Metabolism of glycerol ether-containing lipids in dogfish (*Squalus acanthias*)

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ABSTRACT Dogfish (*Squalus acanthias*) received intrahepatic injections of either palmitic acid-1-^{14}C or chimyl alcohol-1-^{14}C. The lipids of the liver were then analyzed for incorporated radioactivity.

The experiments with labeled palmitic acid demonstrated that fatty acids are reductively incorporated into the alkyl and alkenyl ether chains of glycerolipids. Significantly lower specific activities were found for the diacyl alk-1′-enyl ethers and diacyl glycerol ethers than for other glycerol ether-containing lipids. These compounds may therefore represent terminal points in ether-lipid metabolism.

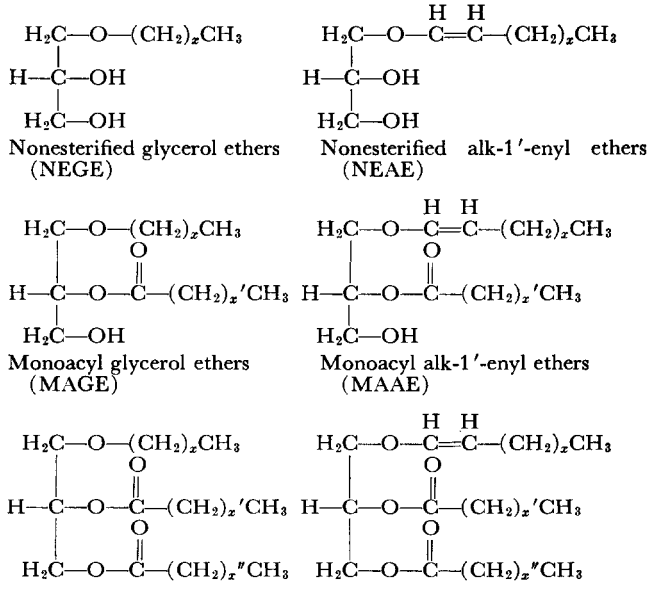
The studies with labeled chimyl alcohol indicate that dogfish liver contains enzymes that have a high capacity for oxidatively cleaving alkyl ether linkages. Furthermore, it is probable that alkyl ethers are converted directly to alkenyl ethers, possibly via a biodehydrogenation reaction.

SUPPLEMENTARY KEY WORDS monoacyl · diacyl · nonesterified · glycerol ethers · liver · analysis · uptake · palmitic acid · chimyl alcohol

THE DIACYL glycerol ethers (DAGE) have been studied in several investigations directed toward a better understanding of pathways by which lipids containing glycerol ethers are metabolized (1–3). The work of Thompson (1) with the terrestrial slug, *Arion ater*, suggests that the DAGE play an important role in initial steps leading to the formation of several other glycerol ethers of the neutral and phosphatide type. These findings are not supported, however, by the investigations of Bickerstaffe and Mead (2) or Ellingboe and Karnovsky (3) with other organisms.

The role DAGE plays in glycerol ether metabolism is worthy of further investigation. The dogfish (*Squalus acanthias*) is a desirable experimental animal for such studies because of the high percentages of DAGE present throughout its body, particularly the liver (4).

In the present work, palmitic acid-1-^{14}C and chimyl alcohol-1-^{14}C were administered to *Squalus acanthias* by intrahepatic injection. The radioactivity incorporated into the DAGE and other glycerol ether-containing lipids of the liver was then determined. The general structures of the ethers investigated, and the names applied to them, are presented below.



This work was conducted at the Torry Research Station, Ministry of Technology, Aberdeen, Scotland, and the Department of Biological Chemistry of the University of Aberdeen.

Abbreviations: GE, glycerol 1-monoether(s); AE, alk-1′-enyl glycerol 1-monoether(s); NE, nonesterified; MA, monoacyl; DA, 2,3-diacyl; TG, triglycerides; DG, diglycerides; MG, monoglycerides; TLC, thin-layer chromatography.

