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Membrane docosahexaenoate is supplied to the developing brain and retina by the liver

(ω3 fatty acids/linolenic acid/phospholipids/docosahexaenoic acid)

BURTON L. SCOTT AND NICOLAS G. BAZAN*

Louisiana State University School of Medicine, Louisiana State University Eye Center, Neuroscience Center and Eye, Ear, Nose and Throat Hospital, New Orleans, LA 70112

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ABSTRACT **Docosahexaenoic acid [22:6ω3; 22:6(4, 7, 10, 13, 16, 19)] is concentrated in phospholipids of cellular membranes from brain and retina. Although linolenic acid [18:3ω3; 18:3(9, 12, 15)] is the major ω3 fatty acid of mouse dams' milk, 22:6 is the prevalent ω3 fatty acid in serum and tissues. Intraperitoneal injection of [1-^{14}C]18:3 into 3-day-old mouse pups resulted in liver and serum lipid labeling that was initially high, followed by a rapid decline. In contrast, labeling of brain and retinal lipids were initially low and increased with time. Labeled 22:6 first appeared in liver 2 hr after injection and later in brain and retina. We suggest that 22:6 synthesized from 18:3 by the liver is secreted into the bloodstream in lipoproteins, taken up by brain and retina, and incorporated into cell membranes. We hypothesize that the 22:6 requirements of membranes (e.g., during synaptogenesis, photoreceptor membrane biogenesis, or repair after ischemic injury or neurodegenerative disorders) are met by a signal that is sent by the appropriate tissues to the liver to evoke the secretion of 22:6-containing lipoproteins.**

The central nervous system requires large amounts of docosahexaenoic acid [22:6ω3; 22:6(4, 7, 10, 13, 16, 19)] during early postnatal development (1, 2) when cellular differentiation, active synaptogenesis, and photoreceptor membrane biogenesis takes place. 22:6 comprises approximately 50% of the acyl groups of phospholipids in photoreceptor membranes (2, 3). Animals lack a desaturase needed to synthesize 22:6 *de novo*. Thus, this polyunsaturated fatty acid must be supplied by dietary intake of 22:6 or by synthesis from dietary 18:3 [18:3ω3; 18:3(9, 12, 15)] (2, 4). Brain and retina avidly retain 22:6, even after lengthy periods of dietary deprivation of essential fatty acids (1, 3, 4), by a mechanism that may involve rapid activation to docosahexaenoyl-coenzyme A (5–7). Nevertheless, prolonged dietary deficiency of ω3 fatty acids eventually decreases brain and retina 22:6 content (8), leading to an altered electroretinogram (3), decreased visual activity (9), and impaired learning ability (10). In addition, 22:6 is acted upon by a lipoxygenase in the retina, which produces oxygenated products of as yet unknown function (11). ω3 fatty acids also modulate the production of oxygenated metabolites of the ω6 series, such as prostaglandins (12, 13).

To trace ω3 fatty acid metabolism during postnatal development of 22:6-rich neural membranes, we have studied the fate of radiolabeled 18:3 administered to suckling mouse pups.

MATERIALS AND METHODS

***In Vivo* Metabolism of [1-^{14}C]18:3.** C57BL/6J mice (The Jackson Laboratory) were maintained on Purina mouse chow. [1-^{14}C]18:3 (55 Ci/mole; 1 Ci = 37 GBq; New England Nuclear) was dried under nitrogen gas, 1 μl of 50 mM $NaHCO_3$ was added per μCi, and the sample was sonicated and vortexed to obtain the sodium salt of the fatty acid. [1-^{14}C]18:3 (4–6 μCi in 5 μl) was injected either intraperitoneally or directly into the stomach through the abdominal wall in 3- to 7-day-old (1.5–4.0 g of body weight) mouse pups by using a 10-μl syringe fitted with a 33-gauge needle. Animals were sacrificed at various times after injection, and liver, brain, and retina were dissected out and rapidly homogenized prior to lipid extraction (14). Blood serum was separated by centrifugation at 2000 × *g* for 20 min, and lipids were extracted and washed (14).

