

Cultured Fish Cells Metabolize Octadecapentaenoic Acid (all-*cis* Δ 3,6,9,12,15-18:5) to Octadecatetraenoic Acid (all-*cis* Δ 6,9,12,15-18:4) via its 2-*trans* Intermediate (*trans* Δ 2, all-*cis* Δ 6,9,12,15-18:5)

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Abbreviations : AS, Atlantic salmon cell line; FBS, fetal bovine serum; GC, gas chromatography; HPLC, high performance liquid chromatography; NMR, nuclear magnetic resonance; 18:5n-3, octadecapentaenoic acid (all-*cis* Δ 3,6,9,12,15-18:5); 2-*trans* 18:5n-3, 2-*trans* octadecapentaenoic acid (*trans* Δ 2, *cis* Δ 6,9,12,15-18:5); 18:4n-3, octadecatetraenoic acid (all-*cis* Δ 6,9,12,15-18:4); PUFA, polyunsaturated fatty acid; SAF-1, gilthead sea bream cell line; TF, turbot fin cell line; TLC, thin-layer chromatography;

ABSTRACT: Octadecapentaenoic acid (all-*cis* Δ 3,6,9,12,15-18:5; 18:5n-3) is an unusual fatty acid found in marine dinophytes, haptophytes and prasinophytes. It is not present at higher trophic levels in the marine food web but its metabolism by animals ingesting algae is unknown. Here we studied the metabolism of 18:5n-3 in cell lines derived from turbot (*Scophthalmus maximus*), gilthead sea bream (*Sparus aurata*) and Atlantic salmon (*Salmo salar*). Cells were incubated in the presence of approximately 1 μ M [U- 14 C] 18:5n-3 methyl ester or [U- 14 C] 18:4n-3 (octadecatetraenoic acid; all-*cis* Δ 6,9,12,15-18:4) methyl ester, both derived from the alga *Isochrysis galbana* grown in H 14 CO $_3$, and also with 25 μ M unlabelled 18:5n-3 or 18:4n-3. Cells were also incubated with 25 μ M *trans* Δ 2, all-*cis* Δ 6,9,12,15-18:5 (2-*trans* 18:5n-3) produced by alkaline isomerization of 18:5n-3 chemically synthesized from docosahexaenoic acid (all-*cis* Δ 4,7,10,13,16,19-22:6; 22:6n-3). Radio- and mass analyses of total fatty acids extracted from cells incubated with 18:5n-3 were consistent with this fatty acid being rapidly metabolized to 18:4n-3 which was then elongated and further desaturated to eicosatetraenoic acid (all-*cis* Δ 8,11,14,17,19-20:4; 20:4n-3) and eicosapentaenoic acid (all-*cis* Δ 5,8,11,14,17-20:5; 20:5n-3). Similar mass increases of 18:4n-3 and its elongation and further desaturation products occurred in cells incubated with 18:5n-3 or 2-*trans* 18:5n-3. We conclude that 18:5n-3 is readily converted biochemically to 18:4n-3 via a 2-*trans* 18:5n-3 intermediate generated by a Δ^3, Δ^2 -enoyl-CoA-isomerase acting on 18:5n-3. Thus, 2-*trans* 18:5n-3 is implicated as a common intermediate in the β -oxidation of both 18:5n-3 and 18:4n-3.

Octadecapentaenoic acid (all-*cis* 18:5n-3) is a fatty acid characteristically present in certain algal groups in marine phytoplankton (1), including dinoflagellates (2), haptophytes (3,4) and prasinophytes (5), all of which have important roles in the marine ecosystem. 18:5n-3 is usually co-associated in these organisms with 22:6n-3. Given that biosynthesis of 22:6n-3 involves peroxisomal chain shortening of its precursor 24:6n-3, it is possible that 18:5n-3 is biosynthesized by chain shortening of 20:5n-3 (see 6). Marine zooplankton and fish ingesting phytoplankton contain little or no 18:5n-3 demonstrating that this fatty acid is readily metabolized by marine animals. Clearly it could be completely catabolized by marine animals by β -oxidation but it may also be directly chain elongated to 20:5n-3. We decided to test the latter possibility in fish cell cultures because marine fish in general have a very limited ability to convert 18:3n-3 to 20:5n-3 and thence to 22:6n-3 (7,8). In some species of marine fish, e.g. turbot, this appears to be due to a deficiency of C₁₈ to C₂₀ fatty acid elongase (9), whereas in others, e.g. sea bream, it appears to be due to a deficiency of Δ 5 fatty acid desaturase (10). The availability of 18:5n-3 can help distinguish between these two possibilities and, in the event of it being a substrate for C₁₈ to C₂₀ fatty acid elongase, algae containing this fatty acid could be useful dietary supplements in marine fish larval culture.

In this study we prepared 18:5n-3 from the haptophycean alga *Isochrysis galbana* and also by chemical synthesis from 22:6n-3, and studied its metabolism in cultured cells from turbot, seabream and Atlantic salmon that differ in their abilities to perform C₁₈ to C₂₀ elongation and Δ 5 fatty acid desaturation reactions. The results show that 18:5n-3 is very readily converted by cells from all three species to 18:4n-3 via a 2-*trans* 18:5n-3 intermediate.

MATERIALS AND METHODS

Fatty acid substrates. The methyl esters of [U-¹⁴C] 18:4n-3 and [U-¹⁴C] 18:5n-3 were prepared from cultures of *Isochrysis galbana* (Parke) (S.M.B.A. strain No. 58 C.C.A.P. strain 927/1) grown in H¹⁴CO₃ as described by Ghioni *et al.* (9). In brief, total lipid was extracted from the

radioactive algal cells, transmethylated by incubation with 1% sulphuric acid in methanol at 50°C for 16 hours to generate fatty acid methyl esters, and the methyl esters of 18:4n-3 and 18:5n-3 isolated by silver nitrate thin-layer chromatography (AgNO_3 – TLC) (11). A total of 5 μCi of methyl esters of both $[\text{U-}^{14}\text{C}]18:4\text{n-}3$ and $[\text{U-}^{14}\text{C}]18:5\text{n-}3$ were obtained, with a specific activity of approximately 12 and 19 mCi/mmol , respectively. The identity of the 18:4n-3 and 18:5n-3 methyl esters was confirmed and their purity (>99%) and specific activity determined by radio-gas chromatography as (GC) as described by Buzzi *et al.* (12).

