Docosahexaenoic acid synthesis from n-3 polyunsaturated fatty acids in differentiated rat brain astrocytes

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Abstract **DHA, the main n-3 PUFA in the brain, is synthesized from n-3 PUFA precursors by astrocytes. To assess the potential of this process to supply DHA for the brain, we investigated whether the synthesis in astrocytes is dependent on DHA availability. Rat brain astrocytes differentiated with dibutyryl cAMP and incubated in media containing 10% fetal bovine serum synthesized DHA from α-linolenic acid ([1-^{14}C]18:3n-3), docosapentaenoic acid ([3-^{14}C]22:5n-3), tetracosapentaenoic acid ([3-^{14}C]24:5n-3), and tetracosahexaenoic acid ([3-^{14}C]24:6n-3). When DHA was added to media containing a 5 μM concentration of these ^{14}C-labeled n-3 PUFA, radiolabeled DHA synthesis was reduced but not completely suppressed even when the DHA concentration was increased to 15 μM. Radiolabeled DHA synthesis also was reduced but not completely suppressed when the astrocytes were treated with 30 μM DHA for 24 h before incubation with 5 μM [1-^{14}C]18:3n-3. These findings indicate that although the DHA synthesis in astrocytes is dependent on DHA availability, some synthesis continues even when the cells have access to substantial amounts of DHA. This suggests that DHA synthesis from n-3 PUFA precursors is a constitutive process in the brain and, therefore, is likely to have an essential function.**—Williard, D. E., S. D. Harmon, T. L. Kaduce, M. Preuss, S. A. Moore, M. E. C. Robbins, and A. A. Spector. **Docosahexaenoic acid synthesis from n-3 polyunsaturated fatty acids in differentiated rat brain astrocytes.** ***J. Lipid Res.*** **2001.** 42: **1368–1376.**

Supplementary key words α-linolenic acid • docosapentaenoic acid • tetracosapentaenoic acid • tetracosahexaenoic acid • cAMP • elongation • desaturation • retroconversion

DHA (22:6n-3), the most abundant n-3 PUFA in the brain, is required by the central nervous system for normal development and function (1–4). DHA is a major component of neural membrane phospholipids and provides an optimum lipid microenvironment for interaction with membrane proteins involved in signal transduction (5–7). Furthermore, DHA is an endogenous ligand for the retinoid X receptor and thereby may have a key role in regulating gene expression in the brain (8). Thus, it is important to understand how the brain obtains DHA and the factors that regulate this process.

Because DHA is an essential fatty acid, it must be either provided to the brain preformed or synthesized in the brain from other n-3 PUFA. Small amounts of DHA and several of its n-3 PUFA precursors normally are present in the plasma (9, 10), and there is evidence that the brain can utilize all of these substrates. A number of studies of experimental animals have shown that plasma DHA, either obtained directly from the diet or synthesized in the liver from n-3 PUFA precursors, is the main source of DHA for the brain (11–16). This is consistent with the observation that fatty acid Δ^6-desaturase, the rate-limiting enzyme in the conversion of n-3 PUFA precursors to DHA (17), decreases in the brain soon after birth (18). However, other studies show that the brain can synthesize DHA from α-linolenic (18:3n-3) and docosapentaenoic acid (22:5n-3) (19–21). The conversion of 18:3n-3 to DHA is increased during essential fatty acid deficiency (22), suggesting that the amount of DHA synthesis from n-3 PUFA precursors in the brain may be regulated by the availability of DHA or other PUFA in the brain tissue or cerebral circulation.

Data obtained with cultured brain cells indicate that DHA synthesis from n-3 PUFA precursors takes place primarily in astrocytes and that these cells supply some of the newly formed DHA to the neurons and blood-brain barrier endothelium (23–26). The purpose of the present study was to gain a more complete understanding of the metabolic conditions that may affect the ability of astrocytes to produce DHA for the brain. Our findings indicate that rat brain astrocytes differentiated in culture with dibutyryl cAMP (27, 28), synthesize DHA from 18-, 22-, and 24-carbon n-3 PUFA. Although synthesis from these substrates decreased when DHA was available in the medium, it was not completely suppressed even when the cells were previ-

Abbreviations: 16:0, palmitic acid; 18:0, stearic acid; 18:3n-3, α-linolenic acid; 20:5n-3, eicosapentaenoic acid; 22:5n-3, docosapentaenoic acid; 22:6n-3 or DHA, docosahexaenoic acid; 24:5n-3, tetracosapentaenoic acid; 24:6n-3, tetracosahexaenoic acid; GFAP, glial fibrillary acidic protein; GLC, gas-liquid chromatography.

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ously loaded with DHA or when they had access to relatively large amounts of DHA during the incubation with these n-3 PUFA. This suggests that DHA synthesis from n-3 PUFA precursors is a constitutive process in differentiated astrocytes and that these glial cells provide at least some DHA for the brain under most metabolic conditions.

MATERIALS AND METHODS

Materials

Streptomycin, penicillin, Eagle's MEM, and L-glutamine were obtained from Life Technologies (Grand Island, NY). Dextrose was obtained from Fisher Scientific (Fair Lawn, NJ), and FBS was obtained from HyClone (Logan, UT). Monoclonal anti-glial fibrillary acidic protein (GFAP) clone G-A-5, DNase, trypsin, trypsin inhibitor, dibutyryl cAMP, and fatty acid-free bovine serum albumin were purchased from Sigma (St. Louis, MO). Hoechst 33,342 was obtained from Molecular Probes (Eugene, OR), and DiI-Ac-LDL (acetylated LDL labeled with 1,1′-dioctadecyl-3,3,3′,3′-tetramethyl-indocarbocyanine perchlorate) was purchased from Biomedical Technologies (Stoughton, MA). α-[1-^{14}C]linolenic acid ([1-^{14}C]18:3n-3) (55 mCi/mmol) was supplied by American Radiolabeled Chemicals (St. Louis, MO), and nonradiolabeled 18:3n-3 was obtained from Cayman (Ann Arbor, MI). [3-^{14}C]docosapentaenoic acid ([3-^{14}C]22:5n-3), [3-^{14}C]tetracosapentaenoic acid ([3-^{14}C]24:5n-3), and [3-^{14}C]tetracosahexaenoic acid ([3-^{14}C]24:6n-3) (50 mCi/mmol), and the analogous fatty acids, were generously provided by H. Sprecher (Department of Medical Biochemistry, Ohio State University, Columbus, OH).

Cell culture

Primary cultures of normal cerebral astrocytes were isolated from 2-day-old Sprague-Dawley rat pups obtained from Harlan (Indianapolis, IN) according to a protocol approved by the University of Iowa (Iowa City, IA) Animal Care and Use Committee. The leptomeninges were dissected from the forebrain gray matter, and the finely divided tissue was disaggregated with trypsin solution (2,725 units/ml) followed by triturating in a DNase solution (109 units/ml) (29, 30). Intact cells were separated from cell debris by centrifugation at 1,000 rpm for 7 min in a 6 mM albumin solution. Astrocytes were seeded in either 10-cm^2 wells or 75-cm^2 vented flasks at 1.0×10^4 cells/cm^2 and grown in an atmosphere containing 5% CO_2 at 37°C with Eagle's MEM containing 10% FBS, 33 mM dextrose, 2 mM glutamine, penicillin (100 units/ml), and streptomycin (100 μg/ml) (23). Any microglial cells were removed by agitating the cell cultures before feeding and experimentation. At least three separate preparations were assayed for GFAP immunofluorescence and Hoechst 33,342 nuclear counterstain. Contaminating microglial cells were detected by the uptake of DiI-Ac-LDL (200 μg/ml) during incubation in growth medium. These assays indicated that the cultures routinely contained at least 95% astrocytes. The protein content of the cultures was measured by the Bradford method (Bio-Rad, Hercules, CA) according to the manufacturer instructions.

