A NEW METABOLIC PATHWAY: BIOSYNTHESIS OF ALKYL ETHER BONDS FROM GLYCERALDEHYDE-3-PHOSPHATE AND FATTY ALCOHOLS BY MICROSOMAL ENZYMES[1]

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We have recently described (1) a microsomal (100,000 *g* pellet) plus soluble protein (100,000 *g* supernatant) complex in preputial gland tumors that makes alkyl ether bonds in glycerolipids from 1-^{14}C-labeled long-chain fatty alcohols. It is the first cell-free system found to form glyceryl ethers. Cofactor requirements for maximum incorporation of the fatty alcohols into C-O-C bonds were ATP, CoA, and Mg^{++}. Fatty acids or fatty aldehydes did not serve as substitutes for the fatty alcohols in this system. Formation of the ether bond in the incubation mixture was documented by (a) isolation of diacyl glyceryl ethers, (b) isolation of ^{14}C-labeled alkylglycerols (free glyceryl ethers) by thin-layer chromatography (TLC) after $LiAlH_4$ reduction of diacyl glyceryl ethers and total lipids, and (c) gas-liquid chromatographic isolation of isopropylidene derivatives of the ^{14}C-labeled O-alkylglycerols (formed by $LiAlH_4$ reduction) having alkyl side chains identical to the fatty alcohol precursors incubated.

The facts that soluble proteins from rat liver could be substituted for the soluble protein fraction from the tumor in stimulating ether biosynthesis and that α-glycerophosphate had no effect on ether biosynthesis suggested to us that some other glycolytic product, perhaps an aldehydogenic glycerol derivative, was the glycerol acceptor in the formation of the alkyl ether bond (1). We have just

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completed a series of experiments with a number of key compounds in the glycolytic pathway to determine the source of the glycerol moiety and the nature of the reaction responsible for glyceryl ether biosynthesis.

METHODS

The biological and analytical methods used were the same as those described in our original communication on this subject (1). Microsomes (unwashed and washed 3 times) were prepared at 100,000 *g* in 0.1 M phosphate buffer containing 0.25 M sucrose (1). Quantities of substrates and cofactors incubated per vial in a final volume of 3 ml (Tables I and II) were 10 or 30 μmoles ATP, 10 or 30 μmoles ADP, 0.3 μmoles CoA, 12 μmoles Mg^{++}, 15 μmoles DL-glyceraldehyde-3-PO_4, 75 mμmoles 1-^{14}C-cetyl alcohol (0.4 μc added in 10 λ of 1% Tween 80), 3 μmoles NADP, 6 μmoles NADPH, 20 μmoles D-glucose-6-PO_4, 1 unit glucose-6-PO_4 dehydrogenase, and 125 μmoles NaF. The commercial sources of cofactors and substrates were the same as before (1). The complete system referred to in the tables includes ≃1.3 mg microsomal protein, 15 μmoles DL-glyceraldehyde-3-PO_4, 30 μmoles ATP, 12 μmoles Mg^{++}, and 0.3 μmoles CoA per vial. All incubations were for 3 h at 37°C in a Dubnoff shaker set at 150 oscillations per min unless otherwise stated. The NADPH generating system consisted of glucose-6-PO_4, glucose-6-PO_4 dehydrogenase, and NADP.

Glyceryl ether lipids were isolated by thin-layer chromatography before and after $LiAlH_4$ reduction of total lipids. The presence of glyceryl ethers in representative samples was confirmed by thin-layer and gas-liquid chromatography of their isopropylidene derivatives; 86-91% of the ^{14}C-activity was associated with 16:0 glyceryl ether peak (chimyl alcohol) after incubating cetyl alcohol.

RESULTS AND DISCUSSION

The results in Table I are typical, and conclusively demonstrate that a microsomal enzyme complex can form a C-O-C bond when long-chain fatty alcohols and glyceraldehyde-3-PO_4 are substrates; ATP, Mg^{++}, and CoA are essential cofactors. Ten μmoles of ATP had the same effect as the 30 μmoles generally used, and ADP can substitute for ATP but not on an equal molar basis at lower concentrations.

