# Polyunsaturated fatty acid metabolism in retinal and cerebral microvascular endothelial cells

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**Abstract** Docosahexaenoic acid (22:6n–3), an n–3 essential fatty acid derived from elongation and desaturation of linolenic acid (18:3n–3), is found in abundant proportion in the brain and the retina. It is generally assumed that the liver is the major source of 22:6n–3 for these organs, although some retinal and cerebral cells, such as retinal pigment epithelium (Wang and Anderson, 1993. *Biochemistry.* **32:**13703–13709) and brain astrocytes (Moore et al. 1991. *J. Neurochem.* **56:**518–524) have the ability to produce 22:6n–3. The aim of the present study was to determine whether retinal and cerebral microvascular endothelium could synthesize 22:6n–3. After incubation of both cultured bovine retinal and rat cerebral endothelial cells with [3-$^{14}$C] 22:5n–3 in presence of serum, radioactivity was primarily recovered in 20:5n–3, indicating active retroconversion reactions in both tissues. However, 22:6n–3, 24:5n–3, and 24:6n–3 were also labeled. All of these metabolites were released in the medium as free fatty acids. Retinal endothelial cells preferentially released labeled 24-carbon metabolites, whereas cerebral endothelial cells released relatively more 20:5n–3 and 22:6n–3. With heat-inactivated serum or no serum, both endothelial cell preparations showed relatively higher retroconversion levels. However, in serum-deprived cells, the elongation/desaturation pattern was affected in retinal cells only, with an accumulation of 24:5n–3 relative to a decrease of 24:6n–3 and 22:6n–3. Fatty acid composition analyses revealed a decrease in long-chain polyunsaturated n–6 and n–3 fatty acids in retinal cells maintained in inactivated serum compared to normal serum, while no change was found in cerebral cells. Taken together, these results suggest that *1*) the synthesis of 22:6n–3 by both retinal and cerebral endothelial cells is independent of a δ4-desaturase; *2*) retinal and cerebral endothelia could be a source of 22:6n–3 for the retina and the brain, respectively; and *3*) retinal endothelial δ6-desaturase, which converts 24:5n–3 to 24:6n–3, could be stimulated by serum components.—**Delton-Vandenbroucke, I., P. Grammas, and R. E. Anderson.** Polyunsaturated fatty acid metabolism in retinal and cerebral microvascular endothelial cells. *J. Lipid Res.* 1997. **38:** 147–159.

**Supplementary key words** docosahexaenoic acid • docosapentaenoic acid • desaturation • chain elongation • retroconversion • fetal bovine serum

Docosahexaenoic acid (22:6n–3), the most highly polyunsaturated n–3 fatty acid in animals, is derived from a series of elongation and desaturation reactions originating from linolenic acid (18:3n–3). In contrast to arachidonic acid (20:4n–6), which is distributed in relatively large amounts in most tissues, 22:6n–3 is found in high proportions primarily in neuronal tissues such as brain (1) and retina (2), and in a few tissues outside the central nervous system such as testis (3). Within the retina of most vertebrate species, 22:6n–3 is heavily concentrated in rod outer segment membrane phospholipids, where it comprises about 50% of the total fatty acids (4). Within the brain, 22:6n–3 is particularly abundant in the gray matter (1). The reason for such high levels of 22:6n–3 is not clearly understood, although changes in retinal and cerebral functions have been reported in rats (5), monkeys (6), and humans (7) fed diets deficient in n–3 fatty acids.

Because of its functional importance, more information is needed about the delivery of 22:6n–3 to these structures. The liver is thought to be the primary site at which 22:6n–3 is formed from dietary 18:3n–3 and longer chain precursors, for distribution to target organs via the circulation (8–10). The role of other tissues in 22:6n–3 synthesis is generally considered to be minor. However, the ability of the rat and dog retinas to form 22:6n–3 in vivo after intravitreal injection of 18:3n–3, eicosapentaenoic acid (20:5n–3), or docosapentaenoic acid (22:5n–3) has been reported (11–13). It has been further demonstrated in the frog that retinal pigment epithelium (RPE) and, to a much lesser extent, the retina could convert 18:3n–3 or 22:5n–3 to 22:6n–3 (14). The ability of the rat brain to synthesize 22:6n–

Abbreviations: RPE, retinal pigment epithelium; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; HBSS, Hank's balanced salt solution; BSA, bovine serum albumin; GLC, gas–liquid chromatography; TLC, thin-layer chromatography; NL, neutral lipids; FFA, free fatty acids; PL, phospholipids; HPLC, high performance liquid chromatography; FAPE, fatty acid phenacyl ester; FAME, fatty acid methyl ester.

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3 has been shown in vivo after intracranial or intraperitoneal injection of 18:3n–3 (15, 16); astrocytes appear to be a major site for 22:6n–3 synthesis (17). Recent studies have shown that 22:6n–3 is found in high proportion in cerebral and retinal microvessels (18, 19). Although the microvascular endothelium is important for regulating nutrient supply in the retina and the brain, the potential role of endothelial cells in 22:6n–3 synthesis has received little attention. In this regard, however, Moore, Yoder, and Spector (20) reported that mouse cerebral endothelium converted 18:3n–3 to 22:5n–3, but could not accomplish the final desaturation step leading to the formation of 22:6n–3.

In the present study, we investigated whether retinal and cerebral microvascular endothelial cells could produce 22:6n–3 from 22:5n–3, to establish a potential role for the microvascular endothelium in the supply of 22:6n–3 to these tissues. In addition, the elongation/desaturation patterns and responsiveness of these processes to serum was compared in the two endothelial cells.

## MATERIALS AND METHODS

### Materials

Fatty acids were supplied by Sigma (St Louis, MO). Radiolabeled [3-$^{14}$C]22:5n–3 (approximately 50 mCi/mmol) was purchased from New England Nuclear (DuPont, Boston, MA). HPLC analyses of the $^{14}$C radioactivity were regularly performed to check that at least 95% of the radioactivity was associated with 22:5n–3.

### Endothelial cell cultures

*Retinal microvascular endothelial cells.* Retinal microvascular endothelial cell cultures were initiated from isolated adult bovine retinal microvessels according to the method of Gitlin and d'Amore (21). The microvessel fragments were plated in Dulbecco's modified Eagle's medium (DMEM, Gibco BRL, Grand Island, NY) supplemented with 20% fetal bovine serum (FBS, Summit Biotechnology, Fort Collins, CO) into 2%-gelatinized 35-mm dishes and allowed to attach for 24 h. At the end of the attachment period, the dishes were rinsed with Hank's balanced salt solution (HBSS, Gibco BRL) and refed with DMEM–20% FBS, the medium being changed every 2 days. At confluence, cells were subcultured by trypsinization. Early cultures (first and second passages) were contaminated by pericytes. At the third passage, the endothelial cells were separated from pericytes by cloning (22). The endothelial origin of the cells was confirmed by the uptake of acetylated low density lipoprotein labeled with 1,1′-dioctadecyl-1,3,3,3′-3′-tetramethyl-indocarbocyanine perchlorate (DiI-Ac-LDL, Biomedical Technologies Inc., Stoughton, MA) (23) and by the absence of significant staining for the α-isoform of smooth muscle actin (Boehringer Mannhein Biochemica, Indianapolis, IN).

*Cerebral microvascular endothelial cells.* Cerebral microvascular endothelial cell cultures were initiated from isolated adult rat cerebral microvessels as previously described (22, 24).

For the experiments reported herein, retinal and cerebral endothelial cell cultures were used between passages 8 and 12.