When 95% confluent, the cultures were incubated for 48 h in growth medium containing 1 mM dibutyryl cAMP. This changed the astrocytes from a flat, polygonal morphology to a stellate appearance with extensive process formation (27, 28). As seen in **Fig. 1**, the differentiated cells stained strongly positive for GFAP. The differentiated morphology was maintained throughout the subsequent period of incubation with fatty acids.

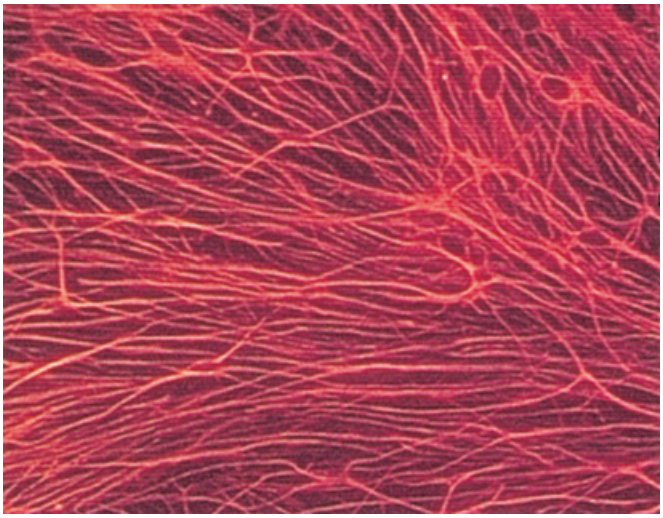

Fig. 1. GFAP immunofluorescence of morphologically differentiated astrocytes. Primary rat brain astrocyte cultures that were 95% confluent were incubated for 48 h in Eagle's MEM containing 10% FBS and 1 mM dibutyryl cAMP. GFAP expression was assessed by immunofluorescence after a 30-min incubation with monoclonal anti-GFAP-Cy3 conjugate (Sigma, St. Louis, MO). The circumference of the cell body and the processes stain intensely for GFAP. A representative culture is shown, but similar morphology, process formation, and GFAP staining were observed repeatedly in the differentiated cultures and were maintained throughout the subsequent incubation with fatty acids.

Incubation and analysis

After the medium containing dibutyryl cAMP was removed, the cultures were washed with warm Eagle's MEM, and the incubations were continued for an additional 48 h in Eagle's MEM containing 10% FBS and a mixture of a radiolabeled and corresponding unlabeled fatty acid (60 dpm/pmol). Control incubations in a cell-free medium demonstrated that there was no breakdown of the radiolabeled fatty acid substrate during the 48-h incubation period.

After incubation, the medium was removed and centrifuged at 3,000 rpm for 10 min to sediment any cell debris. The supernatant solution was acidified to pH 4 with formic acid and extracted three times with 4 volumes of water-saturated ethyl acetate. The combined ethyl acetate extracts were dried under N_2 and the lipids were resuspended in acetonitrile (31). The cell monolayer was washed with warm Dulbecco's phosphate-buffered saline solution containing 0.1 μM albumin, followed by the ice-cold buffer solution alone. The washed cells were scraped into 2 ml of ice-cold methanol containing 1% acetic acid. To extract the cell lipids, 4 ml of chloroform and 1.5 ml of acidified buffer solution were added, and the chloroform phase was isolated. The lipids remaining in the upper phase were extracted a second time with a mixture of chloroform, methanol, and acidified buffer solution (86:14:1, v/v/v), and the combined extracted lipids were dried under a stream of N_2 (31). The lipid residue was resuspended in a 2:1 mixture of chloroform and methanol.

Aliquots of the cell and medium lipid extracts were dissolved in scintillation solution and assayed for radioactivity in a liquid scintillation spectrometer (32). Quenching was monitored with a ^{226}Ra external standard. Additional aliquots of the lipid extracts were dried under N_2 and transesterified at 95°C in acetonitrile with 12% BF_3 in methanol (33). The fatty acid methyl esters were extracted into *n*-heptane and dried under a stream of N_2.

Chromatography

Separation of the radioactive fatty acid methyl esters was achieved by reversed-phase HPLC with an Alltech (Deerfield, IL) 3 μm Adsorbosphere 4.6 × 150 mm reversed-phase C_{18} column and a Gilson (Middleton, WI) dual pump gradient HPLC system equipped with an automatic sample injector (34, 35). The solvent system consisted of acetonitrile and water adjusted to pH 3.4 with phosphoric acid (35). For the samples derived from the [1-^{14}C]18:3n-3 incubations, the gradient began at 76% acetonitrile and was maintained for 45 min, was increased to 100% acetonitrile over 1 min, and then was maintained at 100% acetonitrile for 19 min (gradient I). For all of the other radioisotopes, the gradient was modified to increase stepwise from 76% to 86% acetonitrile over 10 min, was maintained at 86% acetonitrile for 25 min, and then was increased to 100% acetonitrile over 2 min and maintained for 18 min (gradient II). The radioactivity was measured by mixing the effluent with liquid scintillation solution and passing the mixture through an in-line flow scintillation detector. Radiolabeled fatty acid methyl ester standards [palmitic acid (16:0), stearic acid (18:0), 18:3n-3, eicosapentaenoic acid (20:5n-3), 22:5n-3, DHA (22:6n-3), 24:5n-3, and 24:6n-3] were included with each set of chromatograms.

Gas-liquid chromatography (GLC) was used to determine the fatty acid composition of the astrocyte lipids (36). Margaric acid was added to each sample as an internal standard. After extraction and transesterification of the lipids, the fatty acid methyl esters were separated with a Hewlett-Packard (Palo Alto, CA) model 5890-II gas chromatograph with a 1.9 m × 2 mm glass column containing 10% SP2330 on 100–120 mesh Chromsorb WAW (Supelco, Bellefonte, PA). The carrier gas was N_2 at a flow rate of 25 ml/min. The injector temperature was 250°C. The oven temperature gradient began at 165°C and was maintained for 5 min, increased 4°C/min to 217°C, and then ramped to a final temperature of 240°C at 20°C/min. Fatty acid methyl esters were detected by flame ionization at 250°C, and they were identified by comparison of retention times with fatty acid methyl ester standards.

RESULTS

Conversion of 18:3n-3 to DHA

The differentiated rat brain astrocytes converted radiolabeled 18:3n-3, the most abundant unesterified n-3 PUFA ordinarily present in the plasma (10), to DHA. Preliminary studies indicated that the products formed were similar whether or not dibutyryl cAMP remained in the medium during the incubation with fatty acid. Therefore, to simplify the incubation conditions, the dibutyryl cAMP was routinely removed before incubation of the cultures with the medium containing the radiolabeled fatty acid.

HPLC analysis indicated that the differentiated astrocytes converted [1-^{14}C]18:3n-3 to 22:6n-3 and that the 22:6n-3 was incorporated into the cell lipids and released into the medium as free fatty acid (**Fig. 2**). However, the most abundant radiolabeled product contained in the hydrolyzed cell lipid extract after a 48-h incubation was 22:5n-3 (Fig. 2, top). Radiolabeled 20:5n-3, 16:0, and 18:0 also were present in the cell lipids. All of these radiolabeled fatty acids were released from the cells, but the most abundant product in the medium was 22:6n-3 rather than 22:5n-3 (Fig. 2, bottom).

