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A NEW ALDEHYDOGENIC PHOSPHOLIPID—AN ARTIFACT

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SUMMARY

The storing of phosphatide plasmalogens in a solution of chloroform–methanol resulted in the formation of an artifact which was easily detectable by thin-layer chromatography after reduction with lithium aluminum hydride. The reduction product reacted positively to phenylhydrazine–sulfuric acid reagent. This property, together with its chromatographic behavior on thin layers of Silica Gel G suggested the artifact as an addition product of plasmalogens and methanol. The structure of the compound (artifact) was determined by infrared and nuclear magnetic resonance spectroscopy as well as by mass spectrometry. The possible biological significance of such an addition reaction is discussed.

INTRODUCTION

Our recent analysis[1] of ethanolamine phosphatide from hog spinal cord indicated that 90% of it was in the plasmalogen form. Hence its availability as a starting material for the semisynthetic preparation of plasmalogens* (ref. 2) was obvious. However, contrary to expectation, the ethanolamine phosphatide on reduction with lithium aluminum hydride ($LiAlH_4$)[3] and subsequent chromatography (Fig. 1) yielded only small amounts of glyceryl 1-alk-1′-enyl ethers. Instead, a substantially larger amount of a more polar compound than glyceryl 1-alk-1′-enyl ether was the major component. This compound was aldehydogenic in character as indicated by its positive reaction with 2,4-dinitrophenylhydrazine–sulfuric acid reagent[4]. Surprisingly, after acetylation, the infrared spectrum of this compound (Fig. 2) failed to show any vinyl ether absorption at 6.0 μ and showed only ether absorption at 8.9 μ. In the present communication we report the isolation and characterization of this new compound and discuss its possible origin.

* The glyceryl 1-alk-1′-enyl ether obtained from ethanolamine phosphatide of hog spinal cord by $LiAlH_4$ reduction is first acetylated and then interesterified with a known fatty acid methyl ester in presence of sodium methoxide to convert it to neutral plasmalogens.

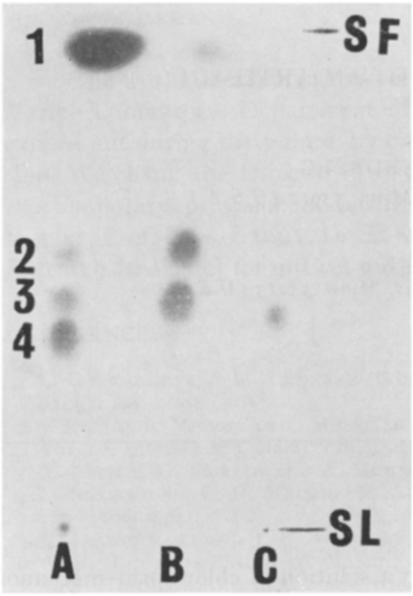


Fig. 1. Thin-layer chromatography of $LiAlH_4$ reduction product of ethanolamine phosphatide from hog spinal cord. Adsorbent: Silica Gel G. Solvent system: petroleum ether–ethyl ether–acetic acid (30:70:1). Spray reagent: 50% aqueous sulfuric acid. Plate charred at 160° for 10 min. Spot identification: 1 = long-chain alcohols; 2 = glyceryl 1-alk-1′-enyl ether; 3 = glyceryl 1-alkyl ether; 4 = unknown compound; Spots 2 and 4 showed positive reaction with acidic 2,4-dinitrophenylhydrazine reagent[4]. A = $LiAlH_4$ reduction products of ethanolamine phosphatide from hog spinal cord; B = standard mixture of glyceryl 1-alk-1′-enyl ether (2) and glyceryl 1-alkyl ether (3); C = standard glyceryl 1-alkyl ether (3). SL = Spotting line; SF = solvent front.

EXPERIMENTAL

Isolation of ethanolamine phosphatides from hog spinal cord

Fresh hog spinal cords (2,000 g, wet weight) collected immediately after the killing of the animals, were transported to the laboratory from the slaughter house on dry-ice. The spinal cords were immediately passed through a meat grinder a number of times, and then the entire material was freeze-dried, yielding 250 g of dry material.