MATERIALS

Six dogfish (*Squalus acanthias*) were obtained from Scottish–Norwegian stock (5) off Noup Head (North Orkney) in the 1st wk of October, 1965, and near Greenstone Point (North Minch) in the 1st wk of February, 1966. The fish, all males, were caught by trawl techniques and then placed in salt-water tanks maintained at 10 ± 2°C. They remained in the tanks for 30 hr before the experiments so that they could adjust to the new environment. The fish were 69 ± 1 cm in length, which suggests that they were 6–7 yr old (6).

The palmitic acid-1-^{14}C was purchased from the Radiochemical Centre, Amersham, Bucks., England and had a specific activity of 143 μc/mg. The chimyl alcohol-1-^{14}C (7.2 μc/mg, a gift from C. R. Houle, U.S. Bureau of Commercial Fisheries Technological Laboratory, Seattle, Wash.) was prepared from palmitic acid-1-^{14}C in high chemical and radiopurity by the method of Baumann and Mangold (7). The purity of this compound was checked by thin-layer chromatography (TLC) and radioautography with techniques described by Mangold, Kammereck, and Malins (8). Triolein was obtained from The Hormel Institute, Austin, Minn. The petroleum ether used throughout this work had a boiling range of 60–80°C.

Glass columns (1.5 × 75 cm) packed with silicic acid (Mallinckrodt, analytical grade) were employed for column separations. The ratio of lipid to adsorbent was 1:30, w/w. Each column was equipped with a solvent reservoir and an attachment to permit the application of a slight pressure of nitrogen during chromatography. Basic adsorbents for the separation of free fatty acids were prepared according to McCarthy and Duthie (9). Layers of Silica Gel G, 250 μ thick, were activated at 135°C for 2 hr before use. Chromatographic fractions were located by chromic-sulfuric acid spray or by iodine vapor. When preparative amounts were isolated, glass sheets partially covered the chromatoplates so that only marker compounds were exposed to indicators.

METHODS

Administration of Labeled Compounds

Each fish was anesthetized by oral application of MS 222 (methanetricanesulphonate, Sandoz Products, London, England) (10) and placed on an operating table, ventral side up. The dorsal side of the fish was packed lightly with ice and refrigerated sea water was run into the mouth throughout the operation. A small midline incision was started just below the pectoral girdle and elongated to a point 2 inches posterior. The right lobe of the liver was then displaced from the body cavity and supported on a piece of sterile gauze covering the skin. Either palmitic acid-1-^{14}C (250 μc) or chimyl alcohol-1-^{14}C (81 and 101 μc) in 0.2 ml of triolein was then injected into the distal part of the lobe 2 inches below the gall bladder. The needle was run just below the hepatic peritoneum and the labeled compounds were injected slowly into the liver. The liver was then returned carefully to the body cavity and a water-tight seal of the skin was made by the use of surgical clips. The fish were replaced in the tank and held until they regained their normal righting reflexes and respiratory movements.

After a prescribed period (3.8–25 hr), the fish were killed and the right distal hepatic lobes were excised. Generally, the weight of these lobes was 31–37 g. In the first experiment, however, approximately half the lobe was taken (13.5 g). Each liver section was placed in a plastic bag which was immediately immersed in liquid nitrogen and stored at −29°C until needed. The lipids were extracted by the method of Hanson and Olley (11).

Analytical Methods

Milligram amounts of lipids were analyzed by the gravimetric technique of Komarek, Jensen, and Pickett (12). Usually, quantities of less than 1 mg were analyzed by the chromic-sulfuric acid method of Amenta (13). The technique of Katz and Keeney (14) was used, however, for the quantification of 2,4-dinitrophenylhydrazone derivatives of aldehydes released from alkenyl ethers. Radioactivity was measured in a Packard Tricarb instrument (Packard Instruments Co., Inc., Downers Grove, Ill.) by the techniques of Snyder and Stephens (15).

Synthesis of Derivatives

Saponification, acetylation, and methylation were as described by Farquhar (16). Dinitrophenylhydrazone derivatives of aldehydes were obtained from alkenyl ethers by the method of Katz and Keeney (14).

Separations

DAGE and TG. These compounds were isolated and characterized by methods previously described by Malins, Wekell, and Houle (4).