### Incubation conditions

The uptake, retroconversion, elongation, and desaturation of [3-$^{14}$C]22:5n–3 were studied in confluent cultures of rat cerebral or bovine retinal endothelial cells grown in DMEM containing 20% FBS in 35-mm dishes. Incubations were carried out in 1.5 ml DMEM containing 1 μCi (20 nmol) of [3-$^{14}$C]22:5n–3 previously conjugated with delipidated bovine serum albumin (BSA, 2:1 molar ratio) in 50 mM $NaHCO_3$, in different serum conditions, for 2 to 24 h. The amount of nonradiolabeled 22:5n–3 originally associated with the 20% serum-containing media (3 nmol, as determined by GLC analysis) was considered negligible and did not significantly modify the specific activity of the added [3-$^{14}$C]22:5n–3. At the end of the incubation period, the medium was removed, the cells were rinsed once with HBSS or versene buffer (for retinal and cerebral cells, respectively), and then harvested by trypsinization.

### Lipid extraction

Prior to lipid extraction, media were centrifuged to remove cellular debris. Lipids from cells and media were extracted twice with chloroform–ethanol 2:1 (v/v). Lipid extracts were dried under nitrogen and resolubilized in a known volume of ethanol. Two aliquots of each lipid extract were taken; one was counted by liquid scintillation (LS60001C, Beckman Instruments, Inc.) for total lipid labeling and the other was plated on silica-gel 60 plates (Merck) to separate total lipids by thin-layer chromatography (TLC), using the developing solvent system hexane–diethyl ether–acetic acid 70:30:1 (v/v), into neutral lipids (NL), free fatty acids (FFA), and phospholipids (PL). Lipids were visualized using iodine vapors and identified by comparison with standards. The areas of interest were scraped off the plate and counted by liquid scintillation to determine the distribution of radioactivity among lipids from cells and media.

### Fatty acid phenacyl ester (FAPE) preparation and HPLC analysis

After extraction as described above, total lipids were saponified (12) and free fatty acids were converted to

FAPEs (25). FAPEs were dissolved in a mixture of acetonitrile–water 98:2 (v/v) and separated by HPLC on a LC-18 reverse-phase column (25 cm × 4.6 mm, Supelco Inc., Bellefonte, PA) using a linear gradient of acetonitrile–water from 80:20 (v/v) to 98:2 at 2 ml/min for 45 min, followed by holding at 98:2 for 10 min. The mobile phase was then brought back to 80:20 in 5 min. The radioactivity profile was monitored by an on-line radioactivity flow detector (Flo-One\Beta, Radiomatic, Tampa, FL) using Ultima-Flo M (Packard Instrument Co., Inc., Downers Grove, IL) at 2.5:1 (v/v) ratio of cocktail to mobile phase. Identities of individual fatty acids were made by comparing elution times to those of FAPEs prepared from fatty acid standards (Sigma).

### Catalytic hydrogenation and HPLC analysis of hydrogenated products

FAPE peaks were collected and FAPEs were extracted twice with hexane, dried, and dissolved in 2 ml ethanol–hexane 2:1. Approximately 6–10 mg of platinum oxide (EM Science, Gibbstown, NJ) was added and the FAPE solution was bubbled with hydrogen for 10 min. The catalyst was pelleted by centrifugation and the pellet was washed once with 1 ml ethanol–hexane. The pooled supernatant and wash were rephenacylated and analyzed on the same column described above using 100% acetonitrile as mobile phase over 35 min.

### Fatty acid methyl ester (FAME) preparation and GLC analysis

Lipid classes separated by TLC as described above were treated with 14% boron trifluoride–methanol (26). The resulting FAMEs were analyzed by gas–liquid chromatography (GLC) (27) using a DB-225 capillary column with helium as a carrier gas, and identified by comparison with appropriate standards.

## RESULTS

### Uptake of [3-$^{4}$C]22:5n–3

Similar amounts of 3-$^{14}$C radioactivity were taken up by retinal and cerebral endothelial cells at each point time examined between 2 h and 24 h (**Fig. 1**). For both cells, the uptake reached a plateau within 16 h, accounting for 47% of the radioactivity initially added to the cultures in retinal endothelial cells and 40% in cerebral endothelial cells. Analyses of radioactivity distribution among lipid classes after 24 h incubation revealed that in retinal endothelial cells, 97% of total radioactivity was recovered in PL, 1.5% in FFA, and 1.5% in NL. In cerebral endothelial cells, PL were also predominantly labeled (91%), with 6.5% of radioactivity being recovered in FFA and 2.5% in NL.

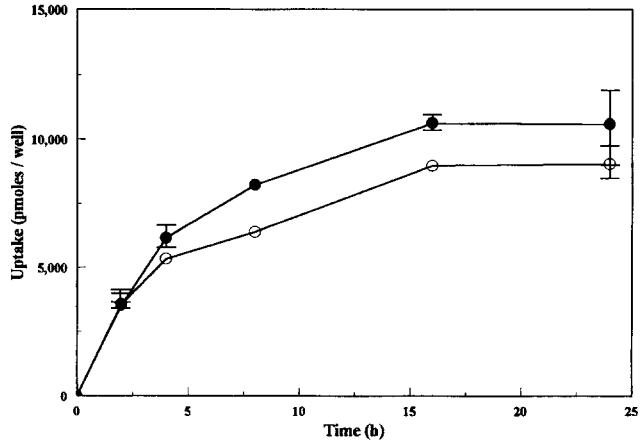


**Fig. 1.** Total uptake of 3-$^{14}$C radioactivity in retinal (●) and cerebral (○) endothelial cells. Confluent cells were incubated with 1 μCi of [3-$^{14}$C]22:5n–3 for 2 h to 24 h in 20% serum-containing medium. Total cellular lipids were extracted and 3-$^{14}$C radioactivity was counted by liquid scintillation. The data have been converted to pmol/well and are the means ±SD of triplicate cells (representative of two separate experiments).

### Retroconversion, elongation, and desaturation of [3-$^{14}$C]22:5n–3

The radiolabeled fatty acids produced by retinal and cerebral endothelial cells after 24 h incubation with [3-$^{14}$C]22:5n–3 are shown in the representative HPLC tracings illustrated in **Fig. 2** and **Fig. 3.** Labeled compounds were identified by comparing their retention times to those of authentic standards, except 24:6n–3 and 24:5n–3, which were compared to the phenacyl esters reported earlier (14). Each peak's identity was further confirmed by HPLC of the hydrogenation products.

In retinal cells, the primary product of 22:5n–3 metabolism, which includes retroconversion, elongation, and desaturation reactions, was 20:5n–3, followed by 22:6n–3, 24:5n–3, and 24:6n–3 (Fig. 2A). Interestingly, all of these metabolites were released into the medium (Fig. 2B). To determine their identity, the lipids in the medium were analyzed by TLC, which revealed that 90% of the radioactivity was associated with the free fatty acid fraction. This fraction was then analyzed by HPLC and the corresponding radioactivity tracings were similar to these obtained from media total lipids (Fig. 2C). Radioactivity was also recovered in saturated compounds such as 16:0 and 18:0, suggesting that some of the 20:5n–3 underwent β-oxidation with reutilization of the released [$^{14}$C]acetate.

