Alternate pathways in the desaturation and chain elongation of linolenic acid, 18:3(n–3), in cultured glioma cells

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Abstract Cultured C6 glioma cells rapidly incorporate and metabolize the essential fatty acids, 18:2(n-6) and 18:3(n-3), to 20- and 22-carbon polyunsaturated fatty acids. Using several deuterated fatty acid substrates we have obtained data that suggest alternate pathways, one possibly involving Δ^8-desaturation, may exist in glioma cells for formation of 20:5(n-3) and 22:6(n-3) from 18:3(n-3). With 18:3(n-3)-6,6,7,7-d_4 practically no 18:4(n-3)-6,7-d_2 or 20:4(n-3)-8,9-d_2 was detected whereas 20:3(n-3)-8,8,9,9-d_4 accounted for 3.4% and $\Delta^{5,11,14,17}$-20:4-8,8,9,9-d_4 for 21.1% of the total deuterated fatty acids recovered in phospholipids after a 16 h incubation; 20:5(n-3)-8,9-d_2, 22:5(n-3)-10,11-d_2, and 22:6(n-3)-10,11-d_2 accounted for 42.4%, 13.2%, and 2.8% of deuterated acyl chains, respectively. When added exogneously, 20:3-8,8,9,9,-d_4 was extensively converted to $\Delta^{5,11,14,17}$-20:4(n-3)-8,8,9,9-d_4 (45%) and 20:5(n-3)-8,9-d_2 (24%); a small amount (4%) of 18:3(n-3)-d_4 also was detected. Both 20:4(n-3)-8,9-d_2 and 18:4(n-3)-12,13,15,16-d_4 were also converted to 20:5(n-3) and 22:6(n-3) with 8 and 0% of the respective original deuterated substrate remaining after 16 h. A possible pathway for 18:3(n-3) metabolism in glioma cells is described whereby an initial chain elongation step is followed by successive Δ^5 and Δ^8 desaturation reactions resulting in 20:5(n-3) formation and accounting for the ordered removal of deuterium atoms. Alternatively, extremely effective retroconversion may occur to chain shorten 20:3(n-3)-d_4 to 18:3(n-3)-d_4 followed by rapid conversion through the classical desaturation and chain elongation sequence. The relative contribution of these possibilities cannot be quantitated with labeled isomers currently available. Whether these alternate routes of polyunsaturated fatty acid metabolism have physiological significance in other cell types or tissues, or may relate to the nutritional state, such as essential fatty acid deficiency, remains unresolved but such possibilities for 20:5(n-3) formation should be considered particularly when the classical pathway may be impaired—**Cook, H. W., D. M. Byers, F. B. St. C. Palmer, M. W. Spence, H. Rakoff, S. M. Duval, and E. A. Emken.** Alternate pathways in the desaturation and chain elongation of linolenic acid, 18:3(n-3), in cultured glioma cells. *J. Lipid Res.* 1991. **32:** 1265–1273.

Supplementary key words essential fatty acid metabolism • deuterated fatty acids

Polyunsaturated fatty acids (PUFA) of the (n-6) and (n-3) families or their precursors are essential dietary components for mammalian species (1, 2). The essential fatty acids, linoleic [18:2(n-6)] and linolenic [18:3(n-3)] acids, are metabolized to longer chain PUFA by a combination of desaturation and chain elongation reactions (3–5). The major products that accumulate in most tissues, esterified to complex neutral and phospholipids, are primarily arachidonate [20:4(n-6)] and docosapentaenoate [22:5(n-6)] from 18:2(n-6) and eicosapentaenoate [20:5(n-3)] and docosahexaenoate [22:6(n-3)] from 18:3(n-3). All major 20- and 22-carbon PUFAs appear to be necessary components of membrane phospholipids for optimal biological function particularly in specialized cells and tissues such as brain, retina, testes, heart, liver, and kidney (6). The constitutive properties of PUFA in membranes generally are considered in terms of their contributions to the fluidity or integrity of membrane bilayer structures (7). In addition, the major (n-6) product, 20:4(n-6), is a prime precursor of eicosanoids and other oxygenated derivatives with specific biological activities (8). Increasing evidence for competitive interactions between PUFA of the (n-6) and (n-3) fatty acid families at the level of formation and action of thromboxanes, prostacyclins, prostaglandins, and leukotrienes appear to relate directly to potential benefits attributed to an appropriate dietary balance of the two families of fatty acids (9, 10).

Classical pathways for conversion of both essential fatty acid precursors have been described whereby a sequence of desaturation (Δ^6, then Δ^5 and Δ^4) reactions, alternating

Abbreviations: PUFA, polyunsaturated fatty acids; GLC-MS, gas–liquid chromatography–mass spectrometry.

with intervening 2-carbon chain elongation, accounts for all primary products that accumulate from the 18-carbon dietary acids. Competitive studies (11–14) have supported existence of common enzymes used by the main precursors and intermediates of the (n–6) and (n–3) families. A generally higher affinity of (n–3) acids for these enzymes results in (n–3) acids being more rapidly metabolized than their (n–6) counterparts, particularly when present in excess or in equimolar mixtures (13–16).

