An octaene fatty acid, 4,7,10,13,16,19,22,25-octacosaoctaenoic acid (28:8n–3), found in marine oils

Colleen K. Van Pelt, Meng-Chuan Huang, Carolyn L. Tschanz, and J. Thomas Brenna[1]

Division of Nutritional Sciences, 116 Savage Hall, Cornell University, Ithaca, NY 14853-6301

Abstract **We report structure determination of an octaene fatty acid, 4,7,10,13,16,19,22,25-octacosaoctaenoic acid (28:8n–3). The molecular weight and double bond locations were determined using acetonitrile chemical ionization mass spectrometry (MS) and MS/MS and were confirmed by MS of hydrogenated and deuterogenated 28:8 and by argentation thin-layer chromatography. 28:8n–3 was 1.2 ± 0.1%, in oil derived from the heterotrophic dinoflagellate *Crypthecodinium cohnii* and a commercial polyunsaturated fatty acid concentrate derived from fish oils (0.16 ± 0.01%), both components of human dietary supplements. It was not found in whole bovine retina, cultured Y79 human retinoblastoma cells, or neonate baboon cerebral cortex. The long chain polyunsaturates present in the *C. cohnii* oil suggest a possible route for 28:8n–3 biosynthesis similar to that for biosynthesis of 22:6n–3.**—Van Pelt, C. K., M-C. Huang, C. L. Tschanz, and J. T. Brenna. **An octaene fatty acid, 4,7,10,13,16,19,22,25-octacosaoctaenoic acid (28:8n–3), found in marine oils.** ***J. Lipid Res.*** **1999.** 40: **1501–1505.**

Supplementary key words n–3 fatty acids • dinoflagellate • fish oil concentrate • *Crypthecodinium cohnii* • gas chromatography/mass spectrometry/mass spectrometry • highly unsaturated fatty acids • microalgae • dietary supplement • octaenoic acid

The omega-3 (or n–3) fatty acids are one of two families of fatty acids recognized as essential for the diet of mammals. They have carbon lengths from 14 (1, 2) to 40 carbons atoms and normally are considered to have 3 to 6 double bonds (trienes to hexaenes) (3, 4) arranged in all-*cis*, methylene-interrupted configuration, with a double bond 3 carbons from the methyl terminus. Pentaene and hexaene n–3 fatty acids with 20 and 22 carbons have been the subject of considerable study over the past 2 decades because of their roles in cardiovascular disease and most recently because of their requirement for development of mammalian retina and brain. For several years infant formulas supplemented with the hexaene 22:6n–3, presumed to be particularly important for neural development of preterm infants (5, 6), have been available commercially in Europe and elsewhere. Prior to this year, heptaenes were the most unsaturated fatty acids to be described, the first example of which was 28:7n–3 found in Baltic herring caught in the spring of 1967 in Finnish waters (7). Heptaenes have also been reported in mammals, specifically ringed seal blubbers (8).

In mammals, n–3 fatty acids or chain lengths C14–24 are inter-convertible through enzyme-mediated elongation, desaturation, and oxidation. Over the past decade, there has been considerable attention to the specific pathways associated with n–3 long chain polyunsaturate (LCP) synthesis prompted by proposal of a revised pathway of mammalian hexaene LCP synthesis (9). Heptaene fatty acids of n–3 structure (38:7n–3 and 40:7n–3) have been described in human brains of patients with genetic defects of peroxisomal functions (10), and a ω6 heptaene (28:7n–6) has been detected in the lipids of herring muscle (11) and in dinoflagellates (12); however, the pathway for the introduction of a seventh double bond, presumably between carbons 4 and 5, is unknown. Very recently an analogous octaene n–3 fatty acid with a double bond between carbons 4 and 5, 28:8n–3, was detected in the lipids of seven dinoflagellates (13).

During investigation of extremely long chain fatty acids we detected a long chain fatty acid of significant concentration but not previously reported in dietary sources of 22:6n–3. We report here results of an investigation to determine its structure and its prevalence in several highly unsaturated lipids, and suggest a possible biosynthetic mechanism.

