Biosynthesis of Wax Esters in Fish. Reduction of Fatty Acids and Oxidation of Alcohols*

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ABSTRACT: Biosynthesis of wax esters in roe of gouramis (*Trichogaster*) was studied. When methyl esters of ^{14}C-labeled palmitic, oleic, linoleic, and other fatty acids were fed to adult female gouramis, between 20 and 60% of the radioactivity was recovered in the total lipids of the fish and approximately two-thirds of that was in roe wax esters. Analyses of the constituents of the wax esters showed that the saturated and monounsaturated esters had been extensively reduced to the respective alcohols. Less reduction occurred with more highly unsaturated esters, but some alcohol was formed even from docosahexaenoate. Palmitic acid was reduced as efficiently as its methyl ester. When labeled palmityl or oleyl alcohol was fed, the incorporation was similar to that from the corresponding methyl esters. The equilibria of alcohol and acid of these chain structures also were the same after feeding alcohols as after feeding methyl esters.

The reduction and oxidation of carboxyl and alcohol groups seem to apply to all common lipid chains. Elongation and desaturation as well as degradation and resynthesis of the chains took place only to a minor extent. However, [1-^{14}C]acetate was incorporated to 17% in lipids. Most of this radioactivity was in the saturated and monounsaturated alcohols of the roe wax esters.

Wax esters have been known for a long time as prominent lipids of some aquatic animals and a survey of the literature (Nevenzel, 1969) indicates their rather widespread occurrence in such sources. Predominance of wax esters in roe, but not in body lipids was encountered first with the mullet (*Mugil cephalus*) (Iyengar and Schlenk, 1967). More recently we found that wax esters are also the major lipids in roe of gouramis (*Trichogaster*) (Sand and Schlenk, 1969). Gourami egg oil, like that of the mullet, contains more than 80% wax esters. Gouramis are smaller than mullets, mature faster, and live in fresh water. Therefore, they are more practical experimental animals than mullets for investigating the biosynthesis of wax esters in fish. A laboratory colony to supply fish under controlled conditions was desirable. Opaline gouramis (*Trichogaster cosby*) were more successfully bred than several other varieties and they were selected for these studies.

Our previous investigations had shown that double bonds in alcohols and acids of roe wax esters are in the same positions. For example, the structures 9,12-18:2[1] and 9,12,15-18:3 and others occur in both moieties. Therefore, alcohols and acids appear to be in a close biosynthetic relationship. We are reporting here the results from feeding radioactive fatty acids and alcohols which give proof for such surmise.

Materials and Methods

Opaline Gourami (T. cosby). Fish were obtained commercially and maintained in 15-gal tanks at 24° in groups of 10–30 specimens according to size. Although gouramis are surface breathers, the aquaria were equipped with bubble filters for aeration and cleanliness of the water. Deionized water was used with 48 mg of $NaHCO_3$, 40 mg of $CaSO_4$, 30 mg of $MgSO_4$, and 3 mg of KCl per l. Commercial aquarium gravel served as source of trace minerals. The water was maintained at pH 7.0 and 15 ml of a 1% solution of sodium sulfathiazole was added to the tanks to minimize bacterial growth.

The fish were fed twice daily with commercial shrimp pellets or frozen brine shrimp. The lipids of these materials contain the common fatty acids including those of the linoleic and linolenic types, but they do not contain wax esters. Mature opaline gourami weigh up to 8 g and consume about 100 mg of food per day.

For breeding, each pair was placed into a 15-gal tank at 27°, without aeration, in water containing one-fourth the concentration of the above-listed salts and without sulfathiazole. Several spawnings occurred over a period of 3–6 hr. The eggs floated to the surface and were placed, by the male, in a bubble nest which he had prepared. The female was then taken from the tank and 2 days later, after the eggs hatched, the male was removed. The fry received the first extraneous food when 2 days old and the fish matured within 4–5 months.

Compounds Fed. Uniformly labeled palmitic acid, methyl palmitate, oleate, linoleate, arachidonate, and 4,7,10,13,16,-19-docosahexaenoate had been obtained in this laboratory from microorganisms or plants fed $^{14}CO_2$ or [1,2-^{14}C]acetate (Mangold and Schlenk, 1957; Gellerman and Schlenk, 1965, 1969; Schlenk *et al.*, 1969). Palmityl and oleyl alcohols were prepared from methyl esters of the radioactive acids by reduc-

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[1] The usual abbreviated form of the Geneva nomenclature is used for the structures of alcohol and acid chains; *e.g.*, 16:0 for palmityl alcohol or palmitic acid, 9,12-18:2 for 9,12-octadecadienyl (linoleyl) alcohol or 9,12-octadecadienoic (linoleic) acid.

