Biosynthesis of Eicosapentaenoic Acid in the Sea Urchin *Psammechinus miliaris*

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ABSTRACT: The sea urchin *Psammechinus miliaris* (Gmelin) (Echinodermata: Echinoidea) was shown by using a deuterated tracer (D_5-18:3n-3) and quantitation by negative chemical ionization gas chromatography–mass spectrometry to convert 18:3n-3 to 20:5n-3. The rate of conversion was very slow, corresponding to 0.09 μg/g tissue/mg 18:3n-3 eaten over 14 d. Deuterated arachidonic acid (D_8-20:4n-6) was also included in the diet to give a measure of the relative amounts of diet eaten by the different animals. The recovery of this fatty acid in tissue lipids was 33.7% compared with only 0.95% recovery of D_5-18:3n-3 and its anabolites, indicating that the majority of the D_5-tracer was catabolized. Considerable elongation of D_5-18:3n-3 into 20:3n-3 and a trace of 22:3n-3 was found, and these were accompanied by minor amounts of the intermediates 18:4n-3 and 20:4n-3. No deuterated 22:6n-3 was found.

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The biosynthesis of polyunsaturated fatty acids (PUFA) in invertebrates is seldom studied, and no quantitative information is available on rates of formation. PUFA are abundant in the marine environment with 16-, 18-, 20-, and 22-carbon PUFA, all found in different classes of phytoplankton, while higher consumers, both invertebrate and vertebrate, retain and concentrate C_{20} and C_{22} PUFA (1). This abundance of dietary C_{20} and C_{22} PUFA may mask biosynthetic capability in consumers. Radiotracer studies have shown that some terrestrial invertebrates can synthesize PUFA *de novo* (2,3) and that *Artemia* can convert 18:2n-6 to 18:3n-3 and then to 20:5n-3 (4). These were formerly functions associated only with photosynthetic organisms.

Tracer experiments using [1-^{14}C]-18:2n-6 and [1-^{14}C]-18:3n-3 have been conducted in a number of invertebrates including the clam *Mesodesma mactroides* (5), the calanoid copepod *Paracalanus parvus* (6), and the prawn *Penaeus japonicus* (7) and have shown small percentage conversions to longer-chain more unsaturated products. However, a drawback of using [1-^{14}C]-labeled substrates is the possible loss of label by β-oxidation and reincorporation of labeled acetate. Thus results may be ambiguous beyond the first elongation step. None of the above studies using radiolabeled tracers quantitated the amounts of fatty acid moving through the pathway.

The sea urchin *Psammechinus miliaris* has recently been identified as a possible aquaculture species (8), as the roe (gonad of both males and females) is a valuable food product. As such there is a requirement for artificial diets which enhance gonad growth. In the wild, *P. miliaris* grazes on living and detached kelp (9), but it also feeds on a wide selection of other algae and encrusting invertebrates (10). Gonad growth in *P. miliaris* was enhanced by commercially prepared salmon feed (11), and gonad growth was also stimulated by diets containing lower levels of lipid (6–8%) of mainly vegetable origin (12). It was unclear whether *P. miliaris* is capable of producing C_{20} and C_{22} PUFA from shorter-chain precursor fatty acids, or indeed whether this species has a requirement for such fatty acids.

In this study we demonstrate, using a deuterated tracer and quantitation by gas chromatography–mass spectrometry (GC–MS), that *P. miliaris* can elongate and desaturate 18:3n-3 to produce 20:5n-3. This method is very sensitive and overcomes the problems of interpretation when using [1-^{14}C]-labeled fatty acids.

MATERIALS AND METHODS

Chemicals. Chloroform, methanol, ethanol, isohexane, and diethyl ether were high-peformance liquid chromatrography-grade from Fisher (Loughborough, Leicestershire, United Kingdom). Butylated hydroxytoluene and standard fatty acids were obtained from Sigma (Poole, Dorset, United Kingdom) and diisopropylamine, anhydrous acetonitrile, and pentafluorobenzyl bromide from Aldrich (Gillingham, Dorset, United Kingdom). Deuterated tracer D_5 (17,17,18,18,18)-linolenic acid and D_8 (5,6,8,9,11,12,14,15)-arachidonic acid were purchased from Cambridge Isotope Laboratories (Andover, MA) as the fatty acid ethyl esters.