Separation of Lipids and Derivatization of Fatty Acids. Phospholipids (PL), free fatty acids (FFA), and triacylglycerols (TG) were isolated by one-dimensional TLC by using silica gel GHL Uni-plates (Analtech) and a solvent system of petroleum ether/diethyl ether/acetic acid, 60:40:2.3 (vol/vol) (14). For determination of total radioactivity in lipid classes, spots resolved by TLC were visualized with iodine vapor and scraped into vials containing water and scintillation fluid, and the radioactivity was determined. Acyl groups were quantitatively converted to fatty acid methyl esters (FAMEs) by heating to 100°C for 60–90 min with 14% (wt/vol) BF_3 in methanol (14).

HPLC and GLC. Radiolabeled FAMEs dissolved in acetonitrile/chloroform/methanol, 6:2:1 (vol/vol) were separated by reverse-phase HPLC by using an elution gradient of acetonitrile/water (15) on a 8-mm × 10-cm Radial-Pak column and Guard-Pak precolumn insert, both containing 10-μm μBondapak C_{18} (Waters). The acetonitrile concentration was 70% (vol/vol) for 55 min, then was increased linearly to 100% during 10 min, and was held for 15 min. Radioactive FAMEs were quantitated with a Flo-One Beta Flow detector (Radiomatic Instruments, Tampa, FL) by using flow rates of mobile phase and scintillation cocktail (Beckman RP Ready Solv) of 2 and 6 ml/min, respectively, and peaks were monitored at 205 nm with a Waters Lambda-Max model 48. To assay endogenous fatty acids, lipid extracts were converted to FAMEs with 14% BF_3/methanol, and FAMEs were separated and quantitated by capillary GLC (16) using a 0.25-mm i.d. × 30-m SP-2330 column (Supelco), with heptadecanoic acid methyl ester as internal standard.

RESULTS

Endogenous ω3 Fatty Acids in Stomach Contents and Serum of Mice During Early Postnatal Development. During the first 3 weeks after birth, 18:3 levels in stomach contents both declined with age and were consistently higher than 22:6 levels (Fig. 1 *Upper*). On the other hand, in pups younger than 3 weeks, the content of 22:6 in serum was 3–6 times higher than that of 18:3 (Fig. 1 *Lower*). The 18:3/22:6 molar

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Abbreviations: PL, phospholipid; FFA, free fatty acid; TG, triacylglycerol; FAME, fatty acid methyl ester.

*To whom reprint requests should be addressed at: LSU Eye Center, 2020 Gravier Street, Suite B, New Orleans, LA 70112.

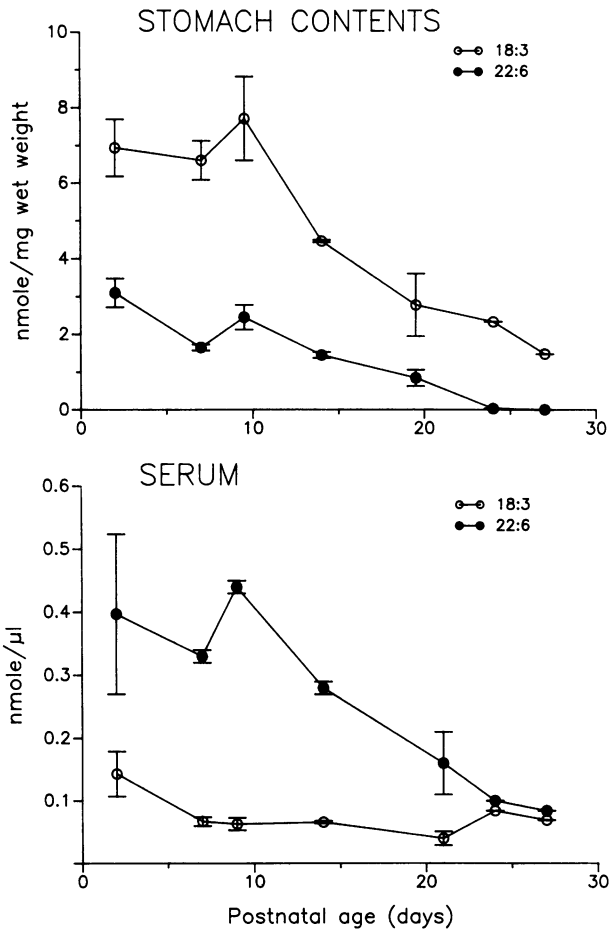

FIG. 1. Endogenous contents of 18:3 (○) and 22:6 (●) in stomach (*Upper*) and serum (*Lower*) of mouse pups during postnatal development. Values are the means of 2–8 samples ± SEM, except for ages older than 20 days, which are values from single samples.

ratio in serum approached 1 during week 4 of life as pups began eating mouse chow. Mouse chow is rich in 18:3 (4440 ± 160 nmol/g) but contains trace amounts of 22:6 (5.9 ± 5.0 nmol/g).

