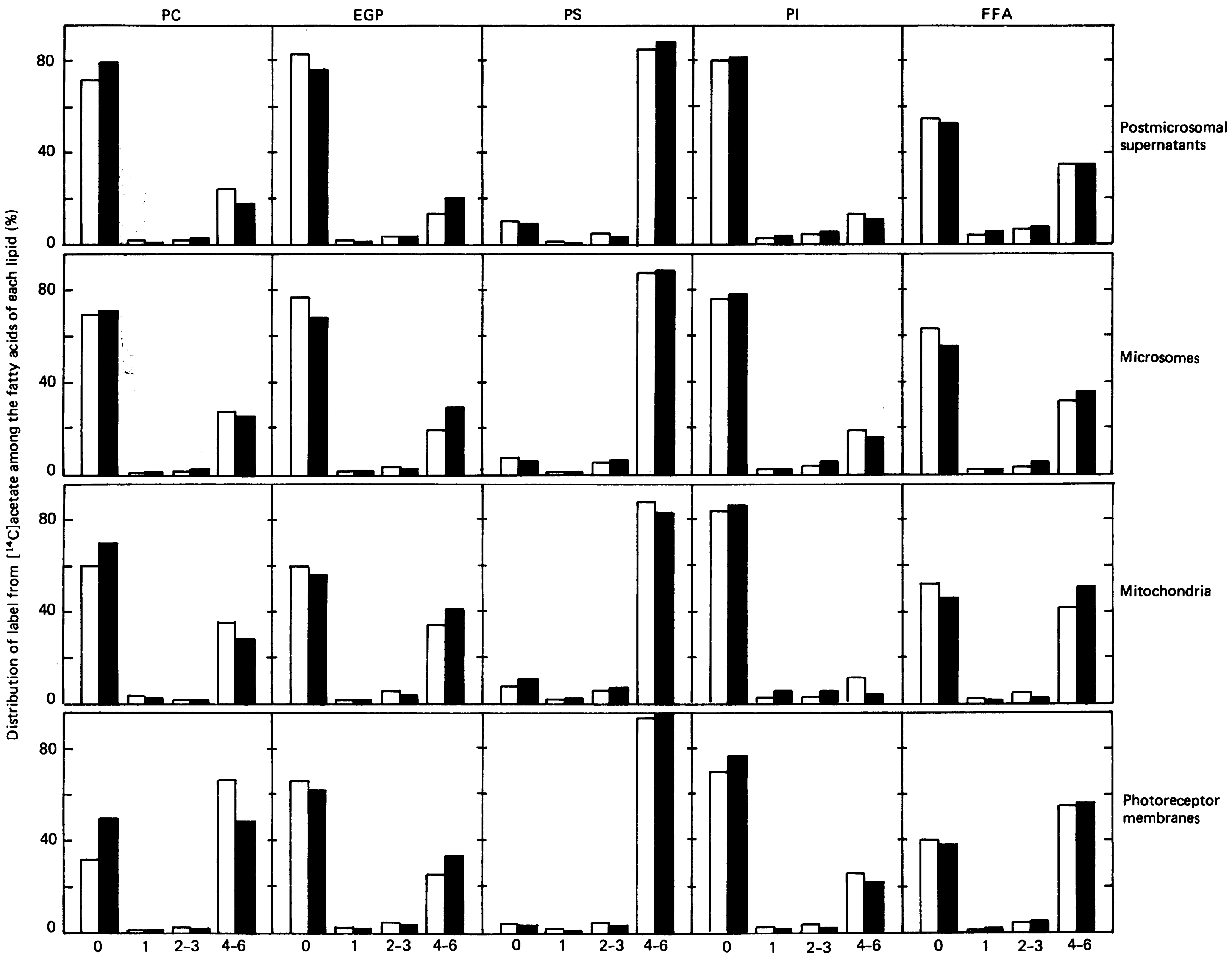

Fig. 3. Distribution of radioactivity among fatty acids of lipid classes from retina subcellular fractions

The depicted lipid classes were isolated from subcellular fractions obtained from retinas which had been incubated with [^{14}C]acetate (Fig. 2). FAME were prepared and resolved by argentation t.l.c. The bars represent the distribution of radioactivity (as a % of the total label recovered in fatty acids of each lipid) after 1 h (□) and 3 h (■) incubations of retinas. 0, saturates; 1, monoenes; 2–3, dienes and trienes; 4–6, tetra-, penta- and hexaenes.

