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STUDIES OF THE α-GLYCERYL ETHER LIPIDS OCCURRING IN MOLLUSCAN TISSUES

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SUMMARY

1. Tissues of *Katherina tunicata*, *Thais lamellosa*, *Octopus dofleini*, and *Protothaca staminea* were examined for α-glyceryl ether-containing lipids.

2. Phospholipids of the species analyzed contain glyceryl ethers amounting to as much as 25 mole % of the total phospholipid phosphorus. The neutral lipids of all species also contain glyceryl ether derivatives.

3. Analysis of the glyceryl ethers by gas–liquid chromatography showed that in most cases chimyl alcohol (1-*O*-hexadecyl glycerol) and batyl alcohol (1-*O*-octadecyl glycerol) predominate, although many variations were noted in the distribution patterns.

4. In some cases cyclic acetal derivatives of the plasmalogens were prepared to allow comparison of the vinyl ether side-chains with those of the saturated ethers. A new method employing $LiAlH_4$ allows the rapid isolation in pure form of cyclic acetals as well as glyceryl ethers.

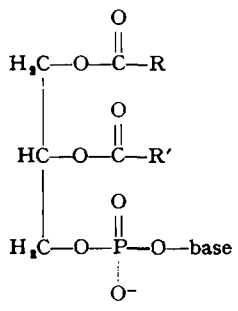
5. The presence of significant levels of glyceryl ether lipids in representatives from the four principal classes of mollusca indicates that these lipids are of widespread occurrence within the phylum.

INTRODUCTION

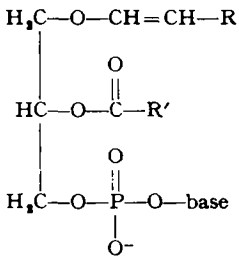
Glycerophospholipids have long been known as important structural components of cells. The classical glycerophospholipid, containing a phosphate diester and two fatty acid ester linkages, may be represented by Structure I. Within recent years it has been discovered that some rather unexpected variants of I exist in nature. Thus the plasmalogens, II, are now recognized as being almost as prevalent in some tissues as are the diacyl phospholipids[1]. In 1958, a third type of glycerophosphatide was identified[2]. This class of lipids, III, has been termed the glyceryl ether phospholipids because of the chemically very stable glyceryl ether backbone of the molecules.

Before 1961, the glyceryl ether phospholipids were known only as trace constituents of natural mixtures. Glyceryl ethers *per se* had been isolated in significant amounts from a number of animal sources[3,4], including some molluscs[4], but it appears

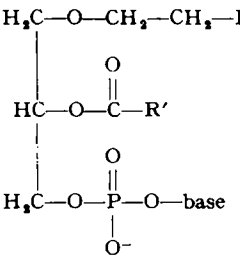
to have been generally accepted that they occur mainly as the neutral diacylated compounds. The discovery by HANAHAN AND WATTS[5] that 15 mole % of bovine erythrocyte phospholipids are of the glyceryl ether type focused new attention on these lipids. The presence of glyceryl ethers in the structural lipids of the cell has been confirmed by other workers. Bovine[6], porcine[7], and rabbit[8] bone marrows have been shown to contain glyceryl ether phospholipids at levels similar to that found in the bovine erythrocyte. The highest concentrations of glyceryl ether phospholipid yet reported were found in two species of terrestrial slug, *Arion ater* and *Ariolimax columbianus*[9]. In these organisms glyceryl ether derivatives comprise over 20 % of the total phospholipids.



I



II



III

The high content in slugs of these hitherto rather unusual lipids raised the question as to whether such animals are anomalous with respect to their lipid distribution. In this paper we have examined representative examples from each of the four principal classes of phylum mollusca. Tissues from a chiton, *Katherina tunicata*, a marine snail, *Thais lamellosa*, a clam, *Protothaca staminea*, and an octopus, *Octopus dofleini*, have all been shown to be rich in glyceryl ether phospholipids.

EXPERIMENTAL

Materials

Column chromatography was carried out with Mallinckrodt reagent-grade silicic acid and Johns Manville hyflo supercel. Silicic acid used for the separation of glyceryl ethers from other products was dried at 100° for at least 24 h immediately before use. Chloroform, methanol, and diethyl ether were reagent-grade products and were used for chromatography without further purification. Diethyl ether for use in the $LiAlH_4$ reductions was dried by distillation over $LiAlH_4$. Hexane (Phillips, high purity) was distilled over $KMnO_4$, retaining the fraction boiling at 67–68°. All solvent mixtures were prepared using volume proportions.