TABLE I: Alcohols and Acids of Gourami Roe Wax Esters.[a]

Structure	Alcohol (%)	Acid (%)
16:0	45.0	1.9
16:1	8.8	10.8
18:0	6.8	0.5
9-18:1	30.1	51.0
9,12-18:2	1.3	13.5
9,12,15-18:3	0.8	4.6
5,8,11,14-20:4	0.1	1.7
4,7,10,13,16,19-22:6	Trace	6.8

[a] For more complete data, see Sand and Schlenk (1969).

tion with $LiAlH_4$. [1-^{14}C]Palmitic acid was purchased (Volk Radiochemical Co.), purified by column chromatography on silicic acid and esterified with diazomethane. Radioactive acetate was fed as palmityl acetate which had been prepared from [1-^{14}C]acetic acid anhydride and nonlabeled palmityl alcohol (Mangold, 1967). The chemical and radioactive purity of all compounds was above 98% according to tests by thin-layer chromatography and gas–liquid phase chromatography. Their activity was close to 0.5 μCi/mg.

Feeding and Recovery of Lipids. The fish used in feeding experiments were selected from one hatch for equal size and healthy appearance, and were between 5- and 9-months old. Two females of known age in a 5-gal. tank were fed in each experiment. They had been trained for several days to eat immediately when powdered dried shrimp was given in an area confined by a floating plastic ring. To determine if age had any effect on lipid metabolism, for each experiment two additional fish from the same hatch were fed [U-^{14}C]-palmitate. All the results from these reference fish were nearly identical which indicated that metabolic patterns did not change within this period.

The compounds fed (10–15 mg, $\sim 10^7$ dpm) were dissolved in hexane, and powdered dried shrimp (30 mg) was suspended in the solutions. The solvent was immediately removed by a stream of nitrogen and, finally, by evacuation. The impregnated particles were then sprinkled on the surface of the feeding area. The two fish ate promptly but some pecking order was observed.

The fish, 24-hr after feeding, were sacrificed by freezing in Dry Ice. Roe and body without intestines were extracted separately in an Omni-Mixer with $CHCl_3$ + CH_3OH (2:1, v/v) and the lipids were recovered as usual.

The radioactivity of these lipids from individual fish was determined by scintillation counting in toluene with 0.5% 2,5-diphenyloxazole + 0.05% 1,4-bis-2-(4-methyl-5-phenyl-oxazolyl)benzene (PPO and dimethyl-POPOP, Packard Instrument Co., Inc.) as fluors at a counting efficiency of 60%. The values for roe and body lipids both reflected the dominance of one fish over the other while eating, but they were sufficiently similar to combine the materials from the two specimens.

Separation of Roe Lipid Classes. Aliquots of about 500 mg of lipids were subjected to column chromatography on silicic acid using chloroform followed by methanol for elution (Sand and Schlenk, 1968). Wax esters, triglycerides, cephalins, and lecithins were separated and the specific activities of these fractions were determined. The same was done with an intermediate fraction which, according to thin-layer chromatography, contained mainly glycerides or esters of similar polarity, smaller quantities of cholesterol and fatty acids and, in lowest amount, alcohols.

Analysis of Alcohols and Acids of Wax Esters. The wax ester fraction contained about 1% hydrocarbons, and traces of sterol esters. Hydrocarbons and sterols were eliminated during the subsequent reactions and fractionations. Acids arising from sterol esters were negligible in amount.

Samples of about 200 mg of wax esters were refluxed for 2 hr in methanol containing 14% BF_3 (Applied Science Laboratories). The resulting mixture of alcohols and fatty acid methyl esters was separated by thin-layer chromatography on silica gel H using 30 g of adsorbant/plate of 20 × 20 cm size (Schmid *et al.*, 1967) with hexane–diethyl ether–acetic acid (85:15:1, v/v) as developing solvent. The bands were detected in ultraviolet light after spraying with dichlorofluorescein, and extracted with chloroform. Alcohols were acetylated by heating to 100° with acetic acid anhydride for 60 min in sealed test tubes. Specific radioactivities of the alcohol acetates and the acid methyl esters were determined by weighing and counting.

Acetates and methyl esters were subjected to gas chromatography on the polar phase, ethylene glycol succinate, in a F & M high-efficiency no. 402 apparatus. It was equipped with a stream splitter leading to a flame ionization detector and to the collecting device. The components were collected and counted.

