THE BIOSYNTHESIS OF ALKYL ETHER BONDS IN LIPIDS BY A CELL-FREE SYSTEM[1]

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Investigators have found the biological significance and the biosynthetic pathways of ether-linked lipids containing glycerol difficult to determine (1). Despite available knowledge concerning enzymes for the biocleavage (2,3), deacylation (4), acylation (5,6), and phosphorylation (7) of glyceryl ethers, the biosynthesis of the ether bond in these compounds has not previously been demonstrated in a cell-free system.

This note describes an active enzyme complex capable of converting intact 1-^{14}C-labeled fatty alcohols into alkyl glyceryl ethers. The reaction has been demonstrated in whole homogenates and in microsomal-plus-supernatant fractions of transplantable preputial tumors in mice. These tumors are typical of neoplasms containing high quantities of alkyl ether linkages in both neutral- and phospho-glycerides (8).

METHODS

Animals and the Preparation of Homogenates and Organelles

The preputial tumors (ESR-586) used in our work were originally purchased from The Jackson Laboratory, Bar Harbor, Maine 04609. Our stock of tumors is maintained by transplanting small portions of minced tumor by subcutaneous injection on the back of C57BL/6 mice, using a Lightwicz antrum trocar attached to a 10-ml syringe.

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Animals were killed by decapitation approximately 14, 34-45, and 58 days after transplanting the tumors. The entire tumor was rapidly removed, trimmed of connective tissue, diced with scissors, and immediately placed in a beaker containing 20 ml ice cold 0.1 M potassium phosphate buffer at pH 7.0. All subsequent steps were carried out in crushed ice until incubation at 37°C was initiated. The tumors were homogenized in a Potter-Elvehjem homogenizer flask using four strokes of a Teflon pestle attached to a Lourdes homogenizing motor set at 50. All homogenates were centrifuged 4000 *g* min at 1°C in an International refrigerated centrifuge (Model PR-1) to remove the whole cells, nuclei, and other debris. In certain experiments, mitochondria (150,000 *g* min pellet), microsomes (6,000,000 *g* min pellet), and supernatant (6,000,000 *g* min supernatant) were prepared in a sucrose buffer using a Beckman Spinco centrifuge (Model L-65B) according to procedures previously described in detail (3).

Incubations

All preparations derived from the preputial tumor were incubated with various cofactors and ^{14}C-labeled substrates (0.4 μc per vial) in a final volume of 3.0 ml per glass vial in a Dubnoff metabolic shaking incubator at 37°C and 150 oscillations per min. The quantity of protein (9) incubated per vial was approximately 15 mg from the homogenates, 4 mg from the mitochondrial fraction, 1 mg from the microsomal fraction, and 7 mg from the supernatant fraction. The quantities of cofactors and substrates (per vial) used in this investigation were: 30 μmoles ATP, 0.3 μmoles CoA, 12 μmoles $MgCl_2$, 3 μmoles NADP, 30 μmoles DL-α-glycerophosphate, 6 μmoles NADH, 78 mμmoles 1-^{14}C lauryl alcohol, 75 mμmoles 1-^{14}C cetyl alcohol, 40 mμmoles 1-^{14}C palmitic acid, 93 mμmoles 1-^{14}C myristic acid, 25 mμmoles 1-^{14}C octanoic acid, and 50 mμmoles 1-^{14}C palmitaldehyde. All ^{14}C-labeled substrates were purchased from New England Nuclear Corp., Inc. and purified by thin-layer chromatography (TLC) to >99% purity as determined by zonal profile scanning (10,11). The exact combination of cofactors and substrates in each vial is described in the

tables. The ATP, CoA, NADP, and NADH were purchased from P-L Biochemicals, Inc. The α-glycerophosphate was from Sigma Chemical Co. and the reagent grade $MgCl_2$ from Fisher Scientific Co.