METHODS

Materials

A commercially available oil of the heterotrophic algae *Crypthecodinium cohnii* (*C. cohnii*), DHASCO™; was obtained from Martek Biosciences, Inc. (Colombia, MD), and a commercial "fish oil concentrate" composed of a mixture of ethyl esters with 30% eicosapentaenoic acid (20:5n–3) and 20% 22:6n–3 was obtained from Arista, Inc. (Stamford, CT). Both oils are used as dietary supplements of LCP for humans. Silver nitrate-impregnated thin-

Abbreviations: LCP, long chain polyunsaturate; TLC, thin-layer chromatography; GC, gas chromatography; CI, chemical Ionization; MS, mass spectrometry.

[1] To whom correspondence should be addressed.

layer chromatography (TLC) plates were purchased from Anal-Tech. Inc. (Newark, DE). Fatty acid standards were obtained from Sigma (methyl 21:0, methyl 28:0) and ("68A," Matreya, Elysian, MN). Platinum (IV) oxide monohydrate was obtained from Aldrich Chemical Co (Milwaukee, WI) and D_2 gas from Cambridge Isotopes, Inc. (Cambridge, MA).

A culture of *C. cohnii* was purchased from American Type Culture Collection (Manassas, VA) and grown in standard recommended media at unregulated room temperature in a dark chamber. Y79 retinoblastoma cells were obtained from the National Institutes of Health (Bethesda, MD) and cultured in RPMI medium supplemented with 10% fetal calf serum and antibiotics. Whole frozen bovine retinas obtained after slaughter were obtained from Lawson, Inc. (Lincoln, NE). Neonatal baboon cortex harvested previously and frozen at −80°C was thawed, homogenized, and extracted according to published methods (14). Briefly, pregnant baboons were maintained on a special balanced casein-based diet with only trace amounts of LCP for the last half of pregnancy. The neonate was maintained from birth on a conventional human infant formula (Enfamil, Mead-Johnson, Evanston, Indiana) with adequate 18:2n–6 and 18:3n–3 but devoid of LCP. At 6 weeks of life the neonate was killed and tissues were harvested for analysis. All animal protocols were approved by the Cornell Institutional Review Board on research animal use.

Fatty acid methyl esters (FAME) were applied to silver nitrate (10%)-impregnated silica gel plates (20 × 20 cm) and the plate was developed twice in a solvent system of hexane–anhydrous diethyl ether–acetic acid 90:10:2. FAME zones were located using iodine vapor followed by charring at 100°C. For GC analysis, duplicate spots were run side-by-side and one lane was used to reveal the position of each spot. Spots of interest were then scraped from the plates and extracted with 5 mL chloroform–methanol 2:1 containing 20% KCl (0.88%) followed by centrifugation. We found that sonication of the silica gel/extraction slurry dramatically improved recovery of extremely unsaturated FAME.

Gas chromatography (GC) and GC/mass spectrometry analysis

Samples were analyzed quantitatively using a Hewlett-Packard 5890 GC and flame ionization detection. A BPX-70 (Restek, Bellefonte, PA) capillary column (60 m × 0.32 mm ID × 0.25 μm) was used for separations. The temperature program was as follows: isothermal 60°C for 1 min; ramp at 50°C/min to 170°C and hold for 6.5 min; ramp at 2.5°C/min to 200°C and hold for 2 min; ramp at 15°C/min to 255°C and hold for 8 min. An equal weight FAME mixture, 68A, was run along with samples to generate instrumental response factors. The response factor for methyl 22:6n–3 was also used for the unknown (methyl 28:8n–3) peak. Methyl 21:0 was added quantitatively to each sample as an internal standard. Compositions are expressed as % of all fatty acids, by weight.

GC/MS and GC/MS/MS were carried out with a Varian Star 3400cx GC interfaced to a Saturn 2000 ion trap mass spectrometer. Electron impact and chemical ionization (CI) with acetonitrile regent gas were used. Under CI conditions, acetonitrile forms an *m/z* 54 ion which adds across double bonds of fatty acids to yield an $(M + 54)^+$ ion. Upon collisional dissociation $(M + 54)^+$ ions yield fragment ions that are characteristic of double bond position. Ion trap parameters for CI–MS and CI–MS/MS are reported elsewhere (15).