Specimens of *K. tunicata*, *T. lamellosa*, *P. staminea*, and *O. dofleini** were all obtained from marine waters near Seattle, Wash.

* *O. dofleini* tissues were a generous gift from Professor A. W. MARTIN, Department of Zoology.

Methods

Whole specimens of *K. tunicata*, *T. lamellosa*, and *P. staminea* were extracted after removing the shells. Samples of *O. dofleini* tentacle and hepatopancreas were analyzed separately. The tissues were extracted 2 times in a Waring blendor with approx. 5 volumes of chloroform–methanol (1:1). The combined extracts were concentrated to a small volume *in vacuo* and were re-extracted twice with chloroform. Water-soluble contaminants were removed by a Folch-type wash involving 8 parts of chloroform extract, 4 parts of methanol, and 3 parts of 0.1 N KCl. The purified lipids were chromatographed on silicic acid–hyflo supercel (2:1, w/w) columns, using a loading ratio of 1 mg phospholipid phosphorus per 2 g silicic acid. The neutral lipids were eluted with chloroform, and phospholipids were eluted with chloroform–methanol (1:1), followed by a small amount of chloroform–methanol (1:9) to assure that all phospholipids were removed. Recoveries of lipid phosphorus after chromatography ranged from 97–100% in all samples used for glyceryl ether analysis.

The resulting fractions were analyzed by thin-layer chromatography, phosphorus assay[10], total-weight determination, and in some cases, plasmalogen assay[11]. Glyceryl ethers were routinely obtained by a modification of the method of HANAHAN AND WATTS[5]. The modification allowed an improved yield of 95–100% of the compounds present in the tissue. According to this method approx. 100 mg of lipid is refluxed for 8 h in 10 ml acetic acid–acetic anhydride (3:2, v/v). The resulting solution is cooled to 4° and 50 ml 6 N KOH in 95% ethanol is slowly added. The resulting solution is 2 N in KOH. After a further 2-h reflux, water is added and the non-saponifiable compounds are extracted with ether.

A second procedure was devised which allows the isolation of both glyceryl ethers and cyclic acetal products derived from plasmalogens. The lipid was treated with $LiAlH_4$ by a modification of the procedure of JANGAARD AND ACKMAN[12]. 10 ml of a diethyl ether suspension of $LiAlH_4$ (30 mg/ml) are added to 5 ml dry diethyl ether in a 250-ml erlenmeyer flask equipped with a magnetic stirring bar and maintained moisture-free by the use of a side-arm drying tube. The flask is immersed in a low-temperature bath of dry ice–acetone. Lipid (50–100 mg) dissolved in 10 ml dry diethyl ether is added dropwise with stirring. After the lipid addition, the flask is brought slowly to room temperature and then refluxed for 30 min. Finally, the lithium aluminum salts are hydrolyzed at dry ice–acetone temperature by the cautious addition of water. Ether-soluble reaction products are purified by ether extraction and water washing of the combined extracts.

Column chromatography of the crude glyceryl ether mixture was carried out on silicic acid columns as previously described[9]. Purity of the resulting fractions was assayed by thin-layer chromatography and infrared spectrophotometry. The quantitative analysis of glyceryl ethers and of cyclic acetals was carried out by determining total weight. Glycerol was measured by the method of HANAHAN AND OLLEY[13].

Analysis of the glyceryl ether side-chain distribution was performed by gas–liquid chromatography of the isopropylidene derivatives[14], using a Barber Colman Model 10 argon chromatograph. Conditions were: a 5-ft column of 15% polyethyleneglycol succinate on Anakrom AB, 60–70 mesh, 12 lb/in² argon inlet pressure, column temperature 175°. Retention times were similar to those reported by HANAHAN *et al.*[14] (*e.g.*, for chimyl alcohol derivative, 24.0 min). Aldehydes, prepared by hydrolysis of the cyclic acetals for 2 h in refluxing 2 N aqueous HCl, were promptly analyzed on

the same gas–liquid chromatographic system except that an inlet pressure of 5 lb/in^2 argon was employed. A typical retention time for standard palmitaldehyde was 8 min.

Hydrogenations were effected in a Parr Instrument Company apparatus with a Pt catalyst, using Merck light petroleum* (b.p. 30–60°)–methanol (1:1) as solvent. To assure basic conditions when cyclic acetals were hydrogenated, two drops of pyridine per 75 ml solvent were added.

