# Regulation of the biosynthesis of 4,7,10,13,16-docosapentaenoic acid

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It is now established that fatty acid 7,10,13,16-22:4 is metabolized into 4,7,10,13,16-22:5 as follows: 7,10,13,16-22:4 → 9,12,15,18-24:4 → 6,9,12,15,18-24:5 → 4,7,10,13,16-22:5. Neither $C_{24}$ fatty acid was esterified to 1-acyl-*sn*-glycero-3-phosphocholine (1-acyl-GPC) by microsomes, whereas the rates of esterification of 4,7,10,13,16-22:5, 7,10,13,16-22:4 and 5,8,11,14-20:4 were respectively 135, 18 and 160 nmol/min per mg of microsomal protein. About four times as much acid-soluble radioactivity was produced when peroxisomes were incubated with [3-$^{14}$C]-9,12,15,18-24:4 compared with 6,9,12,15,18-24:5. Only [1-$^{14}$C]7,10,13,16-22:4 accumulated when [3-$^{14}$C]9,12,15,18-24:4 was the substrate, but both 4,7,10,13,16-22:5 and 2-*trans*-4,7,10,13,16-22:6 were produced from [3-$^{14}$C]6,9,12,15,18-24:5. When the two $C_{24}$ fatty acids were incubated with peroxisomes, microsomes and 1-acyl-GPC there was a decrease in the production of acid-soluble radioactivity from [3-$^{14}$C]6,9,12,15,18-24:5, but not from [3-$^{14}$C]9,12,15,18-24:4. The preferential fate of [1-$^{14}$C]4,7,10,13,16-22:5, when it was produced, was to move out of peroxisomes for esterification into the acceptor, whereas only small amounts of 7,10,13,16-22:4 were esterified. By using $^{2}$H-labelled 9,12,15,18-24:4 it was shown that, when 7,10,13,16-22:4 was produced, its primary metabolic fate was degradation to yield esterified arachidonate. Collectively, the results show that an inverse relationship exists between rates of peroxisomal $\beta$-oxidation and of esterification into 1-acyl-GPC by microsomes. Most importantly, when a fatty acid is produced with its first double bond at position 4, it preferentially moves out of peroxisomes for esterification to 1-acyl-GPC by microsomes, rather than being degraded further via a cycle of $\beta$-oxidation that requires NADPH-dependent 2,4-dienoyl-CoA reductase.

## INTRODUCTION

Several studies have shown that microsomes do not contain an acyl-CoA-dependent 4-desaturase [1–6]. When the fatty acids 7,10,13,16-22:4 and 7,10,13,16,19-22:5 are produced in the endoplasmic reticulum, they are chain-elongated to 9,12,15,18-24:4 and 9,12,15,18,21-24:5 respectively. These two acids are subsequently desaturated at position 6 to yield 6,9,12,15,18-24:5 and 6,9,12,15,18,21-24:6 [1,2]. These endoplasmic reticulum-derived metabolites must then move to a site, most probably the peroxisomes, for partial $\beta$-oxidation [5,7]. The chain-shortened metabolites formed by one or more cycles of $\beta$-oxidation are not esterified into 1-acyl-*sn*-glycero-3-phosphocholine (1-acyl-GPC) by peroxisomes [8], but rather they must move back to the endoplasmic reticulum, where they are used for phospholipid biosynthesis [8,9].

Prior to the discovery that microsomes do not have a 4-desaturase, the biosynthesis of polyunsaturated fatty acids and their incorporation into membrane lipids could be viewed as processes that were both localized primarily in the endoplasmic reticulum [10–12]. The revised pathways of unsaturated fatty acid biosynthesis require considerable movement of fatty acids between microsomes and peroxisomes [13]. Any given fatty acid may be used as a substrate for a variety of different reactions. For example, when [3-$^{14}$C]9,12,15,18-24:4 was incubated with rat hepatocytes, only small amounts were esterified into phospholipids, which is most probably a microsomal process. In addition, it was possible to detect esterified [1-$^{14}$C]22:4,$n-6$ [2]. This finding implies that, when 24:4,$n-6$ is produced in the endoplasmic reticulum (from 22:4,$n-6$), some of it must move into the peroxisomes, where it is partially $\beta$-oxidized to 22:4,$n-6$; this then moves back to the endoplasmic reticulum, where it may be used again as a substrate for either esterification or chain elongation. In this type of study it is not possible to determine how much [3-$^{14}$C]24:4,$n-6$ is metabolized to 20:4,$n-6$ via two cycles of $\beta$-oxidation, because of loss of the label. In any case, when [3-$^{14}$C]24:4,$n-6$ was incubated with hepatocytes, its major measurable fate was desaturation to 24:5,$n-6$ followed by partial $\beta$-oxidation to yield esterified [1-$^{14}$C]22:5,$n-6$ [2]. When [3-$^{14}$C]24:5,$n-6$ was incubated with hepatocytes, only small amounts were esterified into phospholipids. Its major fate was metabolism to yield esterified [1-$^{14}$C]22:5,$n-6$, but again it was not possible to detect product formation after the second cycle of $\beta$-oxidation because of loss of label [2].