Unlabeled methyl esters of 18:5n-3 and 18:4n-3 were prepared by extracting total lipid from *I. galbana* cultures that were not incubated with labeled bicarbonate, transmethylating as described above and recovering the fatty acid methyl ester fraction enriched in polyunsaturated fatty acids (PUFA) by TLC in hexane/diethyl ether/acetic acid (90:10:1, by vol.). Methyl esters of 18:5n-3 and 18:4n-3 were then separated from the fatty acid methyl ester fraction on an ODS C18 HPLC column (diameter 5 mm) by eluting with acetonitrile at 1.5 ml/min, using UV detection at 215 nm. Under these conditions, 18:5n-3 was the first fatty acid eluted. The purity of the methyl esters of both 18:5n-3 and 18:4n-3 was > 99% as determined by GC and GC – mass spectrometry as detailed previously (9,13).

Unlabeled 18:5n-3 was also prepared in greater quantities as the free acid by chemical synthesis according to the method of Kuklev *et al.* (14), which involves a γ -iodo-lactonization of 22:6n-3. The 22:6n-3 used in the synthesis was a concentrate (> 95%) kindly supplied by Croda Universal Ltd., Hull, UK.

The methyl ester of unlabeled 2-*trans* 18:5n-3 was prepared by first saponifying up to 10 mg of all-*cis* 18:5n-3 methyl ester in 1M KOH in ethanol/water (95/5, v/v) at 78°C for 1 h. The solution was then acidified with HCl, extracted with isohexane/diethyl ether (1:1, v/v), evaporated to dryness and transmethylated and extracted as for the other fatty acid samples. The procedure yielded four compounds in constant relative proportions, all of which were confirmed as methyl esters of 18:5n-3 by EI^+ GC – mass spectrometry (13). The four isomers of 18:5n-3

methyl ester were separated by isocratic ODS-HPLC using acetonitrile as eluting solvent as described above. Isomer 1 (25 % of the total) had a retention time on HPLC corresponding to the original all-*cis* Δ 3,6,9,12,15-18:5 (18:5n-3) prepared from *I. galbana* or by chemical synthesis, and its chemical structure was confirmed by ¹H-NMR spectroscopy at 600MHz: δ 0.97(t, 3H, J=7.5 Hz, H-18), 2.07 (quintet, 2H, J=7.5Hz, H-17), 2.60 - 2.83 (4 overlapping t, 8H, H-5, H-8, H-11, H-14), 3.13(d, 2H, J=5.8 Hz, H-2), 3.68 (s, 3H, -OCH₃), 5.34 - 5.44, (8H, overlapping m, alkene-H) 5.53 - 5.62 (2H, overlapping m, alkene-H). Isomer 3 (62 % of the total) was obtained as a pure compound by the ODS-HPLC and identified as *trans* Δ 2, all-*cis* Δ 6,9,12,15-18:5 (2-*trans* 18:5n-3) by ¹H-NMR spectroscopy at 600 MHz: δ 0.96(t, 3H, J=7.5Hz, H-18), 2.07 (quintet, 2H, J=7.5Hz, H-17), 2.20 - 2.29(m, 4H, H-4, H-5), 2.78-2.84(m, 6H, H-8, H-11, H-14), 3.72 (s, 3H, -OCH₃), 5.27 - 5.43 (8H, overlapping m, *cis*-alkene-H), 5.85 (dt, 1H, J=15.6, 1.6 Hz, H-2), 6.96(dt, 1H, J=15.6, 6.6 Hz, H-3). The remaining two isomers, 2 (4 % of the total) and 4 (9 % of the total), could not be obtained in sufficient amounts for ¹H-NMR identification. Complete epoxidation of the isomeric methyl ester mixture with peracetic acid (15) yielded two distinct epoxide species: (a), 89.4% of the total, which accounts for the sum of isomers 1 and 3; (b), 10.6% of the total, which accounts for the sum of isomers 2 and 4.

Cell cultures. The Atlantic salmon (*Salmo salar*) cell line (AS) (16) was originally obtained from Dr. N. Frerichs (Virology Unit, Institute of Aquaculture, University of Stirling, U.K). The turbot (*Scophthalmus maximus*) cell line (TF) was supplied by Dr. B. Hill (Ministry of Agriculture, Food and Fisheries, Fish Diseases Laboratory, Weymouth, UK.). The gilthead seabream (*Sparus aurata* L.) cell line, SAF-1, developed from fin tissue without immortalization, was provided by Dr. M.C. Alvarez (Department of Cell Biology and Genetics, University of Malaga, Spain) (17).

Cell cultures were grown in 75 cm² flasks at 22°C in Leibovitz L-15 medium containing 10 mM HEPES and supplemented with 2mM glutamine, 50 IU/ml penicillin, 50 mg/ml

streptomycin and 10% fetal bovine serum (FBS). Approximately 24 h prior to experimentation the cells were subcultured into fresh medium as above except containing only 2% FBS. The cells were then incubated with fatty acid substrates for 6 days as follows. Labeled substrates were added in 50 μ l ethanol at a radioactive concentration of 0.25 μ Ci per flask containing 15 ml of medium, equivalent to a total mass of 0.021 μ mole (1.4 μ M) and 0.013 μ mole (0.87 μ M) for the methyl esters of [U- 14 C] 18:4n-3 and [U- 14 C] 18:5n-3, respectively. Unlabeled fatty acid methyl esters were added as above at a level of approximately 0.375 μ mole/flask providing a concentration of 25 μ M. All incubations/experiments were conducted in triplicate.

Preliminary experiments incubating AS cells with 25 μ M unlabeled 18:4n-3, added either as the fatty acid salt complexed to bovine serum albumin, or as the methyl ester complexed to bovine serum albumin, or as the methyl ester in ethanol, showed no differences in the metabolism of these substrates via desaturation and elongation to 20:4n-3 and 20:5n-3 (see 9). No methyl esters were detectable in total lipid extracted from cells incubated with methyl esters. Therefore, the methyl esters of both 18:4n-3 and 18:5n-3 (and 2-*trans* 18:5n-3) were used in order to avoid having to saponify the methyl ester of 18:5n-3 to its free fatty acid (see Results).

Lipid extraction and analysis. Methods for the extraction of total lipids from cells, preparing fatty acid methyl esters from total lipid, determining radioactivity in fatty acid methyl esters separated on AgNO₃, and analysing fatty acids by GC and radio – GC were as described in detail previously (9,10,12). The identities of individual radioactive fatty acid methyl esters separated by AgNO₃ for radioassay were confirmed by direct radio – GC analyses (12).