DAAE. These ethers were first detected when a thin-layer chromatogram of the total lipids (petroleum ether–diethyl ether 90:10) was sprayed with HCl–dinitrophenylhydrazine solution. After this treatment, a thin yellow band appeared on the upper edge of the DAGE. Because the DAAE were present in very small amounts, they were concentrated by column chromatography before their isolation by preparative TLC was attempted. For example, a silicic acid column, charged with 3.0 g of the total lipids from the "6-hr" fish, was eluted with petroleum ether–diethyl ether 95:5. The fraction isolated, weighing 1.05 g, was chromatographed on five plates with petroleum ether–diethyl ether 90:10

(4). The upper fifth of the DAGE–DAAE band was then scraped from the plate and eluted with diethyl ether. The DAAE present in this fraction were hydrolyzed in HCl–dinitrophenylhydrazine solution and the crude reaction product was recovered as previously described (14). The hydrazones and diglycerides (DG) were separated from unreacted DAGE by TLC in petroleum ether–diethyl ether–acetic acid 90:10:1. The DG were further purified on another plate in petroleum ether–diethyl ether 75:25. The yields of aldehydes and DG were 0.90 mg (0.09 mole %) and 2.1 mg (0.10 mole %), respectively.

MAAE, MAGE, and DG. The starting material for preparative TLC was obtained by further elution of the silicic acid column with petroleum ether–diethyl ether 75:15. The fraction (500 mg) containing the MAGE, MAAE, and DG was chromatographed on 10 plates in petroleum ether–diethyl ether 70:30 after removal of free fatty acids (9). A section of the adsorbent that included 0.5 cm above and below the area corresponding to marker diolein was scraped from the plate and eluted with diethyl ether. We dissolved the recovered lipid in HCl–dinitrophenyl-hydrazine solution in order to hydrolyze the MAAE (14). Hydrazones and monoglycerides (MG) were separated from a fraction comprising MAGE, DG, and sterols by TLC of the reaction product on a single plate, first with petroleum ether–diethyl ether 80:20 and then with 60:40. The yields of aldehydes and MG were 0.32 mg (0.03 mole %) and 0.44 mg (0.04 mole %), respectively.

The fraction containing the MAGE, DG, and sterols was acetylated and the resulting glycerides were separated on a chromatoplate by development with petroleum ether–diethyl ether 90:10 (4). Each glyceride fraction was saponified and the products were resolved by TLC in petroleum ether–diethyl ether–acetic acid 90:10:1. The plate containing the glycerol ether was developed a second time, in the same direction, with petroleum ether–diethyl ether 10:90. This procedure caused the NEGE to migrate. The weights of glycerol ethers and fatty acids recovered from the acetylated MAGE were 3.5 mg (0.30 mole %) and 3.0 mg (0.31 mole %), respectively. The weight of fatty acids obtained from the acetylated DG was 11.7 mg (1.2 mole %).

NEAE, NEGE, and MG. Finally, the silicic acid column was eluted with diethyl ether after removal of the MAAE, MAGE, and DG as described above. A column fraction (500 mg) was chromatographed on five plates in petroleum ether–diethyl ether 40:60 with monoolein as marker. A section of adsorbent extending 0.5 cm above and below the center of the area corresponding to the marker was scraped from the plate and eluted with diethyl ether. The isolated lipids were treated with HCl–dinitrophenylhydrazine solution and recovered in the usual manner (14). The products of the reaction were chromatographed twice in the same direction with petroleum ether–diethyl ether 85:15 and then 40:60. The hydrazones were separated from a fraction containing NEGE, MG, and sterols. Monoolein and the hydrazone of palmitaldehyde served as marker compounds. To confirm the presence of NEAE, we added selachyl alcohol to the fraction containing the NEGE, MG, and sterols, and again chromatographed the sample with the two solvent systems. The plate was sprayed with HCl–dinitrophenylhydrazine solution and then exposed to iodine vapor. The HCl–dinitrophenylhydrazine-reactive spot was found just above the NEGE, which was more evident because of the increased intensity due to the selachyl alcohol. Thus, the relationship of the NEGE to NEAE is consistent with the observation of Thompson and Lea (17) that NEAE migrate slightly ahead of NEGE on silicic acid plates. The yield of aldehydes was 0.27 mg (0.03 mole %). The fraction containing NEGE, MG, and sterols was methylated and the products of the reaction were chromatographed on thin layers with petroleum ether–diethyl ether 90:10 and then 10:90. Bands corresponding to methyl oleate and selachyl alcohol were then isolated. The yields of methyl esters and NEGE were 0.17 mg (0.02 mole %) and 0.10 mg (0.01 mole %), respectively.