There is, however, evidence to suggest that alternate means or sequences of desaturation and chain elongation may exist under some circumstances. In a recent study with C6 rat glioma cells (17), we observed distinct differences in inhibition of conversion of 18:2(n–6) to 20:4(n–6) compared to 18:3(n–3) conversion to 20:5(n–3), particularly at the Δ^5 desaturation step, when a range of positional isomers of both *cis* and *trans* geometric configuration were tested as competitors. Earlier observations (18–20) of apparently differential interactions of both activating and competing fatty acid isomers indicated a level of complexity beyond identical or simple competition at each step of a common enzyme sequence. Explanations for such observations include the possibilities that alternate enzymes or sequences are utilized or that different modes of regulation are operative in conversion of (n–6) and (n–3) families of fatty acids (17).

In the present study, we have taken advantage of the fact that C6 glioma cells in culture rapidly incorporate exogenous fatty acid from the culture medium and extensively convert 18:2(n–6) and 18:3(n–3) to their primary products, 20:4(n–6) and 20:5(n–3), respectively. We concentrated on the pathway leading to long chain (n–3) PUFA formation using several deuterated (n–3) fatty acids which facilitated assessment of the order of hydrogen (or deuterium) extraction during desaturation reactions relative to chain elongation. This study suggests that, at least in this C6 glioma cell line, alternate pathways for formation of (n–3) PUFA should be considered.

MATERIALS AND METHODS

Materials

Chemicals and reagents used were the finest grade, obtained from commercial suppliers. [1-^{14}C]18:2(n–6) (59 mCi/mmol; 1 Ci = 37 GBq) and [1-^{14}C]18:3(n–3) (55 mCi/mmol) were purchased from Dupont Canada Inc. (NEN Products, Lachine, Que.). Purity of these acids, determined by several analytical procedures described previously (21), was >97%.

Fatty acid isomers containing deuterium substitutions were synthesized as described previously (22–25). Standard 20:4(n–6) and other fatty acids were purchased from Serdary Research Laboratories (London, ON) or Sigma Chemical Co. (St. Louis, MO). The purities of fatty isomers were determined by thin-layer chromatography, gas–liquid chromatography, and mass spectrometry, as previously described (22, 23, 26). 18:2(n–6)-15,15,16,16-d_4, 18:3(n–3)-6,6,7,7-d_4, and 20:3(n–3)-8,8,9,9-d_4 were >97% d_4; 20:4(n–3)-8,9-d_2 was 94.2% d_2 and 4.8% d_4; 18:4(n–3)-12,13,15,16-d_4 was 67.1% d_4, 24.5% d_3, and 4.8% d_2. Fatty acid solutions (20 mg/ml) were maintained under N_2. All solvents used were high performance liquid chromatography grade from Fisher Scientific or BDH Chemicals, Halifax, N.S.

Glioma cell cultures

Rat C6 glioma cells lines were maintained in 150-cm^2 flasks (Corning Glass Works, Corning, NY) in 45 ml Dulbecco's modified Eagle's medium, containing 10% fetal bovine serum (Gibco Canada Ltd., Burlington, Ont.) and penicillin–streptomycin solution (100 U and 100 μg/ml, respectively) (18). Cells were maintained at 37°C in a humidified atmosphere of 95% air–5% CO_2 and subcultured every 5–7 days. For incorporation and metabolism studies, cells were transferred to 60-mm diameter dishes at a density of 0.5 × 10^6–1.0 × 10^6 cells in 5 ml medium. After 72 h, the medium was removed and replaced with 2 ml Dulbecco's modified Eagle's medium without fetal bovine serum. After another 24 h, mixtures of deuterium or [1-^{14}C]labeled fatty acids, with or without unlabeled arachidonate acid, were suspended by sonication at 37°C in sterile 5% bovine serum albumin, and added in a 100 μl volume of the albumin suspension to give a final fatty acid concentration of 50 μM in the incubation medium.

For each separate analysis five dishes of cells were incubated for 16 h at 37°C as indicated above.

Extraction of lipids

Details of the extraction and analyses procedures have been published previously (18, 27–29). In brief, after incubation the cells were harvested, washed with phosphate-buffered saline, and centrifuged. An aliquot was removed for analysis of protein by the method of Lowry et al. (30) with bovine serum albumin as standard. When 1-^{14}C-labeled acids were used as controls, aliquots of medium and cell washes were counted for total medium radioactivity. In all cases the cell pellet was extracted and the lipid extract was separated into neutral lipid and phospholipid fractions by silicic acid chromatography (31). Fatty acyl methyl esters were formed using 10% BF_3 in methanol as previously described (32).

GLC–mass spectrometry analyses

Methyl esters of the phospholipid fractions obtained from lipid extracts of the cultured cells were analysed using a GLC–MS procedure previously published (33).

Methyl esters prepared from *Ephedra campylopoda* seed oil were used to determine GLC retention times for 5c,11c,14c-20:3 and 5c,11c,14c,17c-20:4 (34).