After 24 h incubation, cerebral endothelial cells converted [3-$^{14}$C]22:5n–3 into 20:5n–3, 22:6n–3, 24:5n–3,

24:6n–3, and 16:0 (Fig. 3A). As found in retinal endothelial cells, these products were released into the medium as free fatty acids (Fig. 3B). Indeed, 80% of the radioactivity present in the medium was recovered in the free fatty acid fraction, and the corresponding HPLC profile resembles the HPLC profile of total medium (Fig. 3C). Interestingly, the synthesis of saturated fatty acids was less in cerebral endothelial cells compared to retinal endothelial cells.

Time course studies showed that [3-$^{14}$C]22:5n–3 accumulated in retinal endothelial cells while it decreased in the medium (**Fig. 4A**). Once taken up by cells, [3-$^{14}$C]22:5n–3 was progressively metabolized; after 24 h incubation, less than half initial radioactivity remained associated with 22:5n–3. Fig. 4B clearly shows that retinal endothelial cells perform a high level of retroconversion compared to elongation/desaturation reactions. The relative distribution of label among the different fatty acids was similar during the first 8 h incubation, but then 22:6n–3 became proportionately more labeled. The secretion of free fatty acids into the medium was detectable from the early hours of incubation (Fig. 4C). Interestingly, 20:5n–3 was relatively more retained in cells whereas 24:6n–3 and 24:5n–3 were found primarily in the medium. 22:6n–3 was recovered in similar amounts in cells and medium. The 24-C metabolites were detected in the medium as the largest released products.

In cerebral cells, the uptake and metabolism of [3-$^{14}$C]22:5n–3 also increased with time; after 24 h incubation, 50% of [3-$^{14}$C]22:5n–3 was metabolized (**Fig. 5A**). As found in retinal endothelial cells, cerebral endothelial cells perform retroconversion as a major reaction (Fig. 5B). However, the production of 22:6n–3 showed a marked relative increase after 8 h incubation; at 24 h,

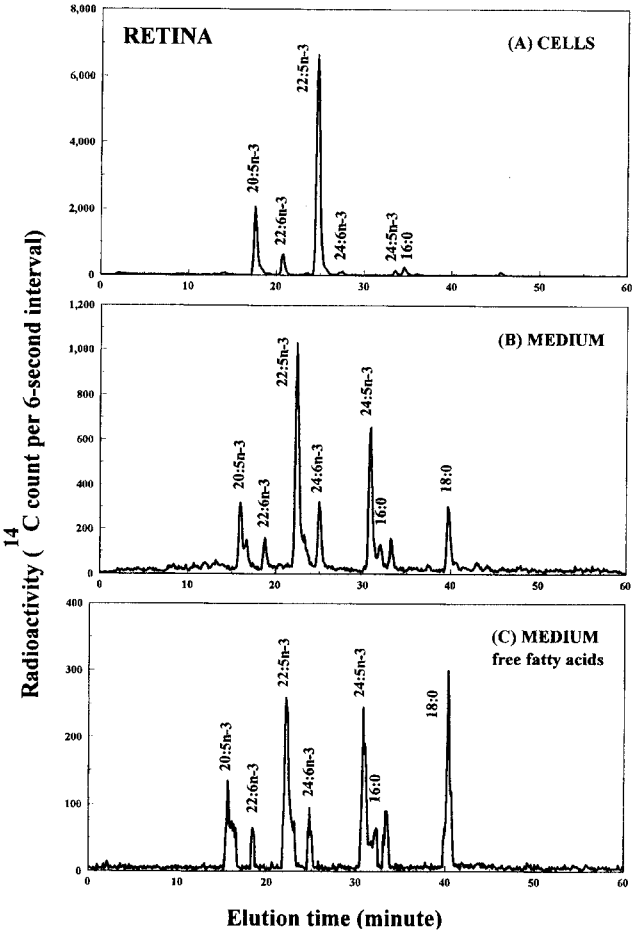


**Fig. 2.** HPLC elution profiles of radiolabeled FAPEs produced and released by retinal endothelial cells after incubation with [3-$^{14}$C]22:5n–3. Confluent retinal endothelial cells were incubated with 1 μCi of [3-$^{14}$C]22:5n–3 for 24 h in 20% serum-containing medium. Total cell lipids (A), total medium lipids (B), and free fatty acids from medium (C) were phenacylated and the resulting FAPEs were separated by reverse-phase HPLC.

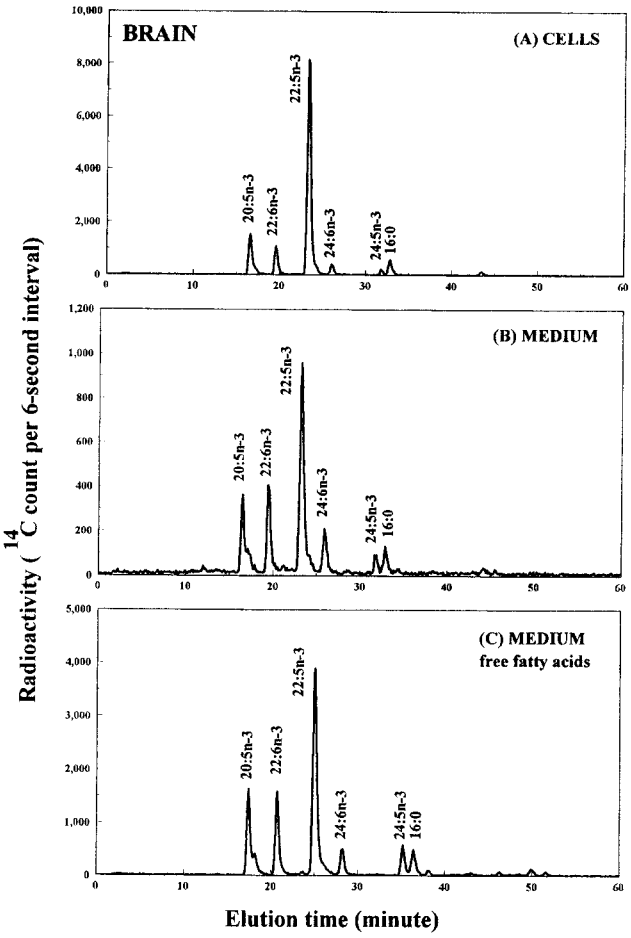


**Fig. 3.** HPLC elution profiles of radiolabeled FAPEs produced and released by cerebral endothelial cells after incubation with [3-$^{14}$C]22:5n–3. Confluent cerebral endothelial cells were incubated with 1 μCi of [3-$^{14}$C]22:5n–3 for 24 h in 20% serum-containing medium. Other details are as in Fig. 2.