Phospholipids. Phospholipids were eluted from silicic acid columns with methanol after the other lipid fractions had been removed. Hydrazone derivatives of aldehydogenic phospholipids were isolated and purified according to the methods of Katz and Keeney (14). Glycerol ethers (NEGE) were obtained from phospholipids by reduction with lithium aluminum hydride (17) and purification by TLC in petroleum ether–diethyl ether 10:90.

RESULTS AND DISCUSSION

Experiments with Palmitic Acid-1-^{14}C

The weights of and radioactivity recovered from the hepatic lipids of fish at the various time intervals are given in Table 1. The maximum radioactivity (1.3%)

TABLE 1 WEIGHTS AND RECOVERED RADIOACTIVITY FROM RIGHT DISTAL HEPATIC LOBES OF FISH INJECTED WITH PALMITIC ACID-1-^{14}C

Sample	Weight of Right Distal Lobe	Total Lipid	Recovered Radioactivity	Injected Dose
hr	*g*	%	*cpm*	%
3.8	13.5	33.4	3.8×10^7	0.6
6.0	31.0	37.6	3.6×10^7	0.6
10.5	34.6	54.2	4.4×10^7	0.8
18.5	36.5	55.0	7.1×10^7	1.3
25.0	32.2	56.3	4.0×10^6	<0.1

TABLE 2 RADIOACTIVITY INCORPORATED INTO THE HEPATIC LIPIDS OF FISH INJECTED WITH PALMITIC ACID-1-^{14}C

	Time in Hr											
	3.8			6.0			10.5			18.5		
Lipid Class	% of injected dose ($\times 10^{-3}$)	mole %	cpm/μmole ($\times 10^{3}$)	% of injected dose ($\times 10^{-3}$)	mole %	cpm/μmole ($\times 10^{3}$)	% of injected dose ($\times 10^{-3}$)	mole %	cpm/μmole ($\times 10^{3}$)	% of injected dose ($\times 10^{-3}$)	mole %	cpm/μmole ($\times 10^{3}$)
TG	66.2	81.3	0.9	280	48.1	2.4	340	74.4	1.2	230	70.9	0.8
DAGE	6.22	15.3	0.4	22.4	46.3	0.2	16.7	23.9	0.2	9.9	27.5	0.1
Alkoxy chain	2.0	13.8	0.1	3.2	45.9	0.03	2.5	21.8	0.03	8.9	24.7	0.1
DAAE	0.15	0.14	1.2	0.32	0.10	1.4	0.37	0.06	1.6	0.16	0.05	0.8
Aldehydes	0.02	0.12*	0.2	0.01	0.09	0.06	0.02	0.06	0.1	0.06	0.03	0.5
DG	0.52	0.53	1.2	2.7	0.54	2.2	4.5	0.36	3.4	1.7	0.45	1.0
MAGE	0.27	0.32	1.0	1.7	0.30	2.6	3.8	0.45	2.4	0.29	0.56	0.1
Alkoxy chain	0.06	0.29	0.3	0.39	0.31	0.5	0.35	0.21	0.4	0.29	0.38	0.2
MAAE	—	—	—	1.2	0.04	8.0	2.4	0.02	14.7	1.8	0.07	3.7
Aldehydes	0.19	0.04	5.5	0.22	0.03	3.0	0.75	0.02	9.7	0.23	0.06	0.1
MG	0.64	0.02	35.0	1.3	0.02	38.5	0.21	0.01	6.8	0.05	0.01	1.4
NEGE												
Alkoxy chain	—	—	—	0.28	0.01	11.7	0.18	0.01	5.6	0.03	1×10^{-3}	4.6
NEAE												
Aldehydes	0.38	0.02	20.7	0.16	0.03	2.6	0.94	0.06	3.8	0.24	0.04	1.6
Phospholipids	24.6	2.72	10.0	57.9	2.2	11.1	27.8	1.9	3.9	140	1.95	18.4
Aldehydes	0.02	0.06	0.5	0.14	0.08	0.7	0.10	0.05	0.5	0.08	0.09	0.3
Free fatty acids	500	0.32	1.8×10^{3}	200	1.6	57.0	370	1.3	78.0	790	2.9	66.0