Lipid Analysis

Lipids were extracted from the incubation mixture by the procedure of Bligh and Dyer (12). Aliquots of the total lipid extracts were chromatographed on Silica Gel G in solvent systems of hexane:diethyl ether:acetic acid (90:10:1 or 80:20:1, v/v) for resolving neutral lipids, or on Silica Gel HR in a solvent system of chloroform:methanol:acetic acid:saline (50:25:8:4, v/v) for resolving phospholipids. Aliquots of total lipids were reduced with $LiAlH_4$, and the products (fatty alcohols and the O-alkyl and O-alk-1-enyl glycerols) were resolved on Silica Gel G in a solvent system of diethyl ether saturated with water (13). In some instances, the free glyceryl ethers were quantitatively eluted from the Silica Gel G with chloroform:methanol (2:1, v/v) and the isopropylidene derivatives of the glyceryl ethers prepared (14). The isopropylidene derivatives were chromatographed on Silica Gel G in a solvent system of hexane: diethyl ether (90:10 or 80:20, v/v). All chromatoplates were radioassayed by liquid scintillation area- or zonal profile-scans (10,11). Gas-liquid chromatography (GLC) of the glyceryl ether isopropylidene derivatives was carried out as previously described (15); radioactivity was collected directly in a scintillation solvent (11) using aluminum tubing as the connecting link between the GLC detector and the counting vial containing the scintillation solvent.

RESULTS AND DISCUSSION

The biosynthesis of the ether bond in glyceryl ether lipids was demonstrated in homogenates and a microsomal-plus-supernatant fraction obtained from preparations of a transplantable preputial tumor in mice. The identification of newly synthesized O-alkylglycerols from 1-^{14}C-labeled fatty alcohols in a cell-free system was based on the TLC isolation of (a) ^{14}C-