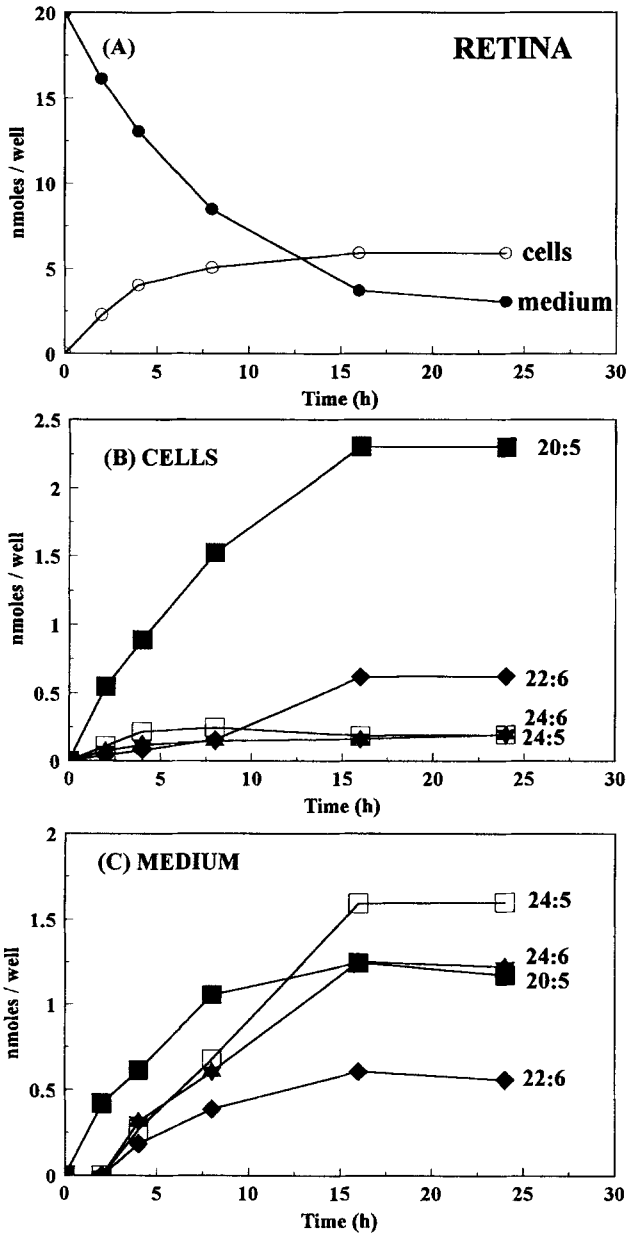


**Fig. 4.** Time dependence of [3-$^{14}$C]22:5n–3 conversion by bovine retinal endothelial cells. Confluent cells were incubated with 1 μCi of [3-$^{14}$C]22:5n–3 for 2 h to 24 h in 20% serum-containing medium. (A) distribution of [3-$^{14}$C]22:5n–3 in cells and medium; (B) metabolized products recovered in cells; (C) metabolized products released in medium. The amounts in nmoles were calculted from the percentage distribution determined by HPLC analyses and the total radioactivity in extracted cell or media lipids (representative of two separate experiments).

its total production was two times higher than in retinal cells (2776 pmol vs. 1182 pmol, accumulated amounts in cells and medium). All of the metabolized products, including 20:5n–3, were preferentially released into the medium (Fig. 5C); the largest released products were 20:5n–3 and 22:6n–3, in amounts more than twice that of 24-C metabolites.

### Effect of serum on retroconversion, elongation, and desaturation of [3-$^{14}$C]22:5n–3

We investigated the effect of serum on the uptake and metabolism of [3-$^{14}$C]22:5n–3 in cerebral and retinal endothelial cells. Cells were first cultured in 20% serum until confluent and then transferred either to the same

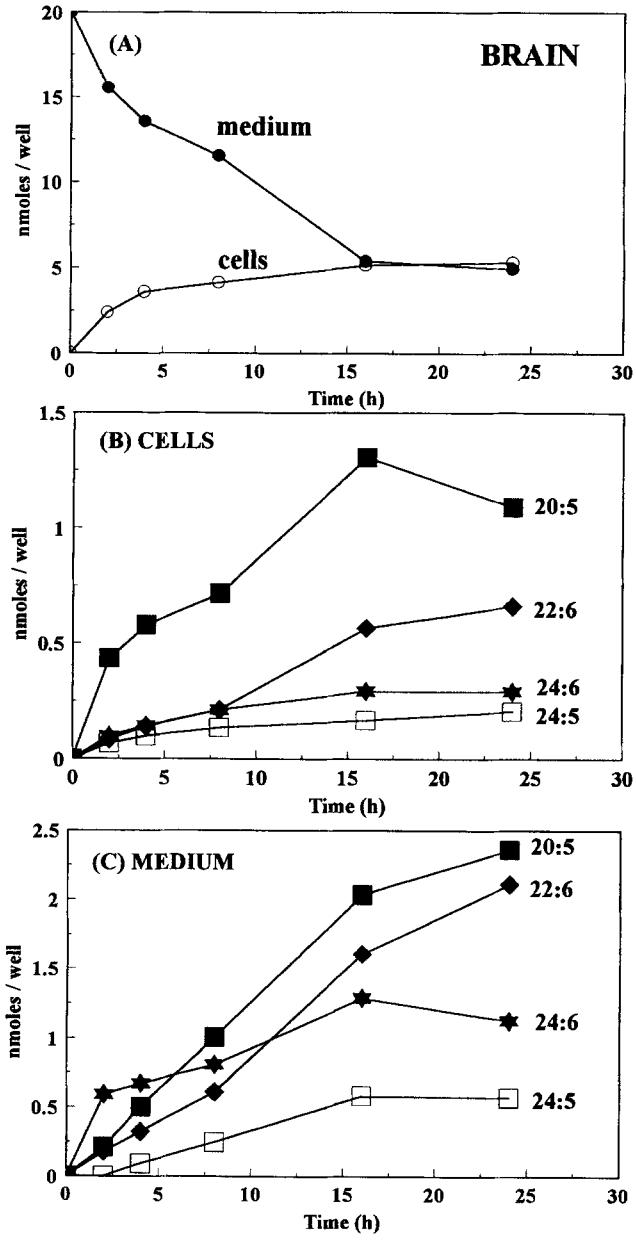


**Fig. 5.** Time dependence of [3-$^{14}$C]22:5n–3 conversion by rat cerebral endothelial cells. Confluent cerebral endothelial cells were incubated with 1 μCi of [3-$^{14}$C]22:5n–3 for 2 h to 24 h in 20% serum-containing medium. Others details are as in Fig. 4.

TABLE 1. Effect of serum of [3-$^{14}$C] 22:5n–3 conversion by bovine retinal endothelial cells

| | 20% Serum | | 20% Inactivated Serum | | No Serum | |
|---|---|---|---|---|---|---|
| | *pmoles* | % | *pmoles* | % | *pmoles* | % |
| Cells | | | | | | |
| Total | 9100 | | 8340 | | 6160 | |
| 22:5 | 5896 ± 30 | 64.8 | 4612 ± 145[a] | 55.3 | 3246 ± 31[a] | 52.7 |
| 20:5 | 2193 ± 26 | 24.1 | 2744 ± 46[a] | 32.9 | 2599 ± 37[a] | 42.2 |
| 22:6 | 309 ± 10 | 3.4 | 225 ± 14[a] | 2.7 | 31 ± 5[a] | 0.5 |
| 24:6 | 146 ± 13 | 1.6 | 104 ± 7[a] | 1.2 | 77 ± 7[a] | 1.2 |
| 24:5 | 309 ± 5 | 3.4 | 326 ± 8 | 3.9 | 123 ± 6[a] | 2.3 |
| Media | | | | | | |
| Total | 10900 | | 11660 | | 13840 | |
| 22:5 | 3750 ± 99 | 34.4 | 3440 ± 150 | 29.5 | 8913 ± 96[a] | 64.4 |
| 20:5 | 1406 ± 58 | 12.9 | 1446 ± 74 | 12.4 | 3252 ± 67[a] | 23.5 |
| 22:6 | 490 ± 14 | 4.5 | 472 ± 30 | 4.0 | 152 ± 9[a] | 1.1 |
| 24:6 | 1035 ± 30 | 9.5 | 944 ± 39 | 8.1 | 360 ± 17[a] | 2.6 |
| 24:5 | 1798 ± 45 | 16.5 | 2938 ± 81[a] | 25.2 | 1093 ± 52[a] | 7.9 |
| Ratio | | | | | | |
| 24:6/24:5 | 0.56 | | 0.32 | | 0.36 | |
| 22:6/24:5 | 0.38 | | 0.21 | | 0.15 | |
| 22:6/24:6 | 0.68 | | 0.66 | | 0.42 | |

Confluent retinal endothelial cells were incubated with [3-$^{14}$C] 22:5n–3 in media containing 20% serum, 20% inactivated serum, or no serum for 24 h. Values are expressed in pmol/well and are means ± SEM of 6 separate wells. Percent distribution of radioactivity was determined by HPLC analyses. Ratios were calculated on total amounts (cells + medium).