Table 4. Labelling of PC fatty acids and free fatty acids of retina subcellular fractions with [^{14}C]acetate

FAME were prepared from PC and from free fatty acids of retina subcellular fractions obtained after 3 h incubations of retinas. They were separated by argentation t.l.c. followed by h.p.l.c. The values represent the % distribution of [^{14}C]acetate among fatty acids. A, Postmicrosomal supernatants; B, microsomes; C, mitochondria; D, photoreceptor membranes. Other details as in Table 2.

	[^{14}C]Acetate label distribution (%) in:							
	Phosphatidylcholine (%)				Free fatty acids (%)			
Fatty acid	A	B	C	D	A	B	C	D
14:0	5.6	1.6	4.1	3.5	7.1	3.5	6.0	—
16:0	66.1	55.0	43.3	38.2	36.7	40.9	31.6	29.5
18:0	7.4	14.4	22.5	7.8	10.2	10.9	8.8	8.5
22:4$(n-6)$	2.0	1.8	1.6	0.6	5.4	6.4	5.6	7.0
22:5$(n-3)+(n-6)$*	1.4	1.9	1.6	1.7	3.7	3.5	10.2	5.6
24:4$(n-6)$	1.0	1.2	1.4	3.7	2.8	3.5	3.5	4.1
24:5$(n-3+(n-6)$*	4.2	6.1	4.9	15.3	16.2	16.6	21.1	31.0
24:6$(n-3)$	0.3	0.9	1.7	1.1	2.6	1.6	6.3	2.9
26:4$(n-6)$	0.1	0.4	0.5	0.3	–	0.6	–	0.3
26:5$(n-3)$	1.0	1.6	1.4	9.9	1.3	1.6	1.8	2.3
26:6$(n-3)$	1.1	1.0	0.7	0.1	0.6	1.9	1.1	1.8
28:5$(n-3)$	0.3	0.7	1.9	0.5	0.2	–	0.7	0.3
28:6$(n-3)$	0.2	0.3	0.1	–	–	0.6	–	–
30:4$(n-6)$	–	–	–	–	–	–	–	–
30:5$(n-3)$	0.8	1.7	1.7	2.8	0.4	–	0.4	0.3
30:6$(n-3)$	0.4	0.6	0.7	0.1	0.4	–	–	0.2
32:4$(n-6)$	–	0.3	0.3	0.2	–	–	–	–
32:5$(n-3)$	1.3	2.6	2.7	3.9	0.4	0.3	0.4	0.3
32:6$(n-3)$	1.7	1.7	3.0	2.9	0.4	–	–	0.3
34:4$(n-6)$	–	0.2	0.2	–	–	–	–	–
34:5$(n-3)$	0.5	1.1	1.1	1.4	–	–	–	–
34:6$(n-3)$	0.3	0.5	0.8	3.5	–	–	–	0.1
36:5$(n-3)$	1.0	0.3	0.1	–	–	–	–	–
36:6$(n-3)$	–	0.4	0.3	–	–	–	–	–
Others	3.2	3.5	3.3	2.4	11.5	8.0	2.9	5.5
VLCPUFA	14.2	21.7	23.5	45.7	25.3	26.7	35.3	43.9
Total $n-6$ polyenes	3.1	4.0	4.0	4.8	8.2	10.5	9.1	11.4
Total $n-3$ polyenes	6.5	10.5	12.9	15.3	34.4	26.1	42.0	45.1

* Mostly accounted for by the $n-3$ isomer.

and mitochondria, for about 20–25%. Among VLCPUFA, $(n-3)$ 24:5, 26:5, 30:5, 32:5 and 32:6 were the most highly labelled acids in all fractions. Fatty acids having 28 or more carbons contributed with 6.5, 10.5, 12.9 and 15.3% to the total radioactivity of cytosolic, microsomal, mitochondrial and photoreceptor membrane PC, respectively. Consistent with the observations in entire retina, the results in Table 4 show that polyenes longer than 22 carbons are mainly responsible for the labelling observed in tetra, penta and hexaenoic fractions of fatty acids of PC (Table 3).