Preparation of diet. The experimental diet was based on a standard fish diet with vegetable meals and vegetable oils substituting for fish meal and fish oil to eliminate preformed C_{20} and C_{22} PUFA. The diet comprised (g/100 g): soy flour 39, wheat meal 31, corn gluten meal 11.4, lysine 0.3, methionine 0.3, mineral mix 4.0, vitamin mix 1.0, carboxymethylcellulose 2.0, and oil 11.0. The oil component consisted of, mg/g dry diet: D_5-18:3n-3 fatty acid ethyl ester 27, D_8-20:4n-6 fatty acid ethyl ester 5.9, and linseed oil 81. The diet was produced as a 1-mm diameter pellet.

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Abbreviations: D_n, deuterated; GC–MS, gas chromatography–mass spectrometry; PUFA, polyunsaturated fatty acid; tri23:0, tritricosanoyl.

Animals. Psammachinus miliaris were collected by SCUBA divers from a depth of 5 m in Loch Creran, Scotland (56°32′20″ N; 5°17′00″ W) in early September. Six urchins (horizontal test diameter 2–3 cm, wet weight 9–12 g) were selected at random and placed in aquaria that had an independent supply of 250 L filtered seawater at ambient temperature (11.8–15.3°C) and salinity. The photoperiod was maintained at a constant 16-h light/8-h dark cycle throughout the experiments. The urchins were fed on the macroalga *Laminaria saccharina ad libitum* for 2 wk to equilibrate their nutritional status. The seawater supply was then exchanged for a 5-µm filtered supply, and the urchins were starved for 48 h prior to feeding the labeled diet. The urchins were fed 0.54 g of labeled diet over the course of 3 h. Urchins were individually presented with pellets that they readily accepted, trapping them with their tube feet and rapidly manipulating them to their jaws. All the diet was ingested. The urchins were returned to a diet of *L. saccharina* and sacrificed 14 d later. They were weighed, the gonad and gut dissected, and the tissues, including the test, weighed. Samples were stored at –70°C prior to analysis.

Lipid extraction. The gonads and guts were thawed on ice then homogenized in 10 mL chloroform/methanol (2:1, vol/vol). Tritricosanoylglycerol (tri23:0 glycerol) standard (30 µg) was added to the gonad homogenates and 10 µg to the gut homogenates. The homogenates were shaken and then stood on ice for 1 h with periodic shaking. The eviscerated tests were crushed in a pestle and mortar, and the slurry was homogenized in 20 mL chloroform/methanol (2:1, vol/vol) with 100 µg tri23:0. The homogenate was left on ice for 2½ h with periodic shaking. The homogenates were then filtered and a Folch extract prepared (13). The final total lipid fraction was weighed and stored under argon at –20°C.

Quantitation of deuterated fatty acids. One milligram of total lipid was saponified with 2 mL of 0.1 M KOH in 95% (vol/vol) ethanol under nitrogen for 1 h at 78°C. Nonsaponifiable material was removed by extracting with isohexane/diethyl ether (2:1, vol/vol), the aqueous phase was acidified then free fatty acids were extracted with diethyl ether. Pentafluorobenzyl esters were then prepared from 100 µg free fatty acid using acetonitrile/diisopropylamine/pentafluorobenzyl bromide (1000:10:1, by vol) at 60°C for 30 min under nitrogen as described by Pawlosky *et al.* (14). Excess reagent and solvent were then removed under nitrogen, samples dissolved in isohexane, and stored at –20°C under argon until analysis. Calibration standards of individual fatty acids with 23:0 were prepared by varying the amount of unknown fatty acid while keeping the 23:0 constant and plotting the peak area ratio against the mass ratio of the fatty acids. Sample volumes for analysis were adjusted such that the amount of 23:0 injected onto the GC–MS was constant. Pentafluorobenzyl esters were chromatographed and quantitated on a Fisons MD 800 GC–MS fitted with an on-column injector and a Chrompack CP wax 52CB column (30 m × 0.32 mm i.d., 0.25 micron film thickness) (Burke Analytical, Alva, Clackmannanshire, United Kingdom) using helium as carrier gas (column head-pressure 7 psi) and running in negative chemical ionization mode with methane as reagent gas (pressure 7 psi). The temperature program was 80–190°C at 40°C/min, 190–240°C at 1.5°C/min, then 240°C for 10 min. Peaks were identified by selective ion scanning for the required masses using a dwell time of 80 ms and cycle time of 20 ms and quantitated by reference to the appropriate standard fatty acid.