[a] $P \leq 0.05$ vs. 20% serum (representative of three separate experiments).

medium or medium containing 20% heat-inactivated serum or no serum for 24 h, followed by incubation with 1 μCi of [3-$^{14}$C]22:5n–3 for another 24 h.

Cell counts showed that the absence of serum affected retinal cell growth ($0.6 \times 10^6$ cells/well) compared to cultures in 20% serum ($1.2 \times 10^6$ cells/well) or 20% inactivated serum ($1.0 \times 10^6$ cells/well). Consistently, the uptake of label was decreased in retinal cells cultured without serum compared to those cultured with 20% serum or 20% inactivated serum (**Table 1**). However, the relative distribution of radioactivity among lipid classes was not changed (results not shown). In cerebral cells, the total uptake was higher when cells were incubated in absence of serum although cell growth was not affected (**Table 2**). In addition, the incorporation into PL slightly decreased (84% vs. 92%) relative to NL (9% vs. 2%) (results not shown).

The metabolism of 22:5n–3 by retinal cells was different in those maintained in 20% serum compared to those maintained in 20% inactivated serum or without serum. In the latter two conditions, there was an increase in retroconversion and a decrease in elongation/desaturation reactions (Table 1). The HPLC profiles clearly show the differences in fatty acid radioactivity patterns in cells incubated in the three media (**Fig. 6**). The absence of serum also affected the pattern of released fatty acids. After incubation in 20% inactivated serum, we found a marked increase of 24:5n–3 and a slight decrease in 24:6n–3 and 22:6n–3 (Table 1 and Fig. 6). Without serum, all elongation/desaturation products were decreased; however, there was also a relative accumulation of 24:5n–3 compared to 24:6n–3 and 22:6n–3. In addition, no radiolabeled saturated 16:0 and 18:0 were detected.

As found in retinal endothelial cells, cerebral endothelial cells incubated with 20% inactivated serum or without serum have greater retroconversion compared to those maintained in 20% serum. However, there was no difference in the elongation/desaturation patterns, as indicated by similar percentage distribution of radioactivity among 24:5n–3, 24:6n–3, and 22:6n–3 in the three serum conditions, in both cells and media (Table 2 and **Fig. 7**). In absence of serum, the cellular amounts, expressed in pmoles, of these latter fatty acids were significantly increased.

### Effect of serum on fatty acid composition

To determine whether the fatty acid composition of retinal and cerebral endothelial cells had changed during culture in different serum conditions, experiments were performed as previously described, but without incubation with labeled 22:5n–3. The fatty acid composition of total cellular lipids was determined by GLC.

Compared to retinal cells incubated in 20% serum, those incubated in 20% inactivated serum or without serum showed a decrease in their total n–6 and n–3 fatty acid levels, compensated by an increase in their total n–9/n–7 fatty acid levels (**Table 3**). In particular, with inactivated serum, there was a 25% decrease in 20:4n–6 while the level of 18:2n–6 was not significantly af-

TABLE 2. Effect of serum of [3-$^{14}$C] 22:5n–3 conversion by rat cerebral endothelial cells

| | 20% Serum | | 20% Inactivated Serum | | No Serum | |
|---|---|---|---|---|---|---|
| | *pmoles* | % | *pmoles* | % | *pmoles* | % |
| Cells | | | | | | |
| Total | 5800 | | 6600 | | 8400 | |
| 22:5 | 4007 ± 37 | 69.1 | 4323 ± 99[a] | 65.5 | 3814 ± 80 | 45.4 |
| 20:5 | 487 ± 29 | 8.4 | 772 ± 27[a] | 11.7 | 2218 ± 160[a] | 26.4 |
| 22:6 | 382 ± 13 | 6.6 | 416 ± 14 | 6.3 | 605 ± 17[a] | 7.2 |
| 24:6 | 197 ± 21 | 3.4 | 238 ± 8 | 3.6 | 260 ± 7[a] | 3.1 |
| 24:5 | 186 ± 14 | 3.2 | 244 ± 17[a] | 3.7 | 370 ± 72[a] | 4.4 |
| Media | | | | | | |
| Total | 14200 | | 13400 | | 11600 | |
| 22:5 | 7881 ± 107 | 55.5 | 6325 ± 160[a] | 47.2 | 4257 ± 147[a] | 36.7 |
| 20:5 | 2300 ± 55 | 16.2 | 2841 ± 65[a] | 21.2 | 2796 ± 208[a] | 24.1 |
| 22:6 | 1747 ± 79 | 12.3 | 1675 ± 54 | 12.5 | 1357 ± 168 | 11.7 |
| 24:6 | 795 ± 40 | 5.6 | 804 ± 64 | 6 | 708 ± 23 | 6.1 |
| 24:5 | 667 ± 35 | 4.7 | 710 ± 49 | 5.3 | 650 ± 60 | 5.6 |
| Ratio | | | | | | |
| 24:6/24:5 | 1.16 | | 1.09 | | 0.95 | |
| 22:6/24:5 | 2.49 | | 2.19 | | 1.92 | |
| 22:6/24:6 | 2.15 | | 2.01 | | 2.03 | |

Confluent cerebral endothelial cells were incubated with [3-$^{14}$C[22:5n–3 in media containing 20% serum, 20% inactivated serum, or no serum for 24 h. Values are expressed in pmol/well and are means ± SEM of 6 separate wells. Percent distribution of radioactivity was determined by HPLC analyses. Ratios were calculated on total amounts (cells + medium).

[a] $P \leq 0.05$ vs. 20% serum (representative of three separate experiments).

fected. Without serum, the decrease in 20:4n–6 was twice higher (−50%) and 18:2n–6 was also significantly diminished (−25%). In both of the latter conditions, all of the fatty acids derived from elongation and desaturation of 18:3n–3, including 22:6n–3, were found in significantly lower proportions, the decreases being more pronounced without serum.

In contrast to what was observed with retinal endothelial cells, the fatty acid composition of cerebral endothelial cells did not change when raised in 20% inactivated serum compared to 20% serum (**Table 4**). Without serum, there was a significant decrease in n–6 fatty acids, in particular 18:2n–6 and 20:4n–6, to an extent similar to that found in retinal cells (−25% and −40% respectively), compensated by an increase in n–9/n–7 fatty acids, but no significant change in n–3 fatty acids was found.

## DISCUSSION

The present work was initiated to determine the ability of retina and cerebral endothelial cells to produce 22:6n–3. In standard culture conditions (20% FBS), fatty acid compositions of both types of endothelial cells were comparable. Saturated fatty acids were the most abundant, accounting for about 40% of total fatty acids. Polyunsaturated fatty acids were present in the form of long chain fatty acids, with 20:4n–6 and 22:6n–3 exceeding their respective fatty acid precursors. As previously reported, the proportion of 22:6n–3 dropped in both retinal and cerebral endothelial cell cultures compared to the initial amounts in microvessels, where 22:6n–3 is found at the same extent as 20:4n–6 (18, 19). The reason for this decrease in 22:6n–3 content is probably the same as that proposed by others, i.e., the relative paucity of n–3 compared to n–6 fatty acids in serum (data not shown).