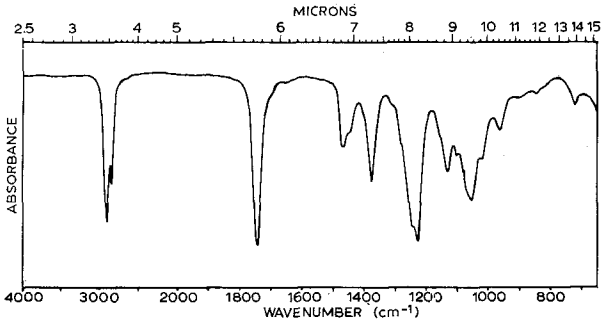


Fig. 2. Infrared analysis of the unknown compound (acetylated product).

One hundred grams of the freeze-dried material was extracted successively with a total volume of 4000 ml of a chloroform–methanol (2:1) mixture (four extractions with 1000 ml each time). The chloroform–methanol extract was then evaporated to dryness on a rotary evaporator and the residue (70 g of lipids), after dissolving in absolute chloroform, was subjected to chromatography on 400 g of acid-washed florosil column with the following dimensions: height 80 cm and internal diameter 6 cm.

The neutral lipids were first eluted with 2 l of chloroform and discarded. This was followed by further elution with chloroform–methanol mixtures (5 l) containing from 0% up to 15% of methanol. These eluates were combined and analyzed by thin-layer chromatography. The thin-layer analysis indicated a mixture containing cerebrosides and ethanolamine and serine phosphatides. The combined eluates were freed of the solvent on a rotary evaporator under suction, the residue dissolved in a minimum amount of diethyl ether and left at 0° in a cold room for 48 h. This favored the precipitation of the cerebrosides from the lipid mixture. After precipitation they were removed by cold filtration. After two successive reprecipitations, the crude ethanolamine phosphatides were rechromatographed on acid-washed florosil columns under conditions similar to those described earlier and yielded 15 g of a fairly pure fraction of ethanolamine phosphatide. This was repurified by preparative thin-layer chromatography before subjecting it to $LiAlH_4$ reduction.

Reduction of ethanolamine phosphatide with $LiAlH_4$[3]

Three grams of $LiAlH_4$ were suspended in 100 ml of absolute anhydrous diethyl ether in a three-necked, 250 ml round-bottom flask and refluxed for 30 min. This suspension was then slowly cooled to −60° in a dry-ice acetone bath, and then a solution of 5 g of ethanolamine phosphatide dissolved in 50 ml of anhydrous diethyl ether was slowly and cautiously added. After bringing the reaction mixture to room temperature, it was refluxed for 2h. The reaction mixture was then lowered to a temperature of −10° in a dry-ice acetone bath, and the unreacted excess $LiAlH_4$ destroyed by cautious addition of 7.5 ml of ethyl acetate. The reaction mixture was then transferred to a separatory funnel containing one liter of water and subsequently extracted with diethyl ether. The emulsions that resulted during the extractions were broken down by centrifugation.

After evaporating the ether extract on a rotary evaporator under vacuum, the residual lipids were redissolved in dry chloroform and then filtered through anhydrous sodium sulfate. The filtrate was then taken in a 50 ml round-bottom flask and evaporated to dryness on a rotary evaporator under vacuum, yielding 3.25 g of residue.

The residual lipids were then acetylated at room temperature overnight in a mixture of 20 ml pyridine and 10 ml acetic anhydride under continuous agitation with a magnetic stirrer. After overnight acetylation, excess of the reagents was removed mostly on a rotary evaporator under vacuum, with intermittent addition of benzene, and any residual reagents still remaining were subsequently removed under high vacuum. The acetylation products were fractionated by preparative thin-layer chromatography in the system Silica Gel G/petroleum ether–diethyl ether (85:15). The new (unknown) compound, which was observed with 2,7-dichlorofluorescein reagent (0.2% in ethanol) under ultraviolet light, was scraped off and extracted with chloroform–methanol (2:1). This extract was washed with water to remove the fluorescein and methanol. The chloroform extract, after drying over anhydrous sodium

sulfate, on removal of the solvent on a rotary evaporator yielded 1.0 g of acetylated product, which was subjected to instrumental analysis.

Thin-layer chromatography

Glass plates (20 × 20 cm) coated with Silica Gel G were air-dried for 30 min and then activated for 1 h at 110° before use.

Infrared spectroscopy

Infrared spectra were determined with a Perkin-Elmer Model 21 double beam infrared spectrometer equipped with sodium chloride optics. All the spectra were determined as liquid films.