Labelling of fatty acids from other lipid classes of retina subcellular fractions

The pattern of labelling of fatty acids markedly differed among lipid classes (Fig. 3). Saturated and, in a second place, highly unsaturated fatty acids contributed with large proportions to the radioactive fatty acids of ethanolamine glycerophospholipid. In contrast to PC, in this phospholipid class, the contribution of polyenes increased over that of saturates at longer incubation times. Polyenes concentrated most of the [^{14}C]acetate incorporated in phosphatidylserine. The opposite was found in phosphatidylinositol, where most of the label was present in saturates in all fractions (only ROS showed slightly higher levels of [^{14}C]polyenes in this lipid). Polyunsaturated free fatty acids were highly labelled in mitochondria and ROS, while cytosol and microsomes showed larger proportions of radioactivity in saturated free fatty acids. In the lipids other than PC shown in Fig. 3, the label observed in polyenes was mostly accounted for by 22- to 26-carbon fatty acids (not shown). Among these polyenes, pentaenes were the most highly labelled, followed by tetraenes, and hexaenes. Thus, 65% of the polyenes of ethanolamine glycerophospholipid and 60–80% of those of phosphatidylserine, phosphatidylinositol and free fatty acids were pentaenes – mainly 22:5 and 24:5$(n-3)$ after 3 h incubation.

The distribution of label among individual free fatty acids of the various subcellular fractions studied (Table 4) shows that the most highly labelled free fatty acids were 16:0, 24:5$(n-3)$ and 18:0. The radioactivity in free VLCPUFA ranged from 25 to 45% of the total ^{14}C in the free fatty acid pool. This was, however, mainly contributed by 24-carbon polyenes: note that, in contrast to PC, there were negligible proportions of label in

free polyenes longer than 26 carbons (1–2% of the total ^{14}C present in the free fatty acids).

DISCUSSION

The presented results show that the retina actively incorporates [^{14}C]acetate *in vitro*, and uses it to synthesize fatty acids which are efficiently esterified into lipids. Considering the major groups of fatty acids present in retina, saturates on the one hand, and tetra, penta and hexaenes on the other, concentrate most of the label from the precursor. Palmitate and VLCPUFA are found to be mainly responsible for the incorporation observed in the former and latter group respectively. Considering the individual lipid classes, an exceedingly high percentage of the fatty acid label is concentrated in PC. Its very long chain polyenes are found to be actively synthesized, as indicated by their specific radioactivities, which exceed that of palmitate. PC was the only phospholipid class containing labelled polyenes of more than 26 carbons, consistent with the demonstration (Aveldaño, 1987) that such fatty acids occur virtually exclusively in PC.

The VLCPUFA of retina PC belong to familiar fatty acid series (Aveldaño & Sprecher, 1987). Thus, tetraenes are of the $n-6$ series, as arachidonate, hexaenes are of the $n-3$ series, as docosahexaenoate, and there are pentaenes from both the $n-3$ and $n-6$ series (in bovine retina, $n-3$ pentaenes predominate over $n-6$ pentaenes; Aveldaño, 1987). The first members of the major series of tetra, penta and hexaenes of retina are thus $20:4(n-6)$, $20:5(n-3)$, $22:5(n-6)$ and $22:6(n-3)$ [the first two are known to be synthesized by ($\Delta5$) desaturation of $20:3(n-6)$ and $20:4(n-3)$ respectively, and the last two by ($\Delta4$) desaturation of $22:4(n-6)$ and $22:5(n-3)$ respectively]. These first four fatty acids showed negligible labelling from [^{14}C]acetate in bovine retina in comparison with longer fatty acids of each series. The results indicate that the major very long chain tetra, penta, and hexaenes present in retina are synthesized *in situ* by elongation of pre-existing fatty acids of each of the series of polyenes. This conclusion is supported by previous work in rat testicular cells, where $(n-6)$ 26- to 30-carbon tetraenes were shown to be labelled from [^{14}C]$20:4(n-6)$ (Grogan, 1984). In human endocytes, Rosenthal & Hill (1984), also showed radioactivity from [^{14}C]20:3 and [^{14}C]-$20:4(n-6)$ in 24- and 26-carbon tetraenes. The labelling of familiar long chain polyenes was consistent with what is known in other tissues of the biosynthesis of fatty acids (Sprecher, 1981; Brenner, 1981), since those which are produced by desaturation reactions were much less labelled than those produced by elongations. Thus, $22:5(n-3)$, which is produced by a 2-carbon addition to $20:5(n-3)$ (Sprecher, 1981; Bazán *et al.*, 1982) showed much higher specific radioactivity than $22:5(n-6)$, the desaturation product of $22:4(n-6)$, [which was labelled in the present case, since it is produced by elongation of $20:4(n-6)$].

