THE BIOSYNTHESIS OF PLASMALOGENS IN A CELL-FREE SYSTEM

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1. Introduction

Until now, the biosynthesis of plasmalogens has not been demonstrated in a cell-free system. In our earlier studies on the enzymic synsthesis of *O*-alkyl bonds in glycerolipids [1–5], we suggested that the formation of the *O*-alkyl linkage from fatty alcohols and dihydroxyacetone-P might be the initial step in plasmalogen biosynthesis. *In vivo* data obtained with *O*-alkylglycerols [6, 7] and fatty alcohols [5, 8, 9] labeled with ^{14}C, ^{3}H, and ^{18}O indicate that the *O*-alkyl glycerolipids are precursors of *O*-alk-1-enyl glycerolipids.

This report describes the biosynthesis of plasmalogens (*O*-alk-1-enyl glycerolipids) in homogenates and mitochondrial supernatants prepared from Ehrlich ascites cells. Substrates and cofactors (dihydroxyacetone-P, fatty alcohols, CoA, ATP, and Mg^{2+}) required for the biosynthesis of *O*-alkyl lipids [1–5], plus $NADP^{+}$, were used for optimal incorporation of radioactivity from 1-^{14}C-hexadecanol into the *O*-alk-1-enyl moiety. Almost all the radioactivity in the plasmalogens of the phospholipids was present in the ethanolamine-containing fraction.

2. Methods

Except for the much higher specific activity of the 1-^{14}C-hexadecanol (46 mCi/mmole) and the use of the dimethylacetal procedure [10, 11], the sources of materials (chemicals, animals, and tumor cells), methods, and most other experimental conditions are identical to those described previously [1–3].

The tumor homogenates were prepared immediately before each incubation and maintained near 0° until incubated. 10 ml of packed Ehrlich ascites cells (collected 6 or 8 days after transplantation) were suspended in 15 ml of 0.1 M phosphate or 0.1 M tris-buffer (pH 7.1) containing 0.25 M sucrose and sonicated for 2 min in a Branson Cell Disrupter Sonifier at an output of 70 W. Low-speed centrifugation (10 min at 800 *g*) removed unbroken cells, nuclei, and other debris from the original homogenate. The sediment was discarded and the supernatant was used in the incubations. The absence of intact cells from the supernatant was verified by light microscopy. In some instances, we prepared mitochondrial supernatants (10 min at 15,000 *g*). Protein was determined by the method of Lowry et al. [12]. Incubations were carried out up to 4 hr and were terminated by extraction of the total lipids [13]. Table 1 describes the incubation systems in detail. To be certain that free fatty aldehydes did not interfere in subsequent analyses, we removed all neutral lipids from the samples specified, on small silicic acid columns with chloroform; the phospholipids were eluted with methanol [14]. Specific phospholipid classes were isolated from some samples by thin-layer chromatography [2, 3].

Identification of plasmalogens was based on the isolation of various derivatives obtained from the total phospholipids or purified ethanolamine–phospholipid fraction. These derivatives included dimethylacetals (HCl–methanol treatment [10, 11]), *O*-alk-1-enylglycerols ($LiAlH_4$ reduction [15]), free fatty aldehydes (released from *O*-alk-1-enylglycerols with concentrated HCl [16]), hydrogenated *O*-alk-1-enylglycerols (H_2 over Adams catalyst [17]), and the isopropylidene derivative of the hydrogenated *O*-alk-1-enylglycerols (acetone–$HClO_4$ [18]). The purified *O*-alk-1-enylglycerols used for hydrogenation were contaminated with less than 3% ^{14}C-*O*-alkylglycerols. The dimethylacetals, the isopropylidenes, and the free

Table 1
Biosynthesis of plasmalogens in cell-free homogenates of Ehrlich ascites cells.

Incubation system[a]	% of radioactivity recovered as dimethylacetals from total lipid extract[b]		Total radioactivity (cpm) incorporated into *O*-alk-1-enyl moieties	
1) Complete	2.51;	2.23	22,340;	23,550[c]
2) minus NaF	2.38;	2.10	21,200;	22,020[c]
3) minus $NADP^+$	0.42;	0.57	3,020;	3,870
4) minus $NADP^+$, NaF	0.44;	0.35	4,260;	3,430
5) minus CoA, ATP, Mg^{2+}, $NADP^+$	0.28;	0.37	2,880;	3,870
6) minus CoA, ATP, Mg^{2+}, $NADP^+$, NaF	0.48;	0.45	4,580;	4,560
7) Complete except homogenate was heated in boiling water bath for 15 min	0.10;	0.13	1,100;	1,400