The data on the fish killed after 25 hr are not included because the incorporated radioactivity and specific activities were very low.
* The discrepancy between the mole % of the total lipids and the ether chains is due to experimental error. The values for intact glycerol ether-containing lipids were obtained by calculation from the components derived by hydrolysis.

recovered after the administration of 250 μc of palmitic acid-1-^{14}C was found after 18.5 hr.[1] The results may be compared with those obtained by Kayama, Tsuchiya, and Tohoku (18) after administration of stearic acid-1-^{14}C to carp by intraintestinal intubation. They found a maximum in the specific activity of total liver lipids after 6 hr; after 10 hr only 0.97% of the administered radioactivity was recovered from the liver, compared to 47% from muscle and skin. Their findings suggest that the labeled fatty acid was distributed throughout the body via the portal blood and only later deposited in the liver from the circulatory system.

The radioactivity data for all lipid classes isolated in this work are presented in Table 2. Triglycerides, DAGE, and phospholipids accounted for most of the incorporated radioactivity (88% at 6 hr). The detection of significant radioactivity in the alkyl and alkenyl ether chains demonstrates a reductive incorporation from fatty acid. By contrast, Friedberg and Greene (19) were unable to obtain the incorporation of labeled fatty acid in vitro into the glycerol ethers of the intestine of either *Squalus acanthias* or the skate (*Raja erinacea*).

The specific activities of the aldehydes derived from the DAAE always exceeded the values for the alkoxy chains of the DAGE (Table 2). This difference was particularly great after 10.5 and 18.5 hr. The specific activities of the alkoxy chains of the DAGE were low at all times in comparison to the ether chains of other lipids. With the exception of the relationship of the DAAE to MAGE in the fish after 18.5 hr, the specific activities of the ether chains of the NEGE, NEAE, MAGE, and MAAE were significantly higher than those of the corresponding diacyl derivatives (DAGE and DAAE). Furthermore, the specific activities of the aldehydes of the phospholipids exceeded those of the alkoxy chains of the DAGE, reaching a ratio of 23:1 in the 6-hr fish. It is of interest to compare these findings to those reported by Thompson (1) with *Arion ater*. In studies with palmitic acid-1-^{14}C and tritium-labeled glycerol ethers, Thompson concluded that the DAGE are formed very rapidly and that the radioactivity of these compounds declined markedly as that of the glycerol ether phosphatides increased. Thompson proposed a tentative pathway in which the DAGE give rise to the glycerol ether phosphatides by replacement of the ester group on the 3-position of glycerol with a phosphoryl base. The glycerol ether phosphatides are then converted to phosphatide plasmalogens. Also suggested was the possibility that DAGE give rise to DAAE.

If a *discrete* pool of highly radioactive DAGE can be ruled out, the data obtained in the present experiments preclude the possibility that DAGE are metabolized to either DAAE or glycerol ether phosphatides as proposed

[1] Examination of an aqueous extract (6 hr) of the liver indicated that an insignificant amount of radioactivity (about 1% of the value obtained for the total lipids) was associated with water-soluble products.