Materials. Sodium [14 C]bicarbonate (~ 50 mCi/mmol) was purchased from ICN Biomedicals Ltd. (Thame, UK). Thin layer chromatography (TLC) (20 cm x 20 cm x 0.25 mm) and high-performance TLC (10 cm x 10 cm x 0.15 mm) plates, pre-coated with silica gel 60, were obtained from Merck, (Darmstadt, Germany). All solvents were of HPLC grade (Rathburn

Chemicals, Walkerburn, Peebleshire, Scotland). Ecoscint A was purchased from National Diagnostic (Atlanta, Georgia). Leibovitz L-15 Medium, Hanks' balanced salt solution, FBS, Glutamine/penicillin/streptomycin (200 mM L-glutamate, 10000 U penicillin and 10 mg streptomycin per ml of 0.9% NaCl), HEPES buffer, fatty acid-free bovine serum albumin, trypsin/EDTA and standard octadecatetraenoic acid (all-*cis* 18:4n-3) were obtained from Sigma Chemical Co. Ltd. (Poole, UK).

Statistical Analysis. All results are presented as means \pm S.D. of three experiments. The statistical significance of differences between mean values in Table 1 obtained for each cell line were analysed by the Student's t-test with differences reported as significant if $P < 0.05$ (18).

RESULTS

The initial experiments in this study were performed with radiolabeled 18:4n-3 and 18:5n-3 isolated from the alga *Isochysis galbana* grown in the presence of radioactive bicarbonate. The fatty acids were prepared as methyl esters following transmethylation of total lipid extracted from the alga. In preparing the free acid by saponifying the methyl ester of 18:5n-3 we noted by TLC analyses of the reaction products that all-*cis* 18:5n-3 was present in low yield and accompanied by other unknown compounds. We compared the incorporation of 18:5n-3 free acid and its methyl ester into lipids of the three cultured cell lines chosen for study, adding the substrates to the cells in ethanol. No differences were found in the incorporation patterns for the free acid and its methyl ester. Therefore, to maximise the use of the limited amounts of 18:5n-3 methyl ester prepared from *I. galbana*, we routinely used the methyl ester of 18:5n-3 in subsequent experiments.

The results of the incorporation of the methyl esters of [U-¹⁴C] 18:4n-3 and [U-¹⁴C] 18:5n-3 into the three cell lines are shown in Table 1. In these experiments fatty acid methyl

esters were prepared from total lipid isolated from the cell cultures and then separated by AgNO_3 TLC for radioassay, with the results being confirmed by radio-GC. In no case was radioactive 18:5n-3 recovered from the cells. Rather, 18:4n-3, 20:4n-3 and 20:5n-3 were the major fatty acids labeled after incubation with either $[\text{U}-^{14}\text{C}]$ 18:4n-3 or $[\text{U}-^{14}\text{C}]$ 18:5n-3 in all three cell lines. The percentage distribution of radioactivity between 18:4n-3, 20:4n-3 and 20:5n-3 differed in the different cell lines depending upon the relative activities of C_{18} - C_{20} elongase and $\Delta 5$ desaturase. In the turbot cell line (TF) the percentages of radioactivity recovered in 18:4n-3, 20:4n-3 and 20:5n-3 were the same in cells incubated with $[\text{U}-^{14}\text{C}]$ 18:4n-3 and $[\text{U}-^{14}\text{C}]$ 18:5n-3, with 18:4n-3 containing a high percentage of radioactivity, 20:5n-3 a modest percentage and 20:4n-3 a minor percentage. A similar result was obtained for the seabream cell line (SAF-1) except that the percentages of radioactivity in 20:4n-3 and 20:5n-3 were essentially reversed compared to the turbot cell line. The Atlantic salmon (AS) cell line gave a different result in that the highest percentage of radioactivity was recovered in 20:5n-3, with both 18:4n-3 and 20:4n-3 having moderate percentages of radioactivity. The species (cell line) differences were emphasised by presenting the data as the summed products for each enzymic step in the pathway, with the marine fish cells clearly showing considerably lower C_{18} – C_{20} elongase activity and, in the case of sea bream cells, lower $\Delta 5$ desaturase activity compared to the salmon cells (Table 2). However, as with the marine fish cell lines, the percentage distribution patterns in the salmon cell line were the same for cells incubated with $[\text{U}-^{14}\text{C}]$ 18:4n-3 and cells incubated with $[\text{U}-^{14}\text{C}]$ 18:5n-3. Thus, the two radioactive substrates yielded the same result in a given cell line irrespective of the pattern of desaturation/elongation expressed by the cell line. Table 1 also shows that of the 0.25 μCi of radioactive fatty acid substrate added, averaged values of 32% and 23% of radioactivity were recovered as fatty acids from cells incubated with $[\text{U}-^{14}\text{C}]$ 18:4n-3 and $[\text{U}-^{14}\text{C}]$ 18:5n-3, respectively, from the three cell lines. However, the difference between the two substrates was significant only for the Atlantic salmon cell line.

An obvious explanation for the findings in Table 1 is that 18:5n-3 is rapidly converted to 18:4n-3 in the cells. This possibility, together with the apparent lability of 18:5n-3 during alkaline saponification of its methyl ester prompted us to examine the stability of 18:5n-3 in more detail. Therefore, we synthesised 18:5n-3 as the free acid from 22:6n-3 using the γ -iodo-lactonization of 22:6n-3 method as described by Kuklev *et al.* (14). Treating the 18:5n-3 product with aqueous alkaline generated four compounds, two major and two minor, as shown by HPLC analysis (Materials and Methods). The two major products were identified by ^1H NMR and GC as all-*cis* 18:5n-3 (25% of the total products) and 2-*trans* 18:5n-3 (62% of the total products). We then studied the metabolism of the methyl esters of the two isomers of 18:5n-3 (and 18:4n-3) in the Atlantic salmon cell line. As there was insufficient isotopically labeled 18:5n-3 available to produce labeled 2-*trans* 18:5n-3 using the method above, the experiment was performed with unlabeled fatty acid substrates at higher concentrations (25 μM) to enable analysis by conventional GC methods. The results showed that cells incubated with 25 μM of all three fatty acid substrates showed increased proportions of 18:4n-3, 20:4n-3 and, to a lesser extent, 22:4n-3 and 20:5n-3 in their lipids over time up to 24 h after supplementation (Table 3). There was no change in the proportions of 22:6n-3 or 22:5n-3 in cell cultures incubated with any of the three fatty acid substrates. The increased concentration of 18:4n-3 in cellular lipids was somewhat less with 2-*trans* 18:5n-3 than all-*cis* 18:5n-3, but the patterns were similar for all three substrates and broadly the same as those observed with the methyl esters of [$\text{U-}^{14}\text{C}$] 18:4n-3 and [$\text{U-}^{14}\text{C}$] 18:5n-3 in Table 1. The results in Table 3 also show that no 18:5n-3 was detected in lipids recovered from the cells incubated with any of the fatty acid substrates.

