On the analysis of long-chain alkane diols and glycerol ethers in biochemical studies

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SUMMARY The chromatographic behavior of 1,2-, 1,3-, 1,4-, and 1,12-long-chain alkane diols and 1-*O*-alkylglycerols and their derivatives has been compared. Thin-layer chromatography on Silica Gel G gives poor separations of the 1,2-, 1,3-, and 1,4-alkane diols, *O*-alkylglycerols, and some of their isopropylidene derivatives. However, gas–liquid chromatography on 10% EGSS-X (coated on 100–120 mesh Gas-Chrom P) resolves the isopropylidenes of the alkane diols and *O*-alkylglycerols.

We also document the formation of 1,3-alkane diols (after $LiAlH_4$ reduction) from 1-^{14}C-labeled fatty acids incubated with mitochondrial fractions from heart and liver of rats. The labeled 1,3-alkane diol was identified by gas–liquid chromatography of its isopropylidene derivative and by its behavior after periodate oxidation. These results serve to caution investigators in the glycerol ether field against incorrect interpretation of data obtained on the incorporation of labeled fatty acids into alkyl ether bonds of glycerolipids. The methodology described points out a technique for distinguishing several types of alkane diols from *O*-alkylglycerols.

SUPPLEMENTARY KEY WORDS alkane diols · *O*-alkylglycerols · fatty acids · glycerol ethers · $LiAlH_4$ reduction · mitochondria

The presence of alkane diol lipids in lipid preparations can present difficulties in the analysis of *O*-alkylglycerols, especially in metabolic experiments where minute quantities of ^{14}C- or ^{3}H-labeled products are formed. The problem is apparent since 1,2-alkane diols (unless derivatives are made) are not separated from glycerol ethers by thin-layer chromatography (1). Furthermore, compounds corresponding to 1,3-alkane diols obtained after $LiAlH_4$ reduction of lipids formed from fatty acids incubated with mitochondria have been proposed (2). These findings have pointed out the need for a reliable protocol to distinguish the various types of alkane diols from *O*-alkylglycerols.

Materials. The alkyl glycerol ethers were purchased from Analabs, Inc., North Haven, Conn., and Western Chemical Industries, Ltd., Vancouver, Canada. 3-Hydroxypalmitic acid and 4-hydroxypalmitic acid were generous gifts from Dr. N. Nicolaides, University of Southern California, Los Angeles. 2-Hydroxypalmitic acid and ricinoleic acid were purchased from Applied Science Laboratories Inc., State College, Pa. The various hydroxy acids were reduced with $LiAlH_4$ (3) to form the corresponding 1,2-, 1,3-, and 1,4-hexadecyl diols, and 1,12-octadecenyl diol.

Abbreviations: TCL, thin-layer chromatography; GLC, gas–liquid chromatography.

TABLE 1 2-hr Incubation* of Palmitic Acid-1-^{14}C with Tissue Fractions of Heart and Liver from Rats

Fraction	Percent of Total Lipid ^{14}C in "Glycerol Ether-like" Compound Based on TLC†	
	Heart	Liver
Homogenate*	26.4, 27.1	10.6, 11.6
Mitochondrial fraction*	45.0, 49.2	43.9, 48.0
Microsomal + soluble fractions*	3.9, 4.3	20.7, 21.5
Boiled mitochondrial fraction*	2.6, 2.5	1.6, 1.7
Mitochondrial fraction minus cofactors	3.0, 3.2	1.8, 1.8
Mitochondrial fraction + 2 μmoles NAD*	4.5, 5.3	17.3, 17.7

* Contained 0.4 μCi of palmitic acid-1-^{14}C (100 nmoles dissolved in 1% Tween-80), CoA (0.3 μmole), ATP (30 μmoles), and $MgCl_2$ (13 μmoles) in a final volume of 3.0 ml in 0.1 M potassium phosphate buffer at pH 7.0. Each fraction incubated was equivalent to that found in 200 mg of tissue based on wet weight. All incubations were carried out in glass vials placed in a Dubnoff metabolic shaker at 37°C and 150 oscillations per min.

† GLC of isopropylidene derivatives proved that the activity *was not* associated with *O*-alkylglycerols, but instead with the 1,3-diols of hexadecane.