In the study reported here, unsaturated fatty acids, labelled with $^{2}$H at the last two carbon atoms, as well as $^{14}$C-labelled acids, were used to determine how peroxisomes and microsomes interact to regulate the synthesis and subsequent esterification of long-chain $n-6$ fatty acids into membrane lipids using 1-acyl-GPC as an acceptor.

## MATERIALS AND METHODS

### Materials

ATP, NAD$^{+}$, NADPH, CoASH, dithiothreitol and essentially fatty acid-free BSA were obtained from Sigma. Lactate dehydrogenase and Nycodenz (Accudenz) were obtained from Boehringer Mannheim and Accurate Chemicals and Scientific Corp. respectively. 1-Palmitoyl-2-*sn*-[1-*oleoyl*-$^{14}$C]glycero-3-phosphocholine was from DuPont NEN. 1-Palmitoyl-2-*sn*-[6,9,12,15-heneicosatetrenoyl]glycero-3-phosphocholine was syn-

Abbreviation used: 1-acyl-GPC, 1-acyl-*sn*-glycero-3-phosphocholine.

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thesized and purified as described by Paltauf and Hermetter [14]. [19,19,20,20-$^2H_4$]Ethyl arachidonate [15] was converted into [23,23,24,24-$^2H_4$]9,12,15,18-tetracosatetraenoic acid via a sequence of reactions whereby the ethyl ester of labelled arachidonic acid was reduced to the alcohol with $LiAlH_4$. The resulting alcohol was converted into the mesylate, which was displaced with cyanide. Following hydrolysis with 5% anhydrous HCl in methanol, the methyl ester of the labelled $C_{21}$ acid was obtained [16]. This sequence of reactions was repeated three more times to yield the desired product. All 1-$^{14}$C- and 3-$^{14}$C-labelled acids were made by total organic synthesis [16].

### Isolation of rat liver peroxisomes and microsomes

Male Sprague–Dawley rats (200–250 g) were fed on Purina chow without or with 0.5% (w/v) clofibrate for 8 days prior to being killed. In essence, the procedure of Das et al. [12], as previously described [17], was used to isolate peroxisomes. Briefly, liver was homogenized in a medium containing 0.25 M sucrose, 1 mM EDTA, 0.1% ethanol and 10 mM Tes, pH 7.5. Nuclei and the heavy mitochondrial fraction obtained by centrifugation at 600 ***g*** and 3300 ***g*** respectively were discarded. The light mitochondrial pellet obtained by centrifugation at 25000 ***g*** for 17 min was resuspended in the homogenization buffer and centrifuged at 25000 ***g*** for 15 min. One additional wash was done under the same conditions. The resulting pellet was then resuspended in the homogenization buffer in a volume corresponding to 1 ml for every 2 g of liver. A 2 ml portion of this suspension was then layered on to 15 ml of 35% (w/v) Nycodenz containing 10 mM Tes, 0.1% ethanol and 1 mM EDTA, and centrifuged at 56800 ***g*** for 45 min. The resulting peroxisomal pellet was suspended in incubation medium, which contained 130 mM KCl and 20 mM Hepes, pH 7.2 [18]. The protein concentration was adjusted to 3 mg/ml, as determined by using the Coomassie Blue reagent (Pierce) with BSA as a standard. In order to prepare microsomes, the 25000 ***g*** supernatant was centrifuged at 110000 ***g*** for 45 min. The microsomal pellet was suspended in 0.6 M KCl/0.1 M phosphate buffer, pH 7.4. After centrifugation at 110000 ***g*** for 45 min, the microsomal pellet was suspended in 130 mM KCl/20 mM Hepes, pH 7.2, and stored at −80 °C.

### Fatty acid activation

Peroxisomes (10 or 50 $\mu$g of protein) were incubated in a total volume of 0.2 ml in a medium that contained 130 mM KCl, 20 mM Hepes, 10 mM MgATP, 0.4 mM CoASH and the sodium salt of the radioactive fatty acid (2 Ci/mol) bound to BSA in a 2:1 molar ratio (assuming an $M_r$ of 66000 for BSA). Reactions were initiated by the addition of peroxisomes. The fatty acid concentration varied from 12.5 to 150 $\mu$M. After 2 min the reactions were terminated by the addition of 2.5 ml of isopropyl alcohol/heptane/0.05 M $H_2SO_4$ (40:10:1, by vol.), followed by 1.5 ml of n-heptane and 1 ml of water. The upper layer, containing the unreacted fatty acid, was discarded and the bottom layer was washed twice with 2 ml of heptane [19]. The bottom layers, containing the acyl-CoAs, were transferred to scintillation vials and radioactivity was counted in 10 ml of ACS II (Amersham). Maximum rates of activation were calculated from double-reciprocal plots.

### Microsomal acylation of 1-acyl-GPC

The CoA derivatives of fatty acids were synthesized via their mixed anhydrides [20]. Maximum rates of acylation were determined spectrophotometrically [21]. Incubations contained 50 $\mu$M acyl-CoA, 300 $\mu$M 1-acyl-GPC, 2 mM 5,5-dithiobis-(2-nitrobenzoic acid) and 25–200 $\mu$g of microsomal protein in 1 ml of 0.1 M Tris/HCl (pH 7.4).