Nuclear magnetic resonance spectroscopy

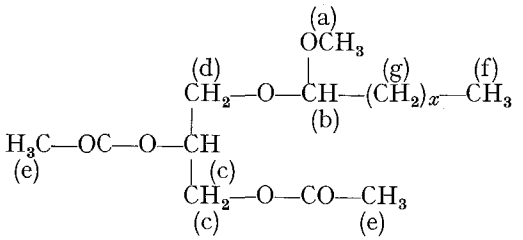
An NMR spectrum was obtained using a Varian Associate high-resolution spectrometer, Model A-60, with a probe temperature of 33°. Deuterochloroform was used as a solvent (solution concentration 10%) and tetramethylsilane was the internal standard.

Mass spectrometry

The mass spectrum was determined on a consolidated Electrodynamic Corporation Model 21-103 mass spectrometer. The experimental conditions were as follows: ionizing current, 15 μA; magnetic current, 0.56 A (for low mass) and 1.12 A (for high mass); the heated inlet, maintained at 280°, had a 3 l reservoir; ionizing voltage, 70 eV; and chamber temperature, 250°.

RESULTS AND DISCUSSION

The aldehydogenic property of the unknown compound, together with an ether absorption at 8.9 μ and *absence* of a vinyl ether absorption at 6.0 μ in the infrared spectrum of its acetyl derivative, suggested as possibilities either a cyclic acetyl or an addition product of the plasmalogen at the vinyl ether linkage[5,6]. However, the R_F-value of the acetyl derivative of the unknown compound relative to the acetyl derivative of the glyceryl 1-alk-1'-enyl ether (Fig. 3) eliminated the cyclic acetal[7] possibility. Hence the following structure seemed reasonable:



(I)

x = homologs containing 30% of saturated 14-carbon chain, 25% of saturated 16-carbon chain, and 45% monounsaturated 16-carbon chain.

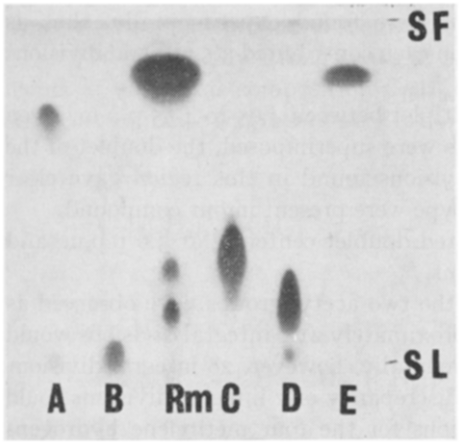


Fig. 3. Thin-layer chromatography of the unknown compound obtained by $LiAlH_4$ reduction of ethanolamine phosphatide of hog spinal cord. Adsorbent: Silica Gel G. Solvent system: petroleum ether–diethyl ether (85:15). Spray reagent: 50% aqueous sulfuric acid. Plate charred at 160° for 10 min. Spot identification: A = tripalmitin; B = triacetin; Rm = $LiAlH_4$ reduction products of ethanolamine phosphatide of hog spinal cord after acetylation; C = mixture of glyceryl 1-alk-1'-enyl ether and glyceryl 1-alkyl ether; D = unknown compound after acetylation (isolated from Rm); E = palmityl acetate. SF = Solvent front; SL = spotting line.

This was confirmed by nuclear magnetic resonance spectroscopy and mass spectrometric analysis.

Nuclear magnetic resonance spectroscopy

For structure I the 'a' protons were observed as a sharp singlet (Fig. 4) at 3.28 p.p.m. The spectrum integral for this singlet was 10.5 divisions, *i.e.*, 3.5 divisions per proton. The same factor was used throughout in calculating the number of protons in each group.

The 'b' proton in the 5.0 to 5.5 p.p.m. region could not be clearly distinguished because of interference with the chain olefinic protons. Approximately 50% of the chains were monounsaturated. With two hydrogens per unsaturated bond, about 3.5 integral divisions should be contributed by these hydrogens to the 5.0 to 5.5 p.p.m.

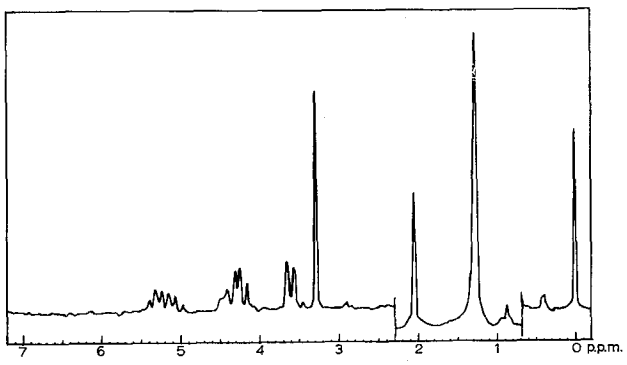


Fig. 4. Nuclear magnetic resonance spectrum of the acetylated derivative of the unknown compound.

region. Approximately 7.0 integral divisions were found experimentally, thus as expected the olefinic protons and the 'b' proton each contributed 3.5 integral divisions to the 5.0 to 5.5 p.p.m. region.