Thin-layer chromatography was carried out on silica gel G plates using the following solvent systems: neutral lipids, light petroleum (b.p. 30–60°)–diethyl ether–acetic acid (90:10:1); phospholipids, chloroform–methanol–water (95:35:4); glyceryl ethers and cyclic acetals, light petroleum (b.p. 30–60°)–diethyl ether–acetic acid (30:70:1). The location of lipid spots was determined by spraying the plates with H_2SO_4 and heating them at 300° for 10 min. Glycerol was chromatographed using *n*-butanol–acetic acid–diethyl ether–water (9:6:3:1). It was detected with a basic $AgNO_3$ spray.

RESULTS

The purified lipid extracts were first examined by thin-layer chromatography. The patterns obtained were generally similar to each other and to those previously found for the slug, where "triglycerides" and cholesterol make up most of the neutral lipids and choline, ethanolamine, serine, and inositol phospholipids account for most of the lipid phosphorus[9]. An exception was the neutral lipid fraction of the Octopus tentacle, which contained only traces of triglyceride.

TABLE I

THE CONTENT OF VARIOUS LIPID CONSTITUENTS OF MOLLUSCAN TISSUES

Species	*Fresh wt. of tissue (g)*	*Total lipid (mg)*	*Phospho-lipid (mg)*	*Glyceryl ethers in neutral lipids (mg)*	*Glyceryl ethers in phospholipids (mg)*	*Glyceryl ethers in phospholipids (average mole %*)*
K. tunicata	205	2125	829	52.0 54.2	60.1 70.4	24.5
T. lamellosa	117	5280	1990	63.6** 47.8	140.2 —	20.0
P. staminea	226	2340	1540	15.1 14.7	56.0*** 44.6	9.4
O. dofleini (hepatopancreas)	87	3380	1580	34.2 43.0	104.0 102.8	17.8
O. dofleini (tentacle)	480	2250	1650	trace trace	159 138	24.8

* Based on total lipid phosphorus.
** Small amount of non-glyceryl ether contaminant.
*** Small amount lost.

The total extracts were separated into neutral lipids and phospholipids by silicic acid column chromatography. Aliquots of these lipid fractions were analyzed for glyceryl ethers using the acetolysis–saponification procedure. It is clear from the data in Table I that in all cases both the phosphoglycerides and the neutral glycerides are

* Baker and Adamson light petroleum appeared to contain an inhibiting contaminant.

rich in glyceryl ether derivatives. Only traces of glyceryl ethers were isolated from hydrolysates of the triglyceride-poor Octopus-tentacle neutral lipids.

Samples of the glyceryl ethers were submitted to gas–liquid chromatography as the isopropylidene derivatives. The distribution of ether side-chains is shown in Table II. The identification of unsaturated ethers was made by chromatographing

TABLE II

DISTRIBUTION OF GLYCERYL ETHERS IN MOLLUSCAN TISSUES

Values are in mole per cent. Number preceding colon in Column 1 refers to number of carbon atoms; number following colon indicates number of double bonds. NL, neutral lipids; PL, phospholipids.

Side chain	*K. tunicata*		*T. lamellosa*		*Octopus (hepatopancreas)*		*Octopus (tentacle)*	*P. staminea*	
	NL	*PL*	*NL*	*PL**	*NL***	*PL*	*NL**** *PL*	*NL**** *PL*	
14:0		2	11	13		1	2	3	5
15:br				1		2	1	1	3
15:0	4	8	4	5		1	2	1	3
16:br							1		1
16:0	72	75	49	37	34	30	38	23	35
16:1			5				7	5	6
17:br					5	6	3	8	11
17:0				2	4	6	7	3	3
18:br(?)					4	4	4	4	3
18:0	8	6	21	12	34	41	20	36	21
18:1	16	10			9	6	7	6	4
19:br			10			4			
20:0				13				7	
20:1							8		4

* 19:unsatd.(?), 16%.
** 19:2(?), 7%.
*** 15:1, 1%; 22:br, 5%.
**** Traces only.

aliquots of the samples after catalytic hydrogenation. The major components in most cases are chimyl alcohol (1-*O*-hexadecyl glycerol) and batyl alcohol (1-*O*-octadecyl glycerol). Selachyl alcohol (1-*O*-octadecenyl glycerol), the other glyceryl ether found commonly in nature, is a major constituent only in *K. tunicata*.