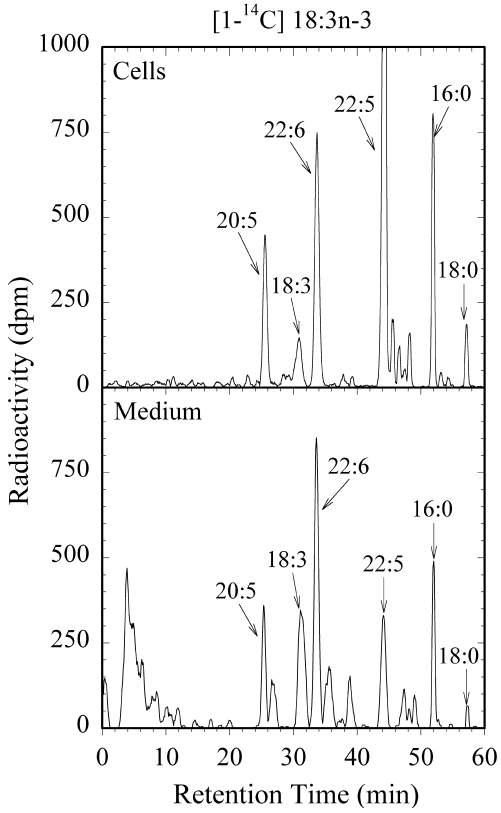

Fig. 2. HPLC analysis of the radiolabeled fatty acids produced by astrocytes from [1-^{14}C]18:3n-3. Primary cultures of rat brain astrocytes were morphologically differentiated as described in Fig. 1. The medium containing dibutyryl cAMP was removed, and the cultures were washed and then incubated for an additional 48 h in a medium containing 10% FBS and 5 μM [1-^{14}C]18:3n-3. The lipids were extracted from the cells and medium, transmethylated, and separated by HPLC using gradient I as described in Materials and Methods. HPLC tracings of the cell lipid extract (top) and culture medium (bottom) are shown. Chromatograms from a single culture are shown, but similar data were obtained from the cell lipids and medium of two additional cultures in each case.

Effect of DHA availability on the uptake of 18:3n-3

We investigated whether the availability of DHA in the medium during the incubation with radiolabeled fatty acids would affect the ability of the differentiated astrocyte cultures to take up and convert 18:3n-3 to radiolabeled 22:6n-3. In these studies DHA concentrations between 2.5 and 15 μM were added to media containing 10% FBS. **Figure 3** illustrates the amount of DHA incorporated by the astrocytes in a 48-h incubation and the extent to which these concentrations of DHA reduced the uptake of 18:3n-3 in a corresponding 48-h incubation. A 3-fold increase in DHA uptake occurred when the DHA concentration was raised from 2.5 to 15 μM (Fig. 3, top). HPLC analysis indicated that essentially all of the [1-^{14}C]22:6n-3 radioactivity incorporated into the cell lipids remained as 22:6n-3. Corresponding cultures of the differentiated astrocyte were incubated in media containing 5 μM [1-^{14}C]18:3n-3 and either 10% FBS alone, or 10% FBS supplemented with 2.5 to 15 μM unlabeled DHA. Although the total radiolabeled fatty acid uptake decreased when DHA was present, the

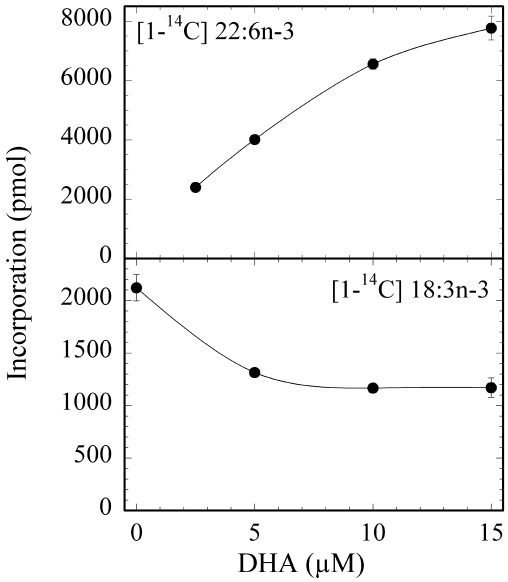

Fig. 3. Effect of DHA concentration on fatty acid uptake by astrocytes. Top: Differentiated astrocyte cultures were incubated for 48 h in a medium containing 10% FBS and 2.5 to 15 μM [1-^{14}C]22:6n-3. After the medium was removed and the cultures were washed, the total radioactivity contained in the cell lipid extract was determined by liquid scintillation counting. Bottom: Astrocyte cultures were incubated for 48 h in a medium containing 10% FBS, either no DHA supplement (DHA = 0) or 2.5 to 15 μM unlabeled DHA, and 5 μM [1-^{14}C]18:3n-3. The total radioactivity incorporated into the cell lipids was determined as described above, and the amounts (pmol) were calculated on the basis of the specific activity of the [1-^{14}C]-22:6n-3 and [1-^{14}C]18:3n-3, respectively, added to the cultures. Each point represents the mean of values obtained from three separate cultures, and standard error bars are shown where they are larger than the data point.

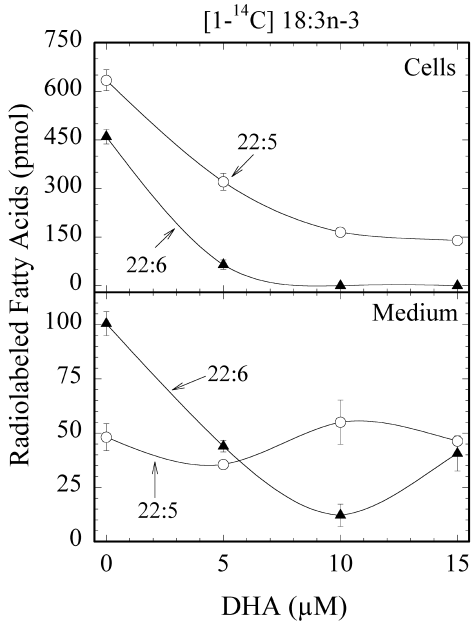

Fig. 4. Effect of unlabeled DHA concentration on the conversion of [1-^{14}C]18:3n-3 to 22:5n-3 and 22:6n-3. Differentiated astrocyte cultures were incubated for 48 h in a medium containing 10% FBS, 5 μM [1-^{14}C]18:3n-3, and either no DHA supplement (DHA = 0) or 2.5 to 15 μM unlabeled DHA as described in Fig. 3. The radiolabeled fatty acids contained in the cell lipids and medium were determined by HPLC as described in Fig. 2. Top: Radiolabeled 22:5n-3 and 22:6n-3 content of the cell lipid extract. Bottom: Amounts of these radiolabeled fatty acids in the culture medium. The amounts (pmol) are calculated on the basis of the specific activity of the [1-^{14}C]18:3n-3 added to the cultures. Each point represents the mean of values obtained from three separate cultures, and standard error bars are shown only where they are larger than the data point.

reduction was only 40% even when the medium contained 15 μM unlabeled DHA (Fig. 3, bottom).

Effect of DHA availability on the conversion of 18:3n-3 to DHA

The radiolabeled fatty acid products contained in the cell lipids and medium of the astrocytes after the 48-h incubation with [1-^{14}C]18:3n-3 were determined by HPLC, and the results for 22:5n-3 and 22:6n-3 are shown in **Fig. 4**. The data points at the DHA concentration of zero are the values obtained when no supplemental DHA was added to the medium. On the basis of the cell lipid analysis, conversion of [1-^{14}C]18:3n-3 to 22:6n-3 appeared to be almost entirely suppressed when the unlabeled DHA concentration in the medium exceeded 5 μM (Fig. 4, top). However, analysis of the medium indicated that a small amount of radiolabeled 22:6n-3 was formed and released from the cells even when 15 μM DHA was present (Fig. 4, bottom). The amount of radiolabeled 22:5n-3 in the cell lipids was reduced but not completely suppressed when unlabeled DHA was present in the medium, and the amount of radiolabeled 22:5n-3 in the medium was unchanged.