Hydrogenation/deuterogenation

Aliquots of *C. cohnii* oil were hydrogenated or deuterogenated catalytically to saturation. Ten mg platinum (IV) oxide monohydrate (Aldrich, Milwaukee, WI) was added to a 50 mg/ml hexane solution of previously methylated algal oil. Either H_2 or D_2 was bubbled through the mixture at a rate of 5 ml/min for 2 min, then the vial was capped. The mixture was heated to 60°C for 3 h, and H_2 or D_2 was bubbled through every 20 min. Solvent was added to replace that lost through evaporation.

Derivatization

Preparation of FAME from natural oils for GC was by saponification/methylation using standard methods reported previously (16, 17). Briefly, samples were treated with methanolic NaOH and 14% BF_3/methanol and extracted into hexane. Internal standard was added to the FAME mixtures.

RESULTS AND DISCUSSION

Figure 1A is a GC/MS chromatogram obtained under CI conditions of methyl esters of fatty acids derived from the heterotrophic marine dinoflagellate *C. cohnii* (18) from the commercial oil. This oil is well known to be dominated by 45–50% 22:6n–3, with negligible amounts of shorter chain n–3 fatty acids. The chromatogram intensity axis is expanded to emphasize low abundance fatty acids. The large peak appearing at 34 min was previously unreported in this oil. This peak consistently yields a strong mass spectral peak at *m/z* 476 as shown in Fig. 1B;

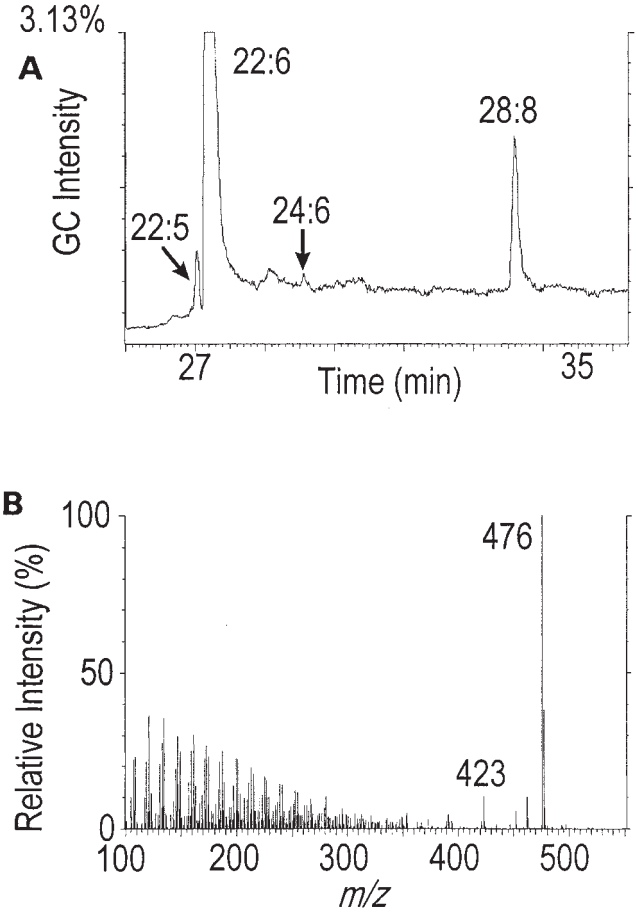

Fig. 1. A) A section of the GC–CI(acetonitrile)/MS total ion chromatogram from a FAME mixture of an extract of *C. cohnii* oil. B) Mass spectrum from the latest eluting peak in A, producing a base peak at *m/z* 476, the $(M + 54)^+$ ion of 28:8.