Relatively few studies on the synthesis of fatty acids in retina are available. Homogenates of dog retinas incubated with labelled malonyl-CoA were reported to synthesize fatty acids (Futterman *et al.*, 1968). These were nearly 80% saturates and 20% polyenes. Of the label detected in polyenes, less than 10% was in $20:4(n-6)$ and $22:6(n-3)$, for most of the radioactivity was in fatty acids which were unknown at that time, 'tentatively identified on the basis of retention time as intermediates in the desaturation and chain-elongation pathways'. The present findings are consistent with such results and extend such interpretation. More recently, rat retinas were shown to synthesize polyunsaturated as well as saturated fatty acids from [1-^{14}C]acetate using an approach quite similar to that described here, namely incubation of retinas in Krebs–Ringer bicarbonate buffer followed by separation of FAME by argentation t.l.c. (Dudley, 1976). This author demonstrated that the formation of tetra, penta and hexaenoic fatty acids is quite active, and showed interesting effects of essential fatty acid deficiency on the relative incorporation of the precursor into each group of polyenes *in vitro*. The results of the present work suggest that the fatty acids mainly labelled in each of their FAME fractions may also have been VLCPUFA. The rat retina was shown to contain VLCPUFA (Aveldaño, 1987; Rotstein *et al.*, 1987). Interestingly, unlike the cattle, the rat retina contains higher proportions of very long chain $n-3$ hexaenes than $n-3$ pentaenes.

The subcellular fractionation of retinas which had been incubated with [^{14}C]acetate showed that labelled VLCPUFA in PC were present in all fractions even at relatively short intervals of incubation. In general, the incorporation of acetate in saturated fatty acids of PC continued to increase in the 1–3 h interval, while that in polyenes was relatively faster, since on a percentage basis polyene labelling was higher after 1 h than after 3 h incubation. Taking into account that VLCPUFA represent 0.3, 0.5, 1.1 and 13.0% of the fatty acids of PC in cytosol, microsomes, mitochondria and photoreceptor membranes respectively (Rotstein & Aveldaño, 1987*a*,*b*), while palmitate accounts for 48, 42, 32 and 26% of the fatty acids of this lipid in the same fractions, it is apparent that the VLCPUFA of PC had exceedingly high specific radioactivities in all subcellular fractions in comparison with saturates, as was determined in Table 2 for entire retina. The relative specific radioactivities of the very long chain polyenes of PC [i.e. the percentage of label incorporated in VLCPUFA (Table 4) divided by the above-mentioned percentages of VLCPUFA in PC] were higher in cytosol, microsomes and mitochondria than in photoreceptor membranes (in that order). This suggests a role of the endoplasmic reticulum and/or mitochondria in the synthesis of VLCPUFA-containing PCs which, through cytosol, may be sent to photoreceptor membranes. This possibility, however, cannot be ascertained with the present approach. Not only the synthesis of fatty acids but their esterification into lipids in each membrane, as well as the exchange of recently synthesized [^{14}C]phospholipids and free fatty acids among subcellular fractions, were apparently very active. Note that labelled lipids were present in all fractions including cytosol, even after only 1 h incubation. To determine the contribution of mitochondria and endoplasmic reticulum to the synthesis of the VLCPUFA of PC obviously first requires the answers to many questions regarding the metabolism of polyenoic fatty acids in general. Using different experimental approaches, such as isolating the fractions and incubating them in conditions which permit the elongation of fatty acids in the presence of labelled acetyl- and malonyl-CoA, as well as the use of individual (radioactive) fatty acid precursors, is likely to provide part of these answers.