RESULTS

Fatty acyl chain profile

When C_6 glioma cells were incubated with deuterated fatty acids, capillary GLC analysis of methyl esters obtained from the total phospholipid fraction, coupled with continuous mass spectroscopy detection of the column effluent, allowed qualitative analysis of both the natural abundance acyl chains and those derived from the exogenous deuterated acids. A typical total ion profile of the polyunsaturated fatty acyl chains obtained by GLC-MS is illustrated in **Fig. 1** and quantitation of phospholipid acyl chain endogenous to the C6 cells is shown in **Table 1**. Under standard growth conditions, saturated and monoenoic acids accounted for approximately 80% of phospholipid acyl chains. Glioma cells contained a relatively low (<1%) amount of the essential fatty acids 18:2(n-6) and 18:3(n-3). Arachidonic acid, 20:4(n-6), was the predominant polyunsaturated fatty acid that accumulated in the membrane phospholipids. Presence of 20:3(n-9) suggested insufficient availability of essential fatty acids during some stages of growth of the cells. Polyunsaturated fatty acids of the (n-3) family were primarily of 22-carbon length (22:5(n-3) and 22:6(n-3)) with relatively little

TABLE 1. Profile of total fatty acyl chains in the phospholipid fraction of cultured glioma cells, with or without a 16-h preincubation in the presence of 40 μM 20:4 (n-6)

Fatty Acyl Chain	Control	40 μM 20:4(n-6) Preincubation
14:0	0.35 ± 0.15[a]	0.39 ± 0.36[b]
16:0	7.77 ± 2.80	19.18 ± 3.55
16:1[c]	4.36 ± 0.42	4.48 ± 0.82
17:0	0.68 ± 0.35	0.77 ± 0.16
17:1	1.24 ± 0.16	1.00 ± 0.25
18:0	16.24 ± 0.86	15.63 ± 0.66
18:1(n-7) and (n-9)	39.62 ± 2.32	35.59 ± 2.54
18:2(n-6)	0.82 ± 0.37	0.86 ± 0.11
18:3(n-6)	0.07 ± 0.07	0.09 ± 0.09
20:0	0.18 ± 0.04	0.12 ± 0.06
20:1(n-9)	1.34 ± 0.22	0.92 ± 0.22
20:2(n-6)	0.31 ± 0.22	0.18 ± 0.16
20:3(n-6)	0.49 ± 0.06	0.46 ± 0.07
20:3[d]	0.17 ± 0.17	0.21 ± 0.21
20:3(n-9)	3.60 ± 2.59	2.18 ± 2.23
20:4(n-6)	5.76 ± 1.87	11.71 ± 1.07
20:5(n-3)	0.20 ± 0.08	0.21 ± 0.04
22:0	0.21 ± 0.09	0.14 ± 0.09
22:1(n-9)	0.39 ± 0.21	0.11 ± 0.12
22:3(n-3)	0.03 ± 0.04	0.02 ± 0.04
22:4(n-6)	0.84 ± 0.29	1.50 ± 0.45
22:5(n-3)	1.38 ± 0.50	1.00 ± 0.61
22:5(n-6)	0.29 ± 0.06	0.53 ± 0.21
22:6(n-3)	1.98 ± 0.49	1.79 ± 0.46
24:0	0.42 ± 0.18	0.26 ± 0.07
24:1	1.15 ± 0.40	0.63 ± 0.44

[a]Mean ± SD; n = 6.
[b]Mean ± SD; n = 4.
[c]Two isomer forms, probably (n-9) and (n-7).
[d]Primarily $\Delta^{11,14,17}$-20:3(n-3) but could include $\Delta^{8,14,17}$-20:3.

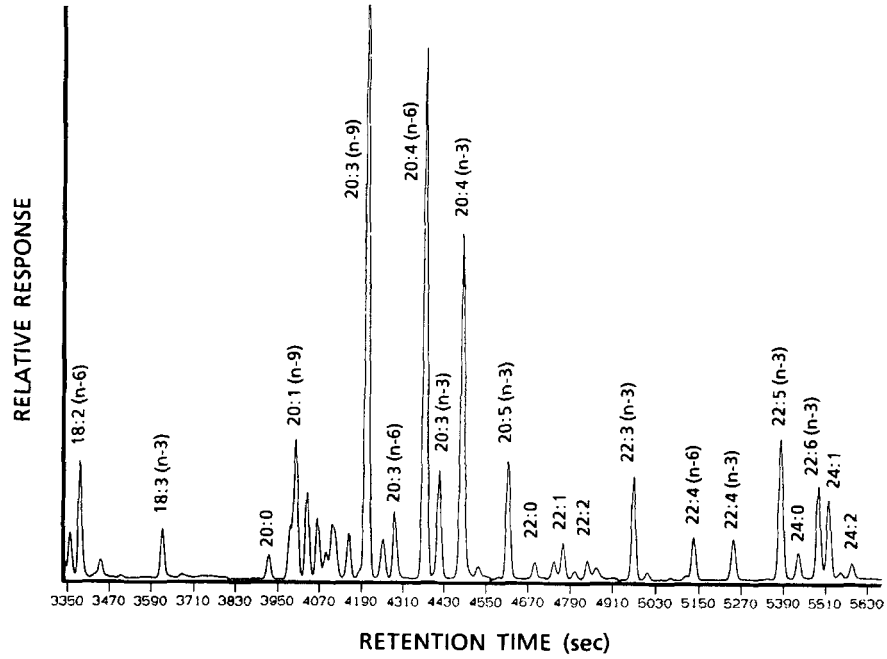

Fig. 1. Polyunsaturated portion of gas-liquid chromatography profile of total ion detection of methyl esters from glioma phospholipids. C6 glioma cells were incubated with 50 μM 20:3(n-3)-8,8,9,9-d_4 for 16 h and methyl esters were obtained from phospholipids of the cell extract. Methyl esters were separated on a 30-m Supelcowax-10 column and quantitated using total ion detection by MS as described under Materials and Methods.