Results

Composition of Roe Lipids. Fish weighing 6 g had ~0.8 g of roe and yielded ~0.35 g of lipids from the roe and ~0.4 g from the body. Wax esters were not found in the latter lipids whereas they are the major lipid of the roe. Typical recoveries from a column chromatogram of roe lipids were: wax esters, 84.1%; triglycerides, 3.5%; cephalin, 3.3%; lecithin, 5.7%; and the intermediate fraction described above, 5.2%. The composition of alcohols and acids of wax esters is given in Table I as far as they are pertinent here. Saturated chains, 16:0 and 18:0, make up about one-half of the alcohols, but very little of the acids. Monounsaturated compounds, mainly 18:1, are at levels more nearly equal in the two moieties. Polyunsaturated chains are more prominent among the acids than among the alcohols.

Incorporation of ^{14}C Compounds. In all cases, more radioactivity was found in the roe than in the body lipids (Table II) and total incorporations of radioactive lipids were between 20 and 60% of the amount which had been placed in the tank. Feeding as methyl ester, acid, or alcohol may bring about minor differences which possibly are due to more or less efficient absorption from the intestinal tract.

Minimum and maximum values of incorporation into roe lipids other than wax esters were encountered with [U-^{14}C]-16:0 and [U-^{14}C]20:4 and these data are listed in footnotes to Table II. Data from other experiments were within these rather narrow limits and are omitted.

Conversion and Synthesis of Chains. Analyses of the alcohol and acid constituents of wax esters showed little elongation

TABLE II: Incorporation of Radioactivity.

Compound Fed[a]	% ^{14}C Recovered in Body Lipids	% ^{14}C Recovered in Roe Lipids	% ^{14}C of Roe Lipids in Wax Esters
16:0 Methyl ester	9.2	27.4	92.8[b]
[1-^{14}C]16:0 Methyl ester	10.2	31.8	89.1
16:0 Acid	3.5	16.7	86.1
16:0 Alcohol	7.2	20.2	88.9
18:0 Methyl ester	3.2	17.5	91.2
9-18:1 Methyl ester	17.0	40.7	91.8
9-18:1 Alcohol	11.9	29.3	90.2
[1-^{14}C]Acetate of 16:0 alcohol	3.4	13.5	87.1
9,12-18:2 Methyl ester	20.9	28.3	90.1
9,12,15-18:3 Methyl ester	13.8	44.3	92.4
5,8,11,14-20:4 Methyl ester	7.0	18.3	71.7[c]
4,7,10,13,16,19-22:6 Methyl ester	12.9	34.3	81.5

[a] Uniformly ^{14}C-labeled chains except when indicated otherwise. [b] Triglycerides, 2.0%; cephalin, 0.8%; lecithin, 3.8%; intermediate fraction, 0.6% ^{14}C. [c] Triglycerides, <0.1%; cephalin, 6.2%; lecithin, 11.1%; intermediate fraction, 11.1% ^{14}C.

or desaturation of the compounds that had been fed. Desaturation was highest with 18:0 methyl ester of which 11.3% was converted into 18:1 alcohol and acid chains (Table III).

Synthesis of wax esters from acetate was investigated by feeding palmityl [1-^{14}C]acetate. As indicated in Table II, *de novo* synthesis of chains was quite active. These data are supplemented in Table IV with details on the distribution of ^{14}C among endogenous alcohols and acids.

Reduction and Oxidation of Acid and Alcohol Groups. Table V lists specific data only for alcohols and acids of the structure which had been fed. The low values for products of chain conversion justify omission of detailed data on compounds of other structure.

TABLE III: Distribution of ^{14}C From [U-^{14}C]18:0 Methyl Ester in Wax Esters.

Chain	% ^{14}C in Alcohol	% ^{14}C in Acid
16:0	1.1	0.3
16:1	0.1	0.1
18:0	75.6	9.7
18:1	4.1	7.2

TABLE IV: Syntheses from [1-^{14}C]Acetate.[a]

Chain	% ^{14}C of Waxes in Alcohol	% ^{14}C of Waxes in Acid
16:0	55.5	5.0
16:1	1.5	2.2
18:0	9.7	0.5
18:1	5.1	17.2

[a] Fed as ester of nonlabeled palmityl alcohol.

Discussion

Little is known about the biosynthesis of fish wax esters in which the origin of the alcohol moiety is of particular interest. Malins (1966) briefly reported that after intrahepatic injection of [1-^{14}C]palmitic acid in dogfish (*Squalus acanthiae*), small amounts of long-chain alcohols were radioactive and that the rate of esterification to wax esters was probably quite significant. Nevenzel and Kayama (1968), working with lantern fish (*Myctophidae*), recovered, after intramuscular injection of radioactive palmitic or oleic acid, 2% or less of the radioactivity in total alcohols of wax esters. Labeled palmityl alcohol yielded 0.2% of the radioactivity in the acid moiety (Nevenzel and Kayama, 1968; Nevenzel, 1969). Although based on only modest experimental evidence, these results indicated conversions which were very obvious in our experiments with gouramis, and have been expanded to polyunsaturated structures.