### Peroxisomal metabolism

The sodium salts of fatty acids bound to BSA in a 2:1 molar ratio were preincubated at 37 °C in a medium that contained 130 mM KCl, 20 mM Hepes (pH 7.2), 0.1 mM EGTA, 0.5 mM $NAD^+$, 0.2 mM NADPH, 0.4 mM CoASH, 10 mM MgATP, 0.1 mM dithiothreitol, 20 mM pyruvate and 2 units of lactate dehydrogenase [17,18]. Reactions were initiated by the addition of 300 $\mu$g of peroxisomal protein. Other incubations also contained 300 $\mu$g of microsomal protein and 100 nmol of 1-acyl-GPC. The final incubation volume was always 1 ml. At various times, 200 $\mu$l aliquots were removed and mixed with an equal volume of 5% $HClO_4$. After 30 min at 4 °C, the samples were centrifuged and 200 $\mu$l aliquots were counted to measure acid-soluble radioactivity.

In order to quantify the esterification of metabolites into 1-acyl-GPC, the reactions were terminated by the addition of 3.3 ml of chloroform and 6.7 ml of methanol, followed by the addition of 100000 d.p.m. of 1-palmitoyl-2-*sn*-[1-$^{14}$C-*oleoyl*]glycero-3-phosphocholine as an internal standard. The tubes were centrifuged and the bottom layer was taken to dryness under a stream of $N_2$. The lipids were dissolved in chloroform, and neutral lipids were separated from phospholipids by sequential elution of Pasteur pipettes packed with Unisil (Clarkson Chemicals) with 10 ml each of chloroform and methanol. The methanol was removed under $N_2$ and the phospholipids were inter-esterified by stirring them with 5% anhydrous HCl in methanol overnight. The methyl esters were recovered by extraction with hexane and separated by reverse-phase HPLC on a 0.46 cm × 25 cm Zorbax ODS column using acetonitrile/water (9:1, v/v), at 1 ml/min, as the eluting solvent. The effluent was mixed with ScintiVerse LC (Fisher) at 3 ml/min, and radioactivity was detected with a Beckman 171 radioisotope detector. The esterified metabolites were quantified (nmol) from the amount of radioactivity that was associated with methyl oleate. When [23,23,24,24-$^2H_4$]24:4,$n-6$ was used as a substrate, the reaction was again terminated by the addition of chloroform and methanol, but 235 nmol of 1-palmitoyl-2-*sn*-[6,9,12,15-heneicosatetraenoyl]glycero-3-phosphocholine was added as an internal standard. The phospholipids were then saponified by stirring them with 4% KOH in methanol/water (96:4, v/v). After acidification with HCl, the non-esterified fatty acids were recovered by extraction with hexane/diethyl ether (9:1, v/v). The fatty acids were then derivatized by heating them at 60 °C for 30 min with 100 $\mu$l each of acetonitrile and *N*-methyl-*N*-(t-butyldimethylsilyl)trifluoracetamide. The excess derivatizing reagent was removed under a stream of $N_2$ and the compounds were dissolved in iso-octane.

MS was carried out with a Hewlett–Packard model 5970 mass selective detector and a 5790 gas chromatograph containing a 30 m × 0.25 mm inside DB-5ms capillary column (J. and W. Scientific, Rancho Cordoba, CA, U.S.A.). Compounds were injected in the splitless mode at 70 °C. After 3 min the oven was programmed to increase to 250 °C at 15 °C/min. The amounts of various metabolites were quantified by monitoring the appropriate $M-57$ ions [22].

## RESULTS

### Microsomal esterification of fatty acids into 1-acyl-GPC

Our previous studies have shown that arachidonic acid is metabolized via 22:4,$n-6$ to 24:4,$n-6$ and 24:5,$n-6$ in the

**Table 1 Rates of acylation of fatty acids into 1-acyl-GPC by liver microsomes from rats fed on Purina chow with and without 0.5% (w/v) clofibrate**

Microsomes were incubated with acyl-CoAs and 1-acyl-GPC in the presence of 5,5′-dithiobis-(2-nitrobenzoic acid). Rates of reaction were determined spectrophotometrically, and are means ± S. E. M. of three separate experiments.

| | Acylation (nmol/min per mg of microsomal protein) | |
|---|---|---|
| Acyl-CoA | Chow diet | Chow diet + 0.5% clofibrate |
| 20:4,*n*−6 | 52.7 ± 2.2 | 159.8 ± 8.8 |
| 22:4,*n*−6 | 6.0 ± 1.6 | 18.4 ± 4.1 |
| 22:5,*n*−6 | 49.8 ± 5.4 | 134.9 ± 3.3 |
| 24:4,*n*−6 | 1.3 ± 0.2 | 1.0 ± 0.4 |
| 24:5,*n*−6 | 1.1 ± 0.3 | 0.9 ± 0.4 |

**Table 2 Rates of activation of fatty acids by peroxisomes from rats fed on Purina chow with or without 0.5% (w/v) clofibrate**

Results are means ± range of two separate experiments.