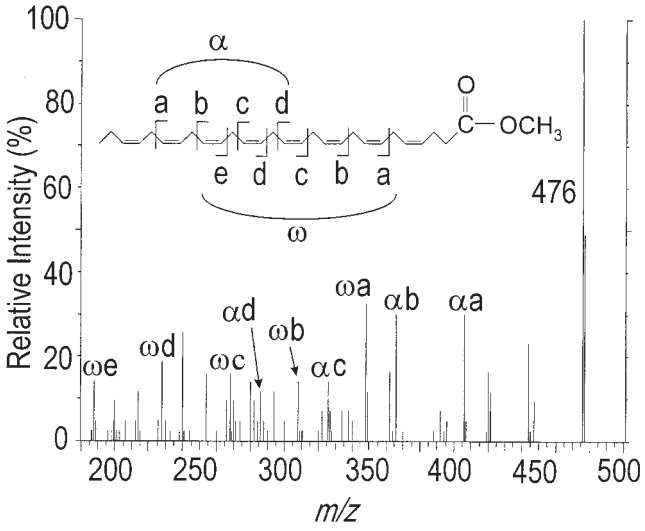

Fig. 2. The CI(acetonitrile)/MS/MS spectrum shows the fragmentation of *m/z* 476 peak observed in Fig. 1. The fragments are consistent with methylene-interrupted omega-3 fatty acid as shown; α fragments carry the ester and ω fragments carry the terminal (ω) carbon, as indicated.

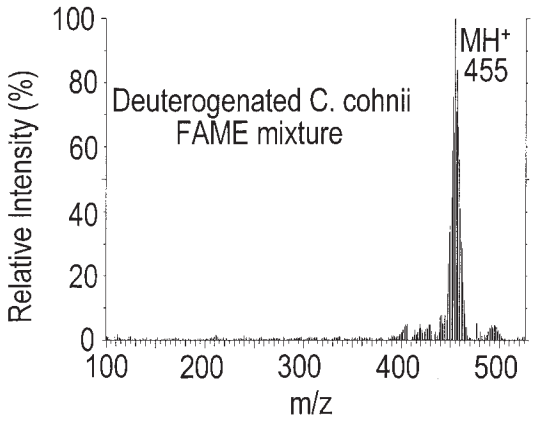

Fig. 3. Single stage mass spectrum of the peak resulting from deuterogenation of 28:8n−3. The isotopic cluster centered at *m/z* 455 corresponds to the MH^+ ion of 28:8n−3 (mol mass 422) plus 16 D atoms (32 daltons).

subtracting 54 daltons due to the reagent group yields a FAME of molecular mass 422 corresponding to 28 carbons and 8 double bonds, 28:8.

MS/MS was performed on the isolated $(M + 54)^+$ ion at *m/z* 476. The results in **Fig. 2** show that collisional dissociation produces a series of 9 fragments corresponding to cleavage between the double bonds. These data strongly indicate the structure of 28:8n−3.

Prior to 1999, no octaene fatty acid had been reported in spite of considerable research on marine oils (19, 20). Very recently 28:8n−3 was detected in the lipids of several photosynthetic dinoflagellates, but not in a heterotrophic dinoflagellate species, such as *C. cohnii* (13). In view of its novelty, the assignment was confirmed in several ways. Separate aliquots of the *C. cohnii* mixture were hydrogenated or deuterogenated catalytically over Pt catalyst for 3 h. GC of the hydrogenated mixture yielded a series of peaks of fully saturated fatty acids, including a peak of the same retention time as genuine methyl 28:0 standard and of a relative intensity similar to that of methyl 28:8n−3 of the untreated mixture. The CI mass spectrum of the methyl 28:0 peak of the deuterogenated peak is presented in **Fig. 3**. The envelope of isotopically substituted peaks is centered at 16 daltons greater than the hydrogenated compound. For comparison, the 22:6n−3 peak is centered at 12 daltons greater than hydrogenated 22:6n−3. The envelope of peaks evidently results from a statistical distribution of D catalytically exchanged for H, and in both cases yields the average mass from the addition of the expected number of D.

As a final check on the degree of unsaturation of this compound, a FAME mixture from the heterotrophic single cell organism *C. cohnii* was separated by degree of unsaturation by argentation TLC. The results are shown in **Fig. 4**. The hexaene spot from 22:6n−3 is dominant as expected. No spot for heptaenes is observed; however, a peak for octaene is clear. This spot was scrapped off and the results of GC analysis are shown in the inset to Fig. 4. The peak in question dominants the chromatogram, again confirming the assignment and degree of unsaturation.