Lipid and Fatty Acid Labeling in Liver, Serum, Brain, and Retina During Early Postnatal Development. Three-day-old pups, 18 hr after intraperitoneal injection of 6 μCi of [1-^{14}C]18:3, had no radioactivity associated with tissues of the peritoneal cavity. In the rest of the carcass (after removal of liver, brain, and retinas), ≈40% of the label was in 18:3, 9% was in 22:6, and the remainder was in 16:0 and in unidentified HPLC peaks (data not shown).

Injection of radiolabeled 18:3 into 3-day-old pups initially resulted in accumulation of 18:3 in liver followed by increased synthesis of 22:6. Labeling of liver and serum lipids declined simultaneously with a shift in radioactivity from precursor (18:3) to end product (22:6) as can be seen in Fig. 2 *A* and *B*. Two hr after injection, 77% of labeling in liver lipids was in unconverted 18:3, 8% was in 22:6, and 10% was in the ω3 elongation and desaturation intermediates, 20:5 and 22:5 (Figs. 2*B* and 3). Two percent of the label was found in 16:0 indicating that some carbon recycling through β-oxidation had taken place. The relative proportion of liver label remaining in 18:3 declined with time, whereas labeled 22:6 increased steadily, reaching 70% at 72 hr (Fig. 2*B*). At 24 hr, ≈30% of labeling of liver lipids was in PL, and most of the remainder was in TG (Fig. 2*C*); however, by 72 hr, 50% of the label was in PL and 45% was in TG (Fig. 2*C*). At both time points, labeled 22:6 accounted for most of the PL radioactivity and for only a fraction of the TG radioactivity,

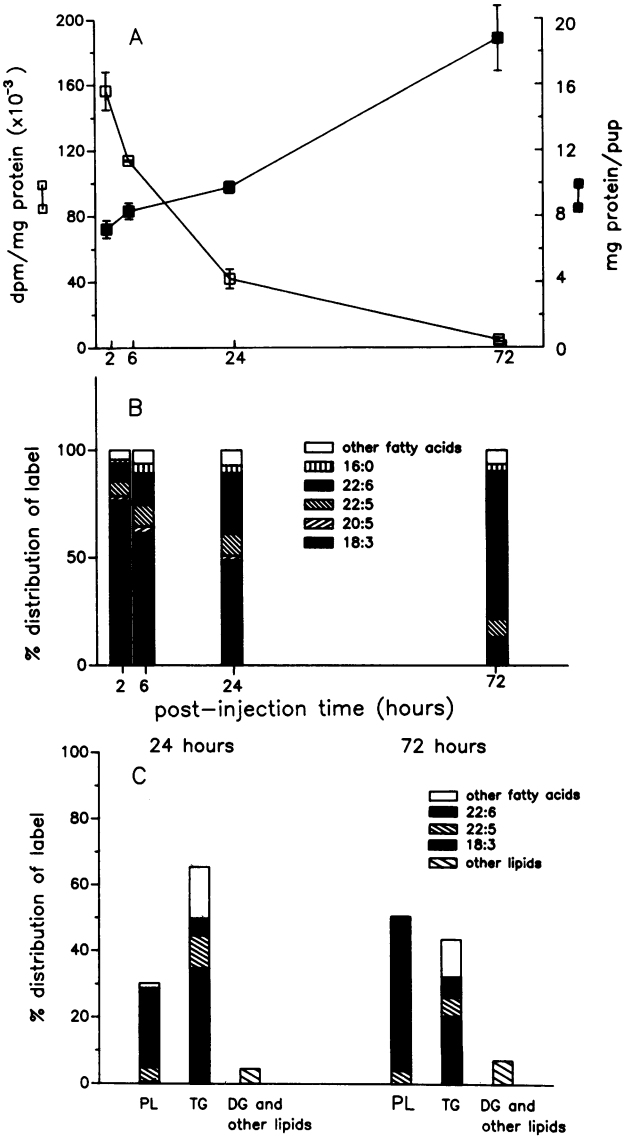

FIG. 2. Time course of labeling of liver lipids after intraperitoneal injection of [1-^{14}C]18:3 into 3-day-old mouse pups. (*A*) Total labeling of liver lipids (□) and total protein content (■). (*B*) Distribution of label among FAMEs separated by HPLC. (*C*) Distribution of label among PL, TG, and diacylglycerols (DG) plus other lipids at 24 and 72 hr after injection of [1-^{14}C]18:3 into 4- to 7-day-old pups. Also, the distribution of label among fatty acyl groups in PL and TG is shown. Data represent averages of at least two samples, each containing tissue from two pups.