| | Activation (nmol/min per mg of peroxisomal protein) | |
|---|---|---|
| Fatty acid | Control | + Clofibrate |
| [1-$^{14}$C]20:4 | 185 ± 19 | 314 ± 27 |
| [1-$^{14}$C]22:4 | 49 ± 12 | 103 ± 6 |
| [1-$^{14}$C]22:5 | 45 ± 2 | 86 ± 8 |
| [3-$^{14}$C]24:4 | 9 ± 4 | 18 ± 2 |
| [3-$^{14}$C]24:5 | 7 ± 1 | 9 ± 1 |

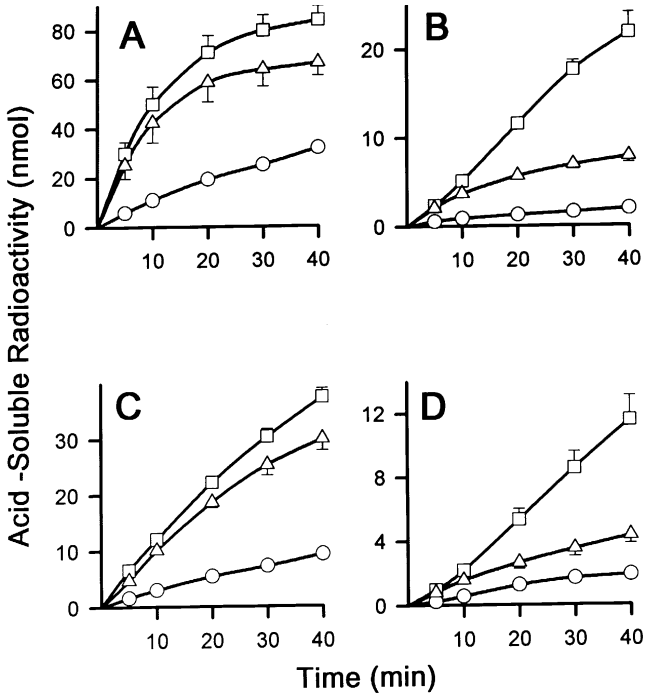


**Figure 1 Time-dependent β-oxidation of [1-$^{14}$C]7,10,13,16-22:4 (A), [1-$^{14}$C]4,7,10,13,16-22:5 (B), [3-$^{14}$C]9,12,15,18-24:4 (C) and [3-$^{14}$C]-6,9,12,15,18-24:5 (D)**

Peroxisomes (300 μg of protein) from rats fed on a diet of Purina chow (○) or one containing clofibrate (□) were incubated with 100 μM substrate. In addition, 300 μg of peroxisomal protein from rats fed clofibrate were incubated with 300 μg of microsomal protein and 100 μM 1-acyl-6PC (△). Results are means ± range for two separate experiments.

endoplasmic reticulum [2]. If either of these two $C_{24}$ acids were used for membrane lipid biosynthesis where they are produced, it would curtail their rapid movement to a site for partial β-oxidation. The results in Table 1 show that neither $C_{24}$ acid was esterified into 1-acyl-GPC by microsomes from rats fed on diets with or without clofibrate. When arachidonic acid and the two $C_{22}$ acids were the substrates, there was an approx. 3-fold increase in rates of acylation with microsomes from rats fed on a clofibrate-containing diet compared with microsomes from rats fed on chow.

### Fatty acid activation

Since 24:4,*n*−6 is a poor substrate for acylation into 1-acyl-GPC, the above results imply that, *in vivo*, it must either be desaturated at position 6 to yield 24:5,*n*−6 or move to peroxisomes for partial β-oxidation to 22:4,*n*−6. The primary metabolic fate of 24:5,*n*−6 must be to move to a site for partial degradation. The true substrates and products for a number of microsomal desaturation and chain-elongation reactions are the acyl-CoAs [23–25]. Presumably when $C_{24}$ acids are produced in the endoplasmic reticulum, they also are acyl-CoAs. Cytosol contains acyl-CoA hydrolases [26,27]. It is not known whether acyl-CoAs can move directly into peroxisomes, or whether they are hydrolysed and subsequently re-activated by the subcellular organelle in which they are to be metabolized. The results in Table 2 show that peroxisomes have the ability to activate $C_{20}$, $C_{22}$ and $C_{24}$ acids, albeit at markedly different rates. As was observed for rates of microsomal acylation, the addition of clofibrate to the diet increased the rates of activation. This effect was somewhat greater for $C_{20}$ and $C_{22}$ acids than for the $C_{24}$ compounds. Microsomes, mitochondria and peroxisomes all contain a long-chain acyl-CoA synthetase [28,29]. Peroxisomes and microsomes, but not mitochondria, contain an enzyme that is designated as a very-long-chain acyl-CoA synthetase [30]. The above studies do not discriminate between these two enzyme activities. Since all of the *n*−6 acids were activated by peroxisomes, all subsequent studies were carried out by generating acyl-CoAs *in situ*, which may well be the case *in vivo*.

### Peroxisomal fatty acid β-oxidation and the esterification of chain-shortened acids into 1-acyl-GPC by microsomes

The results in Figure 1 show that with all four *n*−6 acids the addition of clofibrate to the diet increased the rate of peroxisomal β-oxidation. When clofibrate was included in the diet, about 30 nmol of acid-soluble radioactivity was produced from [1-$^{14}$C]7,10,13,16-22:4 at 5 min (Figure 1A), compared with only about 2 nmol when [1-$^{14}$C]4,7,10,13,16-22:5 was the substrate (Figure 1B). Similar differences existed for the two $C_{24}$ acids: after 5 min, about eight times as much acid-soluble radioactivity was produced from [3-$^{14}$C]9,12,15,18-24:4 as from [3-$^{14}$C]-6,9,12,15,18-24:5 (Figures 1C and 1D respectively).