TABLE 2. Conversion of 18:2(n-6)-15,15,16,16-d_4 during a 16-h incubation in cultured glioma cells, with or without 40 μM 20:4(n-6) in the incubation

Fatty Acyl Chain	Control	40 μM 20:4 (n-6)
18:2(n-6)-d_4	21.3[a]	14.8
18:3(n-6)-d_4	1.1	0.8
20:2(n-6)-d_4	2.1	2.8
20:3(n-6)-d_4	8.7	5.7
20:3-d4[b]	1.9	1.9
20:4(n-6)-d_4	58.2	62.6
22:4(n-6)-d_4	5.4	8.0
22:5(n-6)-d_4	1.0	3.1

[a]Values are percentage of total deuterated acyl chains and are the mean of duplicate determinations.

[b]Unidentified isomer; suspected to be $\Delta^{5,11,14}$-20:3-d_4 based on retention time compared to seed oil standard.

eicosapentaenoic acid (20:5(n-3)). Supplementation of the medium with 20:4(n-6) followed by a 16-h incubation (Table 1) increased the 20:4(n-6) content about twofold and increased the amount of 22:4(n-6) and 22:5(n-6) that accumulated.

Metabolism of deuterated 18:2(n-6)-d_4

Previous studies with [1-^{14}C]18:2(n-6) (15-18, 35) that showed that >70% of label from 18:2(n-6) was inside the cells by 16 h and that >80% of this label was esterified to phospholipids were confirmed by parallel experiments with [1-^{14}C]18:2(n-6) in this study also; <1% of the label was as free fatty acid with the remainder predominantly in triacylglycerol. The major metabolite of 18:2(n-6)-15,15,16,16-d_4 formed by desaturation and chain elongation was 20:4(n-6)-d_4. Some further elongation and desaturation of 20:4(n-6)-d_4 to 22:4(n-6)-d_4 and 22:5(n-6)-d_4 was observed as well as accumulation of 20:3(n-6)-d_4 an anticipated intermediate between 18:2(n-6)-d_4 and 20:4(n-6)-d_4. Other minor intermediates and products were also detected.

Our previous studies (18-20) showed that simultaneous incubation with exogenous 20:4(n-6) actually enhanced conversion of [1-^{14}C]18:2(n-6). This was also observed with 18:2(n-6)-d_4 as exogenous substrate. Conversion of 18:2(n-6)-d_4 to 20:4(n-6)-d_4, 22:4(n-6)-d_4 and 22:5(n-6)-d_4 was greater in the presence of 40 μM exogenous 20:4(n-6) (**Table 2**) but extensive additional activation

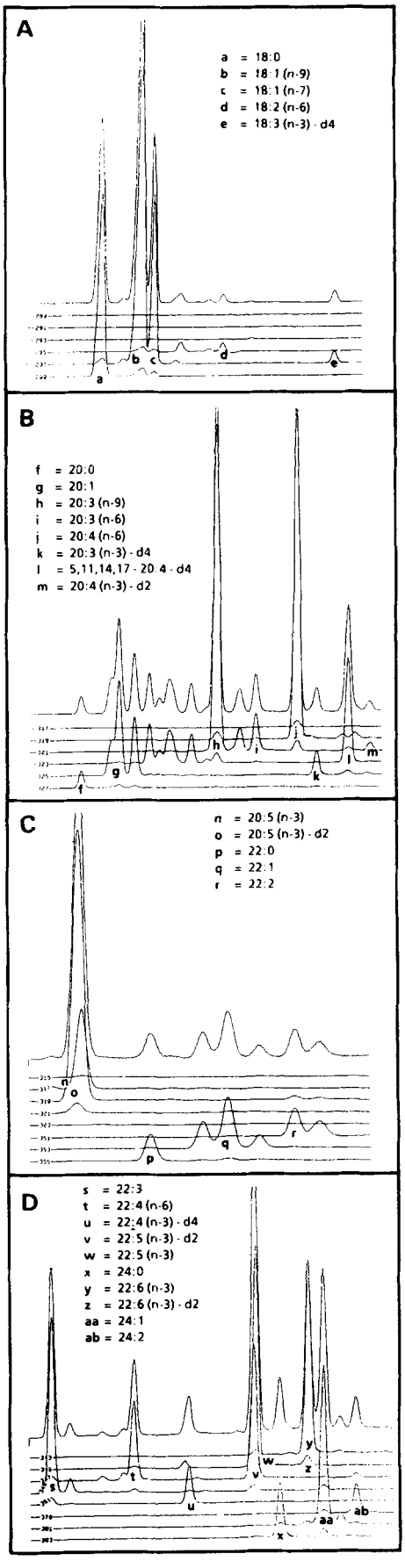

Fig. 2. Portions of the gas-liquid chromatography profile of methyl esters from glioma cells incubated with 18:3(n-3)-6,6,7,7-d_4. C6 glioma cells were incubated for 16 h with 50 μM 18:3(n-3)-6,6,7,7-d_4 and methyl esters were obtained from total phospholipids of the cell extract. Methyl esters were separated by GLC and profiles for selected molecular weights, indicated at the left side of each separate profile within panels, were obtained. The top tracing in each panel is the total mass (sum of all molecular weights) for that retention time. Approximate corresponding retention times (min) for each panel were: panel A, 50-60; panel B, 60-75; panel C, 75-82; panel D, 82-94.