Lipids from two species, those with the lowest and with the highest level of phospholipid glyceryl ethers, were analyzed for plasmalogens. The amounts found are reported in Table III. A large loss of plasmalogen or other material giving a plasmalogen-like reaction occurred during chromatography. The total content of ether phos-

TABLE III

PLASMALOGEN CONCENTRATIONS IN TOTAL LIPIDS AND CHROMATOGRAPHED FRACTIONS

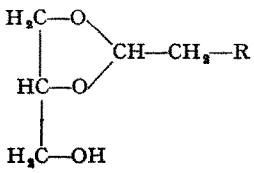
Measured by the procedure of GOTTFRIED AND RAPPORT[11]. After analysis of the total lipid of Octopus (1780 μmoles phospholipid P and *P. staminea* (1640 μmoles phospholipid P), each was chromatographically separated into neutral lipid and phospholipid before analysis was repeated.

	μmoles plasmalogen		
	Total lipid	*Neutral lipid*	*Phospholipid*
Octopus tentacle	269	10	192*
Protothaca	357	0	188

* Recovery of P only 91%.

pholipid in these tissues would be, summing the glyceryl ether level and the plasmalogen level, 21 mole % for Protothaca and 37 mole % for the Octopus tentacle.

Reduction of the lipid extracts with $LiAlH_4$ allows the isolation of both the glyceryl ethers and a degradation product of the plasmalogens, namely, cyclic acetals (IV) of long-chain aldehydes bound to glycerol. A virtually complete separation of these two components can be obtained by chromatography of the $LiAlH_4$ product on



IV

silicic acid columns. First, the fatty alcohols, produced by $LiAlH_4$ from free and esterified fatty acids, are eluted with hexane–diethyl ether (3:1). Next, hexane–diethyl ether (3:2) allows the elution of cyclic acetals and then glyceryl ethers. An excellent separation can be achieved by the use of an automatic fraction collector. Fig. 1 indicates the purity of the pooled fractions. Also shown is the thin-layer pattern of an unknown material found in all the molluscs examined except *K. lamellosa*. This material is eluted from silicic acid with diethyl ether and appears, from its infrared spectrum, to contain an ether linkage.

The yields of glyceryl ethers using the $LiAlH_4$ reaction were similar to those obtained by the acetolysis–saponification procedure. Gas–liquid chromatographic patterns of glyceryl ethers prepared by the two procedures were essentially identical. Cyclic acetals were also obtained in high yield. The octopus-tentacle phospholipids, containing 11.7 mole % (based on moles of lipid phosphorus) of plasmalogens according to the spectrophotometric assay of GOTTFRIED AND RAPPORT[11], yielded in duplicate analyses 10.3 mole % and 8.0 mole % cyclic acetal as measured by weight. The infrared spectrum of the acetal products is similar to that of glyceryl ethers[14], the main differences being a stronger ether absorption peak near 9.0 μ and a weaker hydroxyl peak in the 2.8 μ vicinity. The hydrocarbon side chains of the acetals can be freed by hydrolysis in aqueous HCl. The resulting long-chain aldehydes, when placed directly onto a gas–liquid chromatographic column, provide information about the

TABLE IV

DISTRIBUTION OF ALDEHYDES DERIVED FROM PHOSPHOLIPID CYCLIC ACETAL DERIVATIVES

Side chain	*Octopus (tentacle)*	*Protothaca*
14:1	—	5
15:br	18	—
16:0	14	4
17:br	2	5
Unknown	—	4
17:0	5	3
18:br	4	10
18:0	41	58
18:1	—	4
19:br	8	—
20:1	—	6
21:0	8	—

plasmalogen vinyl ether side chains. Table IV contains the distributions found in the Octopus tentacle and Protothaca. It was discovered that catalytic hydrogenation of the cyclic acetals results in a moderately good yield of glyceryl ethers. Gas–liquid chromatography of these ethers as the isopropylidene derivatives confirmed the data in Table IV.

The $LiAlH_4$ procedure was further tested by applying it to beef-heart lipids, which are rich in plasmalogens but almost devoid of glyceryl ether lipids. As expected, cyclic acetals with properties similar to those from molluscan sources were obtained.