whereas labeled 18:3 was present in only trace amounts in PL and accounted for about half of the TG radioactivity (Fig. 2*C*).

The labeling profile of serum resembled that of liver in that total labeling was highest 2 hr after injection and then declined (Fig. 4*A*). Initially, a large proportion of serum lipid labeling was in unconverted 18:3; however, by 72 hr no labeled 18:3 was detectable (Fig. 4*B*). Labeled 22:6 in serum was present in small amounts at 2 hr and rose to 70% of the total by 72 hr (Fig. 4*B*). Labeling of ω3 fatty acid intermediates peaked by 6 hr, when 16% of the label was found in 20:5 and 11% was found in 22:5 (Fig. 4*B*). Labeling of 16:0 was also maximal at 6 hr, when it represented 6% of the total radioactivity in serum lipids (Fig. 4*B*). PL and TG contained most of the radioactivity in serum; FFA were only slightly labeled (Fig. 4*C*).

The time courses of labeling in brain (Fig. 5) and retinal lipids (Fig. 6) differed from those of liver (Fig. 2) and serum

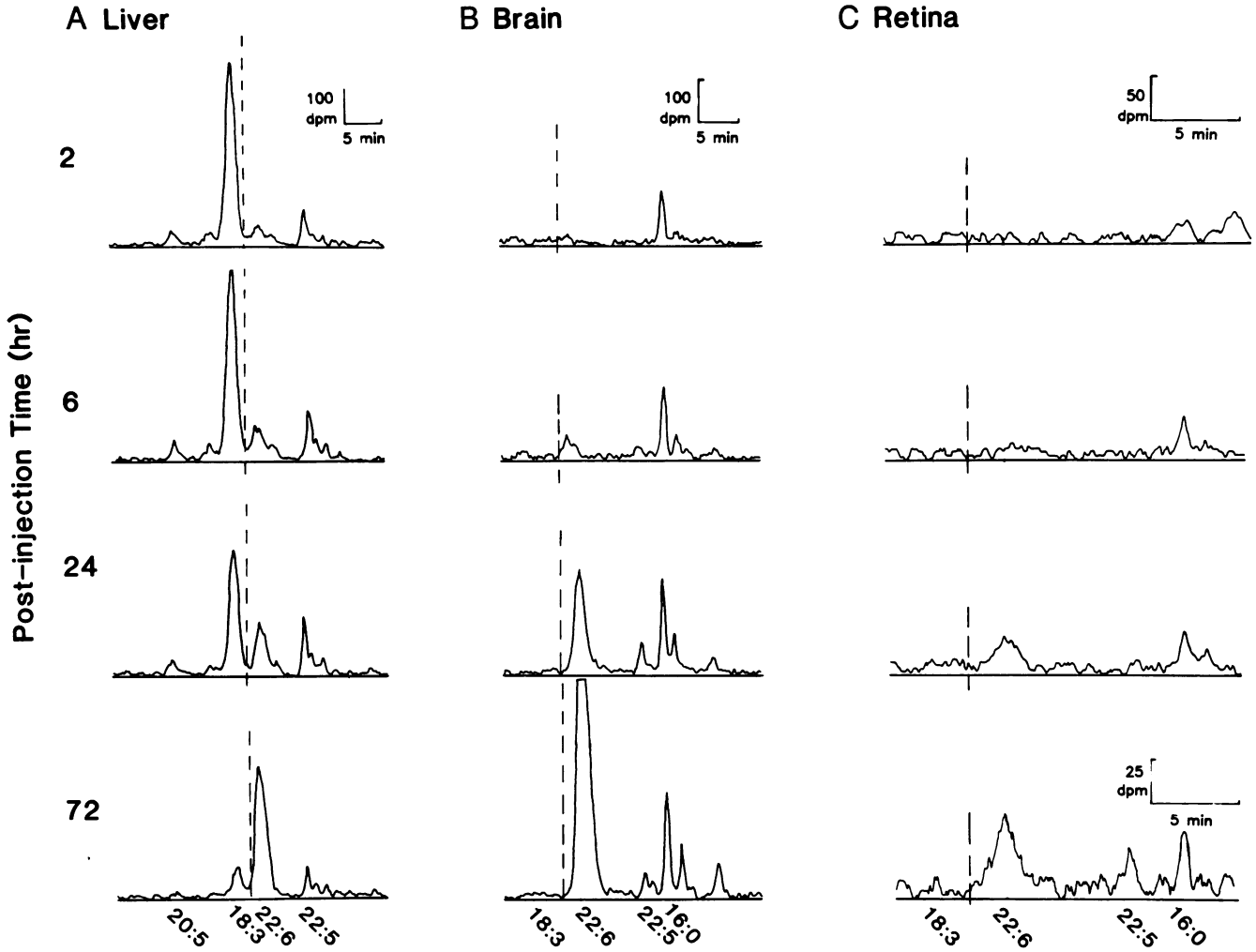

FIG. 3. Reverse-phase HPLC of radiolabeled FAMEs from liver (*A*), brain (*B*), and retina (*C*) at 2, 6, 24, and 72 hr after intraperitoneal injection of [1-^{14}C]18:3 into 3-day-old mouse pups. The total dpm of FAMEs injected onto the HPLC column ranged from 12,000 to 17,500 for liver, 3330 to 22,000 for brain, and 810 to 4020 for retina.

(Fig. 4). In brain and retina, labeling increased until 24 hr after injection and reached a plateau, whereas liver and serum labeling fell steadily. At 10 min after injection, brain and retina contained radiolabeled 18:3 and 16:0, but no detectable radiolabeled 22:6 (data not shown). At 2 hr, nearly one-half the total labeling in brain and retinal lipids was in 16:0; the proportion of label in 16:0 declined thereafter (Figs. 5*B* and 6*B*). By 2 hr after injection, 18:3 labeling in brain declined to undetectable levels, whereas labeled 22:6 increased steadily with time (Figs. 3*B* and 5*B*). Nearly 85% of the label in brain and retina at 24 and 72 hr was in PL (Figs. 5*C* and 6*C*); TG represented <2% of the radioactivity. Most of the label in brain PL was in 22:6 (Fig. 5*C*), but 16:0 also was significantly labeled in PL of both brain and retina (Figs. 3 and 5*C*).