The concentration of 28:8n−3 was investigated quantitatively in two dietary supplement sources with a high abundance of 22:6n−3. Commercial *C. cohnii* oil was highest with 1.20 ± 0.06% (mean ± SD, by weight) of all fatty acids as 28:8n−3. A published fatty acid composition of this oil specifically lists 5 fatty acids of below 1% abundance (21). The fatty acids of *C. cohnii* cultured in our laboratory had 0.23 ± 0.04% of fatty acids as 28:8n−3. The origin of this difference is unclear and may be traceable to strains, growing conditions, or lipid extraction and processing. Finally, a sample of fish oil concentrate commonly used as a dietary supplement contained 0.16 ± 0.01% 28:8n−3. Though the detailed treatment of this product is not known to us, such products are typically distilled

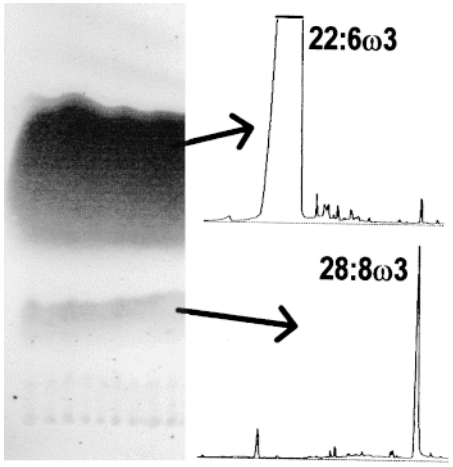

Fig. 4. Argentation TLC plate and associated gas chromatograms demonstrating that the slower migrating band corresponds to the GC peak identified as 28:8n−3.

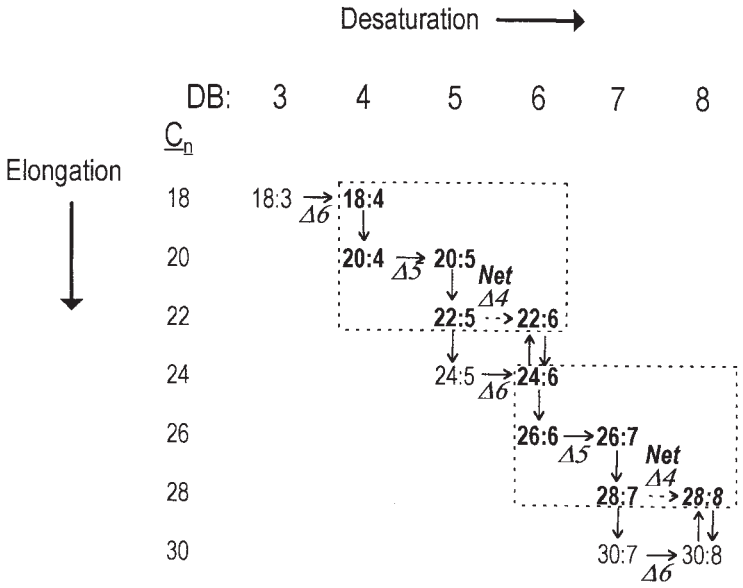

Fig. 5. Diagrammatic representation of the pathway of n−3 synthesis, with desaturation steps represented as horizontal steps and elongations/beta-oxidations as vertical steps. The dashed box including 22:6n−3 includes a series of accepted biochemical transformations leading to 22:6n−3 synthesis. The pathway leading to 28:8n−3 is presented as an hypothesis of the possible synthetic route. The dashed box including 28:8n−3 encloses the identical four final steps leading to 22:6n−3, and suggests that 28:8n−3 may be synthesized by a repeat of the 22:6n−3 pathway starting with 24:6n−3 as a substrate; DB, number of double bonds per molecule; C_n, number of carbon atoms per molecule.

which would be expected to select against 28:8n−3; thus the concentration in the original oil as a fraction of LCP may be underrepresented in the commercial product.