TABLE 3. Conversion of 18:3(n-3)-6,6,7,7-d_4, 20:3(n-3)-8,8,9,9-d_4, 20:4(n-3)-8,9-d_2 or 18:4(n-3)-12,13,15,16-d_4 during a 16-h incubation with cultured glioma cells

Fatty Acyl Chain	18:3 (n-3) 6,6,7,7-d_4	20:3 (n-3) 8,8,9,9-d_4	20:4 (n-3) 8,9-d_2	18:4 (n-3) 12,13,15,16-d_4
18:3(n-3)-d_4	11.5 ± 1.1[a]	4.2 ± 0.5	ND	ND
20:3(n-3)-d_4	3.4 ± 0.2	11.1 ± 0.3	ND	ND
20:4(n-3)-d_2	0.6 ± 0.3	0.8 ± 0.0	7.6 ± 0.3	0.8 ± 0.1
20:4-d_4[b]	21.1 ± 2.1	45.0 ± 0.4	ND	5.6 ± 0.2
20:5(n-3)-d_2	42.4 ± 2.0	23.8 ± 0.7	63.0 ± 0.1	6.9 ± 0.1
20:5(n-3)-d_4	0.4 ± 0.0	0.4 ± 0.1	ND	59.7 ± 0.2
22:4(n-3)-d_2	ND	ND	0.5 ± 0.0	ND
22:4(n-3)-d_4	4.2 ± 0.5	6.7 ± 0.8	ND	0.3 ± 0.0
22:5(n-3)-d_2	13.3 ± 2.0	6.6 ± 0.5	23.2 ± 0.4	2.0 ± 0.5
22:5(n-3)-d_4	ND	ND	ND	19.9 ± 0.2
22:6(n-3)-d_2	2.8 ± 0.5	1.3 ± 0.0	4.4 ± 0.1	1.4 ± 0.1
22:6(n-3)-d_4	ND	ND	ND	3.3 ± 0.1

[a]Values are mean ± SD with 18:3(n-3)-d_4 (n = 3) and mean ± ½ range for other deuterated substrates (n = 2); ND, none detected.
[b]This product was identified as $\Delta^{5,11,14,17}$-20:4.

would not be predicted in a 16-h incubation period when much of the original substrate also was converted under control conditions.

Incubation with deuterated 18:3(n-3)-6,6,7,7-d_4

Previous results (19, 20) that showed [1-^{14}C]18:3(n-3) was rapidly incorporated into glioma cells and metabolized predominantly to 20:5(n-3) were confirmed in this study. By 2 h, >80% of the ^{14}C-label was in phospholipid and by 4–12 h this label was mainly associated with 20:5(n-3). With 18:3(n-3)-6,6,7,7-d_4, most of the 18:3(n-3)-d_4 was metabolized so that <12% unaltered substrate was esterified in the phospholipid fraction (**Fig. 2**, panel A and **Table 3**). Selective mass detection in increment of 2 mass units (see Fig. 2) allowed distinction of potential d_4- or d_2-forms of the products from 18:3(n-3)-d_4 with minimal interference of natural abundance ^{13}C-forms of the major endogenous fatty acids. In Fig. 2, panel B, for example, the more abundant $\Delta^{5,11,14,17}$-20:4-d_4 methyl ester peak at mass 323 was clearly distinct from the smaller amount of 20:4(n-3)-d_2 at mass 321. In Fig. 2, panel C, 20:5(n-3)-d_2 at mass 319, clearly was the predominant deuterated 20:5 isomer detected.

Quantitation of deuterated polyunsaturated fatty acids after incubation of 18:3(n-3)-6,6,7,7-d_4 (Table 3) indicated only small amounts of 20:4(n-3)-d_4 resulted from removal of the 6,7 deuteriums from 18:3(n-3)-d_4; <1% of total deuterated fatty acid was 20:4(n-3)-d_2 and no 18:4(n-3)-d_2 or 18:4(n-3)-d_4 was detected. On the other hand, 21% of total deuterated polyunsaturated fatty acids accumulated as 20:4-d_4 and 3.4% was found as 20:3(n-3)-d_4. More unsaturated products [20:5(n-3) and other PUFA], however, all contained only two deuterium atoms. Some direct elongation of 20:4(n-3)-d_4 to 22:4(n-3)-d_4 also occurred.