DISCUSSION

Octadecapentaenoic acid (all-*cis* 18:5n-3; Fig. 1) is present in classes of algae that have major roles in the marine environment (6). Thus, dinoflagellates can form large toxic blooms; the

haptophycean *Phaeocystis pouchetti* is a major source of scum polluting beaches and inshore waters of mainland European coasts; the haptophycean coccolithophore *Emiliana huxleyi* is a major source of marine CaCO₃ deposits; the latter two organisms are major sources of dimethyl sulphide implicated as a source of “acid rain”. 18:5n-3 can account for more than 15% of the total fatty acids in these organisms, being present in glycolipids where it can account for 66% of the total fatty acids (6). However, it is seldom if ever detected in significant amounts in phytoplankton – consuming animals (1), i.e. in zooplankton and some fish, consistent with its being rapidly metabolized by marine animals.

18:5n-3 coexists in the aforementioned organisms with 22:6n-3 and it is unusual in sharing with 22:6n-3 the property of having the maximum number of double bonds possible in a carbon chain. However, unlike 22:6n-3 whose terminal double bond is 4 carbons from the carboxyl terminus, the terminal double bond in 18:5n-3 is 3 carbons from the carboxyl terminus. The insertion of the last (Δ 4) double bond in 22:6n-3 occurs by a mechanism whereby 24:5n-3 is Δ 6 desaturated to 24:6n-3 which is then chain shortened in peroxisomes to 22:6n-3 (19). Similarly, the biosynthesis of 18:5n-3 is not thought to be possible solely through the action of desaturases and elongases with the conventional pathway from 18:3n-3 to 20:5n-3 proceeding via 18:4n-3 then 20:4n-3. However, by analogy with the biosynthesis of 22:6n-3, the formation of 18:5n-3 is probably straightforward, at least in principle, in that it can be formed directly from 20:5n-3 by chain shortening as postulated previously (6). The precise subcellular location(s) of these putative chain shortening steps in the biosynthesis of 22:6n-3 and 18:5n-3 in these marine phytoplankton is not known (20).

As its biosynthesis requires a special mechanism, so the catabolism of 22:6n-3 requires a special mechanism in that the 2-*trans*, 4-*cis* intermediate formed by the initial direct dehydrogenation of 22:6n-3 is converted by a 2,4 - dienoyl reductase to a 3-*trans* intermediate, which is then converted by a Δ^3, Δ^2 - enoyl isomerase to a 2-*trans* intermediate (21,22). The latter two enzymes are auxiliary enzymes to the multifunctional enzymes of β -oxidation required

for the β -oxidation of polyunsaturated fatty acids in both mitochondria and peroxisomes (23,24). The *2-trans* 22:6n-3 intermediate can then continue in the conventional β -oxidation pathway through hydration across the 2,3 *trans* double bond to yield the 3-hydroxy and then the 3-keto intermediate. Similarly, catabolism of 18:5n-3 requires, in principle, the operation of a Δ^3 , Δ^2 -enoyl isomerase to generate the *2-trans* intermediate required for entry into the β -oxidation pathway though, to our knowledge, this reaction has never been studied with 18:5n-3 (Fig.1).

The results here establish the ease with which all-*cis* 18:5n-3 can be converted to its *2-trans* isomer. Thus, all-*cis* 18:5n-3, chemically synthesised from 22:6n-3, was readily converted in high yield to its *2-trans* isomer by treatment with alkali in aqueous ethanol. The protons at C-2 of all-*cis* 18:5n-3 are both allylic to the *cis*-3,4-double bond and α - to the terminal carboxyl group, and hence should experience a reduction in pK_a (relative to, for example, the corresponding protons in 18:4n-3). These combined effects would be expected to result in an increased tendency of the 18:5n-3 to undergo enolization, particularly in protic solvents. Enolization results in the conjugation of the 3,4-double bond with the enol and hence the configurational stability of the 3,4-double bond may be compromised. Furthermore, when reprotonation of the intermediate enol occurs, it can do so either at C-2 (regenerating 18:5n-3 with either *cis*- or *trans*-stereochemistry at C-3), or at C-4, which would result in the conjugation of the double bond with the carboxylic acid. The latter process is expected to be thermodynamically preferred and would be expected to result in the preferential formation of the *trans* Δ^2 , all-*cis* $\Delta^6,9,12,15$ -18:5n-3 acid. The chemical isomerization of all-*cis* 18:5n-3 to *trans* Δ^2 , all-*cis* $\Delta^6,9,12,15$ -18:5n-3 has not, to our knowledge, been reported before. However, it may have been observed but misinterpreted in that, in reducing all-*cis* 18:5n-3 with hydrazine to determine double bonds, Napolitano *et al.* (25) noted a loss of 18:5n-3 and mentioned the occurrence of an extra peak of 18:4n-3 in gas chromatograms of the reaction products. It is possible that the extra peak was a *2-trans* 18:5n-3 isomer whose formation was prompted by availability of protons in the reaction.

In contrast to 18:5n-3, which is an intermediate in the conventional β -oxidation pathway of 22:6n-3 and 20:5n-3, 18:4n-3 is not, as one cycle of β -oxidation of 18:5n-3 results in the formation of 16:4n-3 via 2-*trans* 18:5n-3, 3-hydroxy 18:4n-3 and 3-oxo 18:4n-3 (26) (Fig.1). However, the first step in the β -oxidation of 18:4n-3 is the action of dehydrogenase to yield 2-*trans* 18:5n-3. Therefore, 2-*trans* 18:5n-3 is a common intermediate in the β -oxidation of both 18:5n-3 and 18:4n-3 (Fig.1).