Uptake of Injected [1-^{14}C]22:6 by Brain and Retina. As was observed for pups injected with [1-^{14}C]18:3, animals injected with [1-^{14}C]22:6 (data not shown) exhibited a rapid, transient burst of liver labeling. The subsequent loss of labeled 22:6 from liver and serum within 6 hr after injection was sufficient to account for the labeled 22:6 accumulated by brain and retina. Furthermore, between 6 and 24 hr postinjection, loss of labeled 22:6 from liver and serum was equivalent to the gain of labeled 22:6 in brain and retina.

DISCUSSION

Our results suggest that the liver plays a major role in supplying 22:6 to the central nervous system during early postnatal development since most of the intraperitoneally injected [1-^{14}C]18:3 is rapidly taken up by the liver where it is elongated and desaturated to [^{14}C]22:6 and then supplied by way of the blood to brain and retina.

Throughout early postnatal development, 18:3 is the most abundant ω3 fatty acid in the diet. After weaning, the 18:3/22:6 ratio rises, which reflects increasing intake by the pups of 18:3-rich, 22:6-poor mouse chow. In contrast, the 18:3/22:6 molar ratio in serum is <1 at all ages, indicating that the developing animals readily convert dietary 18:3 into 22:6. This rapid conversion explains the fact that tissue lipids contain low amounts of 18:3 (2–4), despite the relatively high quantities of dietary 18:3. Moreover, 22:6 is the major ω3 fatty acid present in serum of suckling rat pups whose mothers were provided 18:3-supplemented diets (17). The low 18:3 and higher 22:6 contents observed in serum support the identification of 22:6 as the major ω3 fatty acid provided by serum to developing brain and retina.