Our labeling studies with [3-$^{14}$C]22:5n–3 as a precursor of 22:6n–3 show that both retinal and cerebral microvascular endothelial cells readily take up 22:5n–3 when available in the extracellular fluid and metabolize it in various manners including retroconversion, elongation and desaturation, and esterification into cellular lipids. The label uptake, the distribution of labeling among lipid classes, and the conversion pattern of 22:5n–3 do not significantly differ between the two cell types. Most important are the findings that both retinal and cerebral endothelial cells can synthesize 22:6n–3, that this synthesis appears to be independent of a δ4-desaturase, and that the formed 22:6n–3 and its 24–C precursors are released into the medium. The total production of 22:6n–3 was higher in cerebral cells than in retinal cells, accounting, respectively, for 28% and 11% of total metabolized products.

In contrast to our present results, Moore et al. (20, 28) have previously reported the inability of cerebral endothelium to substantially form 22:6n–3. This difference may be explained by the use of 18:3n–3 as precursor in their study while we used 22:5n–3, as 22:5n–3

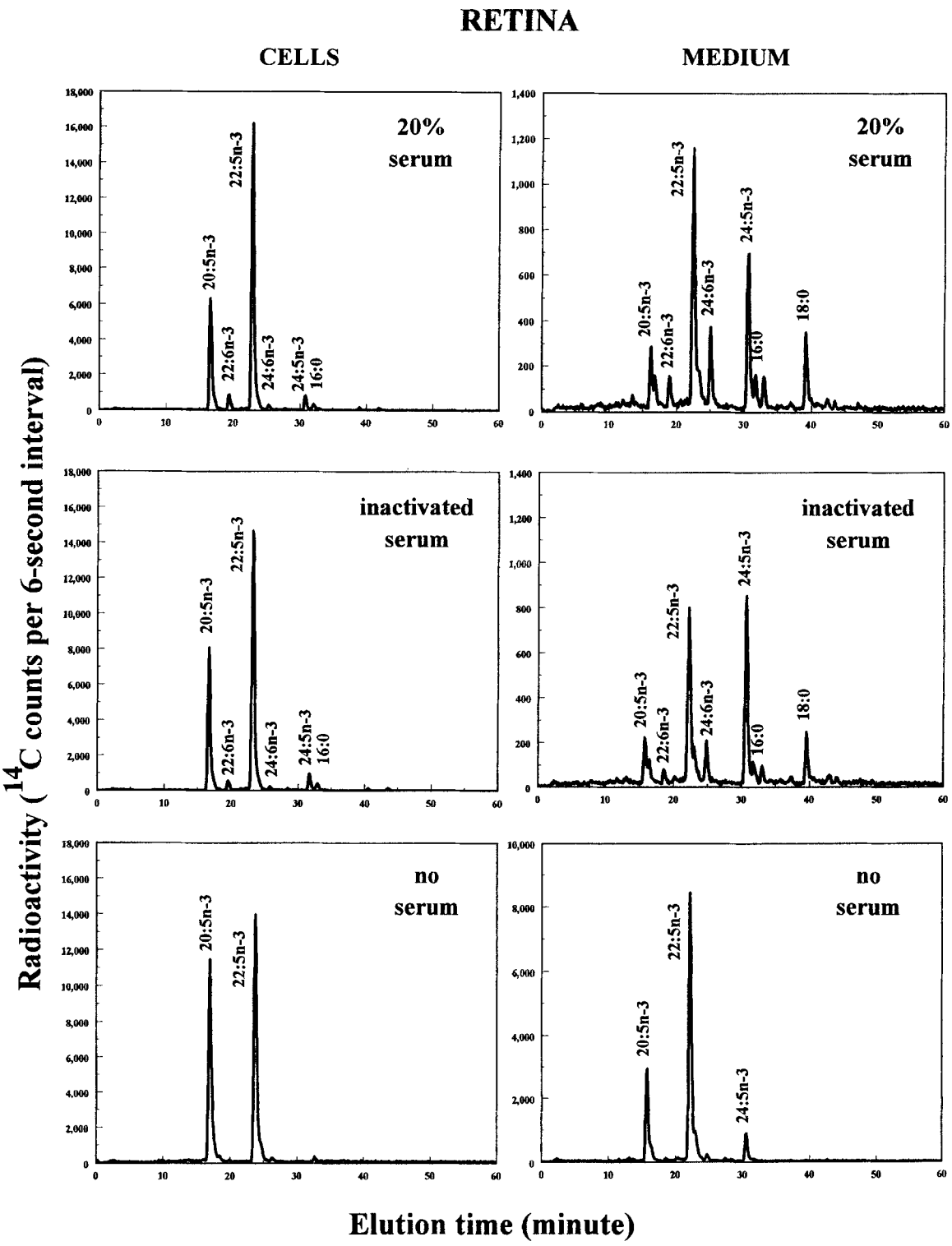


**Fig. 6.** HPLC elution profiles of radiolabeled FAPEs produced and released by retinal endothelial cells after incubation with [3-$^{14}$C]22:5n–3 in different serum conditions. Confluent retinal endothelial cells were incubated with 1 μCi of [3-$^{14}$C]22:5n–3 in medium containing 20% serum, 20% inactivated serum or no serum, for 24 h. Others details are as in Fig. 2 (representative of three separate experiments).