Biochemical Procedures. Heart tissue and livers from female rats of the Charles River CD strain were the source of the subcellular fractions used in the incubations. The tissues were homogenized in a Potter-Elvehjem homogenizer flask using four strokes of a Teflon pestle attached to a Lourdes homogenizing motor set at 50. Because cell-free preparations of heart muscle were difficult to prepare by conventional means, these homogenates were sonicated near 0°C for 3 min at 55 w in a Branson Sonifier Cell Disrupter (model W185D). Mitochondrial (0.15×10^6 *g-min* pellet), microsomal (6×10^6 *g-min* pellet), and soluble (6×10^6 *g-min* supernatant) fractions were prepared using a Beckman Spinco centrifuge (model L2-65B). Incubations were carried out as described in Table 1. The sources of the cofactors used have been described previously (4). Lipids were extracted from the incubation mixture by the procedure of Bligh and Dyer (5), and aliquots of total lipids were analyzed as described in the following section.

Analytical Procedures. The ether-linked lipids and diol lipids derived from the standard hydroxy acids and total lipid extracts after $LiAlH_4$ reduction were resolved on Silica Gel G layers in a solvent system of diethyl ether–acetic acid 100:1 (v/v). The lipids were quantitatively eluted from the Silica Gel G with chloroform–methanol 2:1 (v/v). An aliquot of this solution was subjected to periodate oxidation (6, 7), and a separate aliquot was used for the preparation of the isopropylidene derivatives (8). The isopropylidene derivatives were chromato-

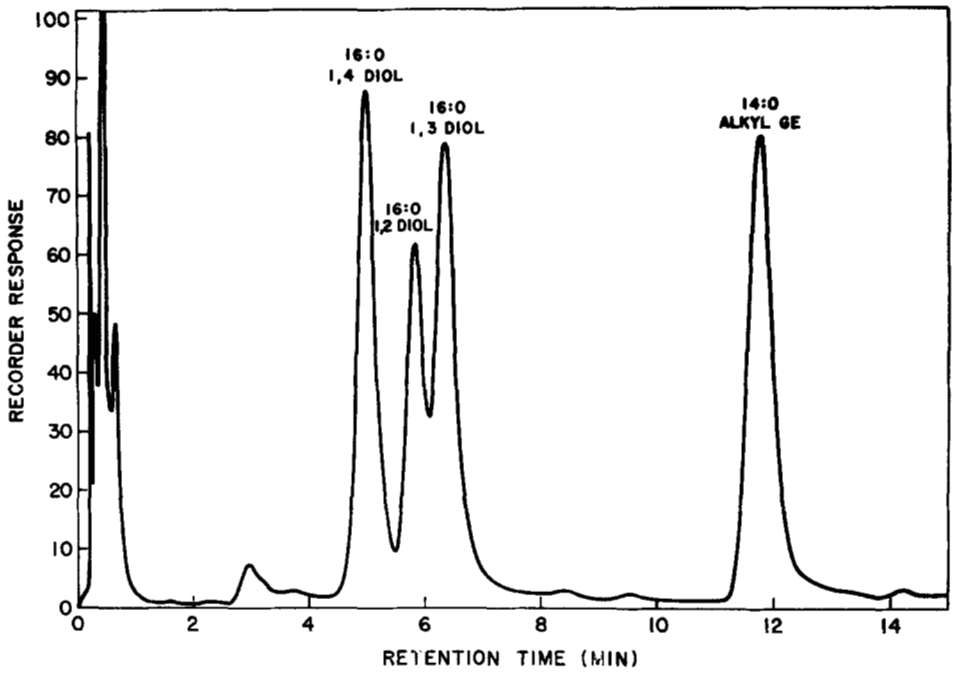

FIG. 1. Gas–liquid chromatogram of the isopropylidene derivatives of various alkane diols and an *O*-alkylglycerol (*14:0 alkyl GE*). Samples were chromatographed on 6 ft × $^1/_8$ in. columns packed with 10% EGSS-X on 100–120 mesh Gas-Chrom P and programmed at 160–190°C at 2°C/min.

graphed on Silica Gel G in solvent systems of hexane–diethyl ether 90:10 or 80:20 (v/v). All chromatoplates were radioassayed by area or zonal profile scans (9). GLC of the isopropylidene derivatives of the glycerol ethers and the alkane diols was carried out, essentially as previously described, on 10% EGSS-X coated on 100–120 mesh Gas-Chrom P (1). Fractions were collected in Pasteur pipettes containing glass wool and $CHCl_3$ (4), and they were assayed for radioactivity in a liquid scintillation solvent (10) with a Packard scintillation spectrometer.

Results and Discussion. The 1,2-, 1,3-, and 1,4-diols of hexadecane have essentially the same R_F as *O*-hexadecylglycerol in a solvent system (diethyl ether–acetic acid) that separates *O*-alkylglycerols from *O*-alk-1-enylglycerols (1). However, when the two hydroxyl groups are sufficiently apart from each other (e.g., the 1,12-octadecenyl diol), the decrease in polarity permits good resolution from the more polar alkane diols and the *O*-alkylglycerols.