Labeled 16:0 and cholesterol appear in brain lipids after intraperitoneal injection of [1-^{14}C]18:3 into 12- to 13-day-old rat pups (18, 19), suggesting that a portion of the injected fatty acid is broken down by β-oxidation and its carbons are recycled. Thus, the sudden rise in serum [^{14}C]18:3 that we observe immediately after injection of [^{14}C]18:3 into developing mice probably results in some of the fatty acid crossing the blood–brain and blood–retinal barriers. Radioactive carbon released by β-oxidation of the fatty acid can then be recycled into newly synthesized 16:0.

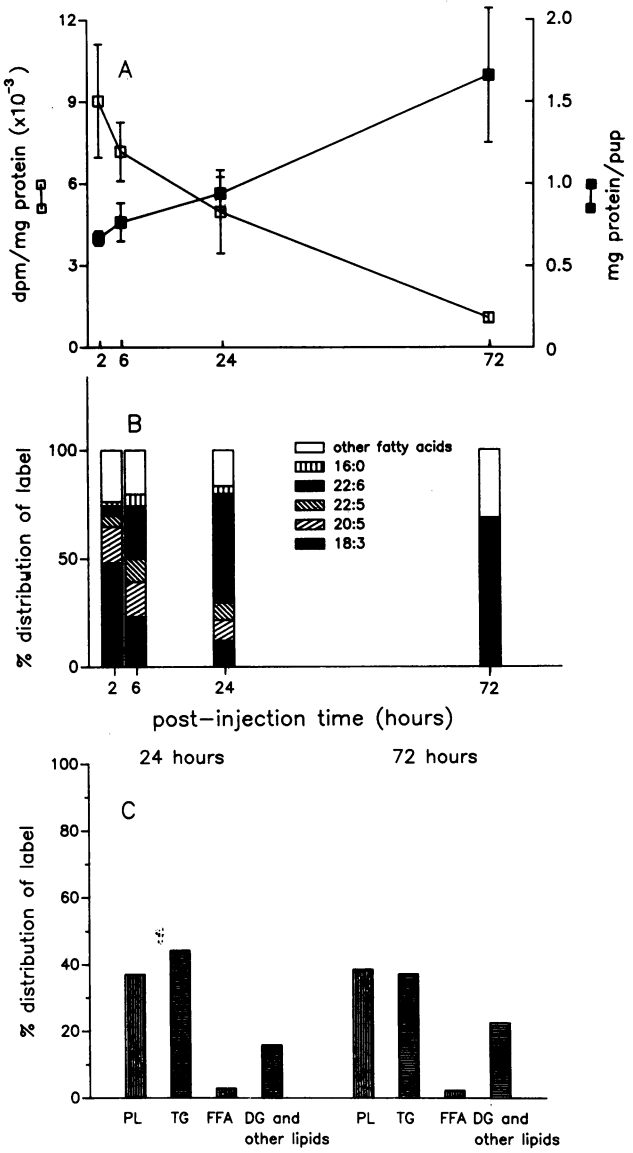

FIG. 4. Time course of labeling of serum lipids after intraperitoneal injection of [1-^{14}C]18:3 into 3-day-old mouse pups. (*A*) Total labeling of serum lipids (□) and total protein content (■). (*B*) Distribution of label among FAMEs separated by HPLC. (*C*) Distribution of label among lipid classes at 24 and 72 hr after injection of [1-^{14}C]18:3. DG, Diacylglycerol.