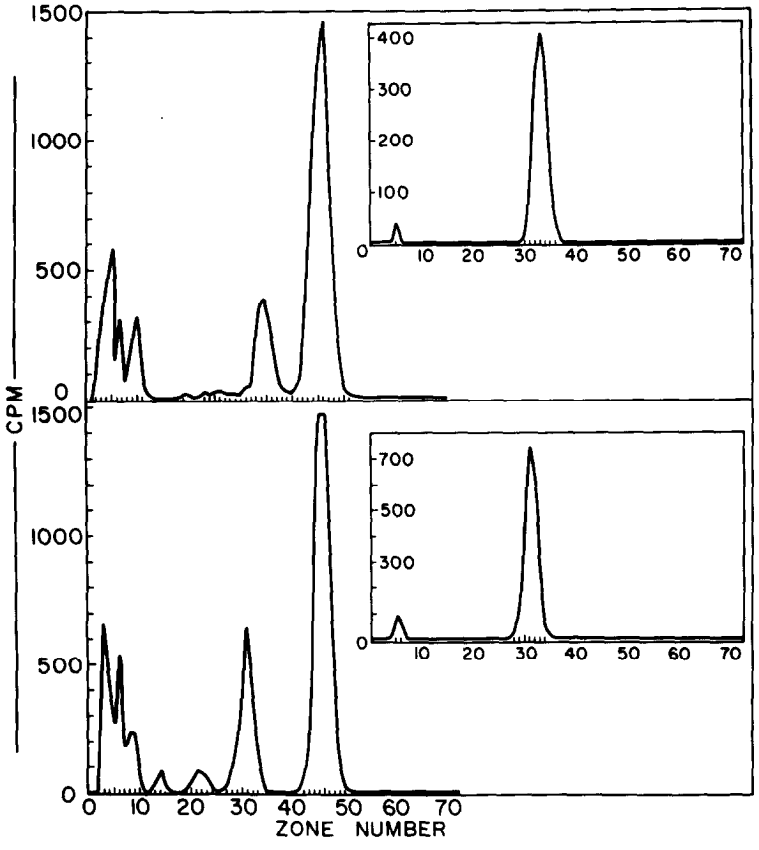

Figure 1 -- Zonal profile scans of total lipid extract obtained after a 3-hr incubation of 1-^{14}C cetyl alcohol (upper scan) and 1-^{14}C lauryl alcohol (lower scan) with homogenates of preputial tumors and cofactors (ATP, CoA, NADP, and α-glycerophosphate). Peak identification of resolved components is according to zone number: free fatty acids (13-16), triglycerides (18-24), diacylglyceryl ethers (26-35), wax esters (40-50). The diacylglyceryl ethers were isolated by preparative TLC and reduced with $LiAlH_4$; about 75% of the ^{14}C in this fraction was associated with the O-alkylglycerols and the remainder with fatty alcohols derived from the acyl moieties. The zonal profile scans depicted in the insert were obtained by chromatography of the isopropylidene derivatives of the O-alkylglycerols liberated from total lipids by $LiAlH_4$ reduction (see text for details).

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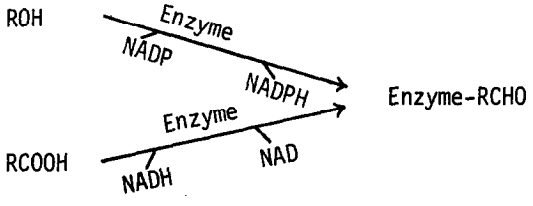
diacylglyceryl ethers (Fig. 1), (b) ^{14}C-O-alkylglycerols after $LiAlH_4$ reduction of total lipid extracts (Tables I and II) and of purified glyceryl ether diesters (Fig. 1), and (c) isopropylidene derivatives of the O-alkylglycerols isolated after $LiAlH_4$ reduction (Fig. 1), and on the GLC isolation of isopropylidene derivatives of the ^{14}C-labeled O-alkylglycerols liberated from total lipids by $LiAlH_4$ reduction.

Figure 1 depicts zonal profile scans of thin-layer chromatograms of an extract obtained from the homogenates which were incubated with $^{14}C_{12:0}$ and $^{14}C_{16:0}$ fatty alcohols and cofactors. The solvent system used resolves diacyl glyceryl ethers (zones 26-35) and triacylglycerols (zones 18-24). Glyceryl ether biosynthesis is relatively slow, but as much as 46% of 78 mμmoles 1-^{14}C lauryl alcohol was incorporated into ether linkage with glycerol during a 5-hour period. Gas-liquid chromatography of the ^{14}C-labeled O-alkylglycerol isopropylidene derivatives prepared from the homogenate samples revealed that 90% and 89% of the ^{14}C were associated with the $C_{12:0}$ and $C_{16:0}$ glyceryl ethers. Such data indicate that the $C_{12:0}$ and $C_{16:0}$ fatty alcohols were attached to the glycerol moiety intact and that neither degradation nor chain elongation occurred in our system.

Our experiments with the homogenates (Fig. 1) were also designed to rule out the presence of glyceryl ethers in neoplasms simply as the result of a reversibility of the ether-cleaving enzymes in rat liver (2,3).



The enzyme aldehyde complex could react with a glycerol intermediate to form a hemiacetal structure which yields glyceryl ethers by dehydration.

However, we soon established that only 1-^{14}C fatty alcohols could be rapidly incorporated into O-alkylglycerols: 1-^{14}C-labeled $C_{8:0}$, $C_{14:0}$, or $C_{16:0}$ fatty acids (in the presence of NADH) and 1-^{14}C palmitaldehyde were not substrates. Although the free 1-^{14}C palmitaldehyde was not incorporated into O-alkylglycerols (Table I), it did not rule out the possibility that a tightly bound aldehyde-enzyme complex resulting from the oxidation of alcohol to aldehyde could form the active enzyme complex. But when we found that NADP, a hydrogen acceptor necessary for the oxidation of an alcohol to an

TABLE I

BIOSYNTHESIS OF ALKYL ETHER BONDS OF LIPIDS IN HOMOGENATES AND SUBCELLULAR FRACTIONS OF PREPUTIAL TUMORS FROM MICE

Sample	Incorporation of 1-^{14}C fatty alcohols† into O-alkylglycerols††	
	Cetyl alcohol	
	mμmoles	% ^{14}C
1. Homogenate†††	10	13
2. Mitochondria	1	1
3. Microsomes	2	3
4. Supernatant	1	2
5. Mitochondria + supernatant	4	5
6. Microsomes + supernatant	11	15
7. Microsomes + boiled (15 min) supernatant	1	2
8. Boiled (15 min) microsomes + supernatant	1	2
9. Microsomes + supernatant from rat liver	11	15

†Incubated for 3 hr with ATP, CoA, and Mg^{++} as described in methods section.