When the various cell lines were incubated with [U-¹⁴C] 18:5n-3 methyl ester, no radioactive 18:5n-3 was detected in total lipid extracted from the cells. However, radioactivity was readily detected in 18:4n-3 and its elongated and further desaturated products, 20:4n-3 and 20:5n-3. Moreover, for a given cell line, the distributions of radioactivity in 18:4n-3, 20:4n-3 and 20:5n-3 from cells incubated with [U-¹⁴C] 18:5n-3 were essentially the same as those generated from the same cells incubated with [U-¹⁴C] 18:4n-3. Similarly, the increases in the mass % of 18:4n-3, 20:4n-3 and 20:5n-3 in Atlantic salmon cells incubated with 25 μ M 18:4n-3 were similar in cells incubated with 25 μ M all-*cis* 18:5n-3 and also with 25 μ M 2-*trans* 18:5n-3. The observed patterns are in accord with our previous findings and conclusions that Atlantic salmon cells convert 18:4n-3 to 20:5n-3 more readily than either turbot or sea bream cells, due to a relative deficiency in the C₁₈-C₂₀ fatty acid elongase in turbot cells and a relative deficiency in the Δ^5 fatty acid desaturase in the sea bream cells (9,10). Consequently, production of radiolabelled 22:5n-3 was higher in the salmon cells incubated with labelled 18:4n-3 and 18:5n-3 than in turbot and sea bream cells, but although 22:5n-3 is a poor substrate for retroconversion to 20:5n-3 and serves as a substrate for 22:6n-3 formation (27), there was virtually no production of 22:6n-3 in salmon cells similar to the marine fish cell lines. This is not unexpected as most established cells in culture lack the ability to produce substantial amounts of 22:6n-3 although the precise reason in enzymic terms is unknown (20). The results are also consistent with all-*cis* 18:5n-3 being readily converted to 18:4n-3 in all the cell lines, via a Δ^3 , Δ^2 -enoyl isomerase generating a 2-*trans* 18:5n-3 intermediate followed by a hydrogenase

(reversed dehydrogenase activity) operating on the *2-trans* 18:5n-3 intermediate to generate 18:4n-3 (Fig.2). The 18:4n-3 so produced is then available for elongation and desaturation reactions to generate 20:4n-3 and 20:5n-3 (Fig.2).

As noted earlier, some 32% and 23% of the radioactivity added as [U-¹⁴C] 18:4n-3 and [U-¹⁴C] 18:5n-3, respectively, was recovered from cellular total lipid as 18:4n-3, 20:4n-3 and 20:5n-3, the remainder presumably being converted to β -oxidation products (Fig.1). That 18:5n-3 is more readily β -oxidised than 18:4n-3 by the cells implies that the isomerisation of added all-*cis* 18:5n-3 to *2-trans* 18:5n-3 proceeds more readily in the cells than the 2,3 dehydrogenation of added 18:4n-3 to *2-trans* 18:5n-3. This may reflect the fact that the cells studied here were actively growing and dividing and, therefore, as much concerned with directing exogenous polyunsaturated fatty acids into biosynthetic pathways directed towards membrane lipid formation as into β -oxidation.

Irrespective of how the relevant anabolic and catabolic pathways are controlled, the ease of conversion of all-*cis* 18:5n-3 to *2-trans* 18:5n-3 and thence to 18:4n-3, demonstrated here in fish cells, readily accounts for the absence of 18:5n-3 from marine trophic levels higher than the algae.

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REFERENCES

1. Mayzaud, P., Eaton, C.A., and Ackman, R.G. (1976). The Occurrence and Distribution of Octadecapentaenoic Acid in a Natural Plankton Population. A Possible Food Chain Index, *Lipids* 11, 858-862.

2. Joseph, J.D. (1975) Identification of 3,6,9,12,12-Octadecapentaenoic Acid in Laboratory-Cultured Photosynthetic Dinoflagellates, *Lipids* 7, 395-403. 916.
3. Volkman, J. K., Smith, J.D., Eglinton, G., Forsberg, T.E.V., and Corner, E.D.S. (1981) Sterol and Fatty Acid Composition of Four Marine Haptophycean Algae, *J. Mar. Biol. Assoc. U.K.* 61, 509-527.
4. Sargent, J.R., Eilertsen, H.C., Falk-Petersen, S., and Taasen, J.P. (1985) Carbon Assimilation and Lipid Production in Phytoplankton in Northern Norwegian Fjords, *Mar. Biol.* 85, 109-116.
5. Dunstan, G.A., Volkman, J.K., Jeffrey, S.W., and Barrett, S.M. (1992) Biochemical Composition of Microalgae From the Green Algal Classes Chlorophyceae and Prasinophyceae. 2. Lipid Classes and Fatty Acids, *J. Exp. Mar. Biol. Ecol.* 161, 115-134.
6. Sargent, J.R., Bell, M.V., and Henderson, R.J. (1996) Protists as Sources of (n-3) Polyunsaturated Fatty Acids for Vertebrate Development in Protistological Actualities. Proc. 2nd Europ. Conf. Protistology and 8th Europ. Conf. Ciliate Biology. Clermond-Ferrand, France 21-26 July 1995. (Brugeroll, G., and Mignot, J.-P., eds.) pp. 54-64.
7. Sargent, J.R., Henderson, R.J., and Tocher, D.R. (1989) The Lipids in *Fish Nutrition* (Halver, J., ed.) 2nd Edition, pp.153-218, Academic Press, New York.
8. Sargent, J.R., Bell, J.G., Bell, M.V., Henderson, R.J., and Tocher, D.R. (1995) Requirement Criteria for Essential Fatty Acids, *J. Appl. Ichthyol.* 11, 183-198.
9. Ghioni, C., Tocher, D.R., Bell, M.V., Dick, J.R., and Sargent, J.R. (1999) Low C₁₈ to C₂₀ Fatty Acid Elongase Activity and Limited Conversion of Stearidonic Acid, 18:4n-3, to Eicosapentaenoic Acid, 20:5n-3, in a Cell Line from the Turbot, *Scophthalmus maximus* *Biochim. Biophys. Acta* 1437, 170-181.
10. Tocher, D.R., and Ghioni, C. (1999) Fatty Acid Metabolism in Marine Fish: Low Activity of