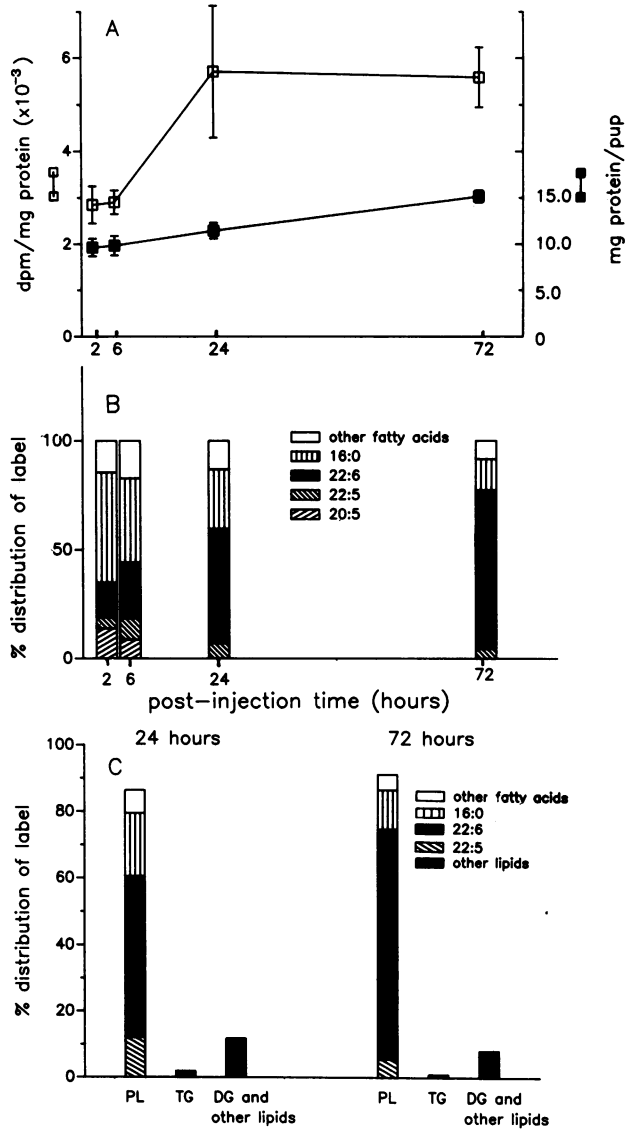

FIG. 5. Time course of labeling of brain lipids after intraperitoneal injection of [1-^{14}C]18:3 into 3-day-old mouse pups. (*A*) Total labeling of brain lipids (□) and total protein content (■). (*B*) Distribution of label among FAMEs separated by HPLC. (*C*) Distribution of label among lipid classes at 24 and 72 hr after injection of [1-^{14}C]18:3. DG, Diacylglycerol.

Radiolabeled 18:3 given orally or by means of intracerebral injection is also rapidly desaturated and elongated and then acylated into brain lipids (15, 18–20). Feeding radiolabeled 18:3 to pregnant guinea pigs results in progressive decreases in 18:3 labeling and increases in 22:6 labeling during passage from maternal liver to placenta, to fetal liver, and to brain, suggesting that both maternal and fetal tissues contribute to elongation and desaturation of 18:3 *in utero* (21). Both brain and liver can desaturate 18:3 *in vitro* during early postnatal development in the rat (22). The specific activity of this conversion in brain is highest at 4 days of age and declines subsequently, whereas in liver the specific activity of Δ^6-desaturase is low at 4 days of age and then increases rapidly. The total capacities of brain and liver in suckling rats to desaturate 18:3 are similar until day 8, after which time activity increases rapidly in liver and declines in brain (22). Thus, both liver and brain are potentially important sites of 18:3 desaturation during early postnatal development. However, our *in vivo* observations in developing mice, that after injection of labeled 18:3, labeled 22:6 appears in liver and serum before it appears in brain, suggest that liver contributes most of the newly synthesized 22:6 that accumulates in brain and retina.

In liver lipids of mouse pups 24 hr after injection, most of the labeled 18:3 was in TG, and most of the labeled 22:6 was in PL. With increasing time, labeling of liver lipids shifted from [^{14}C]18:3 in TG to [^{14}C]22:6 in PL, suggesting that the liver initially stored at least part of the injected 18:3 as TG and then subsequently desaturated and elongated it to 22:6 for acylation into PL. In contrast, lipids from brain and retina contained nearly all of their label in 22:6 of PL, whereas TG labeling was very low.

Dietary fat absorption involves breakdown of TG to FFA followed by re-esterification of the fatty acid by the intestinal epithelium (23). Lipids are packaged into chylomicrons and very low density lipoproteins and secreted into lymphatics. After passage through the liver and other tissues, the ingested lipids are distributed to circulating lipoproteins. However, part of these physiological events are bypassed when the fatty acid is intraperitoneally injected. In this case, the fatty acid is rapidly absorbed directly into intestinal lymphatics (in