Mammalian retina and cerebrum are rich in LCP, particularly 22:6n−3, and thus are a candidate for presence of 28:8n−3. 28:8n−3 was below our estimated detection limits in a sample of 5 bovine retinas (<17.5 ng/1.76 g tissue) and cultured Y79 human retinoblastoma cells (<7 ng/5 × 10^8 cells). The chemically reducing environment of the rumen is thought to be a harsh environment for unsaturates, typically hydrogenating and isomerizing methylene-interrupted *cis* double bonds to the energetically more favorable *trans* and conjugated forms (22). Thus, despite their reliance on LCP for brain and nervous function, ruminant tissue tends to be low in unsaturates, and these first results indicate that 28:8n−3 is not synthesized under normal conditions in the steer. Y79 cells were cultured using fetal calf serum in the culture media, which was the only source of unsaturated fatty acids. The bovine calf is known to be born with low levels of essential fatty acids, including a high 20:3n−9/20:4n−6 ratio, thus the abundance of ω3 fatty acids is typically low (23).

A baboon neonate was maintained on an LCP-free diet for 6 weeks of life and thus relied on its inherent synthetic capacity to produce LCP. 28:8n−3 was also below detection limits in neonatal baboon cerebral cortex (<35 ng/1.4 g tissue). In all samples of retina or brain, LCP supply was either small or nonexistent. Thus, the failure to detect 28:8n−3 is evidence for a low synthetic capability, but does not address the transport and incorporation of dietary 28:8n−3 in these 22:6n−3-rich tissues, or the possible synthesis of 28:8n−3 from excess 22:6n−3.

Both 28:8n−3 and 22:6n−3 contain the maximal number of methylene-interrupted double bonds in a straight-chain fatty acid which also has a $-CH_2-CH_2-$ group at both ends of the molecule. They also share a double bond across the 4–5 positions numbered from the carboxyl group. Unless it is consumed from marine foods, mammals synthesize 22:6n−3 from 18:3n−3 in a series of alternating desaturations and elongations, as shown in **Fig. 5**. The five enzymatic steps are 18:3n−3 → 18:4n−3 → 20:4n−3 → 20:5n−3 → 22:5n−3 → 22:6n−3 corresponding sequentially to 6-desaturation, elongation, 5-desaturation, elongation, and a net 4-desaturation which is presently accepted to be a multi-step/multi-organelle process (9). We observe, remarkably, that the final four steps from 18:4n−3 to 22:6n−3 could be repeated for the conversion of 24:6n−3 to 28:8n−3. In Fig. 5 these four repeat steps are set off in dashed boxes. We note that the small peak detected at 29.5 min was identified by molecular weight as 24:6n−3 in Fig. 1, indicating that it is available in this organism, albeit at very low levels. This intriguing parallel between 22:6n−3 and 28:8n−3 synthesis suggests that studies of 28:8n−3 biosynthesis may be a useful tool in elucidation of the details of 22:6n−3 synthesis.

Dietary supplements containing 22:6n−3 are widely consumed around the world across all ages of the life cycle from preterm infants to the elderly. Our data indicate that the increasing consumption of marine oils translates into an increase in 28:8n−3 consumption, a change that warrants investigation of its physiological consequences.

Supported by a grant from the National Institutes of Health (GM49209).

Manuscript received 15 March 1999 and in revised form 21 April 1999.

REFERENCES

1. Cunnane, S. C., M. A. Ryan, K. S. Craig, S. Brookes, B. Koletzko, H. Demmelmair, J. Singer, and D. J. Kyle. 1995. Synthesis of linoleate and α-linolenate by chain elongation in the rat. *Lipids.* **30:** 781–783.
2. Williard, D. E., T. L. Kaduce, S. D. Harmon, and A. A. Spector. 1998. Conversion of eicosapentaenoic acid to chain-shortened omega-3 fatty acid metabolites by peroxisomal oxidation. *J. Lipid Res.* **39:** 978–986.