- Fatty Acyl $\Delta 5$ desaturation in Gilthead Sea Bream (*Sparus aurata*) Cells, *Lipids* 34, 433-440.
11. Christie, W.W. (1982) *Lipid Analysis*, 2nd Edition, Pergamon Press, Oxford.
 12. Buzzi, M., Henderson, R.J., and Sargent, J.R. (1996) The Desaturation and Elongation of Linolenic Acid and Eicosapentaenoic Acid by Hepatocytes and Liver Microsomes from Rainbow Trout (*Oncorhynchus mykiss*) Fed Diets Containing Fish Oil or Olive Oil, *Biochim. Biophys. Acta* 1299, 235-244.
 13. Tocher, D.R., Dick, J.R., and Sargent, J.R. (1995) Occurrence of 22:3n-9 and 22:4n-9 in the Lipids of the Topminnow (*Poeciliopsis lucida*) Hepatic Tumor Cell Line, PLHC-1, *Lipids* 30, 555-565.
 14. Kuklev, D.V., Latyshev, N.A., and Bezuglov, V.V. (1991) Synthesis of All-*cis*-Octadeca 3,6,9,12,15-Pentaenoic Acid, *Bioorganicheskaya Khimiya* 17, 1433-1436.
 15. Emken, E.A. (1971) *cis* and *trans* Analysis of Fatty Esters by Gas Chromatography: Octadecenoate and Octadecadienoate Isomers, *Lipids* 7, 459-465.
 16. Nicholson, B.L., and Byrne, C. (1973) An Established Cell Line from the Atlantic Salmon (*Salmo salar*), *J. Fish. Res. Bd. Can.* 30, 913-916.
 17. Bejar, J., Borrego, J.J., and Alvarez, M.C. (1997) A Continuous Cell Line from the Cultured Marine Fish Gilthead Seabream (*Sparus aurata* L.), *Aquaculture* 150, 143-153.
 18. Zar, J.H. (1984) in *Biostatistical Analysis*, Prentice-Hall, Englewood Cliffs, New Jersey.
 19. Voss, A. Reinhart, M., Sankarappa, S., and Sprecher, H. (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.
 20. Tocher, D.R., Leaver, M.J., and Hodgson, P.A. (1998) Recent Advances in the Biochemistry and Molecular Biology of Fatty Acyl Desaturases, *Prog. Lipid Res.* 37, 73-117.

21. Kilponen, J.M., Palosaari, P.M., Sormunen, R.T., Vihinen, M., and Hiltunen, J.K. (1992) Isoenzymes of Δ^3, Δ^2 -Enoyl-CoA Isomerase in Rat Liver in *New Developments in Fatty acid Oxidation* (Tanaka, K., and Coates, P.M., eds.) pp. 33-40, Alan R. Liss, New York.
22. Kilponen, J.M., and Hiltunen, J.K. (1993) β -Oxidation of Unsaturated Fatty Acids in Humans. Isoforms of Δ^3, Δ^2 -Enoyl-CoA-Isomerase, *FEBS Lett.* 322, 299-303.
23. Hiltunen, J.K., Filppula, S.A., Koivuranta, K.T., Siivari, K., Qin, Y.-M., and Hayrinen, H.M. (1996) Peroxisomal β -Oxidation and Polyunsaturated Fatty Acids, *Ann. N. Y. Acad. Sci.* 804, 116-128.
24. Gurvitz, A., Wabnegger, L., Yagi, A.I., Binder, M., Hartig, A., Ruis, H., Hamilton, B., Dawes, I.W., Hiltunen, J.K., and Rottensteiner, H. (1999) Function of Human Mitochondrial 2,4-Dienoyl-CoA Reductase and Rat Monofunctional Δ^3 - Δ^2 -Enoyl-CoA Isomerase in β -oxidation of Unsaturated Fatty Acids, *Biochem. J.* 344, 903-914.
25. Napolitano, G.E., Ratnayake, W.M.N., and Ackman, R.G. (1988) All-*cis*-3,6,9,12,15-Octadecapentaenoic Acid: A Problem of Resolution in the GC Analysis of Marine Fatty Acids, *Phytochemistry* 27, 1751-1755.
26. Gurr, M.I., and Harwood, J.L. (1991) in *Lipid Biochemistry: An Introduction*, 4th Edition, Chapman Hall, London.
27. Voss, A., Reinhart, M., and Sprecher, H. (1992) Differences in the interconversion between 20-carbon and 22-carbon (n-3) and (n-6) polyunsaturated fatty acids in the rat liver, *Biochim. Biophys. Acta* 1127, 33-40.

Legends to Figures

FIG.1. Section of the β -oxidation pathway for n-3 polyunsaturated fatty acids showing the position of 2-*trans* 18:5n-3 as a common intermediate in the β -oxidation of 18:5n-3 and 18:4n-3.

FIG. 2. Reaction scheme whereby 18:5n-3 is converted to 18:4n-3 and thence to 20:5n-3 in cultured fish cells.