The effect of loading the astrocytes with DHA before incubation with [1-^{14}C]18:3n-3 also was tested. After incubation with dibutyryl cAMP, the astrocyte cultures were incubated for 24 h in a medium containing either 10% FBS supplemented with 30 μM unlabeled DHA, or 10% FBS alone (control cultures). Previous studies have shown that supplementation of cultured cells with DHA under these conditions, substantially increases the intracellular DHA content (36–38). Consistent with these findings, analysis of the astrocyte fatty acid composition by GLC indicated that the DHA content increased from 2.8% (3.9 μg) of the total fatty acids in the control cultures to 21.6% (42.9 μg) in the cultures supplemented with DHA (average values from three cultures).

After these media containing DHA were removed and the cultures were washed, the cells were incubated for 48 h with 5 μM [1-^{14}C]18:3n-3 to determine whether the increase in intracellular DHA content would affect the production of radiolabeled 22:6n-3. The astrocytes enriched with DHA incorporated 32% less radiolabeled fatty acid than the control cells. **Figure 5** illustrates the radiolabeled fatty acids synthesized from the [1-^{14}C]18:3n-3 that were present in the cell lipids and medium at the end of the 48-h incubation. HPLC analysis indicated that 22:6n-3 accounted for 30% of the radioactivity present in the cell lipids of the control cultures (Fig. 5, top left). By contrast, only 15% of the radioactivity was present as 22:6n-3 in the cultures enriched with DHA, and a larger percentage of the incorpo-

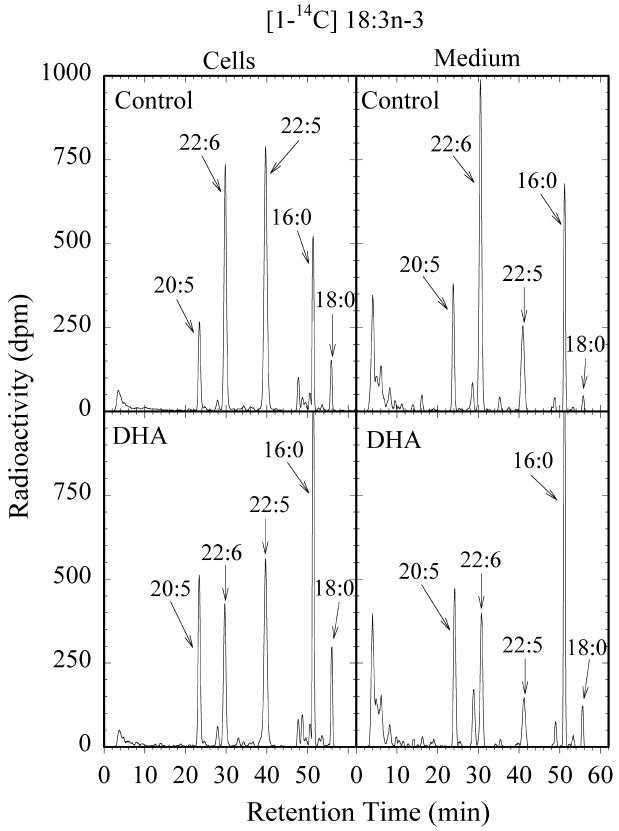

Fig. 5. Effect of intracellular DHA content on radiolabeled fatty acids produced from [1-^{14}C]18:3n-3. After differentiation, the astrocyte cultures were incubated in a medium containing either 10% FBS (control cultures) or 10% FBS supplemented with 30 μM unlabeled DHA. After 24 h, these media were removed and the fatty acid composition of the cells from three control and three DHA-enriched cultures was determined by GLC. The three remaining control and DHA-enriched cultures were incubated for 48 h with 5 μM [1-^{14}C]18:3n-3 in a medium containing 10% FBS. The radiolabeled fatty acids contained in the cell lipids and medium were determined by HPLC using gradient I as described in Fig. 2. The HPLC tracings obtained from the cells are shown on the left, and those from the culture medium are shown on the right. The top panels show the tracings obtained with the cultures not supplemented with DHA (Control), and those initially loaded with DHA are shown in the bottom panels (DHA).

rated radioactivity was present as 16:0, 18:0, and 20:5 (Fig. 5, bottom left). Much more radiolabeled 22:6n-3 also was released into the medium by the control cultures (Fig. 5, top right) than the cultures enriched with DHA (Fig. 5, bottom right). As noted for the cell lipids, the major radiolabeled fatty acid in the medium of the cultures enriched with DHA was 16:0.

Taken together, these results indicate that the differentiated astrocytes continued to convert some 18:3n-3 to DHA and secrete some of the newly formed DHA even when their intracellular DHA content was almost eight times greater than under ordinary culture conditions.

Effect of DHA availability on the conversion of 22:5n-3 to DHA

Additional studies were done to determine whether the availability of DHA would reduce the formation of DHA from 22:5n-3, another n-3 PUFA precursor of DHA that is normally available in the plasma (9, 10). As shown in **Fig. 6**, the differentiated astrocytes readily converted 22:5n-3 to DHA during a 48-h incubation. HPLC analysis indicated that 40% of the [3-^{14}C]22:5n-3 radioactivity incorporated into the cell lipids had been converted to 22:6n-3 (Fig. 6, top). In addition, the cell lipids contained radiolabeled 16:0, 18:0, 20:5n-3, 24:5n-3, and 24:6n-3. HPLC analysis of the medium indicated that, as in the cells, the most abundant radiolabeled product was 22:6n-3 (Fig. 6, bottom). A substantial amount of radiolabeled 20:5n-3 and 16:0 also was present in the medium.

The availability of unlabeled DHA in the medium also reduced but did not completely suppress the conversion of 5 μM [3-^{14}C]22:5n-3 to DHA. Only 30% less radiolabeled fatty acid was taken up by astrocytes incubated for 48 h in media containing 15 μM DHA as compared with cells incubated in media without supplemental DHA. **Figure 7** shows the radiolabeled 22:6n-3 and 20:5n-3 content of the cells and medium at the end of the 48-h incubation. The quantity of 22:6n-3 incorporated into the cell lipids decreased by 70% when 15 μM DHA was present (Fig. 7, top), but the amount released into the medium decreased by only 40% (Fig. 7, bottom). Although the conversion to 22:6n-3 was reduced, there was no appreciable change in

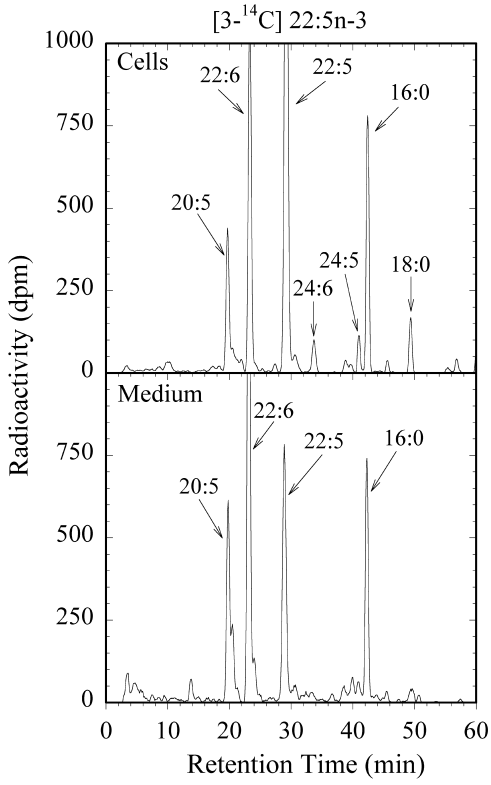

Fig. 6. HPLC analysis of radiolabeled fatty acids produced from [3-^{14}C]22:5n-3. After differentiation, the astrocyte cultures were incubated for 48 h in a 10% FBS medium containing 5 μM [3-^{14}C]-22:5n-3, and the cells and medium lipids were analyzed by HPLC as described in Fig. 2, except that gradient II was used. The HPLC tracings shown are from the cell lipid extract (top) and culture medium (bottom).