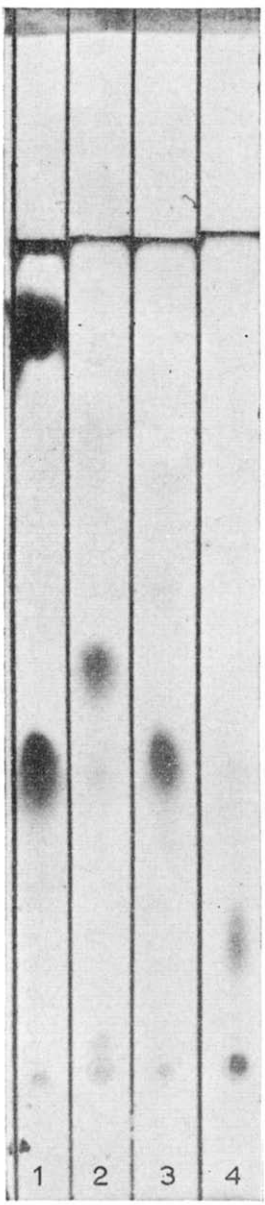


Fig. 1. Thin-layer chromatographic pattern of fractions from the $LiAlH_4$ reaction. 1, standards: (from bottom) selachyl alcohol, cholesterol, palmitic acid and triolein at solvent front: 2, cyclic acetals; 3, glyceryl ethers; 4, unknown fraction.

Hydrolysis and gas–liquid chromatography of the resulting aldehydes showed that the relatively high degree of side-chain unsaturation* was not affected by the $LiAlH_4$ process. The presence of glycerol in the HCl hydrolysate was detected by thin-layer chromatography, and a quantitative glycerol assay indicated a molar glycerol/cyclic acetal ratio of 1.28.

DISCUSSION

In addition to the two species of terrestrial slug examined earlier[9], tissues from four members of phylum Mollusca have beenanalyzed in some detail for glyceryl ethers. In all cases the phospholipids as well as the neutral glycerides contain large concentrations of the glyceryl ether derivatives. Since the species analyzed represent each of the four major classes of mollusca, it is likely that glyceryl ether lipids are of general occurrence throughout the phylum. The presence of these ethers in the phospholipids is of particular interest in view of the probable importance of phospholipids in mediating certain physiological membrane phenomena. Unfortunately, there are at present insufficient data to judge whether a structural difference such as the presence of an ether rather than an ester linkage would promote physiological changes.

If, as is indicated by thin-layer chromatography, the species described in this paper are similar in their lipid composition to the slug, the glyceryl ethers of the phospholipids are distributed among several phospholipid classes, being particularly high in the choline lipids. Those of the neutral lipids occur as the diacyl derivatives. Earlier findings with the slug and the present data concerning Octopus indicate that, in contrast to most higher animals, neutral glycerides are found only in the viscera of molluscs.

The vinyl ether side chains from the plasmalogens of Protothaca and Octopus do not greatly resemble in length the glyceryl ether side chains. The most noticeable difference lies in the predominance of the C_{18} chain. This is also the case in the slug lipids, but analysis showed in that species that the plasmalogens are concentrated in the ethanolamine lipids, and that they do resemble in side-chain distribution the glyceryl ethers of that particular fraction.

Although no data are available from other laboratories on glyceryl ethers of molluscan phospholipids, plasmalogens have been reported to be present in species of this phylum. RAPPORT AND ALONZO[15] found these α,β-unsaturated ethers in relatively high concentrations in the phospholipids of 5 species representing 3 classes of mollusca. Our data indicate that a given species is likely to contain both forms of ether phospholipid. The plasmalogen levels reported here for the chromatographed lipids may be low due to degradation of the labile vinyl ether bond on the column. However, it is clear in the case of the Octopus tentacle that the glyceryl ether derivative strongly predominates over the vinyl ether form. The relationships of the ether phospholipids in other phyla will be of taxonomic interest.

The unpredicted success of $LiAlH_4$ in cleaving both acyl ester and phosphate diester bonds to yield stable and separable products from the ether phospholipids greatly facilitates work in this area. The reaction requires only 0.5 h as compared to 10 h for the acetolysis–saponification procedure. The high yields of cyclic acetals will

* G. A. THOMPSON, unpublished analyses of dimethylacetals.

enable this method to be usefully employed in studying the biosynthesis of plasmalogens, and will be particularly valuable in analyzing their glyceryl moiety, which is usually lost in the isolation of the plasmalogen side chain. Other parameters of this reaction, such as the nature of the products obtained using milder conditions, are presently under examination.

ACKNOWLEDGEMENTS

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NOTE ADDED IN PROOF

Comparison of physical properties, neutral periodate oxidation, hydrogenation products, and determination of α,β-unsaturation[11] of $LiAlH_4$-produced "cyclic acetals" and synthetic cyclic acetals[16] strongly indicate that the former compounds are in fact α,β-unsaturated glyceryl ethers. However, all information obtained assuming the acetal structure remains valid.

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