Incubation with other deuterated (n-3) fatty acids

Product accumulation with incubation of other deuterated substrates also was evaluated (**Fig. 3** and Table 3). With 20:3(n-3)-8,8,9,9-d_4 as substrate, a 20:4-d_4 isomer accounted for 45% of total deuterated acyl chains. Based on relative retention on the capillary column, on relative shifts that occur on this column with such bond arrangements, on comparison of retention times with 5,11,14,17-20:4 prepared from *Ephedra campylopoda* oil, and on actual mass determined by mass spectrometry, this isomer was identified as $\Delta^{5,11,14,17}$-20:4-d_4 and was clearly distinct from the 20:4(n-3) ($\Delta^{8,11,14,17}$) isomer (see Fig. 3A). Direct elongation of $\Delta^{5,11,14,17}$-20:4-d_4 resulted in substantial (7%) formation of $\Delta^{7,13,16,19}$-22:4-d_4. Further Δ^8 desaturation of $\Delta^{5,11,14,17}$-20:4-d_4 could result in release of two deuterium atoms from the 8,9-positions to give 24% 20:5(n-3)-d_2 and, by further elongation, 7% 22:5(n-3)-d_2. A small amount (1.3%) of 22:6(n-3)-d_2 also was detected.

Incubations with 20:4(n-3)-8,9-d_2 indicated that the major products were 63% 20:5(n-3)-d_2, 23% 22:5(n-3)-d_2, and 4% 22:6(n-3)-d_2. Products formed from 18:4(n-3)-12,13,15,16-d_4 confirmed that this fatty acid can serve as a precursor or intermediate in formation of 20:4(n-3)-d_4 (6%), 20:5(n-30)-d_4 (60%), 22:5(n-3)-d_4 (20%), and 22:6(n-3)-d_4 (3%). Small amounts of d_2 polyunsaturated acids, notably 7% 20:5(n-3)-d_2, were detected as well, probably due to <25% non-d_4 forms in the 18:4(n-3)-12,13,15,16-d_4 preparation.

DISCUSSION

Metabolism of 18:2(n-6) and 18:3(n-3) to longer chain, more unsaturated PUFA, considered to proceed by an alternating sequence of desaturation and chain elonga-

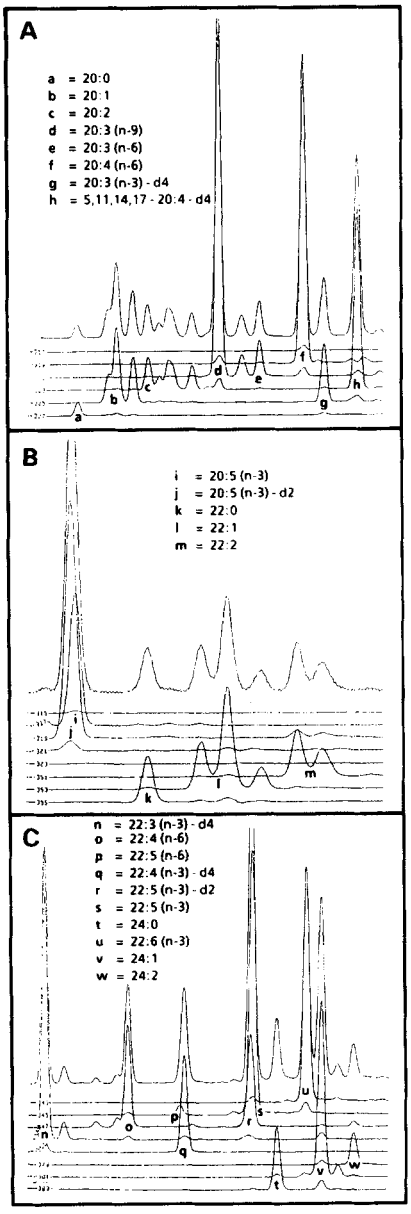

Fig. 3. Portions of the gas-liquid chromatography profile of methyl esters from glioma cells incubated with 20:3(n-3)-8,8,9,9-d_4. C6 glioma cells were incubated for 16 h with 50 μM 20:3(n-3)-8,8,9,9-d_4 and methyl esters were obtained from total phospholipids of the cell extract. Methyl esters were separated by GLC and profiles for selected molecular weights, indicated on the left side of each separate profile, were obtained. The top tracing in each panel is the total mass (sum of each molecular weight) for each retention time. Approximate retention times (min) for each panel were: panel A, 64-75; panel B, 75-82; panel C, 82-94.

tion, is a rapid process in cultured C6 glioma cells. Our earlier studies (17-20, 35) generally supported the view that both essential fatty acids use common desaturation and chain elongation enzymes. Competitive interactions between the two essential fatty acids are clearly evident with 18:3(n-3) having an apparently higher affinity for at least the initial Δ^6 desaturation enzyme (11-20). However, some observations seemed incompatible with this type of shared enzyme system as the only means of PUFA formation from the two essential fatty acid precursors. For example, 20:4(n-6) formation from 18:2(n-6) was markedly stimulated, but 20:5(n-3) formation from 18:3(n-3) was inhibited, by exogenous 20:3(n-6) or 20:4(n-6) (18-20). Simultaneous feeding of deuterated 18:2(n-6) and 18:3(n-3) to human subjects has indicated that conversion of 18:3(n-6) to 20:5(n-3 and 22.6(n-3) is much greater than conversion of 18.2(n-6) to 20:4(n-6) (36). Recent studies with geometric and positional isomers as competitors (17) further led us to conclude that the apparently differential interactions of activating and competing fatty acid isomers supported a level of complexity beyond simple competition at each step of a common enzyme sequence. Whether this means alternate enzymes or different modes of regulation are utilized remains an open question.