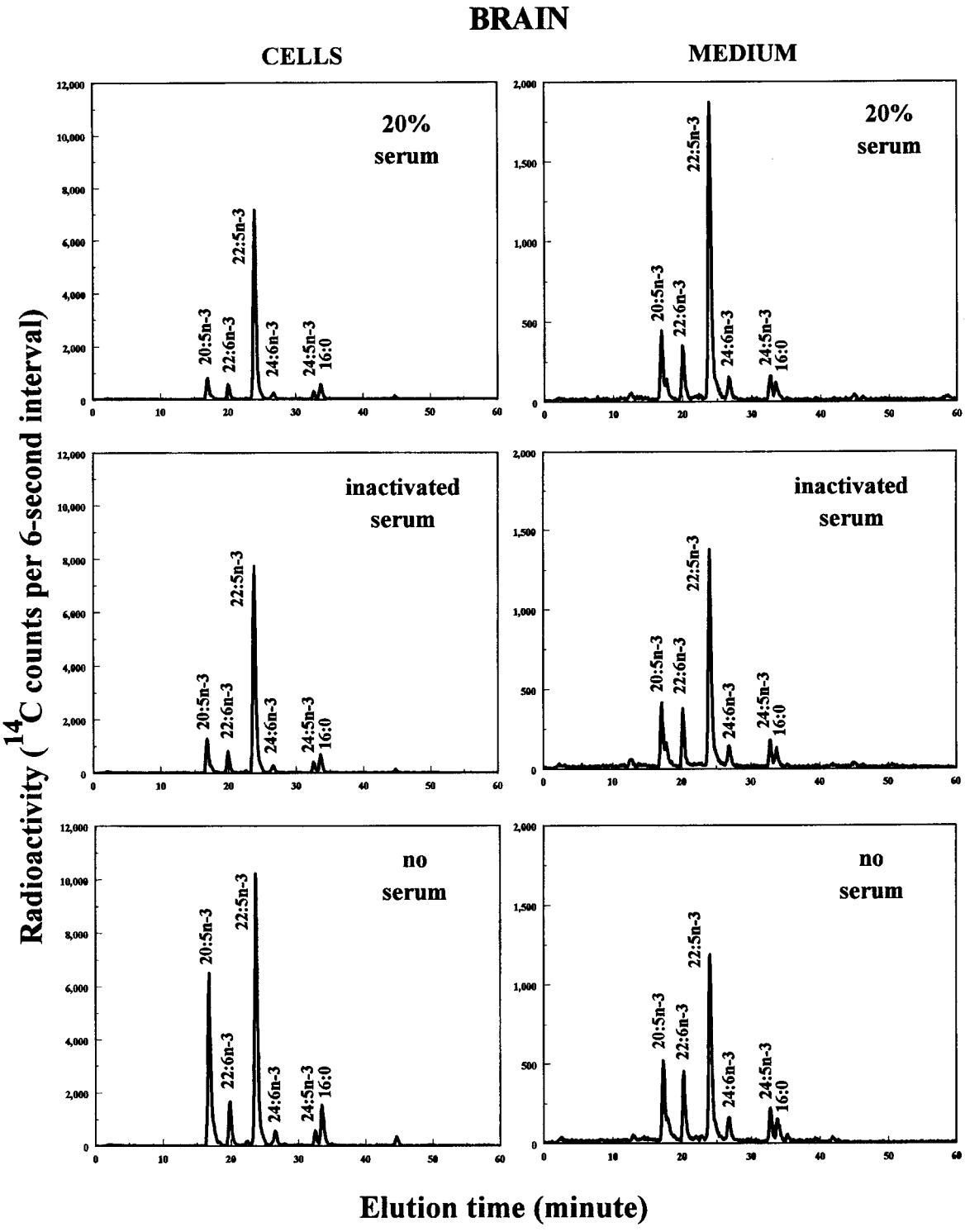


**Fig. 7.** HPLC elution profiles of radiolabeled FAPEs produced and released by cerebral endothelial cells after incubation with [3-$^{14}$C]22:5n–3 in different serum conditions. Confluent cerebral endothelial cells were incubated with 1 μCi of [3-$^{14}$C]22:5n–3 in medium containing 20% serum, 20% inactivated serum, or no serum for 24 h. Others details are as in Fig. 2 (representative of three separate experiments).

TABLE 3. Effect of serum on total lipid fatty acid composition of bovine retinal endothelial cells

| Fatty Acids | 20% Serum | 20% Inactivated Serum | No Serum |
|---|---|---|---|
| | *mol %* | *mol %* | *mol %* |
| Saturate | | | |
| 16:0 | 17.70 ± 0.26 | 18.83 ± 0.15 | 19.33 ± 0.67 |
| 18:0 | 21.77 ± 0.42 | 21.00 ± 0.56 | 22.80 ± 2.05 |
| Total | 42.47 ± 0.71 | 43.17 ± 0.40 | 45.40 ± 2.85 |
| Monoenoic | | | |
| 16:1 (n–9 + n–7) | 3.10 ± 0.10 | 3.77 ± 0.15[b] | 4.77 ± 0.21[c] |
| 18:1 (n–9 + n–7) | 19.30 ± 0.25 | 23.70 ± 0.22[c] | 27.27 ± 1.06[c] |
| 20:1n–9 | 0.77 ± 0.06 | 0.93 ± 0.06[a] | 0.90 ± 0.07[a] |
| 22:1n–9 | 0.50 ± 0.10 | 0.60 ± 0.00 | 0.83 ± 0.12[a] |
| 24:1n–9 | 1.20 ± 0.10 | 1.43 ± 0.06[a] | 1.50 ± 0.00[b] |
| Total | 24.87 ± 0.15 | 30.43 ± 0.31[c] | 35.27 ± 1.36[c] |
| n–6 | | | |
| 18:2n–6 | 4.07 ± 0.20 | 3.70 ± 0.17 | 3.00 ± 0.26[b] |
| 20:3n–6 | 1.80 ± 0.00 | 1.67 ± 0.06[a] | 1.50 ± 0.00[c] |
| 20:4n–6 | 18.53 ± 0.59 | 13.57 ± 0.35[c] | 9.13 ± 1.00[c] |
| 22:4n–6 | 1.87 ± 0.06 | 1.77 ± 0.06[a] | 1.53 ± 0.12[a] |
| 22:5n–6 | 0.53 ± 0.06 | 0.57 ± 0.06 | 0.63 ± 0.06 |
| Total | 26.97 ± 0.64 | 21.30 ± 0.62[c] | 16.13 ± 1.34[c] |
| n–3 | | | |
| 18:3n–3 | ND | ND | ND |
| 20:5n–3 | 0.50 ± 0.00 | 0.33 ± 0.06[b] | ND |
| 22:5n–3 | 2.10 ± 0.10 | 1.73 ± 0.06[b] | 1.40 ± 0.20[b] |
| 22:6n–3 | 2.90 ± 0.10 | 2.47 ± 0.15[a] | 1.80 ± 0.35[b] |
| Total | 5.50 ± 0.15 | 4.53 ± 0.12[c] | 3.20 ± 0.49[c] |

Confluent retinal endothelial cells were kept for 48 h in media containing 20% serum, 20% inactivated serum, or no serum. Fatty acid composition of total lipids was determined by GLC analyses. The values are the means ± SD of 3 separate wells. The totals indicated can be higher than the sum of total indicated fatty acids as minor fatty acids are not included in the table. ND, not detected.

[a] $P \leq 0.05$; [b] $P \leq 0.01$; [c] $P \leq 0.001$ vs. 20% serum (representative of two separate experiments).

provides a shorter route to 22:6n–3 than 18:3n–3. Indeed, in our experiments using 18:3n–3 as precursor (data not shown), the formation of 22:6n–3 was barely detectable, representing, at most, 2% of total radioactivity in our HPLC tracings. Moreover, using 18:3n–3 as precursor, we did not detect labeled 22:6n–3 in the medium, consistent with a very low cellular synthesis. Curiously, however, these authors also found no or only minimal formation of 22:6n–3 when using 20:5n–3 as precursor, although 22:5n–3 was largely formed. It is possible that some parameters in the experimental conditions may account for our dissimilar results, such as serum concentration or the species of cerebral microvessels (rat versus mouse).

The labeling of 24:5n–3 and 24:6n–3 in both retinal and cerebral endothelial cells strongly suggests the existence of the δ4-desaturase independent pathway for the synthesis of 22:6n–3, which consists of the sequential chain elongation of 22:5n–3 to 24:5n–3, followed by δ6-desaturation of 24:5n–3 to produce 24:6n–3, which is finally chain-shortened to 22:6n–3. Since its first demonstration in rat liver by Voss et al. (29), this alternate pathway has been observed in rat retinal pigment epithelium (14), rat astrocytes (28), murine cerebral endothelium (28), and more recently in human skin fibroblasts (30); the latter study also showed that the retroconversion of 24:6n–3 to 22:6n–3 is a peroxisomal-dependent process. Our time-course studies showed that at a certain point, the cellular amount of 22:6n–3 continues to increase relative to its 24-C precursors, consistent with the latter two being intermediates and 22:6n–3 the end product of fatty acid anabolism.