The results in Figure 1 show that, when peroxisomes from rats fed on a clofibrate-containing diet were incubated with microsomes and 1-acyl-GPC, there was only a small decrease in the production of acid-soluble radioactivity from [1-$^{14}$C]7,10,13,16-22:4 (Figure 1A) or [3-$^{14}$C]9,12,15,18-24:4 (Figure 1C). The loss of label from these two fatty acids requires only the enzymes of saturated fatty acid degradation. Conversely, when [1-

**Table 3 Incorporation of radioactive fatty acids into phospholipids on incubation of peroxisomes with 1-acyl-GPC and microsomes**

Peroxisomes (300 $\mu$g of protein) from rats fed on Purina chow containing 0.5% (w/v) clofibrate were incubated with 100 $\mu$M fatty acid, 100 $\mu$M 1-acyl-GPC and 300 $\mu$g of microsomal protein. After 30 min, the incubations were terminated by the addition of chloroform and methanol, followed by the addition of 100000 d.p.m. of 1-palmitoyl-2-[1-$^{14}$C-*oleoyl*]-*sn*-glycero-3-phosphocholine. The phospholipids were separated by silicic acid column chromatography and inter-esterified. The amounts of esterified acids were calculated from the amounts of radioactivity associated with the methyl oleate in the HPLC radiochromatograms. Results are means ± range from two separate experiments.

| | Esterified radioactive fatty acid (nmol) | | | |
|---|---|---|---|---|
| Substrate | 22:4,*n*−6 | 24:4,*n*−6 | 22:5,*n*−6 | 24:5,*n*−6 |
| [1-$^{14}$C]22:4,*n*−6 | 15.8 ± 0.3 | – | – | – |
| [3-$^{14}$C]24:4,*n*−6 | 9.0 ± 1.0 | 3.2 ± 1.4 | – | – |
| [1-$^{14}$C]22:5,*n*−6 | – | – | 39.9 ± 3.3 | – |
| [3-$^{14}$C]24:5,*n*−6 | – | – | 31.8 ± 3.3 | 2.2 ± 0.6 |

$^{14}$C]4,7,10,13,16-22:5 (Figure 1B) or [3-$^{14}$C]6,9,12,15,18-24:5 (Figure 1D) was incubated with peroxisomes, microsomes and 1-acyl-GPC, there was a marked decrease in the production of acid-soluble radioactivity. The first cycle of $\beta$-oxidation of 6,9,12,15,18-24:5 also uses only the enzymes of saturated fatty acid degradation but, as soon as 4,7,10,13,16-22:5 is produced, its continued degradation requires both NADPH-dependent 2,4-dienoyl-CoA reductase and $\Delta^3$,$\Delta^2$-enoyl-CoA isomerase, which is a component of the peroxisomal trifunctional enzyme [31].

Table 3 compares the amounts of labelled acids esterified into phospholipids when peroxisomes were incubated with microsomes and 1-acyl-GPC. When [1-$^{14}$C]22:4,*n*−6 was the substrate, there was only a small decrease in the production of acid-soluble radioactivity when incubations contained microsomes and 1-acyl-GPC (Figure 1A). After 30 min, 15.8 nmol of 22:4,*n*−6 had been esterified into phospholipids (Table 3), which is equal to the difference in the production of acid-soluble radioactivity when peroxisomes were incubated with and without microsomes and 1-acyl-GPC. When [1-$^{14}$C]4,7,10,13,16-22:5 was incubated directly with peroxisomes, microsomes and 1-acyl-GPC, or generated from [3-$^{14}$C]6,9,12,15,18-24:5, its primary fate was esterification rather than continued $\beta$-oxidation. For example, at 30 min about 3 nmol of acid-soluble radioactivity had been produced from [3-$^{14}$C]24:5,*n*−6 (Figure 1D), but 31.8 nmol of 22:5,*n*−6 was esterified (Table 3). Only small amounts of the two $C_{24}$ acids were directly esterified. When [3-$^{14}$C]24:4,*n*−6 was the substrate, the preferred metabolic fate of [1-$^{14}$C]22:4,*n*−6, when it was generated, was continued $\beta$-oxidation rather than esterification, since after 30 min only 9 nmol had been esterified.