RESULTS

After 14 d, all three tissues contained D_5-18:3n-3 and its anabolites together with D_8-20:4n-6 (Table 1). The distribution of labeled fatty acids within the tissues varied between the animals with gonad, gut and test each having the majority of D_5-labeled fatty acids in two animals. However, D_8-20:4n-6 was predominantly found in the test (Table 1) (five out of six animals). When expressed as mg lipid or fatty acid per g tissue, gut was the most lipid-rich tissue for total lipid and labeled fatty acids (Table 1). The total recoveries of labeled fatty acids were 139 µg for D_5-18:3n-3 and metabolites and 1.076 mg of D_8-20:4n-6, corresponding to 0.95 and 33.7% of ingested material, respectively, suggesting that most of the tracer was catabolized.

There was a good correlation between the total D_5-fatty acids and D_8-20:4n-6 recovered in the six animals (r = 0.9917). This indicated that the fatty acids were being processed similarly in the animals. The proportion of D_8-20:4n-6 recovered in individual animals was used to calculate the amount of D_5-18:3n-3 eaten by individuals. The data for fatty acid incorporation were then expressed as µg fatty acid/g tissue/mg D_5-18:3n-3 ingested.

TABLE 1
The Distribution of Lipid and Deuterated Fatty Acids in the Different Tissues (mean ± 1 SD, *n* = 6)

	Tissue			
	Gonad	Gut	Test	Total
Lipid (mg)	12.9 ± 5.7	5.3 ± 0.8	30.5 ± 10.4	48.7 ± 12.9
mg lipid/g tissue	24.8 ± 8.3	28.3 ± 4.5	5.6 ± 1.1	4.7 ± 0.9
Total D_5-fatty acids (µg)	6.4 ± 5.7	9.3 ± 8.8	7.5 ± 2.6	23.2 ± 14.2
µg D_5-fatty acid/g tissue	11.5 ± 8.9	49.2 ± 44.6	1.4 ± 0.3	2.2 ± 1.2
D_8-20:4n-6 (µg)	31.6 ± 26.2	70.6 ± 68.4	77.2 ± 30.4	179.4 ± 89.6
µg D_8-20:4n-6/g tissue	57.0 ± 40.5	373.6 ± 345.6	14.4 ± 4.8	17.1 ± 7.4

TABLE 2
Fatty Acids Derived from D_5-18:3n-3 Present 14 d After a Pulse of 14.6 mg D_5-18:3n-3 (mean ± 1 SD, *n* = 6)

	μg D_5-Fatty acid/g tissue/mg D_5-18:3n-3 eaten	% Recovered
D_5-18:3n-3	0.33 ± 0.07	38.0
D_5-20:3n-3	0.35 ± 0.05	39.9
D_5-22:3n-3	0.021 ± 0.005	2.3
D_5-18:4n-3	0.011 ± 0.004	1.1
D_5-20:4n-3	0.076 ± 0.018	8.4
D_5-22:4n-3	Trace	<0.1
D_5-20:5n-3	0.090 ± 0.015	10.2
D_5-22:5n-3	Trace	<0.1

[a]Trace = <0.001 μg D_5-fatty acid/g tissue/mg D_5-18:3n-3 eaten. Percentage recovered is percentage of total D_5-fatty acid recovered.

The initial substrate D_5-18:3n-3 and its immediate elongation product 20:3n-3 were the predominant labeled fatty acids recovered (Table 2), together accounting for 77.9% of the recovered label. Further elongation to 22:3n-3 accounted for 2.3% of deuterated fatty acids. Octadecatetraenoic acid (18:4n-3), the Δ6-desaturation product of 18:3n-3, was a minor component (Table 2), accounting for 1.1% of label. The subsequent elongation product 20:4n-3 was some seven times more abundant, and its Δ5-desaturation product 20:5n-3 accounted for 10.2% of recovered label. This represented 0.009% of the 18:3n-3 ingested (0.09 μg/g tissue/mg D_5-18:3n-3 eaten). Only trace amounts of 22:4n-3 and 22:5n-3 were found, and we could not detect any D_5-22:6n-3. The samples were analyzed also for the elongation and desaturation products of D_8-20:4n-6. There was some elongation to 22:4n-6 (5.7% of substrate) but no further elongation to 24:4n-6 or desaturation to 22:5n-6.