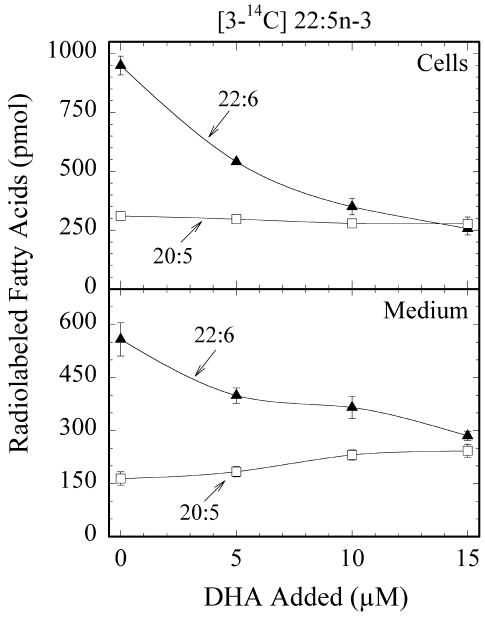

Fig. 7. Effect of unlabeled DHA concentration on the conversion of [3-^{14}C]22:5n-3 to radiolabeled 20:5n-3 and 22:6n-3 by astrocytes. The experimental conditions and HPLC analysis were the same as described in Fig. 6. The graphs show the radiolabeled 20:5n-3 and 22:6n-3 content of the cell lipid extract (top) and culture medium (bottom). The amounts (pmol) are calculated on the basis of the specific activity of the [3-^{14}C]22:5n-3 added to the cultures. Each point is the mean of values obtained from three separate cultures, and standard error bars are shown where they are larger than the data point.

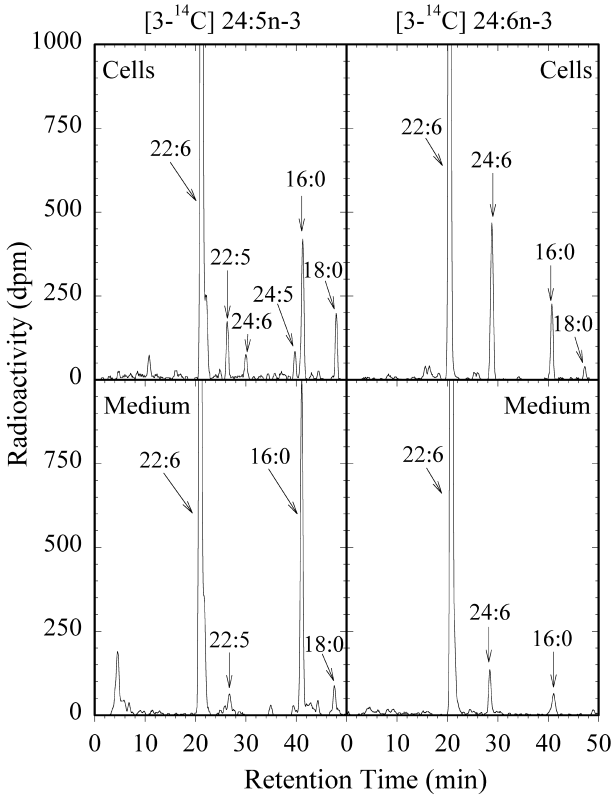

Fig. 8. HPLC analysis of radiolabeled fatty acids produced from 24-carbon n-3 PUFA. After differentiation, the astrocyte cultures were incubated for 48 h in a 10% FBS medium containing 5 μM [3-^{14}C]24:5n-3 or [3-^{14}C]24:6n-3. The cells and medium lipids were analyzed by HPLC as described in Fig. 6. HPLC tracings from cultures incubated with [3-^{14}C]24:5n-3 are shown on the left, and those from cultures incubated with [3-^{14}C]24:6n-3 are shown on the right. The top panels are tracings from the cell lipid extract, and the bottom panels are tracings obtained from the culture medium.

the amount of radiolabeled 20:5n-3 contained in the cell lipids or released into the medium.

Effect of DHA availability on the conversion of 24-carbon n-3 PUFA to DHA

We also determined whether the availability of DHA would suppress the synthesis of DHA from its 24-carbon n-3 PUFA precursors (17, 35, 39). HPLC analysis of the cell lipids and medium indicated that the differentiated astrocyte cultures produced large quantities of DHA from both of these substrates (**Fig. 8**). When the cultures were incubated for 48 h with 5 μM [3-^{14}C]24:5n-3, the main radiolabeled compound contained in the cell lipids was 22:6n-3 (Fig. 8, top left). In addition, a small amount of the radiolabeled 24:6n-3 intermediate was detected, together with 16:0, 18:0, and 22:5n-3. HPLC analysis of the medium indicated that 65% of the radioactivity was present as 22:6n-3 and 25% as 16:0 (Fig. 8, bottom left). When the astrocytes were incubated for 48 h with 5 μM [3-^{14}C]24:6n-3, the only radiolabeled product present in appreciable amounts in the cell lipids (Fig. 8, top right) and medium (Fig. 8, bottom right) was 22:6n-3.

The availability of DHA in the medium reduced the conversion of both 24-carbon n-3 PUFA to DHA. When 15 μM unlabeled DHA was present, the amounts of [3-^{14}C]24:5n-3 and [3-^{14}C]24:6n-3 incorporated by the astrocytes were reduced by 24% and 40%, respectively. **Figure 9** illustrates the effect of DHA availability on the amounts of radiolabeled 22:6n-3 present in the cell lipids and medium as determined by HPLC analysis. In a 48-h incubation with [3-^{14}C]24:5n-3 (Fig. 9, top), the amount of radiolabeled 22:6 present in the cell lipids decreased progressively as the concentration of unlabeled DHA was raised. However, there was no reduction in the amount of radiolabeled 22:6n-3 released into the medium. A similar result was observed when the astrocytes were incubated under these conditions with 5 μM [3-^{14}C]24:6n-3 (Fig. 9, bottom). The radiolabeled 22:6n-3 content of the cell lipids was 50% lower when 15 μM unlabeled DHA was added, but the amount released into the medium was not reduced.