## Characterization of metabolites produced on incubation of [1-$^{14}$C]-4,7,10,13,16-22:5 and [3-$^{14}$C]6,9,12,15,18-24:5 with peroxisomes

When [1-$^{14}$C]22:4,*n*−6 was incubated alone with peroxisomes, it was only possible to detect unmetabolized substrate when the methyl esters, formed by saponification and subsequent esterification, were analysed by HPLC. When [3-$^{14}$C]24:4,*n*−6 was the substrate it was possible to detect both unmetabolized substrate and [1-$^{14}$C]22:4,*n*−6. When [1-$^{14}$C]22:5,*n*−6 was incubated with peroxisomes, three radioactive metabolites were eluted immediately before unmetabolized substrate (Figure 2A). When [3-$^{14}$C]24:5,*n*−6 was incubated under the same conditions, it was possible to detect the same three compounds in addition to [1-

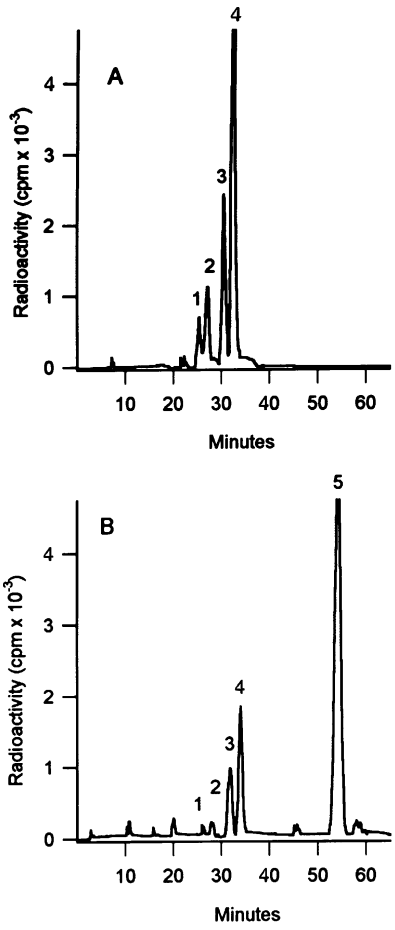


**Figure 2 HPLC radiochromatograms obtained on incubation of 100 $\mu$M [1-$^{14}$C]4,7,10,13,16-22:5 (A) or [3-$^{14}$C]6,9,12,15,18-24:5 (B) with 300 $\mu$g of peroxisomal protein from rats fed on a clofibrate-containing diet**

The incubations were terminated after 30 min, and methyl esters were prepared and analysed by HPLC. Compounds **1** and **2** are isomers of 3,5,7,10,13,16-22:6, and compounds **3**, **4** and **5** are 2-*trans*-4,7,10,13,16-22:6, 22:5,*n*−6 and 24:5,*n*−6 respectively.

$^{14}$C]22:5,*n*−6, i.e. compound **4** in Figure 2(B). The UV spectrum of compound **3** was identical with that of authentic methyl-2-*trans*-4-*cis*-10:2, which was isolated from the seed oil of the Chinese tallow tree [32]. Compound **3** is thus 2-*trans*-4,7,10,13,16-22:6, which is the substrate for NADPH-dependent 2,4-dienoyl-CoA reductase. Compounds **1** and **2** had absorbance maxima at 257, 267 and 277 nm, showing that they contained three double bonds in conjugation, and are thus probably isomers of 3,5,7,10,13,16-22:6 [9]. These compounds are most likely formed from 2-*trans*-4,7,10,13,16-22:6 by peroxisomal $\Delta^{3,5}$,$\Delta^{2,4}$-dienoyl-CoA isomerase [33] operating in the reverse direction.

## Metabolism of $^2$H-labelled 9,12,15,18-24:4

Since the production of acid-soluble radioactivity from [1-$^{14}$C]7,10,13,16-22:4 and [3-$^{14}$C]9,12,15,18-24:4 was not markedly reduced when incubations also contained microsomes and 1-acyl-GPC, subsequent incubations were carried out with [23,23,24,24-$^2H_4$]9,12,15,18-24:4. In these experiments we compared the amount of acid-soluble radioactivity produced when [3-$^{14}$C]9,12,15,18-24:4 was incubated with peroxisomes, microsomes and 1-acyl-GPC with the amount of [19,19,20,20-

$^2H_4$]5,8,11,14-20:4 esterified in phospholipids. In four separate experiments in which 100 nmol of the substrate was incubated for 30 min with 300 $\mu$g each of microsomal and peroxisomal protein and 100 $\mu$M 1-acyl-GPC, it was observed that $19.2 \pm 1.4$ nmol (mean $\pm$ S.E.M.) of acid-soluble radioactivity was produced. With the $^2$H-labelled substrate, $15.2 \pm 1.5$ nmol of labelled arachidonate was esterified. The data clearly show that, when 9,12,15,18-24:4 is the substrate, it is degraded rapidly via two cycles of $\beta$-oxidation, but once arachidonate is produced its primary metabolic fate is to move out of peroxisomes for acylation into 1-acyl-GPC.