Our present evaluation of the relative order of specific desaturation and chain elongation steps has been facilitated by availability of isotope tracer molecules with deuterium atoms at specific, identified positions in the acyl chains, of highly sensitive analyses using capillary GLC in combination with selective mass detection, and of a cell type where adequate isotope enrichment can be reproducibly achieved. Several observations were not anticipated. First, little or no accumulation of 18:4(n-3)-6,7-d_2 and 20:4(n-3)-8,9-d_2 intermediates was observed. This, however, could be explained if conversion were very rapid with chain-elongation and Δ^5 desaturation steps much faster than initial Δ^6 desaturation, compatible with Δ^6 desaturation normally being the rate limiting reaction (3, 5). Even if this were the case, detectable levels of intermediates were anticipated. Secondly, substantial accumulation (21% of total deuterated products) of $\Delta^{5,11,14,17}$-20:4-8,8,9,9-d_4 and 3% 20:3(n-3)-8,8,9,9-d_4 from 18:3(n-3)-6,6,7,7-d_4 is not readily explained by the classical pathway because two deuterium atoms would be lost during initial Δ^6 desaturation.

One explanation, illustrated in **Fig. 4**, would be an initial 2-carbon chain elongation followed by Δ^5-desaturation. This would not remove deuterium atoms from the 8,9 positions and would produce $\Delta^{5,11,14,17}$-20:4-d_4. This is supported by the fact that the $\Delta^{5,11,14,17}$-20:4(n-3)-d_4 isomer elutes on the capillary GLC column at a retention time compatible with a shift due to a Δ^5 *cis* double bond, and identical to $\Delta^{5,11,14,17}$-20:4 analyzed from *Ephedra campylopoda* oil. Further desaturation to produce the primary product that accumulates, 20:5(n-3)-8,9-d_2, would require Δ^8 desaturation and removal of two deuterium atoms. Accordingly, two desaturation reactions operating in succession, with the Δ^8 desaturase effective between two existing double bonds, would be required. Beyond formation of 20:5(n-3)-d_2, accumulation of 13% 22:5(n-3)-d_2 and 3% 22:6(n-3)-d_2 could be accounted for

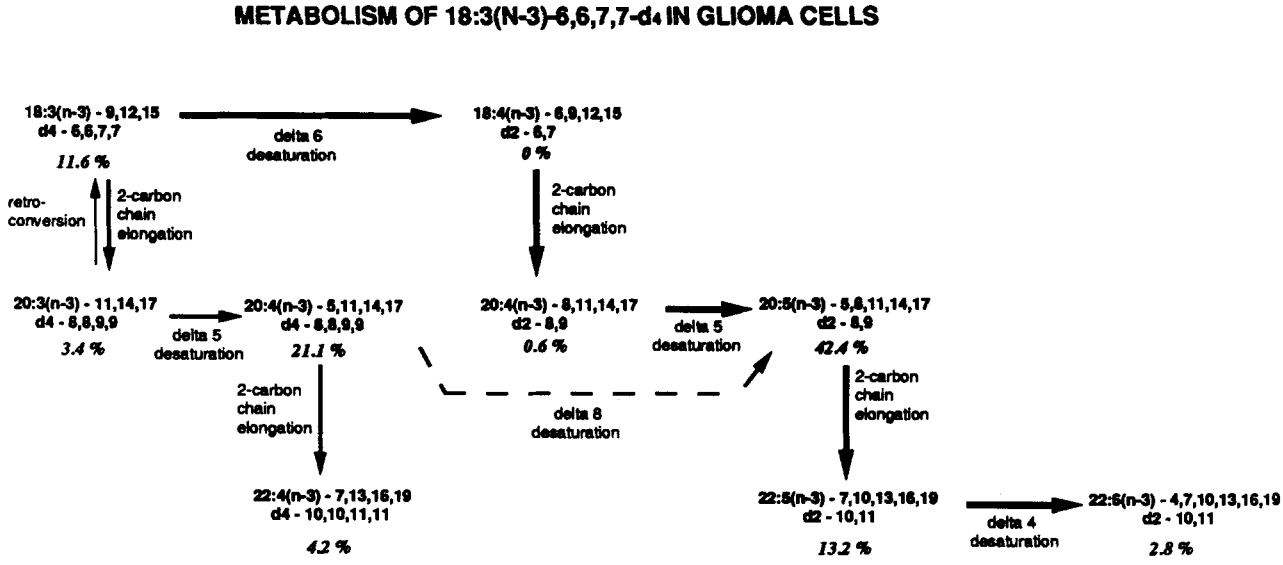

Fig. 4. Proposed alternate pathways for metabolism of 18:3(n-3) in C6 glioma cells. The tetradeuterated substrate might proceed to 20:5 (n-3) by two routes. By the classical pathway (Δ^6 desaturation followed by chain elongation), no significant accumulation of intermediates occurred (indicated by % under each fatty acid). An alternate proposal involves direct chain elongation followed by Δ^5 and Δ^8 desaturation in succession. Some elongation of the intermediate formed by Δ^5 desaturation also occurs. Steps following 20:5(n-3) formation are common to both pathways. Possibly, 20:3(n-3) could be retroconverted to 18:3(n-3) and be further metabolized by the classical pathway.

by chain elongation and Δ^4 desaturation common to both pathways.