DISCUSSION

These results demonstrate that rat brain astrocytes retain the ability to synthesize DHA from n-3 PUFA precursors after they have undergone morphological differentiation in culture. However, the amount of DHA formed was reduced considerably when the astrocytes had access to an extracellular supply of DHA or the intracellular DHA con-

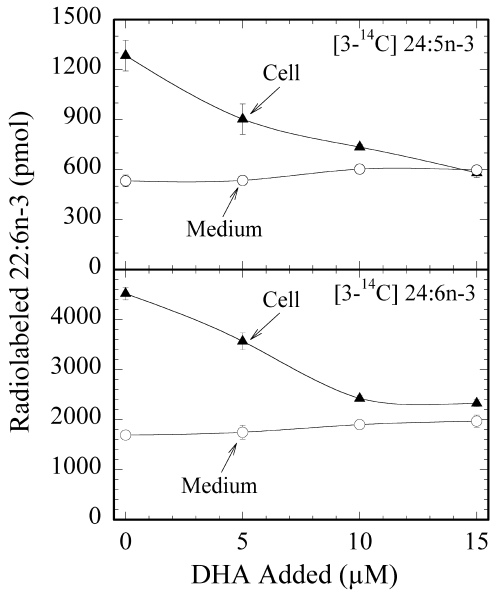

Fig. 9. Effect of unlabeled DHA concentration on the radiolabeled 22:6n-3 produced by astrocytes from [3-^{14}C]24:5n-3 or [3-^{14}C]24:6n-3. The experimental conditions and HPLC analysis were the same as described in Figs. 7 and 8. The graphs show the amounts of radiolabeled 22:6n-3 in the cell lipid extract and medium of the cultures incubated with 5 μM [3-^{14}C]24:5n-3 (top) or 5 μM [3-^{14}C]24:6n-3 (bottom). The amounts (pmol) are calculated on the basis of the specific activity of the [3-^{14}C]24:5n-3 and [3-^{14}C]-24:6n-3, respectively, added to the cultures.

tent was increased. Therefore, the amount of DHA derived from n-3 PUFA precursors appears to be regulated by the availability of preformed DHA. Yet, the astrocytes continued to synthesize a small amount of DHA even when the concentration of DHA in the extracellular fluid was three times higher than the n-3 PUFA substrate. Some conversion of 18:3n-3 to DHA also persisted when the intracellular DHA content was raised to 21.6%, a value 7.7 times greater than in control astrocytes. These findings suggest that DHA synthesis in astrocytes may be a constitutive process that is required to fulfill an essential function in the brain.

Several mechanisms probably contribute to the decrease in DHA production from n-3 PUFA precursors when a supply of DHA is available. Although some decrease in uptake of the radiolabeled n-3 PUFA occurred when DHA was added to the medium (Fig. 3, bottom), this cannot be the only factor because a reduction also occurred when the cells were loaded with DHA before exposure to the radiolabeled fatty acid (Fig. 5). Less conversion of [3-^{14}C]24:6n-3 to DHA occurred when the medium contained DHA (Fig. 9, bottom), indicating that a decrease in peroxisomal retroconversion may be another contributing factor. However, one or more of the chain elongation or desaturation reactions apparently are reduced, too, because less [1-^{14}C]18:3n-3 was converted to 22:5n-3 when an excess of DHA was available (Fig. 4). Alternatively, the decrease in DHA production may be due to increased degradation of the n-3 PUFA substrate or products rather than a direct effect on DHA synthesis. The radiolabeled 16:0 that was formed was derived from the β-oxidation of the n-3 PUFA substrate or its products, followed by recycling of the acetyl-CoA into the lipogenic pathway (11). More radiolabeled 16:0 was formed from [1-^{14}C]18:3n-3 when the cells were loaded with DHA (Fig. 5), suggesting that increased degradation may have contributed to the lesser production of DHA from the n-3 PUFA substrates.

Because 10% FBS was present in all of the incubation media, it is possible that, like DHA, other fatty acids contained in the serum may have affected the conversion of n-3 PUFA precursors to DHA. Detailed studies to determine the effects of these fatty acids have not been done. However, we have obtained some preliminary data with 18:2n-6, the most abundant n-6 PUFA normally present in the plasma free fatty acid fraction (10). In an experiment with media containing 10% FBS, the astrocytes converted 575 ± 20 pmol of [1-^{14}C]18:3n-3 to DHA in 48 h (mean ± SEM, n = 3). Addition of 15 μM unlabeled 18:2n-6 reduced the amount formed by 18%, to 471 ± 73 pmol (D. E. Williard, S. D. Harmon, T. L. Kaduce, M. Preuss, S. A. Moore, M. E. C. Robbins, and A. A. Spector, unpublished observations). Thus, it appears that 18:2n-6 also may reduce DHA production, but to a much lesser extent than DHA.

Previous results indicated that between 75% and 85% of the DHA synthesized by primary cultures of undifferentiated astrocytes is released into the extracellular fluid (23). Additional results demonstrated that neurons cultured under similar conditions do not synthesize appreciable amounts of DHA, but they readily take up DHA when it is available in the medium (23). On the basis of these findings, we formulated a hypothesis that the function of DHA synthesis in astrocytes is to supply DHA to the neurons (24, 25). Subsequent findings indicated that astrocytes also supply DHA to the endothelial cells of the blood-brain barrier (26). Although the present data show that astrocytes retain the ability to release some newly synthesized DHA after they undergo differentiation, the amount is smaller than previously observed in undifferentiated astrocytes. The percentage of radiolabeled DHA recovered in the extracellular fluid in 48-h incubations ranged from only 19% with [1-^{14}C]18:3n-3 to 37% with [3-^{14}C]22:5n-3. In addition to the morphological changes, astrocytes incubated with dibutyryl cAMP express many other characteristics of differentiation (27, 28, 40, 41). It is possible that their membrane lipids, like brain phospholipids (4, 42), may require a higher percentage of DHA than those in undifferentiated astrocytes. Alternatively, the larger DHA incorporation may simply reflect an increase in membrane phospholipid content due to the extensive process formation that occurs when the cells differentiate. Even though a higher percentage was retained, some newly formed DHA still was released by the differentiated astrocytes under all of the conditions tested. Therefore, the paradigm that a major function of DHA synthesis in astrocytes is to supply DHA to the neurons may also apply to the brain after development occurs.

It is generally thought that the final steps in DHA synthesis are elongation of 22:5n-3 to 24:5n-3, Δ^6 desatura-

tion of the 24:5n-3, and retroconversion of the resulting 24:6n-3 to DHA in the peroxisomes (17, 35, 39, 43, 44). This has been challenged, and an alternative mechanism in which 24:6n-3 is an elongation product rather than a precursor of DHA has been proposed (45). Radiolabeled 24:5n-3 and 24:6n-3 were formed when [3-^{14}C]22:5n-3 was converted to DHA (Fig. 6, top), and radiolabeled 24:6n-3 was formed when [3-^{14}C]24:5n-3 was converted to DHA (Fig. 8, top left). These results are consistent with either mechanism. However, no radiolabeled 22:5n-3 was detected when [3-^{14}C]24:6n-3 was converted to DHA (Fig. 8, top right). If 24:6n-3 was an elongation product rather than a precursor of DHA, it would have to be converted to 22:5n-3 in order to form DHA. Therefore, our findings do not support the elongation hypothesis. They are entirely consistent with the mechanism proposed by Sprecher and colleagues (17, 44), in which the 24-carbon n-3 PUFAs are intermediates in the conversion of 22:5n-3 to DHA.

Studies of neonatal and lactating animals indicate that dietary 18:3n-3 is utilized for the synthesis of saturated fatty acids (11, 46, 47). This process, which also occurs in the developing rat brain (48), involves β-oxidation and recycling of the acetyl-CoA into the lipogenic pathway. Production of radiolabeled saturated fatty acids from [1-^{14}C]18:3n-3 was observed previously in undifferentiated astrocytes (23), and the present results indicate that this process continues to occur after the astrocytes undergo differentiation The main product, 16:0, was incorporated into the cell lipids and released into the medium. We estimated the amounts of PUFA products formed during these incubations by assuming that the specific activity of the radiolabeled substrate does not change during the metabolic process. This is a reasonable assumption because the reactions involved are chain elongation, desaturation, or a single β-oxidation cycle. However, a similar assumption cannot be made regarding the utilization of these substrates for saturated fatty acid synthesis, and there is no convenient way to use the radioactivity measurements to calculate the specific activity of the 16:0 or 18:0 that is produced. Therefore, it is not possible to compare the amounts of saturated fatty acid and DHA produced from the n-3 PUFA substrates.