Table I. The Biosynthesis of C-O-C Bonds in Glycerolipids by Microsomes of Preputial Gland Tumors

System	% ^{14}C of 1-^{14}C-cetyl alcohol (75 mμmoles incubated) incorporated into O-alkylglycerols derived from total lipids after $LiAlH_4$ reduction*
1. Complete	31, 26, 19, 28
2. Complete minus ATP	0.9, 1.2
3. Complete minus CoA	1.0, 0.93
4. Complete minus Mg^{++}	1.0, 0.99
5. Complete minus glyceraldehyde-3-PO_4	1.9, 0.75
6. Complete except microsomes were boiled	1.9, 0.75
7. Microsomes and ^{14}C-cetyl alcohol alone	0.64, 2.3

Microsomes were washed 3 times; see text for details on cofactors, substrates, and other conditions.

*Values calculated for individual vials.

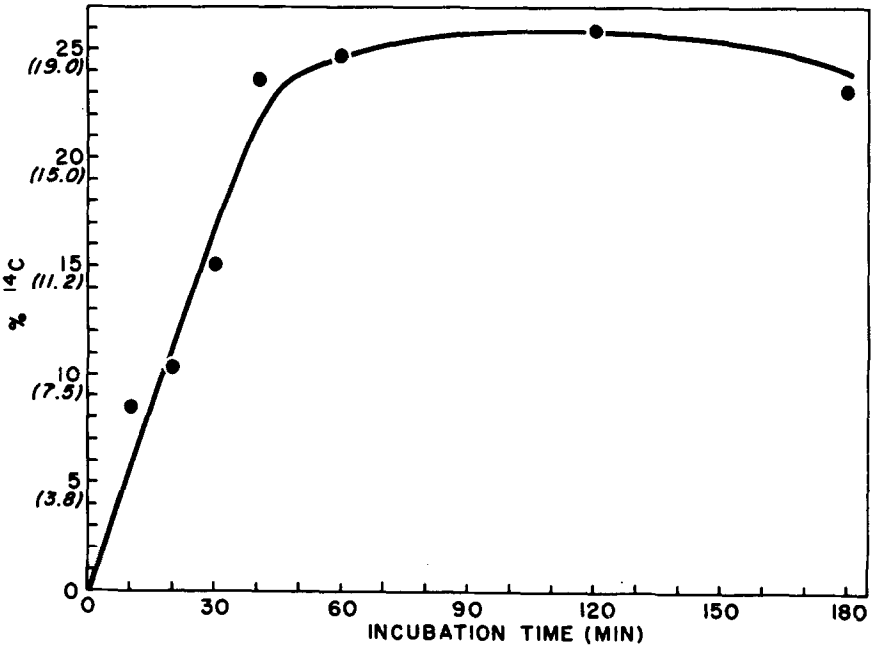

Figure 1 -- Incorporation of 1-^{14}C-cetyl alcohol into O-alkylglycerols of total lipids (after $LiAlH_4$ reduction) by an ether-synthesizing enzyme complex in preputial tumor microsomes. Each value is the mean of duplicate incubations. The values in parentheses below the percentages refer to mμmoles of fatty alcohol incorporated. The complete system, as described in text, was used for this experiment.

NADPH is required at one of the stages before acylation can occur. Fatty acids, fatty aldehydes, or glycerophosphate did not participate as substrates. Nonspecific esterases, requiring no cofactors, incorporate a portion of the fatty alcohols into waxes during incubations.

Previous data obtained with homogenates, in the absence of an added glycerol source, indicated that the reaction for ether biosynthesis proceeded rather slowly (1), and a number of the experiments reported here were also completed with 3-hr incubations. However, recent time curves for ether biosynthesis obtained with the specific microsomal system described in this paper have revealed that maximal incorporation of fatty alcohols occurs between 0.5 and 1 h, and change very little after this period (Fig. 1). The following reaction scheme is indicated