In addition to elongation and desaturation reactions, retroconversion reactions are actively performed by retinal and cerebral cells. In fact, retroconversion of 22:5n–3 to 20:5n–3 is the primary process, even though the endogenous 20:5n–3 content is lower than that of 22:6n–3 (0.5% and 3%, respectively) in both retinal and cerebral endothelial cells. This high production of 20:5n–3 may lead to the subsequent formation of eicosanoids as previously described (20).

The ability of several types of endothelial cells to release long-chain polyunsaturated fatty acids into the culture medium has been well documented (20, 31, 32). In the present study, we report that secretion is actually a major process for newly formed fatty acids. Moore (33) has previously proposed a pathway in which cerebral endothelium would form and release 20:5n–3, which would be then converted by astrocytes to 22:6n–3, which was released for uptake by neurons. Accordingly, we found that cerebral cells released primarily 20:5n–3, but the fact that they secrete 22:6n–3 in high pro-

TABLE 4. Effect of serum on total lipid fatty acid composition of rat cerebral endothelial cells

| Fatty Acids | 20% Serum | 20% Inactivated Serum | No Serum |
|---|---|---|---|
| | *mol %* | *mol %* | *mol %* |
| Saturate | | | |
| 16:0 | 25.03 ± 0.21 | 24.03 ± 0.21 | 23.17 ± 1.59 |
| 18:0 | 13.93 ± 0.91 | 14.60 ± 0.62 | 13.10 ± 0.26 |
| Total | 40.70 ± 0.82 | 40.43 ± 0.72 | 38.00 ± 1.78 |
| Monoenoic | | | |
| 16:1(n–9 + n–7) | 2.63 ± 0.55 | 2.77 ± 0.25 | 7.27 ± 2.07[a] |
| 18:1(n–9 + n–7) | 18.00 + 1.41 | 18.40 ± 0.63 | 28.60 ± 6.00[a] |
| 20:1n–9 | 0.57 ± 0.06 | 0.57 ± 0.06 | 0.73 ± 0.25 |
| 22:1n–9 | 0.47 ± 0.06 | 0.43 ± 0.06 | 0.50 ± 0.00 |
| 24:1n–9 | 2.10 ± 0.36 | 2.07 ± 0.21 | 1.87 ± 0.15 |
| Total | 23.77 ± 1.61 | 24.24 ± 0.60 | 38.97 ± 7.56[a] |
| n–6 | | | |
| 18:2n–6 | 2.50 ± 0.10 | 2.50 ± 0.00 | 1.87 ± 0.29[a] |
| 20:3n–6 | 2.43 ± 0.21 | 2.43 ± 0.06 | 1.67 ± 0.55 |
| 20:4n–6 | 23.60 ± 1.08 | 23.27 ± 0.32 | 13.80 ± 4.39[a] |
| 22:4n–6 | 2.00 ± 0.17 | 2.00 ± 0.10 | 1.80 ± 0.10 |
| 22:5n–6 | 0.80 ± 0.10 | 0.70 ± 0.00 | 0.70 ± 0.20 |
| Total | 31.37 ± 1.36 | 30.90 ± 0.36 | 19.80 ± 5.44[a] |
| n–3 | | | |
| 18:3n–3 | ND | ND | ND |
| 20:5n–3 | 0.20 ± 0.17 | 0.27 ± 0.06 | 0.37 ± 0.21 |
| 22:5n–3 | 1.20 ± 0.10 | 1.27 ± 0.06 | 0.97 ± 0.12 |
| 22:6n–3 | 2.77 ± 0.15 | 3.00 ± 0.10 | 1.97 ± 0.64 |
| Total | 4.17 ± 0.40 | 4.54 ± 0.12 | 3.31 ± 0.96 |

Confluent cerebral endothelial cells were kept for 48 h in media containing 20% serum, 20% inactivated serum, or no serum. Fatty acid composition of total lipids was determined by GLC analyses. The values are the means ± SD of 3 separate wells. The totals indicated can be higher than the sum of total indicated fatty acids as minor fatty acids are not included in the table. ND, not detected.

[a] $P \leq 0.05$ vs. 20% serum (representative of two separate experiments).

portion, and 24-C fatty acids to a lesser extent, suggests that they also could play a role in supplying 22:6n–3 to neurons. Based on such a model, retinal endothelium could particulate in supplying 22:6n–3 to rod other segments. Retinal pigment epithelium could cooperate in this process (14).

That serum can influence the fatty acid composition and the desaturase activities of cultured cells has been previously reported (34). In most cases, supplementing the medium with a higher concentration of serum resulted in a decrease of desaturase activities, one explanation being that serum lipids provide sufficient polyunsaturated fatty acids and especially 20:4n–6 to at least partially inhibit endogenous synthesis (35–37). However, in neuroblastoma cells, 20:4n–6 was actually stimulatory with respect to the desaturation of [$^{14}$C]18:2n–6 (38) and Moore et al. (20) have reported a positive correlation between the desaturase activities and the serum concentration in murine cerebral endothelial cells. In the present study, we clearly show that the metabolic conversion of 22:5n–3 by retinal and cerebral endothelial cells varies depending on the serum condition. In the absence of serum, levels of retroconversion were higher for both cells, especially for retinal cells where 20:5n–3 accounted for 75% of total metabolized products compared to 35% when serum was present. Under this condition, we also interestingly found that the production of 22:6n–3 was markedly depressed in retinal cells but not in cerebral cells. Although the absence of serum did affect retinal cell growth, the cells remained metabolically active as evidenced by their high level of retroconversion. Our data suggest that the δ6-desaturase converting 24:5n–3 to 24:6n–3 in retinal endothelial cells may be stimulated by fetal bovine serum. Thus, in our labeling experiments, we found that the total production of labeled 24:5n–3 increased relative to 24:6n–3 and 22:6n–3 when cells were raised in 20% inactivated serum or without serum compared to normal serum. Consequently, the 24:6n–3/24:5n–3 ratio, an index of δ6-desaturase activity, dropped by about 40% in these two latter conditions (Table 1). Moreover, analyses of the fatty acid composition of cellular lipids revealed that the 20:4n–6/18:2n–6 ratio, an index of total δ6- and δ5-desaturase activities, dropped by 20% in cells incubated in 20% inactivated serum and by 35% in those incubated without serum (Table 3). In the latter condition, the large decrease in 20:4n–6 could primarily result from a lower availability of both 20:4n–6 and its precursor 18:2n–6 in the culture medium, as these fatty acids are found in high proportion in FBS (11% and 7%, respectively, data not shown). That would also explain the decrease in 20:4n–6 observed in cerebral

cells maintained without serum. However, this cannot account for the changes observed in retinal cells maintained in 20% inactivated serum, as the serum fatty acid composition was not affected by heat inactivation (data not shown). Another possibility would consider the eventual change in fatty acid binding to serum proteins after heat inactivation, assuming that some of these proteins could be denatured, together with the recent demonstration that mode of transport of fatty acids to endothelial cells can influence subsequent cellular uptake and metabolism (39, 40). However, this possibility is most unlikely as the decrease in content of long-chain n–3 and n–6 fatty acids after using inactivated serum is only observed in retinal endothelial cells, indicating a specific mechanism. As a possible explanation, we suggest an effect of some serum components such as hormones and growth factors that are known to regulate the desaturase activities (41).

In conclusion, the data presented in this study demonstrate that both retinal and cerebral endothelial cells can synthesize and secrete 22:6n–3 and its 24–C precursors. This is of particular interest for the supply of 22:6n–3 to the brain and the retina. The finding that the regulation of this metabolism by serum is different in these two cells may be relevant for diabetic microangiopathy that occurs in the retinal but not the cerebral microvasculature.

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