DISCUSSION

Deuterated arachidonic acid (D_8-isomer) was added to the diets to give an indication of the amount of diet eaten. The reasoning behind this was that a C_{20} PUFA, which is an important structural fatty acid of cell membranes (a major PUFA in this species, *ca.*10% of total fatty acids) and also a precursor of the eicosanoids (15), is less likely to be heavily catabolized than a C_{18} PUFA, or indeed a saturated or monounsaturated fatty acid. The data supported this hypothesis as D_8-20:4n-6 was retained much better than D_5-18:3n-3 and its metabolites, with recoveries of 33.7 and 0.95%, respectively. The great majority of the D_5-18:3n-3 therefore either was not assimilated or was catabolized. It is likely that "nonessential" fatty acids were being oxidized to provide energy for either somatic growth or gonad recovery. Therefore, we used the relative amounts of D_8-20:4n-6 recovered in the different animals to deduce the amount of diet, and hence D_5-18:3n-3, ingested. Using this correction reduced the variability in the data.

Psammechinus miliaris has an annual reproductive cycle, peak gonad indices being obtained during June and July in the area where these urchins were collected (16). The urchins used in this study were in the postspawning or recovery phase of the reproductive cycle (17), and their gonads were therefore relatively depleted in lipid (2.5% wet weight) compared to 7.1% for urchins of the same species in the premature phase (18). In the recovery phase of the gametogenic cycle, urchins replenish the nutrient stores in the nutritive phagocytes of the gonad (17). Therefore, the urchins used in this study were likely to have been sequestering lipid.

The results clearly showed that the urchins were capable of synthesizing 20:5n-3 from 18:3n-3, even when reared on a natural diet (*L. saccharina*), which contains 20:4n-6 and 20:5n-3 as the major PUFA. The rate of formation of 20:5n-3 was slow, equivalent to only 0.009% of the D_5-18:3n-3 ingested over a 14-d period. In a preliminary experiment, we found that the amounts of D_5-18:4n-3, D_5-20:4n-3, and D_5-20:5n-3 in gonad approximately doubled between day 7 and day 14 post-dose, whereas the amount of substrate D_5-18:3n-3 decreased (data not shown). Of the label recovered from D_5-18:3n-3, 42.2% was in elongation products and 19.7% in Δ6-desaturation products. This indicates an active C_{18}–C_{20} elongase that effectively reduces the substrate availability for the desaturase. There was only limited elongation of C_{20} PUFA to C_{22} PUFA; D_5-22:3n-3 (6.0% of substrate D_5-20:3n-3) and D_8-22:4n-6 (5.7% of substrate D_8-20:4n-6) were measurable while D_5-22:4n-3 and D_5-22:5n-3 arising from D_5-20:4n-3 and D_5-20:5n-3, respectively, were barely detectable. The much larger amounts of D_5-20:3n-3 and D_8-20:4n-6 allowed their elongation products to be measured. Urchins reared on a natural diet of *Laminaria* contained 11.3% 20:4n-6, 10.0% 20:5n-3, 0.1% 22:5n-6, and 1.5% 22:6n-3 (18), supporting the finding that conversion of C_{20} PUFA to C_{22} PUFA is low in this species. The urchins clearly contain an active Δ5-desaturase. This was suggested by earlier fatty acid compositional data (19) which showed a number of unusual Δ5-monounsaturated fatty acids and Δ5-nonmethylene-interrupted dienes. In *P. miliaris* feeding on salmon diet or the detritus from salmon cages that contains C_{20} and C_{22} monounsaturated fatty acids, the corresponding Δ5-20:2 and Δ5-22:2 nonmethylene-interrupted dienes were found (18).

The data show conclusively that *P. miliaris* can synthesize 20:5n-3 from 18:3n-3 and that this species therefore has the genetic apparatus necessary to bring about this conversion, i.e., a Δ6-desaturase, C_{18} to C_{20} elongase, and a Δ5-desaturase. However, further desaturation reactions do not occur while further elongation to C_{22} PUFA is limited. The rate of formation of 20:5n-3 was very slow, about one-twentieth of the rate of 22:6n-3 formation measured in rainbow trout using the same method (Bell, M.V., unpublished data), and was well below that necessary to account for the amount of 20:5n-3 present in the animals which must have been accumulated from the diet. The ability to synthesize C_{20} PUFA when the diet usually contains these fatty acids is perhaps surprising and suggests an essential role for C_{20} PUFA in echinoderms, probably as prostanoid precursors. Several B series prostaglandins were reported in 10 species of Japanese echinoderms (20).

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