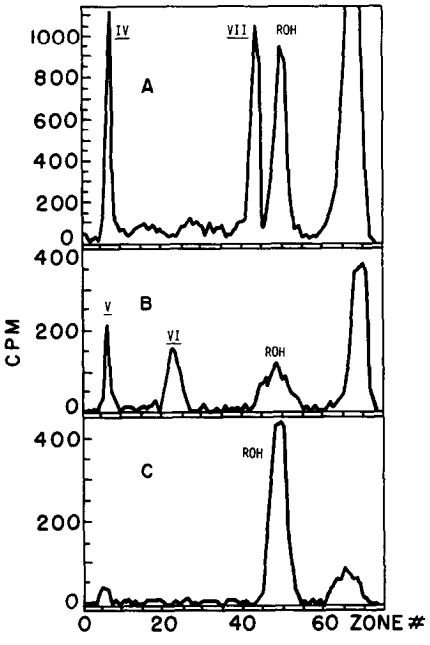

Figure 2 -- Zonal profile scans (2-mm) of thin-layer chromatographic separations of total lipids isolated from the vials described in Table II, i.e., (A) no NADPH, (B) 6 μmoles NADPH added at end of 3 h, and (C) 6 μmoles NADPH added at zero time. Numbers next to peaks are thought to be compounds depicted by same numbers in Fig. 3. The peak at solvent front (>zone 60) is other nonpolar lipids. The chromatogram was developed in diethyl ether:acetic acid, 100:0.5 (v/v). The isopropylidene derivative of the "free" glyceryl ethers (Chromatogram B, peak VI) was isolated by gas-liquid radiochromatography (1); 88% of the ^{14}C was collected as the 16:0 glyceryl ether (chimyl alcohol).

by our incubation of substrates and cofactors (Tables), and by the chromatographic behavior (Fig. 2) of the labeled components found after incubations:

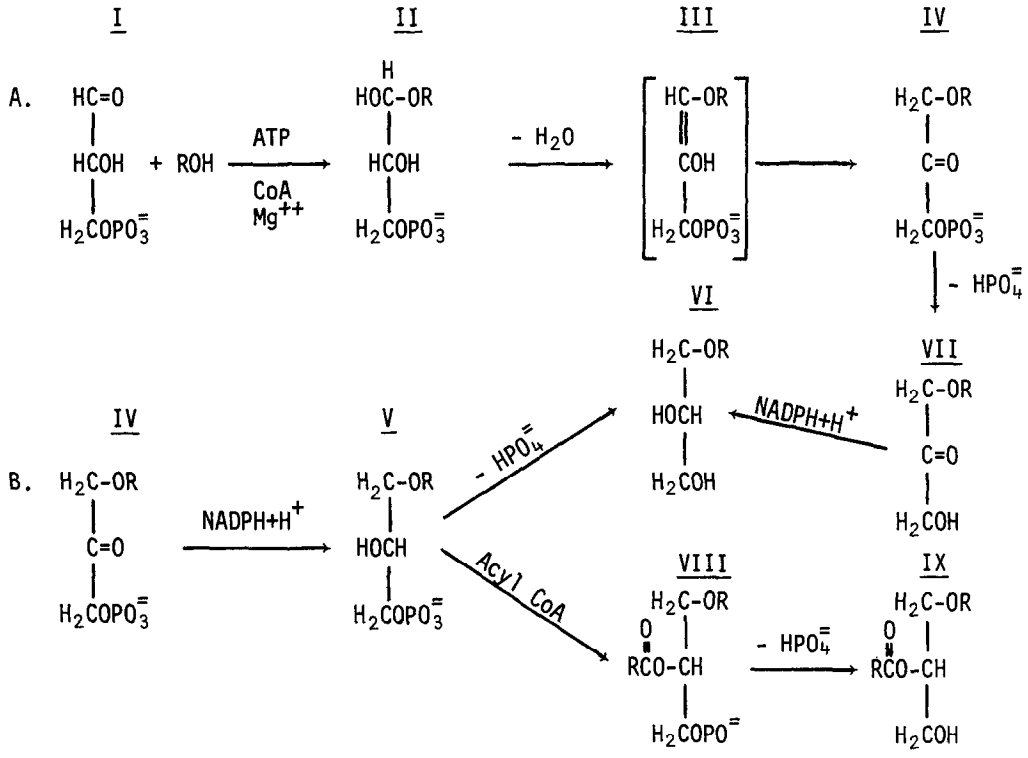

Figure 3. Proposed Biosynthetic Pathway for Alkyl Glyceryl Ethers

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We believe that the first step (Fig. 3,A) is the formation of a hemiacetal (II) and that the keto form (IV) of the glyceryl ether phosphate from dehydration of the hemiacetal occurs, since there is no appreciable biosynthesis of free glyceryl ethers (Table II) or diacyl glyceryl ethers in the absence of NADPH. Furthermore, the O-alkylglycerols isolated after $LiAlH_4$ reduction of samples incubated without NADPH would be expected if the keto form were present. Although NADPH is necessary for the reduction of the ketone intermediates (IV and VII) before acylation can occur, it cannot be added at the beginning of the reaction when reduction of the glyceraldehyde-3-PO_4 can also occur and prevent the formation of C-O-C bonds (Table II). The enzymic reduction of the keto compounds (IV and VII)

Table II. The Effect of NADPH and NaF on the Formation of "Free" O-Alkylglycerols by an Ether-Synthesizing Enzyme Complex in Preputial Tumor Microsomes

System	"Free" glyceryl ethers before $LiAlH_4$ reduction	"Free" glyceryl ethers after $LiAlH_4$ reduction of total lipids
	*% 1-^{14}C-cetyl alcohol (75 mμmoles) incorporated**	
†1. Complete	2.9, 1.6	31, 32
†2. Complete plus NADPH at beginning of incubation	1.3, 1.2	2.4, 3.1
†3. Complete; at end of 3 h, NADPH was added and incubated for 1 more h.	8.1, 8.1	31, 31
†4. Complete; at end of 3 h, NADPH and soluble fraction were added and incubated for 1 more h.	15, 13	33, 34
‡5. Complete; at end of 0.5 h, NADPH generating system added and incubated for 0.5 h.	19, 17	32, 31
‡6. Complete plus NaF; at end of 0.5 h, NADPH generating system added and incubated for 0.5 h.	1.3, 0.33	21, 20

* Values calculated for individual vials.

† Microsomes were washed 3 times; see text for details on cofactors, substrates, and other conditions.

‡ Unwashed microsomes.

would also permit the proper stereochemical orientation of the hydroxyl group at position 2, since naturally occurring glyceryl ethers have the alkyl moiety at the 1-position of glycerol (2) [Hirschmann's nomenclature (3)]. Indeed, the build-up of ^{14}C-labeled free glyceryl ethers (VI) present in the total lipids of samples incubated with NADPH (Table II, Fig. 2) and the build-up of other ^{14}C-components, which we believe to be compounds IV and VII, in the absence of NADPH, support this conclusion (Fig. 2). These data are compatible with the observation that phosphatidate hydrolase can attack lysophosphatidates (4). Furthermore, NaF inhibited the

release of phosphate, preventing the formation of free glyceryl ethers in the presence of NADPH. The enzyme responsible for reduction of the keto compounds appears to be in the supernatant fraction or only loosely bound to microsomes.

Four preparations of homogenates (4,000 *g* min supernatant) in this series formed diacyl glyceryl ethers (23% ^{14}C incorporated) when NADP was added to generate NADPH, confirming our earlier data (1) on the biosynthesis of the diacyl type. Reaction B (Fig. 3) provides the necessary acylation sequence (1) that yields the diacyl glyceryl ethers isolated from homogenates containing an NADPH source. This portion of the scheme resembles the pathway for the biosynthesis of glycerides (5).

The roles of CoA, ATP or ADP, and Mg^{++} in reaction A are not known although it is quite possible that the fatty alcohols require phosphorylation. Kinetic data of the various parameters involved are being accumulated by us while we study the isolation and characterization of intermediates in this new biosynthetic pathway. The direct precursor role of fatty alcohols in glyceryl ether biosynthesis was suggested by earlier data showing that the locations of double bonds in the hydrocarbon moieties of glyceryl ethers and fatty alcohols in normal preputial glands were nearly identical (6).

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