[a] The complete system in a final volume of 3 ml contained 20 nmoles 1-^{14}C-hexadecanol (1 μCi), ATP (10 mM), CoA (100 μM), Mg^{2+} (4 mM), dihydroxyacetone-P (1.66 mM), $NADP^+$ (2 mM), NaF (20 mM), 1 ml of homogenate (34 mg protein), and 0.1 M phosphate buffer (pH 7.1). The results in this table were obtained from samples incubated for 4 hr at 37°.
[b] Based on thin-layer chromatography radioassay (60% counting efficiency for ^{14}C).
[c] Radioactivity in aldehydogenic moiety was verified by gas–liquid chromatography in these samples.

fatty aldehydes were each analyzed by thin-layer and gas–liquid chromatography [1–3, 17]. Fatty aldehydes used as standards were prepared by us [19] or purchased from Supelco, Inc. (Bellefonte, Pa.).

3. Results and discussion

The dimethylacetals, formed by HCl–methanol treatment of the total phospholipid fraction isolated from the complete incubation system, are represented by peak 2 in the thin-layer chromatographic zonal profile scan (fig. 1); the substrate was also incorporated into *O*-alkyl (peak 1) and acyl (peak 3) lipids. Approximately 85% of the dimethylacetals isolated from the phospholipids was obtained from the ethanolamine-containing fraction. The purified ethanolamine-containing phospholipids contained 10% of the radioactivity as plasmalogen (based on dimethylacetals). We purified the dimethylacetal fractions by preparative thin-layer chromatography on Silica Gel G developed in benzene [11]; gas–liquid chromatography subsequently revealed that more than 90% of the radioactivity in these fractions was present as the dimethylacetal of palmitaldehyde (table 2).

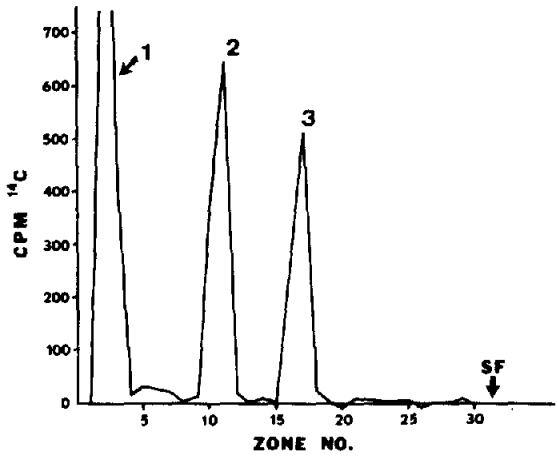

Fig. 1. ^{14}C-Zonal profile scan (5 mm) of a thin-layer chromatogram containing the products formed by HCl–methanol treatment of the total phospholipids isolated from an incubation mixture containing the complete system described in the legend to table 1. The chromatography was done on Silica Gel G layers in a solvent system of benzene. The numbered peaks refer to *O*-alkyl phospholipids (1), dimethylacetals of fatty aldehydes (2), and methyl esters of fatty acids (3). SF = solvent front.

Table 2
Radioactivity in aldehydogenic portion of plasmalogens isolated from a cell-free system[a].

Chain length based on fatty aldehyde standards	Separated as dimethylacetals						Separated as free fatty aldehydes[d]		Isopropylidene of hydrogenated *O*-alk-1-enyl-glycerols[e]	
	Sample 1[b]		Sample 2[b]		Sample 3[c]		Sample 4		Sample 5	
	cpm	%	cpm	%	cpm	%	cpm	%	cpm	%
Up to $C_{16:0}$	66	3.4	48	3.8	19	7.2	15	0.7	3	1.3
Only $C_{16:0}$	1765	92.0	1152	90.3	223	85.2	1850	94.2	221	96.1
Beyond $C_{16:0}$ (through $C_{18:1}$)	87	4.5	75	5.9	20	7.6	105	5.1	6	2.6

[a] Incubation mixture contained the complete system described in the legend to table 1.
[b] Prepared by HCl–methanol treatment of total phospholipids.
[c] Prepared by HCl–methanol treatment of ethanolamine-phospholipids.
[d] Prepared by HCl treatment of *O*-alk-1-enyl glycerols obtained by $LiAlH_4$ reduction of the total lipids.
[e] Prepared after $LiAlH_4$ reduction of ethanolamine-phospholipids.