Fig.1

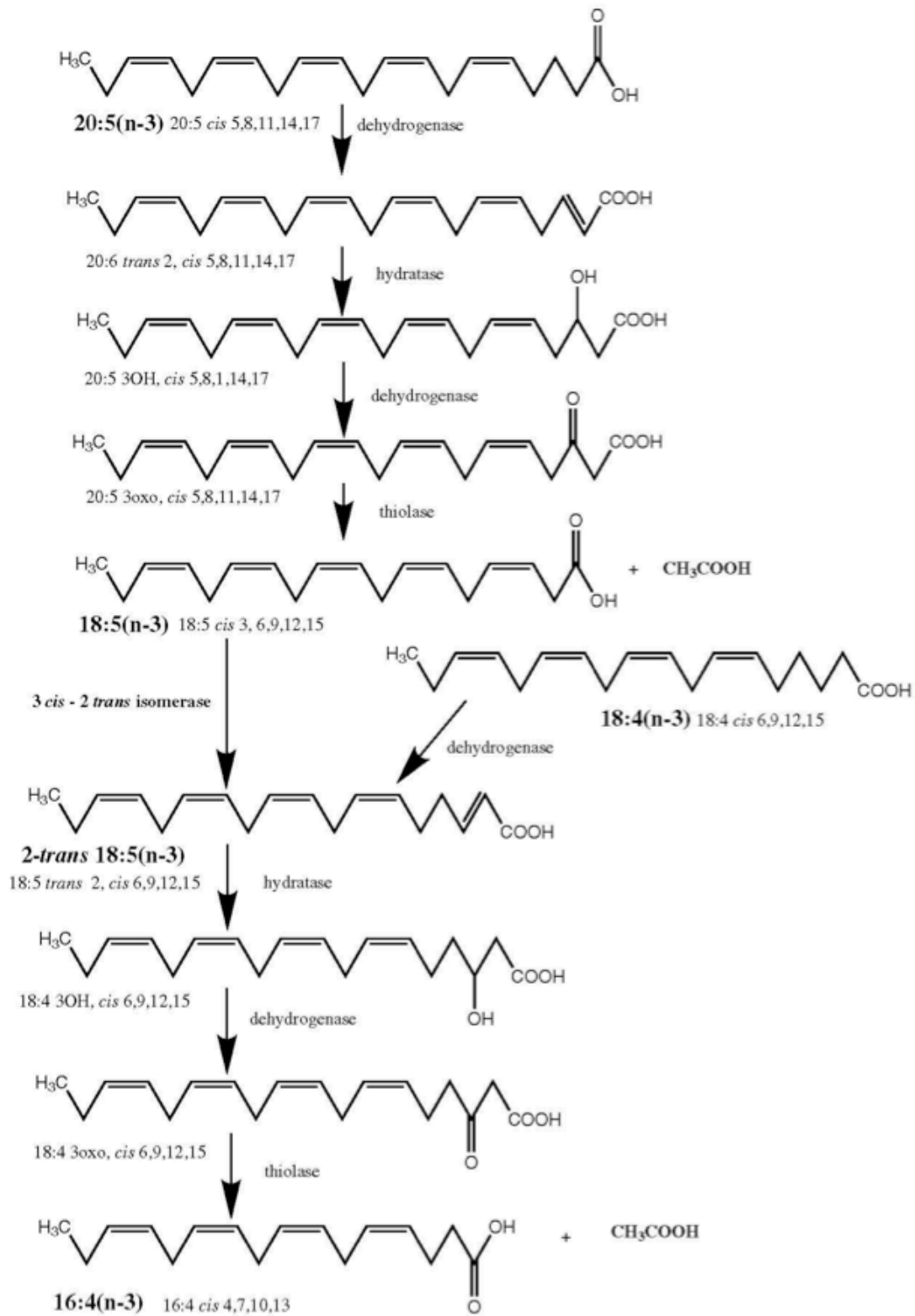


Fig.2

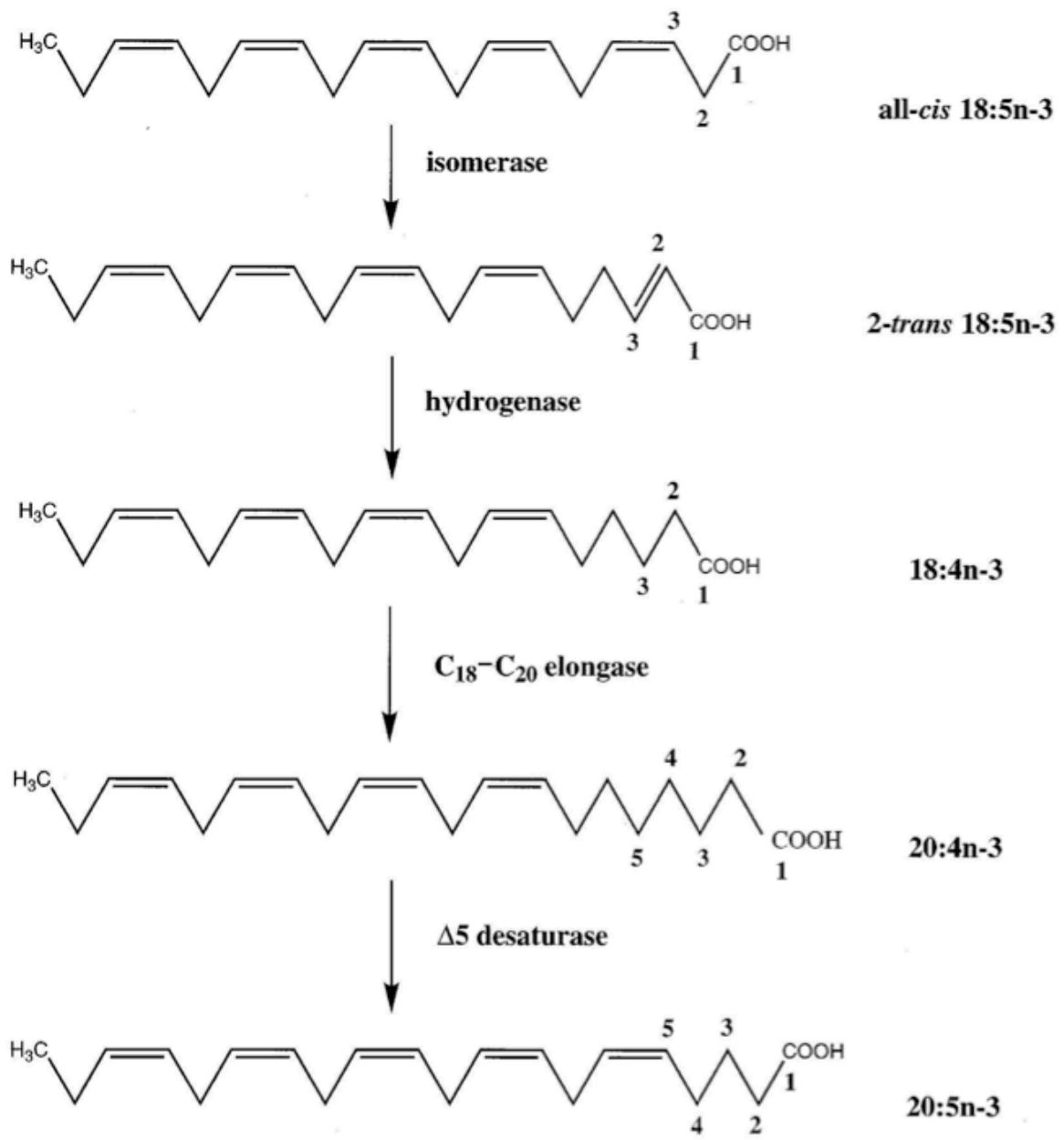


TABLE 1

Incorporation of Radioactivity into Total Lipid and Fatty Acids in Fish Cell Cultures after Supplementation with [U-¹⁴C] 18:4n-3 and [U-¹⁴C] 18:5n-3.