The comparative results indicate that DHA contained in the extracellular fluid is a more effective source of DHA for the astrocytes than an equivalent concentration of an n-3 PUFA precursor. For example, when astrocytes were incubated in media containing either 5 μM DHA or 18:3n-3, 8.8 times more DHA was recovered in the cells incubated for 48 h with DHA than in those incubated with 18:3n-3. Even if the DHA released into the medium by the cells incubated with 18:3n-3 is included in the calculation, the total amount of DHA obtained when preformed DHA was available was seven times greater. These results are consistent with data indicating that plasma DHA is eight times more effective than 18:3n-3 in producing DHA accretion in the fetal baboon brain (49). There is considerable evidence that increases in dietary DHA intake, such as through fish oil supplements, will increase the supply of DHA to the brain (1, 4, 50). The present findings support this conclusion because they suggest that the amount of DHA supplied by such supplements would far exceed any reduction in DHA synthesis from n-3 precursors.

In summary, DHA production from n-3 PUFA precursors in astrocytes appears to be a minor process in quantitative terms as compared with the amount that can be obtained from preformed DHA. This is consistent with in vivo data indicating that although the brain can convert n-3 PUFA precursors to DHA (19–22), most of the DHA in the brain is supplied preformed from the plasma (11–16, 49). However, the fact that some DHA synthesis persists in the astrocytes even when excess DHA is available suggests that endogenously produced DHA may have a vital role in either astrocyte function or interactions between astrocytes and other brain cells.

This study was supported by Program Project grant HL49264 from the National Heart, Lung, and Blood Institute and by RO1 CA82722 from the National Cancer Institute, National Institutes of Health. We thank Dr. Howard Sprecher (Department of Medical Biochemistry, Ohio State University) for supplying the 22- and 24-carbon fatty acids used in this study.

Manuscript received 21 March 2001 and in revised form 1 May 2001.

REFERENCES

1. Connor, W. E., M. Neuringer, and S. Reisbeck. 1992. Essential fatty acids: the importance of n-3 fatty acids in the retina and brain. *Nutr. Rev.* **50:** 21–29.
2. Uauy, R., P. Peirano, D. Hoffman, P. Mena, D. Birch, and E. Birch. 1996. Role of essential fatty acids in the function of the developing nervous system. *Lipids.* **31:** S-167–S-176.
3. Greiner, R. S., T. Moriguchi, A. Hutton, B. M. Slotnick, and N. Salem, Jr. 1999. Rats with low levels of docosahexanoic acid show impaired performance in olfactory-based and spatial learning tasks. *Lipids.* **34:** S-239–S-243.
4. Lauritzen, L., H. S. Hansen, M. H. Jorgensen, and K. F. Michaelsen. 2001. The essentiality of long chain *n*-3 fatty acids in relation to development and function of the brain and retina. *Prog. Lipid Res.* **40:** 1–94.
5. Litman, B. J., and D. C. Mitchell. 1996. A role for phospholipid polyunsaturation in modulating membrane protein function. *Lipids.* **31:** S-193–S-197.
6. Mitchell, D. C., and B. J. Litman. 1998. Molecular order and dynamics in bilayers consisting of highly polyunsaturated phospholipids. *Biophys. J.* **74:** 879–891.
7. Huster, D., K. Arnold, and K. Gawrisch. 1998. Influence of docosahexaenoic acid and cholesterol on lateral lipid organization in phospholipid mixtures. *Biochemistry.* **37:** 17299–17308.
8. Mata de Urquiza, M., S. Liu, M. Sjoberg, R. H. Zetterstrom, W. Griffiths, J. Sjovall, and T. Perlmann. 2000. Docosahexaenoic acid, a ligand for the retinoid X receptor in mouse brain. *Science.* **290:** 2140–2144.
9. Lands, W. E. M., B. Libelt, A. Morris, N. C. Kramer, T. E. Prewitt, P. Bowen, D. Schmeisser, M. H. Davidson, and J. H. Burns. 1992. Maintenance of lower proportions of (n-6) eicosanoid precursors in phospholipids of human plasma in response to added dietary (n-3) fatty acids. *Biochim. Biophys. Acta.* **1180:** 147–162.
10. Conquer, J. A., and B. J. Holub. 1998. Effect of supplementation with different doses of DHA on the levels of circulating DHA as non-esterified fatty acid in subjects of Asian Indian background. *J. Lipid Res.* **39:** 286–292.
11. Sheaff Greiner, R. C., Q. Zhang, K. J. Goodman, D. A. Giussani, P. W. Nathanielsz, and J. T. Brenna. 1996. Linoleate, α-linolenate, and docosahexaenoate recycling into saturated and monounsaturated fatty acids is a major pathway in pregnant or lactating adults and fetal or infant rhesus monkeys. *J. Lipid Res.* **37:** 2675–2686.