### Competition studies with $C_{24}$ fatty acids

All of the above studies were carried out using a single substrate. *In vivo*, when $C_{24}$ acids are produced, the possibility exists that both 24:4,$n-6$ and 24:5,$n-6$ could move into peroxisomes and compete for partial $\beta$-oxidation to yield, respectively, 22:4,$n-6$ and 22:5,$n-6$, both of which have the potential of serving as substrates for continued $\beta$-oxidation or being transferred back to the endoplasmic reticulum for esterification into 1-acyl-GPC. When 100 nmol of [3-$^{14}$C]9,12,15,18-24:4 and [3-$^{14}$C]-6,9,12,15,18-24:5 were incubated together for 30 min with peroxisomes, microsomes and 1-acyl-GPC, $1.8 \pm 0.2$ and $12.4 \pm 2.4$ nmol respectively of 7,10,13,16-22:4 and 4,7,10,13,16-22:5 was esterified in phospholipids. As expected, these esterification values, which are averages ($\pm$ range) of two separate experiments, were lower than those obtained when 100 nmol of [3-$^{14}$C]24:4,$n-6$ or [3-$^{14}$C]25:5,$n-6$ was incubated individually under these conditions (Table 3). When the two 3-$^{14}$C-labelled $C_{24}$ acids are incubated together, the first cycle of $\beta$-oxidation of both substrates requires only the enzymes of saturated fatty acid oxidation, and thus it is expected that, due to substrate competition, smaller amounts of each substrate would be degraded to yield $C_{22}$ acids than when they are incubated alone. However, the important point of these studies is that the ratio of esterified 22:5,$n-6$/22:4,$n-6$ was similar when the two 3-$^{14}$C-labelled fatty acids were incubated together (i.e. $12.2/1.8 = 6.7$) and when they were incubated separately ($39.9/9 = 4.4$).

The synthesis of 22:6,$n-3$ from 24:6,$n-3$ [9] proceeds via a pathway analogous to that for the production of 22:5,$n-6$. When experiments identical to those described above were carried out with [3-$^{14}$C]9,12,15,18,21-24:5 and [3-$^{14}$C]6,9,12,15,18,21-24:6, it was found that $1.6 \pm 1.2$ and $9.0 \pm 0.2$ nmol respectively of 7,10,13,16,19-22:5 and 4,7,10,13,16,19-22:6 was esterified. As with the $n-6$ acids, there was enhanced esterification of the metabolite, i.e. 22:6,$n-3$, whose continued peroxisomal degradation requires NADPH-dependent 2,4-dienoyl-CoA reductase activity.

When 22:5,$n-6$ and 22:6,$n-3$ are produced from their $C_{24}$ precursors, their continued peroxisomal degradation requires NADPH-dependent 2,4-dienoyl-CoA reductase. Experiments were thus carried out to determine if there was preferential esterification of either 22:5,$n-6$ or 22:6,$n-3$ when [3-$^{14}$C]24:5,$n-6$ and [3-$^{14}$C]24:6,$n-3$ were incubated together with microsomes, peroxisomes and 1-acyl-GPC. Because of chromatographic overlap problems, these studies were carried out by incubating 100 nmol of a labelled fatty acid with an equal amount of the appropriate unlabelled analogue. In two separate experiments it was observed that, when 100 nmol of [3-$^{14}$C]24:5,$n-6$ was incubated with an equal amount of unlabelled 24:6,$n-3$, 12.2 and 8.5 nmol respectively of 22:5,$n-6$ was esterified after a 30 min incubation. In the direct crossover study using [3-$^{14}$C]24:6,$n-3$ and unlabelled 24:5,$n-6$, 10.2 and 12.0 nmol respectively of 22:6,$n-3$ was esterified in two separate experiments. Under these conditions there were no differences in the amounts of 22:5,$n-6$ and 22:6,$n-3$ esterified when they were generated by co-incubation of their respective immediate $C_{24}$ precursors.

## DISCUSSION

When hepatocytes were incubated with 3-$^{14}$C-labelled $C_{24}$ $n-3$ [1] and $n-6$ [2] fatty acids, only small amounts of the substrates were esterified directly into phospholipids. In the present study, as well as in an analogous study with labelled $n-3$ fatty acids [9], it was observed that these compounds were poor substrates for esterification into 1-acyl-GPC by microsomal acyl-CoA:1-acyl-GPC acyltransferase. When $C_{24}$ acids are produced in the endoplasmic reticulum, their primary metabolic fate must be to move to another intracellular site for further metabolism. It remains to be determined whether they move as acyl-CoAs or whether they are hydrolysed in the cytosol [26,27] and re-activated at their site of subsequent metabolism. It also remains to be determined whether mitochondria, which lack a very-long-chain acyl-CoA synthetase [30], have the capacity to activate unsaturated $C_{24}$ acids, convert them into acylcarnitines and subsequently degrade them completely, which would appear to be an example of futile metabolite cycling.

The results obtained here, as well as with the corresponding $n-3$ fatty acids [9], generally show that an inverse relationship exists between rates of peroxisomal $\beta$-oxidation and rates of microsomal esterification into 1-acyl-GPC. When [3-$^{14}$C]24:4,$n-6$ was incubated with peroxisomes, the addition of microsomes and 1-acyl-GPC did not markedly decrease the production of acid-soluble radioactivity. The $\beta$-oxidation of 24:4,$n-6$ to 20:4,$n-6$ requires only the enzymes of saturated fatty acid degradation. Neither 24:4,$n-6$ nor 22:4,$n-6$, the product formed after one cycle of $\beta$-oxidation, was readily esterified into 1-acyl-GPC. By using $^2$H-labelled 24:4,$n-6$ it was shown that the primary metabolic fate of 20:4,$n-6$, when it was produced, was esterification rather than continued peroxisomal degradation. The first cycle of [3-$^{14}$C]24:5,$n-6$ $\beta$-oxidation also uses only the enzymes of saturated fatty acid degradation. When [3-$^{14}$C]24:5,$n-6$ was incubated with peroxisomes, microsomes and 1-acyl-GPC, there was a marked decrease in the production of acid-soluble radioactivity, which would be produced during the second cycle of fatty acid degradation. As soon as 22:5,$n-6$ is produced, it is preferentially transferred out of peroxisomes and used as a substrate for esterification in microsomes rather than undergoing continued peroxisomal $\beta$-oxidation. Since peroxisomes do not contain acyl-CoA:1-acyl-GPC acyltransferase [8], the chain-shortened product must then be transferred back to the endoplasmic reticulum for use in membrane lipid biosynthesis.