The pattern of labelling of lipid classes (Fig. 2) and of lipid fatty acids (Fig. 3) differed among fractions. In

general, both for free fatty acids and for lipid fatty-acyl moieties, the saturated-to-polyenoic-fatty-acid labelling ratio was largest in cytosol and lowest in photoreceptor membranes. This runs parallel to the fatty acid composition of lipids in fractions, since all the phospholipids of Fig. 3 showed increasingly higher percentages of saturated-over-polyenoic-fatty-acid ratios in the following order: photoreceptor membranes, mitochondria, microsomes, cytosol (Rotstein & Aveldaño, 1987*a*,*b*). Furthermore, the large differences observed among lipid classes in their patterns of incorporation of [^{14}C]acetate-labelled fatty acids (Fig. 3) are also quite consistent with their fatty acid composition. Thus, phosphatidylserine, which contains very little palmitate and large amounts of 24- and 26-carbon ($n-3$) polyenes in all fractions, showed a high polyenoic-to-saturated-fatty-acid labelling ratio. The opposite was true for ethanolamine glycerophospholipid and phosphatidylinositol, which contain much more palmitate than phosphatidylserine and exceedingly small proportions of polyenes longer than 22 and 20 carbons respectively. In these three phospholipids, most of the radioactivity in polyenes was found in 22- to 26-carbon ($n-3$) pentaenes, ($n-6$) tetraenes and ($n-3$) hexaenes, in that order, the first predominating.

In the free fatty acid pool, polyenes longer than 26 carbons were labelled to considerably lower extents than in PC (Table 4), suggesting that either 1) the esterification of such polyenes in PC as they are synthesized is remarkably active, or 2) that PC is one of the sources of polyenoic fatty acids to be elongated. Acyl-CoA esters are known to be the obligate substrates for fatty acid desaturase- and elongase-catalysed reactions. Such acyl-CoAs may be synthesized from free fatty acids and coenzyme A (by an ATP-dependent condensation) or from phospholipids by an ATP-independent acyltransferase-catalysed reaction (see Giusto *et al.*, 1986):

$$\text{CoA} + \text{phospholipid} \rightleftharpoons \text{acyl-CoA} + \text{lysophospholipid}$$

It might be speculated that PC could provide the polyenes which, as acyl-CoAs, could serve as substrates for elongase systems. Such elongated acyl-CoAs (also substrates of acyl transferases) could be re-introduced in PC by acylating lysoPC at the (yet unknown) site(s) of synthesis of VLCPUFA-containing PCs. This possibility would be consistent with the high specific radioactivities of VLCPUFA in this lipid class.

One of the most intriguing findings of the present experiments was that the amount of labelled VLCPUFA present in photoreceptor membrane PC was significantly high (Table 3) i.e. similar to that of other subcellular fractions after only 1 h incubation. These are short incubation times compared with the half-life of phospholipids in ROS membranes, which are in the order of 18–23 days (Anderson *et al.*, 1980*a*,*b*,*c*). If one agrees with the generally-assumed belief that these highly specialized membranes lack the ability to synthesize, elongate or desaturate fatty acids, then either 1) the incorporation of VLCPUFA (synthesized in other membranes) into ROS PC, or 2) the transport of VLCPUFA-containing PC to the ROS (from other subcellular sites) are surprisingly active. From what is known at present, both possibilities are likely. PC actively incorporates free polyenoic fatty acids like [^{14}C]22:6($n-3$) in isolated ROS and disks incubated in the presence of ATP and CoA (Giusto *et al.*, 1986). Photoreceptor membranes isolated after incubation of retinas in the presence of glucose with various polyenoic free fatty acids {[^{14}C]20:4($n-6$), [^{14}C]22:5($n-3$) and [^{14}C]-22:6($n-3$)} also show substantial incorporations in PC (Rotstein & Aveldaño, 1987*a*,*b*). Moreover, a soluble transfer protein has been identified in bovine retinas that exchanges PC between liposomes and ROS membranes with a transfer activity larger for ROS than for mitochondrial membranes, the phospholipid acceptor generally used in lipid transport studies (Dudley & Anderson, 1978).

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