The less polar isopropylidene derivatives of the alkane diols and *O*-alkylglycerols separate only slightly in a solvent system of hexane–diethyl ether 80:20 (v/v). Formation of the isopropylidenes of 1,3- and 1,4-alkane diols was somewhat unexpected, since isopropylidenes are usually prepared in the analysis of compounds having only adjacent hydroxyl groups. However, construction of molecular models of the isopropylidenes of the 1,3- and 1,4-diols clearly shows that these derivatives are possible, but experimentally their yields are lower than those obtained for 1,2-alkane diols.

The isopropylidene derivatives of the *O*-alkylglycerols and 1,2-, 1,3-, and 1,4-alkane diols are resolved from each other by GLC on 10% EGSS-X coated on Gas-Chrom P (100–120 mesh) with temperature programming (Fig. 1). The alkane diols elute much earlier than the corresponding derivative of *O*-alkylglycerols. The elution order of the isopropylidenes of the alkane diols is 1,4 > 1,2 > 1,3 for the hexadecane series. Critical pairs of these compounds that might be encountered because of unsaturation would obviously need to be separated by argentation TLC before GLC. The use of periodate oxidation to prepare aldehyde derivatives of compounds having two adjacent hydroxyl groups is also helpful in the analysis of complex mixtures.

Table 1 shows typical data obtained from experiments carried out by incubating homogenates and subcellular fractions of rat heart and liver with palmitic acid-1-^{14}C for a period of 2 hr. We have also isolated the 1,3-alkane diols after similar incubations with beef heart homogenates and subsequent reduction with $LiAlH_4$. The TLC analysis reported in the table is based on the quantities of ^{14}C associated with the TLC area corresponding to the *O*-alkylglycerols (glycerol ethers) liberated after $LiAlH_4$ reduction of the total lipids extracted from the incubation mixture. Essentially all the other radioactivity was confined to the fatty alcohols, the other major reduction product. Another significant

product with an ether bond that was liberated by reduction from the endogenous lipids of heart was *O*-alk-1-enylglycerols, but they did not contain any ^{14}C.

GLC and periodate oxidation proved that a labeled compound formed by incubating palmitic acid-1-^{14}C with the mitochondria of heart or liver was reduced by $LiAlH_4$ to a 1,3-alkane diol. The fact that the labeled compound formed an isopropylidene derivative indicated that it must contain two hydroxyl groups; furthermore, its stability to periodate oxidation demonstrated that the two hydroxyls were not adjacent on the hydrocarbon chain. After TLC of the periodate oxidation products, approximately 86% of the radioactivity associated with the glycerol ether area on thin-layer plates before oxidation was recovered in the same TLC area after oxidation. The isopropylidene derivative of the purified ^{14}C-compound from the mitochondrial systems had a GLC retention time that coincided with that of the 1,3-hexadecyl diol, and it eluted much earlier than the isopropylidene derivatives of known glycerol ethers ($C_{14:0}$, $C_{16:0}$, or $C_{18:0}$).

It appears that the product formed in the mitochondrial system is a consequence of β-oxidation and the subsequent reduction step with $LiAlH_4$. In the absence of NAD, β-oxidation would not proceed beyond the β-hydroxy acyl CoA intermediate; reduction of the β-hydroxy acid with $LiAlH_4$ produces 1,3-alkane diols. Although NAD does not normally penetrate intact mitochondria, it can under certain conditions, e.g., after sonication, in the presence of phosphate ions, etc. (11). No effort was made in this study to isolate highly purified intact mitochondria, and the data indicate that NAD entered the heart mitochondrial preparations to a greater extent than the liver preparations under our experimental conditions. 1,3-Alkane diols could also be encountered in extramitochondrial systems since the β-hydroxy and β-keto acyl compounds are also formed as intermediates during fatty acid synthesis.

This note emphasizes the need for combining GLC with TLC in identifying minute quantities of labeled lipid products formed in incubation systems. Moreover, these data demonstrate that when fatty acids are used as substrates some caution must be used in concluding, solely on the basis of $LiAlH_4$ reduction and TLC data, that glycerol ether biosynthesis has occurred. This is not to say that radioactivity from fatty acids cannot be incorporated into the ether-linked side chains of glycerolipids, since the acid can be reduced to the alcohol, and the latter subsequently incorporated into ether lipids.

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