It appears that the low activity of peroxisomal NADPH-dependent 2,4-dienoyl-CoA reductase is a major control point in the regulation of peroxisomal $\beta$-oxidation. When [1-$^{14}$C]22:5,$n-6$ was incubated with peroxisomes, or generated from its $C_{24}$ precursor, 2-*trans*-4,7,10,13,16-22:6 accumulated. Similar results were obtained with the analogous $n-3$ fatty acids, i.e. [3-$^{14}$C]24:6,$n-3$ and [1-$^{14}$C]22:6,$n-3$ [9]. When 3-$^{14}$C-labelled 24:4,$n-6$ and 24:5,$n-6$ were incubated together in the presence of microsomes and 1-acyl-GPC, there was preferential esterification of 22:5,$n-6$ compared with 22:4,$n-6$. Similar results were obtained when 3-$^{14}$C-labelled 24:5,$n-3$ and 24:6,$n-3$ were incubated together. When [1-$^{14}$C]16:3,$n-6$, the product formed after two cycles of arachidonate $\beta$-oxidation, was incubated with peroxisomes, 2-*trans*-4,7,10-16:4 accumulated [9]. It was possible to detect labelled 4,7,10-16:3 in the

medium when control fibroblasts, but not those from patients with Zellweger's syndrome, were incubated with $^{3}$H-labelled arachidonic acid [34]. Collectively, the above findings suggest that, when an acid is produced in peroxisomes with its first double bond at position 4, its further degradation is impaired, since the next cycle requires the action of NADPH-dependent 2,4-dienoyl-CoA reductase. As a result, that fatty acid is preferentially transported out of peroxisomes, via some unknown pathway, for use in another subcellular compartment, such as for rapid acylation, as was observed when 22:5,$n-6$ was produced from 24:5,$n-6$.

It has generally been recognized that desaturation of linoleate and linolenate at position 6 is the rate-limiting step in the biosynthesis of long-chain $n-6$ and $n-3$ fatty acids [35–37]. When rats are raised on a chow diet, their liver phospholipids contain large amounts of arachidonate, but small amounts of both 22:4,$n-6$ and 22:5,$n-6$. Conversely, the same membrane lipids contain little if any 20:5,$n-3$ and small amounts of 22:5,$n-3$, but 22:6,$n-3$ is a major component. Although the pathways for 22:5,$n-6$ and 22:6,$n-3$ biosynthesis are identical, no single reaction rate for any reaction can explain these compositional differences. Reaction rates for the desaturation of 24:4,$n-6$ and 24:5,$n-3$ at position 6 were similar. Moreover these rates were similar to those for the desaturation of linoleate and linolenate at position 6 [38]. Reaction rates for chain elongation of 20:4,$n-6$ and 20:5,$n-3$ were similar to each other, but about 5-fold greater than those for chain elongation of 22:4,$n-6$ and 22:5,$n-3$ [2]. None of these reaction rates in themselves can be used as reliable indicators to predict why membrane lipids accumulate specific fatty acids. In a similar way, the reaction rates for the esterification of analogous $n-3$ and $n-6$ fatty acids into 1-acyl-GPC were similar, and could not be used as predictors as to why membrane lipids accumulate specific unsaturated fatty acids [21]. In the study reported here, it was found that there was no selectivity in the degradation of 3-$^{14}$C-labelled 24:5,$n-6$ and 24:6,$n-3$ to their respective 1-$^{14}$C-labelled fatty acids followed by their movement out of peroxisomes for esterification into 1-acyl-GPC. The fatty acids 22:4,$n-6$ and 22:5,$n-3$ may be viewed as central intermediates in determining how much 22:5,$n-6$ and 22:6,$n-3$ is produced. Both 22:4,$n-6$ and 22:5,$n-3$, when produced in microsomes, may be esterified directly, partially $\beta$-oxidized to yield 20:4,$n-6$ and 20:5,$n-3$, which can be esterified, or metabolized into 24:5,$n-6$ and 24:6,$n-3$, which are esterified following chain shortening. When [3-$^{14}$C]22:4,$n-6$ was injected into the tail vein of rats fed a chow diet, 96 and 4% respectively of the esterified radioactivity in liver phospholipids was arachidonate and unmetabolized substrate. When [3-$^{14}$C]22:5,$n-3$ was injected, 20, 56 and 24% respectively of the esterified radioactivity was 20:5,$n-3$, 22:5,$n-3$ and 22:6,$n-3$ [39]. Clearly there are differences in how these two central intermediates are metabolized *in vivo* that, at present, cannot be explained by any individual reaction in either microsomes or peroxisomes.

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