Not all observations are explained solely by the alternate scheme in Fig. 4. Formation of small amounts of 18:3(n-3)-d_4 from 20:3(n-3)-d_4 apparently involves some form of retroconversation or partial β-oxidation. Accordingly, 20:3(n-3)-d_4 could be shunted into the classical pathway by such retroconversion. If the conversion were extremely active, it would explain formation of 20:5(n-3)-d_2 by removal of the deuteriums initially in the 8,9-position of 20:3(n-3)-d_4, involving Δ^6 desaturation at the chain shortened stage (i.e., 18:3(n-3)-6,6,7,7,-d_4) and subsequent conversion through the classical pathway. In this possibility $\Delta^{5,11,14,17}$20:4-d_4 and 22:4-d_4 could represent dead-end products of alternate metabolism. Against this alternative means of 20:5(n-3)-d_2 formation from 20:3(n-3)-d_4 is the limited retroconversion of 20-carbon to 18-carbon fatty acids compared to retroconversion of 22-carbon fatty acids (37). In numerous previous studies with 1-^{14}C-labeled 20-carbon fatty acids in glioma cells (17–20, 35), we have never observed appreciable loss (<5% with up to 24 h incubations) of carboxyl carbon. Thus, while some retroconversion of 20:3(n-3) apparently occurs and cannot be ruled out as a contributor, it seems unlikely to be quantitatively adequate to explain the extensive formation of 20:5(n-3) from 20:3(n-3). Further, it should be borne in mind that the present study involves substantial mass, not just tracer amounts, of the fatty acid substrates.

Part of the alternate pathway, illustrated in Fig. 4, proposes that chain elongation and Δ^5-desaturation reactions, along with a nonconventional Δ^8-desaturation enzyme, could account for our observations. The presence of a Δ^8-desaturase, that can form 20:3(n-6) ($\Delta^{8,11,14}$ from 20:2(n-6)($\Delta^{11,14}$), has been indicated for rat (38) and human (39) testes as well as normal and cancerous tissues from human ovaries, colon, and bladder (40); however, Δ^8-desaturase seems to be inoperative as an alternate means of 20:4(n-6) or 20:5(n-3) formation in liver (16, 41) and brain (42, 43). Expression of Δ^8 desaturation activity may be restricted to specific tissues, species, or the transformed state. An alternative to an actual Δ^8 desaturase has been proposed (44) whereby Δ^8 double bond formation results from a Δ^9-desaturase with altered specificity depending on the position and geometric configuration of the other double bond in the fatty acyl chain.

In this study, we were unable to assess the potential for an alternate route for 18:2(n-6) metabolism due to the position of the deuterium atoms in the only labeled 18:2(n-6) isomer available to us. A minor $\Delta^{5,11,14}$-20:3-d_4 isomer suggests that alternatives may exist for 18:2(n-6) metabolism as well.

This study also establishes that 22:6(n-3) can be formed by cultured glioma cells and that a Δ^4-desaturase must be present in this transformed cell line. Based on studies with 1-^{14}C-labeled fatty acid substrates, we (18, 35) and others (45) had concluded that Δ^4 desaturase was inoperative in continuous cell lines, in contrast to primary cultures. Inadequate analytical sensitivity required for detection of relatively low levels (1–4%) of 22:6(n-3) formation from radioactive substrates may have misdirected earlier interpretations. Based on more sensitive and

reliable capillary-GLC separations of 22:6(n-3), we conclude that cultured glioma cells can both accumulate 22:6(n-3) from the exogenous medium and synthesize 22:6(n-3) from the parent 18:3(n-3) or intermediates.

It might be argued that the proposed alternate pathway is an artificial situation created as an isotope selection effect in response to the presence of heavier deuterium atoms in the fatty acid substrates. While this cannot be ruled out, an alternate possibility, involving Δ^8-desaturation could be of significance under some physiological conditions. Cultured cells used in these experiments were confluent and hence not growing extensively when conversion of the deuterated fatty acid was assessed. Further, a deficiency of essential fatty acids at some stage of growth was indicated from the accumulation of substantial 20:3(n-9). Our previous studies (17) have shown that 20:3(n-9), the predominant PUFA formed from 18:1(n-9) in an essential fatty acid deficiency state, can inhibit formation of 20:4(n-6) and 20:5(n-3) from their respective 18-carbon precursors; thus, 20:3(n-9) formed as a substitute for inadequate supply of (n-6) or (n-3) acids may exacerbate the deficiency state by further reducing product formation from an already compromised supply of essential fatty acid substrates (17). Whether this might involve interactions with the alternate pathway of essential fatty acid metabolism requires further investigation, feasible with our cell model by altering growth conditions and essential fatty acid status prior to metabolic studies with deuterated precursors. A further consideration is whether expression of the alternate pathway is unique to transformed lines or is operative in primary culture, normal tissue preparations, or in vivo. Unfortunately, the potential contribution of alternate pathways cannot be readily quantitated with deuterated isomers currently available to us. What is evident from this study is that alternate possibilities for 20:5(n-3) formation should be considered particularly in conditions where the classical pathway may be impaired or restricted.

This work was supported by the Medical Research Council of Canada grant PG-16. The technical assistance of Denise Kidson, Scott Sproull, and Robert Zwicker is gratefully acknowledged.
Manuscript received 29 November 1990 and in revised form 19 April 1991.

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