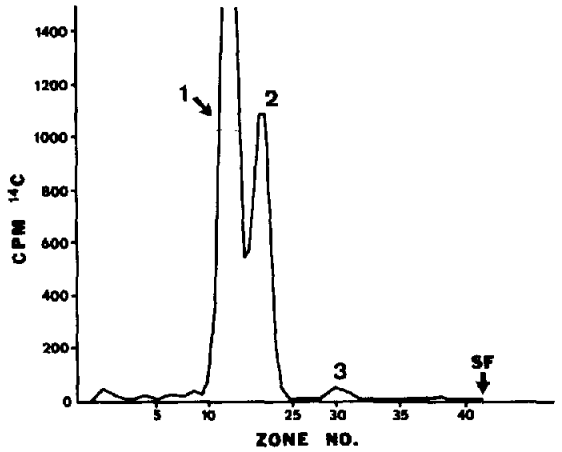

Fig. 2. ^{14}C-Zonal profile scan (2 mm and 5 mm) of a thin-layer chromatogram containing a purified fraction of *O*-alk-1-enylglycerols originally prepared by $LiAlH_4$ reduction of the total lipids isolated from an incubation mixture containing the complete system described in the legend to table 1. ^{14}C-Labeled *O*-alkylglycerols (peak 1) were purposely added as a marker. The chromatography system for isolating the enriched fraction of *O*-alk-1-enylglycerols, and the scan illustrated, was done on Silica Gel G layers in a solvent system of diethyl ether saturated with water. The numbered peaks refer to *O*-alkylglycerols (1), *O*-alk-1-enylglycerols (2), and fatty alcohols (3). The 2 mm portion of the scan was only done for the ether-lipid area (zones 10–28); the areas before and after the ether-lipids were analyzed in 5 mm zones. SF = solvent front.

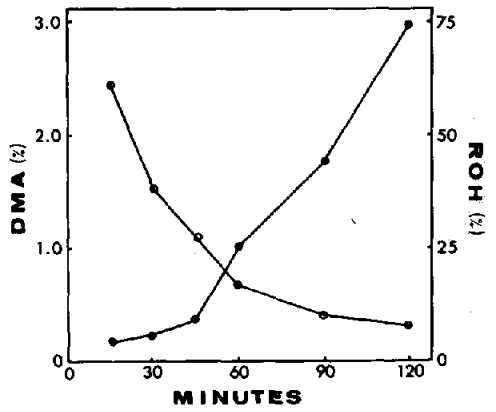

Fig. 3. The biosynthesis of plasma (•——•) and disappearance of 1-^{14}C-hexadecanol (ROH) (○——○) as a function of time. DMA refers to dimethylacetals formed by treatment of total lipids with methanol–HCl. The complete incubation system is the same as that described in table 1, except that 0.1 M tris buffer was substituted for the phosphate buffer.

In one experiment, samples of total lipids and the purified ethanolamine phospholipids containing the ^{14}C-labeled plasmalogens were reduced with $LiAlH_4$. Unlabeled ethanolamine plasmalogen from beef brain was added to facilitate detection of the ^{14}C-labeled *O*-alk-1-enylglycerols liberated; these were purified from the *O*-alkylglycerols by preparative thin-layer chromatography. Although the yield (63% less than that obtained with the dimethylacetal procedure) of *O*-alk-1-enylglycerols was poor, we were able to detect a radioactive peak at the R_f of *O*-alk-1-enylglycerols by thin-layer chromatography (typical results are seen in fig. 2, peak 2). The fatty aldehydes liberated from the *O*-alk-1-enylglycerols with concentrated HCl were also isolated by preparative thin-layer chromatography. Gas–liquid chromatography (done under the same conditions used for the analysis of dimethylacetals) revealed that the palmitaldehyde fraction contained most of the radioactivity. These data (table 2) confirmed by a second independent method that plasmalogens had been biosynthesized. A third method of identification involved the hydrogenation of the *O*-alk-1-enylglycerols (liberated from the purified ethanolamine phospholipids) over Adams catalyst. The *O*-alkylglycerols produced were identified by thin-layer chromatography R_f value and by gas–liquid chromatography retention time of the isopropylidene derivative. More than 95% of the ^{14}C was associated with the isopropylidene of *O*-hexadecylglycerol.

The complete system required for optimal biosynthesis of plasmalogens in homogenates contained ^{14}C-labeled hexadecanol, dihydroxyacetone-P, CoA, ATP, Mg^{2+}, and $NADP^+$ (table 1). The time course for the reaction is shown in fig. 3. Mitochondrial supernatant (microsomes + soluble proteins) appears to be as active in synthesizing plasmalogens as the homogenate. Plasmalogens were not synthesized in enzyme blanks heated in a boiling water bath for 15 min and only minimal synthesis occurred in samples that did not contain CoA, ATP, and Mg^{2+}.

The reaction(s) involved in the formation of *O*-alk-1-enyl lipids and the role of $NADP^+$ in plasmalogen biosynthesis are not known. It is possible that the need for $NADP^+$ is related to the reduction-oxidation of *O*-alkyldihydroxyacetone-P [2, 3] or that it serves as an acceptor of hydrogens from the α, β carbon atoms of the *O*-alkyl moiety [1]. The cell-free system and experimental approach described in this report provide the necessary tools to investigate the specific reactions responsible for the biosynthesis of plasmalogens.

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