The 'c' protons were observed as a multiplet between 4.05 to 4.55 p.p.m. Even though the absorptions for these three protons were superimposed, the doublet of the $-CH_2$-group was clear. The 10.5 integral divisions found in this region gave clear indication that only three protons of the 'c' type were present in our compound.

The 'd' protons appeared as the expected doublet centering at 3.6 p.p.m. and contributed the expected 7.0 integral divisions.

The 'e' protons of the two methyls in the two acetyl groups were observed as the expected sharp singlet at 2.07 p.p.m. Approximately 21.0 integral divisions would be expected for these six hydrogens. Experimentally, however, 28 integral divisions were observed in this region. This apparent discrepancy of 7 integral divisions could be easily explained as follows: the absorptions for the four methylene hydrogens adjacent to the double bond in the chain are superimposed as a broad region under the sharp singlet. Since only about 50% of the chains were unsaturated, approximately 7 integral divisions should be contributed by these methylene protons.

The terminal methyl 'f' protons of the long chains gave a badly resolved triplet centering at 0.88 p.p.m. The 10.5 integral divisions found for these protons indicated that there was only one of these methyl groups per each molecule.

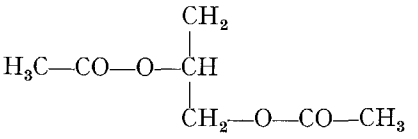
The methylene hydrogens 'g' of the aliphatic chains gave a sharp peak at 1.27 p.p.m. One hundred and four integral divisions were obtained for these protons. This was equivalent to thirty protons or around fifteen methylene groups. This was in good agreement with the proposed structure. (By gas–liquid chromatographic analysis of the aldehydes liberated from this unknown compound by acid hydrolysis, it was observed that the unknown compound contained approximately 30% hexadecanal, 25% octadecanal and 45% octadecenal.)

Mass spectrometry

In agreement with the nuclear magnetic resonance spectroscopy analysis, the mass spectrometric studies confirmed the postulated structure I.

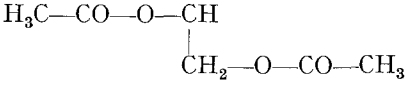
Due to lability of the molecule under electron impact, the molecular ion was not observed. However, strong fragment peaks that were characteristic of the molecule were observed at masses 159, 145, 283, 281 and 255. Using these five fragment peaks, the structure was deduced.

The strongest peak was at mass 159 due to the fragment of elemental composition $C_7H_{11}O_4$. The structure of this fragment could be:



(II)

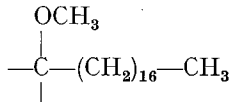
The moderately strong peak at mass 145 was due to the fragment of elemental composition $C_6H_9O_4$. The structure of this fragment could be:



(III)

Thus, the original compound seems to be 1,2-diacetate rather than 1,3-diacetate.

The moderately strong peaks at masses 283, 281 and 255 could be due to fragments of elemental composition $C_{19}H_{39}O$, $C_{19}H_{37}O$ and $C_{17}H_{35}O$, respectively. The structures of these fragments were rationalized as IV, V and VI, respectively: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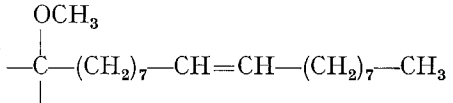
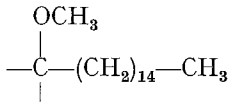
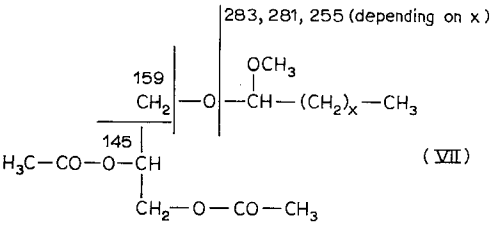

(V)


(VI)

The over-all fragmentation pattern thus appeared to be as follows:



(VII)

Elemental analysis

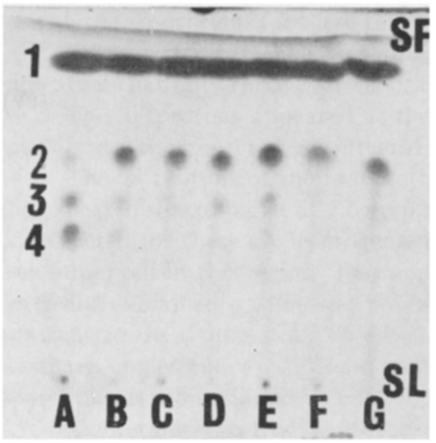
Nuclear magnetic resonance spectroscopy and mass spectrometric analysis were further substantiated by elemental analysis (Table I).