TABLE V: Reduction and Oxidation of Acid and Alcohol Groups.

Compound Fed[a]	% ^{14}C of Wax in Original Chain As Alcohol	% ^{14}C of Wax in Original Chain As Acid	% ^{14}C in Other Chains As Alcohol	% ^{14}C in Other Chains As Acid
16:0 Methyl ester	86.3	7.3	3.3	3.1
[1-^{14}C]16:0 Methyl ester	88.7	5.7	1.8	3.8
16:0 Acid	88.7	6.5	2.7	2.1
16:0 Alcohol	84.9	8.1	3.5	3.5
18:0 Methyl ester	75.6	9.7	5.3	7.6
9-18:1 Methyl ester	35.7	64.7	1.0	0.6
9-18:1 Alcohol	39.6	58.0	1.5	0.9
9,12-18:2 Methyl ester	22.3	75.3	0.6	1.8
9,12,15-18:3 Methyl ester	27.7	67.2	2.8	2.3
5,8,11,14-20:4 Methyl ester	2.6	87.0	5.8	4.2
4,7,10,13,16,19-22:6 Methyl ester	2.7	92.2	2.5	1.6

[a] Uniformly ^{14}C-labeled chains except when indicated otherwise.

Data of Tables II and V give ample proof that incorporation and reduction, as well as oxidation of the functional groups occur in the gourami. High radioactivity of those chain structures which had been fed demonstrates the direct conversion of carboxyl and alcohol groups. From palmitic ester or acid, nearly 90% of the radioactivity is in the alcohol moieties and of this, 97% is in palmityl alcohol whereas only about 3% is in other alcohols. About 60% of the radioactivity from oleyl alcohol is in oleic acid whereas the other acid moieties contain only 1% (Table V).

The extent of reduction or oxidation seems to depend on the structure of the chain. The same ratios, in the order of 10 to 1, of palmityl alcohol to palmitic acid were reached in the wax esters regardless of which of the two compounds had been fed. Similarly, the ratio of about 1:2 is reached for oleyl alcohol to oleic acid from either compound as starting material. The latter proportion was also approximated by 18:1 chains which originated from stearic acid (Table III).

For all structures the radioactive ratios, alcohol to acid, crudely reflect the compositional ratios (Table I). Gourami roe wax esters contain less polyunsaturated alcohols than acids; accordingly, only about 25% of the radioactive 18:2 and 18:3 chains was found as alcohol components and even less of the more highly unsaturated acids had been reduced (Table V). It is generally accepted that fish, like rats and other higher animals, cannot synthesize *de novo* these polyunsaturated structures. Our results show that the polyunsaturated alcohols are not necessarily ingested as such or as wax esters, but can arise from polyunsaturated acids which the fish obtains with the food.

The equilibrium fatty acid $\rightleftarrows$ fatty alcohol is of importance for several metabolic aspects: fatty alcohols, after conversion into acids, can be used as an energy source by β oxidation; polyunsaturated alcohols can be used as a reserve for fatty acids which are essential for development of the fry; alcohols ingested as wax esters with some foods or microorganisms can be adjusted to a suitable composition *via* fatty acids which may undergo elongation or desaturation and then be reduced again to alcohols.

Neither *de novo* synthesis of long-chain alcohols as such, nor direct conversions of their chain have been reported. With present knowledge on lipid synthesis, it is reasonable to consider fatty acids rather than alcohols as the primary product. The relative radioactivities of C_{16} and C_{18} alcohols and acids from [1-^{14}C]acetate (Table IV) conform to radioactive proportions obtained by feeding the corresponding fatty acids or alcohols. Whereas *de novo* synthesis is quite active, the low radioactivities in C_{16} chains after feeding C_{18} or longer chain methyl esters (Tables III and V) show that degradation and subsequent resynthesis through the acetate pool play only a very minor role in the distribution of ^{14}C from long-chain compounds.

The esterification reaction must proceed rapidly since the amounts of free alcohols in the roe are very small. It is uncertain whether alcohols in free form are the end products of the reduction and are necessary intermediates for the esterification. The experiments with palmityl and oleyl alcohols indicate, however, that free alcohols are esterified and that the biological reduction process is not a prerequisite for this reaction.

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