12. Pawlosky, R. J., G. Ward, and N. Salem, Jr. 1996. Essential fatty acid uptake and metabolism in the developing rodent brain. *Lipids.* **31:** S-103–S-107.
13. Su, H. M., L. Bernardo, M. Mirmiran, X. H. Ma, T. N. Corso, P. W. Nathanielsz, and J. T. Brenna. 1999. Bioequivalence of dietary α-linolenic and docosahexaenoic acids as sources of docosahexaenoate accretion in brain and associated organs of neonatal baboons. *Pediatr. Res.* **45:** 87–93.
14. Nouvelot, A., C. Delbart, and J. M. Bourre. 1986. Hepatic metabolism of alpha-linolenic acid in suckling rats, and its possible importance in polyunsaturated fatty acid uptake by the brain. *Ann. Nutr. Metab.* **30:** 316–323.
15. Scott, B. L., and N. G. Bazan. 1989. Membrane docosahexaenoate is supplied to the developing brain and retina by the liver. *Proc. Natl. Acad. Sci. USA.* **86:** 2903–2907.
16. Sinclair, A. J. 1975. Incorporation of radioactive polyunsaturated fatty acids into liver and brain of developing rats. *Lipids.* **10:** 175–184.
17. Sprecher, H., D. L. Luthria, B. S. Mohammed, and S. P. Baykousheva. 1995. Reevaluation of the pathways for the biosynthesis of polyunsaturated fatty acids. *J. Lipid Res.* **36:** 2471–2477.
18. Bourre, J. M., M. Piciotti, and O. Dumont. 1990. Δ^6-Desaturase in brain and liver during development and aging. *Lipids.* **25:** 354–356.
19. Dhopeshwarkar, G. A., and C. Subramanian. 1976. Biosynthesis of polyunsaturated fatty acids in the developing brain. I. Metabolic transformations of intracranially administered 1-^{14}C linolenic acid. *Lipids.* **11:** 67–71.
20. Cook, H. W. 1978. *In vitro* formation of polyunsaturated fatty acids by desaturation in the rat brain: some properties of enzymes in developing brain and comparison with liver. *J. Neurochem.* **30:** 1327–1334.
21. Pawlosky, R., A. Barnes, and N. Salem, Jr. 1994. Essential fatty acid metabolism in the feline: relationship between liver and brain production of long-chain polyunsaturated fatty acids. *J. Lipid Res.* **35:** 2032–2040.
22. Dwyer, B., and J. Bernsohn. 1979. Incorporation of [1-^{14}C]linolenate into lipids of developing rat brain during essential fatty acid deficiency. *J. Neurochem.* **32:** 833–838.
23. Moore, S. A., E. Yoder, S. Murphy, G. R. Dutton, and A. A. Spector. 1991. Astrocytes, not neurons, produce docosahexaenoic acid (22:6ω-3) and arachidonic acid (20:4ω-6). *J. Neurochem.* **56:** 518–524.
24. Spector, A. A., and S. A. Moore. 1992. Role of cerebromicrovascular endothelium and astrocytes in supplying docosahexaenoic acid to the brain. *In* Essential Fatty Acids and Eicosanoids. A. Sinclair and R. Gibson, editors. American Oil Chemists' Society, Champaign, IL. 100–103.
25. Moore, S. A. 1994. Local synthesis and targeting of essential fatty acids at the cellular interface between blood and brain: a role for cerebral endothelium and astrocytes in the accretion of CNS docosahexaenoic acid. *World Rev. Nutr. Diet.* **75:** 128–133.
26. Bernoud, N., L. Fenart, C. Benistant, J. F. Pageaux, M. P. Dehouck, P. Moliere, M. Lagarde, R. Cecchelli, and J. Lecerf. 1998. Astrocytes are mainly responsible for the polyunsaturated fatty acid enrichment in blood-brain barrier endothelial cells in vitro. *J. Lipid Res.* **39:** 1816–1824.
27. Goldman, J. E., and F. C. Chiu. 1984. Dibutyryl cyclic AMP causes intermediate filament accumulation and actin reorganization in astrocytes. *Brain Res.* **306:** 85–95.
28. Imamura, M., and E. Ozawa. 1998. Differential expression of dystrophin isoforms and utrophin during dibutyryl-cAMP-induced morphological differentiation of rat brain astrocytes. *Proc. Natl. Acad. Sci. USA.* **95:** 6139–6144.
29. Murphy, S. 1990. Generation of astrocyte cultures from normal and neoplastic central nervous system. *In* Methods in Neuroscience. Vol. 2. P. M. Conn, editor. Academic Press, New York. 33–47.
30. Dutton, G. R. 1990. Isolation, culture, and use of viable central nervous system perikarya. *In* Methods in Neuroscience. Vol. 2. P. M. Conn, editor. Academic Press, New York. 87–102.
31. Gordon, J. A., S. K. Heller, T. L. Kaduce, and A. A. Spector. 1994. Formation and release of a peroxisome-dependent arachidonic acid metabolite by human skin fibroblasts. *J. Biol. Chem.* **269:** 4103–4109.
32. Williard, D. E., T. L. Kaduce, S. D. Harmon, and A. A. Spector. 1999. Conversion of eicosapentaenoic acid to chain-shortened omega-3 fatty acid metabolites by peroxisomal oxidation. *J. Lipid Res.* **39:** 978–986.
33. Morrison, W. R., and L. M. Smith. 1964. Preparation of fatty acid methyl esters and dimethylacetals from lipids with boron fluoride-methanol. *J. Lipid Res.* **5:** 600–608.
34. Fang, X., T. L. Kaduce, M. VanRollins, N. L. Weintraub, and A. A. Spector. 2000. Conversion of epoxyeicosatrienoic acids (EETs) to chain-shortened epoxy fatty acids by human skin fibroblasts. *J. Lipid Res.* **41:** 66–74.
35. Moore, S. A., E. Hurt, E. Yoder, H. Sprecher, and A. A. Spector. 1995. Docosahexaenoic acid synthesis in human skin fibroblasts involves peroxisomal retroconversion of tetracosahexaenoic acid. *J. Lipid Res.* **36:** 2433–2443.
36. Vartak, S., M. E. C. Robbins, and A. A. Spector. 1997. Polyunsaturated fatty acids increase the sensitivity of 36B10 rat astrocytoma cells to radiation-induced cell kill. *Lipids.* **32:** 283–292.
37. Yorek, M. A., D. K. Strom, and A. A. Spector. 1984. Effect of membrane polyunsaturation on carrier-mediated transport in cultured retinoblastoma cells: alterations in taurine transport. *J. Neurochem.* **42:** 254–261.
38. North, J. A., A. A. Spector, and G. R. Buettner. 1994. Cell fatty acid composition affects free radical formation during lipid peroxidation. *Am. J. Physiol.* **267:** C177–C188.
39. Voss, A., M. Reinhard, S. Sankarappa, and H. Sprecher. 1991. The metabolism of 7,10,13,16,19-docosapentaenoic acid to 4,7,10,13,16,19-docosahexaenoic acid in rat liver is independent of a 4-desaturase. *J. Biol. Chem.* **266:** 19995–20000.
40. Eng, D. L., Y. L. Lee, and P. G. Lal. 1997. Expression of glutamate uptake transporters after dibutyryl cyclic AMP differentiation and traumatic injury in cultured astrocytes. *Brain Res.* **778:** 215–221.
41. McManus, M. F., L-C. Chen, I. Vallejo, and M. Vallejo. 1999. Astroglial differentiation of cortical precursor cells triggered by activation of the cAMP-dependent signaling pathway. *J. Neurosci.* **19:** 9004–9015.
42. Crawford, M. A., M. Bloom, S. Cunnane, H. Holmsen, K. Ghebremeskel, J. Parkington, W. Schmidt, A. J. Sinclair, and C. L. Broadhurst. 2001. Docosahexaenoic acid and cerebral development. *World Rev. Nutr. Diet.* **88:** 6–17.
43. Delton-Vandenbroucke, I., P. Grammas, and R. E. Anderson. 1997. Polyunsaturated fatty acid metabolism in retinal and cerebral microvascular endothelial cells. *J. Lipid Res.* **38:** 147–159.
44. Sprecher, H. 2000. Metabolism of highly unsaturated ω3 and ω6 fatty acids. *Biochim. Biophys. Acta.* **1486:** 219–231.
45. Infante, J. P., and V. A. Huszagh. 1998. Analysis of the putative role of 24-carbon polyunsaturated fatty acids in the biosynthesis of docosapentaenoic (22:5n-6) and docosahexaenoic (22:6n-3) acids. *FEBS Lett.* **431:** 1–6.
46. Cunnane, S. C., C. R. Menard, S. S. Likhodii, J. T. Brenna, and M. A. Crawford. 1999. Carbon recycling into de novo lipogenesis is a major pathway in neonatal metabolism of linoleate and α-linolenate. *Prostaglandins Leukot. Essent. Fatty Acids.* **60:** 387–392.
47. Cunnane, S. C. 2001. New developments in α-linolenate metabolism with emphasis on the importance of β-oxidation and carbon recycling. *World Rev. Nutr. Diet.* **88:** 178–183.
48. Menard, C. R., K. Goodman, T. Corso, J. T. Brenna, and S. C. Cunnane. 1998. Recycling of carbon into lipids synthesized de novo is a quantitatively important pathway of [U-^{13}C]-α-linolenate utilization in the developing rat brain. *J. Neurochem.* **71:** 2151–2158.
49. Su, H-M., M-C. Huang, N. M. R. Saad, P. W. Nathanielsz, and J. T. Brenna. 2001. Fetal baboons convert 18:3n-3 to 22:6n-3 in vivo: a stable isotope tracer study. *J. Lipid Res.* **42:** 581–586.
50. Gibson, R. A., M. A. Neumann, and M. Makrides. 1996. Effect of dietary docosahexaenoic acid on brain composition and neural function in term infants. *Lipids.* **31:** S-177–S-181.