TABLE 3 INCORPORATION OF RADIOACTIVITY INTO LIPIDS AFTER ADMINISTRATION OF CHIMYL ALCOHOL-1-^{14}C*

Lipid Class	Time in Hr: 4.0 — % of injected dose (×10⁻³)	4.0 — mole %	4.0 — cpm/μmole (×10³)	10.5 — % of injected dose (×10⁻³)	10.5 — mole %	10.5 — cpm/μmole (×10³)
TG	—	—	—	170	84.0	0.2
DAGE	—	—	—	4.25	11.4	0.04
Alkoxy chain	—	—	—	1.4	10.5	0.01
DAAE	—	—	—	0.30	4×10^{-3}	6.6
Aldehydes	—	—	—	0.04	4×10^{-3}	0.9
DG + MAGE fatty acids	5.2	1.2	0.3	6.28	0.12	4.6
MAGE						
Alkoxy	5.7	0.24	1.6	42.5	0.36	10.4
MAAE	—	—	—	0.73	1×10^{-3}	50.8
Aldehydes	0.28	5×10^{-3}	3.5	0.47	1×10^{-3}	30.0
MG	2.8	0.04	5.0	20.7	0.11	18.9
NEGE						
Alkoxy chain	49.3	0.12	28.8	820	0.13	6×10^{2}
NEAE						
Aldehydes	0.02	1×10^{-4}	15.4	0.77	2×10^{-4}	2.7×10^{2}
Phospholipids	—	—	—	140	2.5	4.8
Aldehydes	0.82	0.23	0.2	1.9	0.02	6.51
Alkoxy chain	—	—	—	32.5	0.47	6.0
Free fatty acids	160	9.9	1.1	270	2.8	84.0

* In the 4.0 hr and 10.5 hr experiments, 101 μc and 81 μc were administered, respectively.

by Thompson (1). In contrast to the findings with *Arion ater* (1), it appears that the DAGE represent primarily a terminal point in the biosynthesis of glycerol ethers. Such an argument is supported by the fact that the ether chains of the small percentages of NEGE, NEAE, MAGE, and MAAE had considerably higher specific activities in comparison to the DAGE. The DAGE are apparently the major form in which the ether linkage is "stored" in the liver (4). It is likely that the metabolism of these compounds is related in a fundamental way to the unique function of the liver as a hydrostatic organ (20).

Experiments with Chimyl Alcohol-1-^{14}C

In the 4-hr and 10.5-hr experiments, 0.3% and 3.9% of the administered radioactivity was recovered in the total lipids, respectively. As with the palmitic acid-1-^{14}C experiments, this increase of recovered radioactivity with time can be explained in terms of deposition from the circulatory system.

One of the most striking features of the present data is the evidence for rapid and extensive oxidative cleavage of the ether bond of chimyl alcohol (Table 3). At 10.5 hr, free fatty acids account for 68% of the radioactivity of the total lipids. There is ample evidence to establish the presence in nature of enzymes capable of cleaving the ether linkage of glycerol ethers (21–23). Lewis (24) suggested, however, that elasmobranch fish, which contain large amounts of glycerol ethers in their tissues, may not have developed the enzyme systems necessary to metabolize the ether linkage. Furthermore, the studies of Pfleger, Piantadosi, and Snyder (25) and of Thompson (26) suggest that organisms rich in glycerol ethers are poor in ether-cleaving enzymes. The extensive oxidative cleavage of the chimyl alcohol-1-^{14}C in the present experiments does not suggest such an inverse relationship between glycerol ether content and enzyme activity. It appears that glycerol ethers, present in high percentages, are oxidized and resynthesized at a rapid rate in the liver of *Squalus acanthias*.

The relatively low specific activity of the DAGE 10.5 hr after injection supports the conclusion drawn from the palmitic acid work that the DAGE are not important precursors in the formation of other glycerol ethers in this species.

The high ratios of specific activities of NEGE:NEAE (2:1 at both time periods) (Table 3) suggest that part of the labeled chimyl alcohol was converted directly to the analogous plasmalogen (NEAE), possibly via a biodehydrogenation reaction. This observation is consistent with the findings of Thompson (1, 26) which indicate a conversion from alkyl to alkenyl ether in *Arion ater*. A possible source of the NEAE could be free or esterified fatty acids. In both experiments, however, the specific activities of the NEAE were significantly higher than the specific activities of any fatty acids examined (Table 3).

No evidence was found for the presence of glycerol ether phosphatides in the experiments with palmitic acid-1-^{14}C. Nevertheless, small percentages of NEGE

were isolated from the phospholipid fractions by lithium aluminum hydride reduction (17) after the administration of labeled chimyl alcohol. A capacity to phosphorylate glycerol ethers was therefore demonstrated, but it is uncertain if such a conversion is an important intermediary step in the metabolism of glycerol ethers.

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