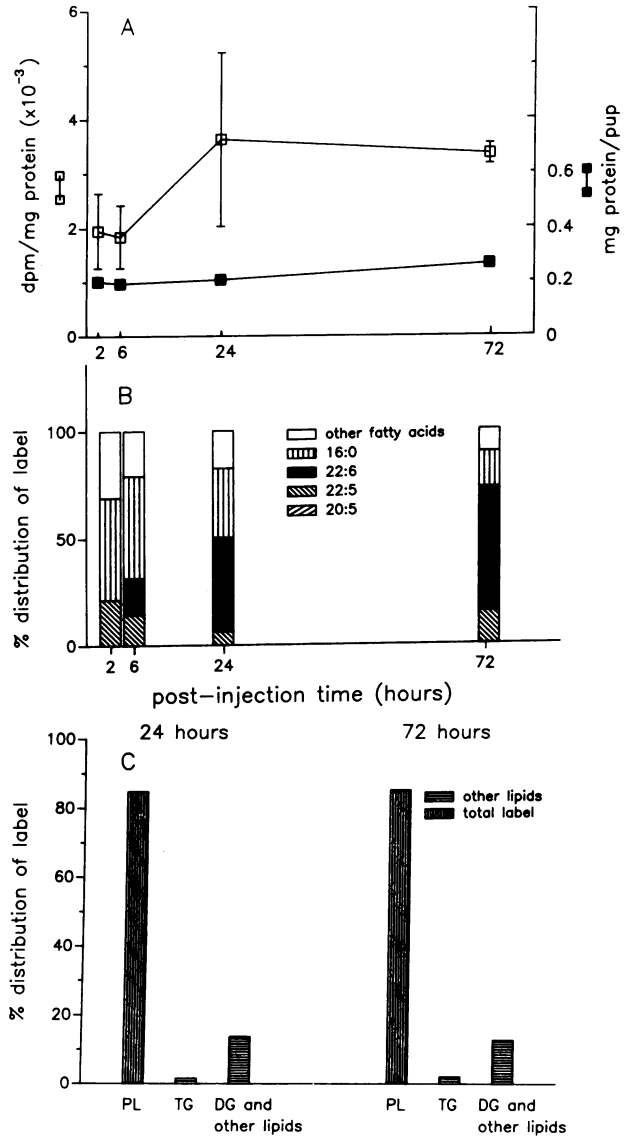

FIG. 6. Time course of labeling of retinal lipids after intraperitoneal injection of [1-^{14}C]18:3 into 3-day-old mouse pups. (*A*) Total labeling of retinal lipids (□) and total protein content (■). (*B*) Distribution of label among FAMEs separated by HPLC. (*C*) Distribution of label among lipid classes at 24 and 72 hr after injection of [1-^{14}C]18:3. DG, Diacylglycerol.

addition to capillaries feeding into the portal system), without necessarily being acted upon by the intestinal epithelium. The intraperitoneally injected fatty acid, however, still becomes packaged into circulating lipoproteins (unpublished observations).

We have also compared intraperitoneal injection to direct injection of [1-^{14}C]18:3 into the stomachs of pups (data not shown), an approach that simulates lipid intake, and we find that the patterns of tissue labeling are similar between the two injection routes. By using either method, labeling rapidly rises and falls in liver and serum as radioactive 22:6 gradually increases in brain and retina.

Our results indicate that newly synthesized 22:6, primarily acylated in PL, may be secreted by liver into blood in the form of lipoproteins. In this form, they are distributed mainly to brain and retina for membrane PL synthesis. One can speculate that synthesis and secretion of 22:6-containing lipoproteins by liver is controlled by a signal from neural tissues requiring 22:6, as during synaptogenesis or photoreceptor membrane biogenesis. Early postnatal development particularly allows the study of this issue because a burst in the 22:6 requirement occurs. Likewise, 22:6 may also be required for membrane maintenance throughout life. During ischemic injury or convulsions, the central nervous system releases free 22:6 (24–26), and also 22:6 is peroxidized. In these pathological conditions, the need for 22:6 replenishment of excitable membranes may be signaled to the liver for supply of 22:6. Loss of 22:6 in neurodegenerative disorders and aging may result from a failure in the replenishment system. A number of mechanisms conceivably contribute to maintaining 22:6 levels in tissues, such as cellular (27, 28) and extracellular matrix fatty acid-binding proteins (29), docosahexaenoyl-coenzyme A synthetase (5–7), and likely a relative enrichment in receptors (fatty acid-binding proteins for uptake of 22:6) that allows brain and retina (including choriocapillaries and retinal pigment epithelium) to take up 22:6 from serum carriers. A putative 22:6 receptor could explain why the central nervous system contains much higher quantities of 22:6 than other tissues, despite being exposed to similar levels of 22:6 circulating in the blood.

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