3. Sprecher, H. 1981. Biochemistry of essential fatty acids. *Prog. Lipid Res.* **20:** 13–22.
4. Innis, S. M. 1991. Essential fatty acids in growth and development. *Prog. Lipid Res.* **30:** 39–103.
5. Gibson, R. A., and M. Makrides. 1998. The role of long chain polyunsaturated fatty acids (LCPUFA) in neonatal nutrition. *Acta Paediatr.* **87:** 1017–1022.
6. Crawford, M. A., K. Costeloe, K. Ghebremeskel, and A. Phylactos. 1998. The inadequacy of the essential fatty acid content of present preterm feeds [published erratum appears in *Eur. J. Pediatr.* 1998 Feb;**157:**160]. *Eur. J. Pediatr.* **157 Suppl 1:** S23–S27.
7. Linko, R. R., and H. Karinkanta. 1970. Fatty acids of long chain length in Baltic Herring lipids. *J. Am. Oil. Chem. Soc.* **47:** 42–46.
8. Kakela, R., R. G. Ackman and H. Hyvarinen. 1995. Very long chain polyunsaturated fatty acids in the blubber of ringed seals (*Phoca hispida* sp.) from Lake Saimaa, Lake Ladoga, the Baltic Sea, and Spitsbergen. *Lipids.* **30:** 725–731.
9. Voss, A., M. Reinhart, 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 4-desaturase. *J. Biol. Chem.* **266:** 19995–20000.
10. Poulos, A., K. Beckman, D. W. Johnson, B. C. Paton, B. S. Robinson, P. Sharp, S. Usher, and H. Singh. 1992. Very long-chain fatty acids in peroxisomal disease. *Adv. Exp. Med. Biol.* **318:** 331–340.
11. Rezanka, T. 1990. Identification of very long polyenoic acids as picolinyl esters by Ag+ ion-exchange high-preformance liquid chromatography, reversed-phase high-performance liquid chromatography and gas chromatography-mass spectrometry. *J. Chromatogr.* **513:** 344–348.
12. Volkman, J. K., S. M. Barrett, S. I. Blackburn, M. P. Mansour, E. L. Sikes, and F. Gelin. 1998. Microalgal biomarkers: a review of research developments. *Org. Geochem.* **29:** 1163–1179.
13. Mansour, M. P., J. K. Volkman, D. G. Holdsworth, A. E. Jackson, and S. I. Blackburn. 1999. Very-long-chain (C28) highly unsaturated fatty acids in marine dinoflagellates. *Phytochemistry.* **50:** 541–548.
14. 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:** 1–7.
15. Van Pelt, C., and J. T. Brenna. 1999. Acetonitrile chemical ionization tandem mass spectrometry (CI-MS/MS) to locate double bonds in polyunsaturated fatty acid methyl esters. *Anal. Chem.* **71:** 1981–1989.
16. Sheaff, R. C., H-M. Su, L. A. Keswick, and J. T. Brenna. 1995. Conversion of α-linolenate to docosahexaenoate is not depressed by high dietary levels of linoleate in young rats: tracer evidence using high precision mass spectrometry. *J. Lipid Res.* **36:** 998–1008.
17. Su, H. M., L. A. Keswick, and J. T. Brenna. 1996. Increasing dietary linoleic acid in young rats increases and then decreases docosahexaenoic acid in retina but not in brain. *Lipids.* **31:** 1289–1298.
18. Beach, D. H., G. W. Harrington, J. L. Gellerman, H. Schlenk, and G. G. Holz, Jr. 1974. Biosynthesis of oleic acid and docosahexaenoic acid by a heterotrophic marine dinoflagellate *Crypthecodinium cohnii*. *Biochim. Biophys. Acta.* **369:** 16–24.
19. Ackman, R. G. 1989. Marine Biogenic Lipids, Fats, and Oils. Vol. 1. Boca Raton, FL, CRC Press.
20. Ackman, R. G. 1989. Marine Biogenic Lipids, Fats, and Oils. Vol. 2. Boca Raton, FL, CRC Press.
21. Behrens, P. W., and D. J. Kyle. 1996. Microalgae as a source of fatty acids. *J. Food Lipids.* **3:** 259–272.
22. Harfoot, C. G. 1981. Lipid metabolism in the rumen. *In* Lipid Metabolism in Ruminant Animals. W. W. Christie, editor. Pergamon Press, New York. 35–42.
23. Noble, R. C. 1981. Lipid metabolism in the neonatal ruminent. *In* Lipid Metabolism in Ruminent Animals. W. W. Christie, editor. Pergamon Press, New York. 432–433.