††O-Alkylglycerols liberated from total lipids by $LiAlH_4$ reduction.

†††Essentially none of the radioactivity from 1-^{14}C palmitaldehyde, 1-^{14}C octanoic acid, or 1-^{14}C myristic acid (incubated under identical conditions) was incorporated into O-alkylglycerols.

aldehyde (3), inhibited the incorporation of the fatty alcohols into O-alkyl glycerols (Table II), it seemed that reversibility of the ether-cleaving enzyme system was not responsible for glyceryl ether biosynthesis.

The biosynthesis of glyceryl ethers was also studied in subcellular fractions incubated with 1-^{14}C-labeled fatty alcohols. The data in Table I showed that the microsomes-plus-supernatant fraction was essential for the biosynthesis of ether bonds; neither microsomes nor supernatants alone were sufficient. Boiled supernatants added to microsomes and boiled microsomes

TABLE II

EFFECT OF COFACTORS AND SUBSTRATES ON THE BIOSYNTHESIS OF ALKYL ETHER BONDS OF LIPIDS IN A MICROSOME-PLUS-SUPERNATANT FRACTION OBTAINED FROM MOUSE PREPUTIAL TUMORS

Cofactor additions	Incorporation of ^{14}C into O-alkylglycerols†,††		
	Substrate	mμmoles	% ^{14}C
1. Complete system†††	1-^{14}C cetyl alcohol	17	23
2. Complete system minus CoA	1-^{14}C cetyl alcohol	8	10
3. Complete system minus Mg^{++}	1-^{14}C cetyl alcohol	8	10
4. Complete system minus CoA and Mg^{++}	1-^{14}C cetyl alcohol	6	8
5. Complete system + NADP	1-^{14}C cetyl alcohol	8	11
6. Complete system + α-glycero-phosphate	1-^{14}C cetyl alcohol	13	17
7. Complete system + α-glycero-phosphate; microsomes + boiled (15 min) super-natant	1-^{14}C cetyl alcohol	2	2
8. No cofactors	1-^{14}C cetyl alcohol	1	1
9. Complete system + NADH	1-^{14}C palmitic acid	1	3

†Incubated for 3 hr as described in methods section.

††O-Alkylglycerols liberated from total lipids by $LiAlH_4$ reduction.

†††Each vial contained ATP, CoA, Mg^{++}, and microsomes-plus-supernatant in the amounts described in the methods section.

added to supernatants were inactive, but a supernatant fraction obtained from rat liver was able to replace the active supernatant derived from the tumor. Glyceryl ether biosynthesis could not be demonstrated in preparations obtained from old tumors (58 days) which grossly appeared necrotic.

The cofactor requirements for maximum incorporation of 1-^{14}C fatty alcohols into glyceryl ethers were ATP, CoA, and Mg^{++} (Table II). α-Glycerophosphate does not appear to be the glycerol source in this reaction. The results obtained from our experiments suggest that, instead, an aldehydogenic glycerol derivative reacts with the fatty alcohols to form glyceryl ethers. This glycerol derivative could be thought of as a phosphorylated intermediate, since ATP and Mg^{++} are necessary as cofactors. The stimulatory effect of CoA is probably related to an enhanced utilization of the initial product in acylation reactions (also requiring ATP for thiokinase), a system that is active in this tumor (6). Further investigation of the system to establish the identity of the glycerol moiety reacting with fatty alcohols is currently under way.

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