TABLE I

ELEMENTAL ANALYSIS OF THE ACETYL DERIVATIVE OF THE UNKNOWN COMPOUND

Element	*Theory*	*Obtained*
C	67.91	68.10
H	10.68	10.69
O	21.41	20.94

The formation of this type of compound in high amounts from ethanolamine phosphatides of hog spinal cord suggested investigations of their formation from other tissue phosphatides high in plasmalogens. On testing seven such samples, which also contained both choline- and ethanolamine plasmalogens, it was observed (Fig. 5)



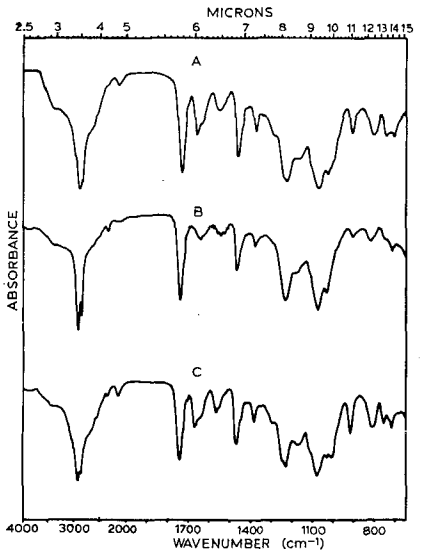


Fig. 5. Thin-layer chromatography of seven phosphatides. Adsorbent: Silica Gel G. Solvent system: petroleum ether–diethyl ether–acetic acid (30:70:1). Spray reagent: 50% aqueous sulfuric acid. Plate charred at 160° for 10 min. Spot identification: 1 = long-chain alcohols; 2 = glyceryl 1-alk-1′-enyl ether; 3 = glyceryl 1-alkyl ether; 4 = unknown compound. Spots 2 and 4 reacted positively toward acidic 2,4-dinitrophenylhydrazine reagent. A = $LiAlH_4$ reduction product of ethanolamine phosphatide of hog spinal cord; B = $LiAlH_4$ reduction product of ethanolamine phosphatide of hog brain; C = $LiAlH_4$ reduction product of ethanolamine phosphatide of hog heart; D = $LiAlH_4$ reduction product of choline phosphatide of hog heart; E = $LiAlH_4$ reduction product of ethanolamine phosphatide of beef spinal cord; F = $LiAlH_4$ reduction product of ethanolamine phosphatide of beef heart; G = $LiAlH_4$ reduction product of choline phosphatide of beef heart. SF = Solvent front; SL = spotting line.

Fig. 6. Infrared spectra of (A) ethanolamine phosphatide of beef spinal cord, (B) ethanolamine phosphatide of hog spinal cord (artifact), and (C) ethanolamine phosphatide of hog spinal cord (no artifact).

that only the ethanolamine phosphatides isolated from hog spinal cord on $LiAlH_4$ reduction could produce this unusual compound in high proportions. Did this mean that the ethanolamine phosphatides isolated from hog spinal cord differed from the ethanolamine phosphatides isolated from other tissues? This was verified by comparing the infrared spectrum of ethanolamine phosphatide isolated from hog spinal cord (Fig. 6B) with the infrared spectrum of ethanolamine phosphatide isolated from beef spinal cord (Fig. 6A). As anticipated the infrared spectrum of the ethanolamine phosphatide isolated from hog spinal cord was devoid of vinyl ether absorption, while the infrared spectrum of the ethanolamine phosphatide isolated from beef spinal cord showed a strong vinyl ether absorption, thus confirming our TLC (Fig. 5) and infrared (Fig. 2) observations. However, the absence of vinyl ether absorption in the infrared spectrum of the former compound contradicted our unpublished observation made during the analytical studies of ethanolamine phosphatides of hog spinal cord[1]. Thus it was clear that the artifact had developed during the isolation of ethanolamine phosphatide from hog spinal cord.