	TF			SAF-1			AS		
	¹⁴ C-18:4	¹⁴ C-18:5		¹⁴ C-18:4	¹⁴ C-18:5		¹⁴ C-18:4	¹⁴ C-18:5	
Total lipid									
Total μ Ci	0.07 \pm 0.01	0.06 \pm 0.01		0.08 \pm 0.02	0.06 \pm 0.01		0.09 \pm 0.01	0.05 \pm 0.01*	
pmol/mg total lipid	2.6 \pm 0.3	2.7 \pm 0.3		3.4 \pm 0.9	2.7 \pm 0.3		3.0 \pm 0.3	2.5 \pm 0.1	
Fatty acid									
18:4n-3	74.1 \pm 0.8	76.7 \pm 0.5*		81.0 \pm 2.0	82.6 \pm 0.6		18.8 \pm 1.0	24.0 \pm 2.5*	
18:5n-3	n.d.	n.d.		n.d.	n.d.		n.d.	n.d.	
20:4n-3	4.4 \pm 0.0	4.5 \pm 0.3		13.2 \pm 1.4	10.3 \pm 1.2		23.6 \pm 1.3	23 \pm 1.7	
20:5n-3	16.4 \pm 0.5	14.8 \pm 0.8		0.7 \pm 0.5	1.1 \pm 0.6		48.4 \pm 1.7	46 \pm 2.2	
22:4n-3	0.8 \pm 0.1	1.1 \pm 0.3		5.1 \pm 0.2	6.0 \pm 0.5		1.2 \pm 0.3	1.1 \pm 0.3	
22:5n-3	1.6 \pm 0.3	1.2 \pm 0.1		n.d.	n.d.		4.5 \pm 0.5	3.3 \pm 0.7	
22:6n-3	n.d.	n.d.		n.d.	n.d.		1.7 \pm 0.3	n.d.*	

The [U-¹⁴C]18:4n-3 and [U-¹⁴C]18:5n-3 had specific activities of approximately 12 and 19 mCi/mmol, respectively, and were added to cell cultures as methyl esters in 50 μ l ethanol at an isotopic concentration of 0.25 μ Ci per flask containing 15 ml of medium, equivalent to 0.021 μ mole (1.4 μ M) and 0.013 μ mole (0.87 μ M) for [U-¹⁴C]18:4n-3 and [U-¹⁴C]18:5n-3, respectively. Data for incorporation into fatty acid fractions are percentages of total radioactivity recovered in fatty acids. All results are presented as means \pm S.D. of triplicate experiments. The statistical significance of differences between mean values obtained for each cell line were analysed by the Student's t-test with differences reported as significant if $P < 0.05$. n.d., not detected; AS, Atlantic salmon cells; SAF-1, sea bream cells; TF, turbot cells.

Enzyme activity	TF						SAF-1						AS								
	¹⁴ C-18:4			¹⁴ C-18:5			¹⁴ C-18:4			¹⁴ C-18:5			¹⁴ C-18:4			¹⁴ C-18:5					
C18-20 elongase	25.9	±	0.8	21.6	±	0.4	*	19.0	±	2.1	17.4	±	0.5	81.2	±	1.0	73.8	±	2.4		
delta-5 desaturase	19.5	±	0.5	17.7	±	0.9		0.7	±	0.4	1.1	±	0.6	56.4	±	2.6	51.7	±	2.8		
C20-22 elongase	5.1	±	0.5	2.3	±	0.4	*	5.1	±	0.3	6.0	±	0.5	*	9.2	±	0.7	4.5	±	0.9	*
delta-6' desaturase		n.d.			n.d.				n.d.			n.d.		1.7	±	0.3		n.d.		*	

All results (percentage of total radioactivity recovered) are presented as means ± S.D. of triplicate experiments. The statistical significance of differences between mean values obtained for each cell line were analysed by the Student's t-test with differences reported as significant if P < 0.05 (denoted by *). delta-6' desaturase, formerly termed delta-4 desaturase; n.d., not detected; AS, Atlantic salmon cells; SAF-1, sea bream cells; TF, turbot cells.

TABLE 3

Selected Fatty Acids in Composition of Total Lipid of Atlantic Salmon (AS) Cells Harvested at Different Times after Addition of 18:4n-3, all-cis18:5n-3 and 2-trans 18:5n-3.

Fatty acid	18:4n-3					all-cis18:5n-3					2-trans 18:5n-3		
	1h	3h	5h	24h	48h	1h	3h	5h	24h	48h	5h	24h	48h
18:4n-3	1.0 ± 0.2	2.3 ± 0.4	5.3 ± 0.8	8.9 ± 1.1	3.8 ± 1.4	1.0 ± 0.2	1.7 ± 0.4	6.9 ± 1.6	11.8 ± 0.4	4.3 ± 1.2	1.4 ± 0.1	3.9 ± 0.6	3.7 ± 2.5
18:5n-3	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
20:4n-3	0.1 ± 0.1	0.2 ± 0.1	0.6 ± 0.1	1.3 ± 0.4	1.1 ± 0.4	n.d.	n.d.	0.5 ± 0.2	1.4 ± 0.1	0.8 ± 0.5	0.4 ± 0.1	1.2 ± 0.1	1.5 ± 1.1
20:5n-3	1.3 ± 0.3	1.5 ± 0.2	0.6 ± 0.2	2.0 ± 0.5	1.4 ± 0.6	1.5 ± 0.1	1.2 ± 0.3	1.6 ± 0.5	2.0 ± 0.1	1.0 ± 0.4	1.5 ± 0.1	1.5 ± 0.2	1.1 ± 0.8
22:4n-3	0.5 ± 0.4	0.6 ± 0.3	0.8 ± 0.1	1.0 ± 0.5	0.7 ± 0.3	1.0 ± 0.2	1.1 ± 0.3	0.7 ± 0.2	1.1 ± 0.4	0.5 ± 0.2	0.5 ± 0.1	0.9 ± 0.6	0.8 ± 0.6
22:5n-3	3.3 ± 0.5	3.1 ± 0.4	3.2 ± 0.2	3.4 ± 0.4	3.1 ± 0.4	3.1 ± 0.1	3.6 ± 0.3	3.5 ± 0.4	3.5 ± 0.6	2.8 ± 0.5	3.4 ± 0.2	3.4 ± 0.4	2.8 ± 0.6
22:6n-3	5.1 ± 0.6	4.5 ± 0.4	4.9 ± 0.3	5.0 ± 0.6	4.9 ± 0.5	4.8 ± 0.3	5.1 ± 0.4	5.4 ± 0.7	4.9 ± 0.5	4.8 ± 0.7	4.8 ± 0.5	5.0 ± 0.6	4.6 ± 0.7

All supplemented fatty acids were unlabeled and added as methyl esters in 50 µl ethanol at a fatty acid concentration of 25 µM as described in detail in Materials and Methods. Results are expressed as % of weight of total fatty acids and are mean values ± S.D. of three determinations. n.d., not detected.