This isolation, as compared to that of other tissue phosphatides, alone was

carried out on a large scale for the purpose of acquiring sufficiently larger amounts of glyceryl 1-alk-1'-enyl ethers as starting material for the plasmalogen synthesis[2] and hence had remained in contact with a mixture of chloroform–methanol (2:1) for a longer period (about six weeks); supposedly this could favor development of an artifact[5,6].

To confirm this point, 100 g of fresh hog spinal cord, after grinding and freeze-drying, was immediately extracted with chloroform–methanol (2:1). The chloroform–methanol extract was concentrated and fractionated preparatively on layers of Silica Gel G with chloroform–methanol–ammonia (70:30:5) as the developing solvent, and the separated ethanolamine phosphatide isolated without any delay. The infrared analysis of this sample (Fig. 6C) showed strong vinyl ether absorption similar to that of beef spinal cord ethanolamine phosphatides (Fig. 6A), and its $LiAlH_4$ reduction product showed a complete absence of the new compound, but presence of larger amounts of glyceryl 1-alk-1'-enyl ether (Fig. 7). These observations confirmed that the procedure previously employed led to the formation of the artifact from the plasmalogens.

Compounds of similar chromatographic and infrared properties were recently reported by THOMPSON AND LEE[3] in four out of five mollusca examined after $LiAlH_4$ reduction of their lipids. However, they did not characterize these compounds. Very possibly their compounds also were artifacts.

RAPPORT[5] observed that lipids containing a high concentration of plasmalogens lost their unsaturated ether linkage (as determined by specific iodination reaction[8])

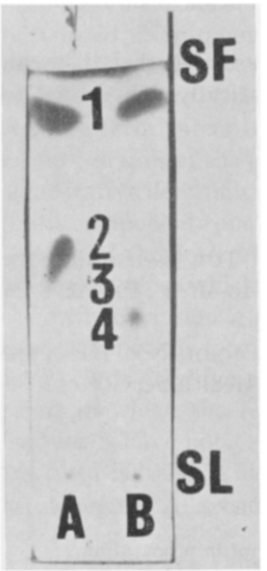


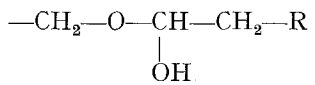
Fig. 7. Thin-layer chromatography of $LiAlH_4$ reduction product of ethanolamine phosphatides from hog spinal cord. Adsorbent: Silica Gel G. Solvent system: petroleum ether–diethyl ether–acetic acid (30:70:1). Spray reagent: 50% aqueous sulfuric acid. Plate charred at 160° for 10 min. Spots identification: 1 = long-chain alcohols; 2 = glyceryl 1-alk-1'-enyl ether; 3 = glyceryl 1-alkyl ether; 4 = unknown compound. Spots 2 and 4 reacted positively with acidic 2,4-dinitrophenylhydrazine reagent. A = $LiAlH_4$ reduction product of ethanolamine phosphatide from hog spinal cord (no artifact); B = $LiAlH_4$ reduction product of ethanolamine phosphatide from hog spinal cord (artifact). SF = Solvent front; SL = spotting line.

on standing at 20–24° in a chloroform–methanol solution for several days but still fully retained their capacity to generate higher fatty aldehydes as determined by *p*-nitrophenylhydrazine formation[9]. Hence he suggested an addition reaction of the unsaturated ether producing a mixed acetal. The present report not only confirmed RAPPORT's observation, although by chromatographic technique, but also confirmed his suggestion of mixed acetal formation by I.R.- and N.M.R.-spectroscopy and by mass spectrometry.

RAPPORT[5], in the same communication, suggested that the function of plasmalogens may be related to their capacity to add water-soluble compounds to the activated double bond. THIELE[10] indicated redox equilibrium between the ester phosphatides and plasmalogens as follows:

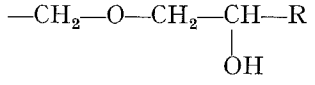
$$-CH_2-O-CO-CH_2-R + 2\,H \leftrightharpoons -CH_2-O-CH=CH-R + H_2O$$

It appears possible that an intermediate exists in this equilibrium reaction as follows:



(VIII)

or



(IX)

The former structure (VIII) may account for the abnormal compound we have observed and the latter structure (IX) may explain the occurrence of methoxy-substituted glyceryl ethers in shark liver oil[11].

Work is now in progress to label these intermediates to see whether they can be enzymatically oxidized to diester phosphatides or enzymatically dehydrated to alkenyl acyl phosphatides in various biological systems.

ACKNOWLEDGEMENTS

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