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Nonphosphatide Fatty Acyl Esters of Alkenyl and Alkyl Ethers of Glycerol*

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Evidence has been presented, in a preliminary communication from this laboratory (1), and by Schogt, Begemann, and Koster (2), to substantiate the previously postulated existence (3, 4) of nonphosphatide plasmalogens. This evidence was based mainly on observations made with unsaponifiable fractions obtained after treatment of neutral lipid with alkali. In our preliminary communication (1), for example, the neutral lipid of the digestive gland of the starfish (*Asterias forbesi*) was saponified. Fatty aldehyde was found to be released from the unsaponifiable fraction by mild acid treatment, and was detected with fuchsin.

The color reaction used by Norton (5) to detect $\alpha\beta$-vinyl ethers[1] of the phosphatide series (phosphatide plasmalogens) was also applied to the unsaponifiable fraction and was positive. The implication that the aldehydogenic substance was an alkenyl ether of glycerol was confirmed by the fact that glycerol was released with the aldehyde during mild acid hydrolysis, the molar ratio of glycerol to fatty aldehyde being 1.1 (1). Hydrogenation, or halogenation of the lipid by a method specific for the double bond adjacent to the oxygen bridge, eliminated the release of these two moieties by acid (1). Finally, the position of the alkenyl ether linkage to glycerol was found to be on a primary carbinol group of glycerol. This information was obtained by allowing the unsaponifiable fraction to react with periodate, and then cleaving by mild acid treatment. Glycolaldehyde was isolated rather than glycerol; this situation could prevail only if the unsaponifiable fraction contained alkenyl ethers of glycerol linked to one of the primary carbinol groups (1).

In the preliminary communication, some data were also reported on the *intact* neutral lipid of the starfish. The neutral lipid fraction contains large amounts of glycerol ethers of the batyl alcohol type, *i.e.* alkyl ethers of glycerol. Some concentration of both alkenyl and alkyl ethers of glycerol was obtained by reversed phase paper chromatography, followed by silicic acid chromatography. A fraction was prepared that was 50% triglyceride, *i.e.* triesters of glycerol, and 50% glycerol monoether diester. Of the glycerol ethers, only 5% was alkenyl ether (neutral plasmalogen); the remainder was alkyl ether (diesters of batyl alcohol and its analogues). Mild acid treatment of this neutral lipid fraction to cleave the vinyl ether yielded a substance tentatively identified by chromatography and chemical analysis as a diglyceride.

The present communication will not further elaborate the studies on unsaponifiable fractions previously given in our preliminary communication (1). The objectives of this paper are (*a*) to amplify, where necessary, descriptions of the methods used; (*b*) to report on the enrichment of neutral lipid fractions with respect to the concentration of neutral plasmalogens, and the preparation of a fraction containing *only* ether diesters of glycerol, of which approximately 85% were alkenyl ether diesters; (*c*) to use this concentrate to strengthen and extend the preliminary data on the intact glycerol ether diesters, especially with respect to the molar ratios of the various moieties; (*d*) to extend information on the occurrence of these substances, especially in mammalian tissues.

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[1] Since the discovery of the nature of the aldehydogenic linkage in the plasmalogens (6, 7), these compounds have been referred to by many authors as $\alpha\beta$-vinyl ethers of glycerol. This terminology could conceivably cause confusion in that the carbon atoms of the glycerol moiety are often referred to as α, β, and α' in assignments of positions of attachment of various entities to the glycerol backbone.

In this paper we shall use the term "alkenyl" to refer to the vinyl fatty substituent that yields a fatty aldehyde on acid treatment of the plasmalogen (*cf.* (8)). More specifically, if the carbon atoms of this fatty chain are numbered 1′, 2′, etc., we are referring to an α-glycerol (1′-alk-1′-enyl) ether; α here refers to a primary carbinol group of glycerol. The "ene" refers to the double bond of the fatty chain adjacent to the oxygen bridge.

The long chain entity that is *not* cleaved from glycerol with mild acid treatment—as in those α-glycerol ethers known for many years, for example, in batyl or selachyl alcohol—will be referred to as the "alkyl" substituent. Again, specifically, we have an α-glycerol (1′-alkyl) ether, and there is no double bond between carbon atoms 1′ and 2′ of the fatty alcohol moiety. There may, of course, be double bonds in several other positions in this fatty chain (9, 10).

From the work of Baer and Fischer (11), the α-glycerol alkyl ethers are assigned the D-configuration. The work of Kiyasu and Kennedy (12) indicates that the α-alkenyl ether β-acyl esters of glycerol obtained by enzymatic cleavage of phosphoryl choline from a phosphatide plasmalogen are also in the D-configuration.

Thus the compounds studied in our paper may be referred to as acyl esters of D-α-alkenyl (or D-α-alkyl) ethers of glycerol.

If one applies the nomenclature system of Hirschmann for glycerol derivatives (13), based on the system of Cahn, Ingold, and Prelog (14), in which L-α-glycerophosphate is simply glycerol 3-phosphate, then the ether esters studied by us are acyl esters of glycerol 1-alkenyl (or -alkyl) ethers. More specifically, they are 1-*O*-(1′-alk-1′-enyl)glycerol and 1-*O*-(1′-alkyl)glycerol 2,3-diacyl esters, where 1, 2, 3 refer to the carbon atoms of glycerol, and 1′, 2′, etc., to those of the fatty moiety in ether linkage.

We shall use the concise forms "glycerol alkenyl ether (diesters)" and "glycerol alkyl ether (diesters)" throughout, to refer to these compounds with the ether link to a primary carbinol group of glycerol, and presumably of the D-configuration as outlined above.

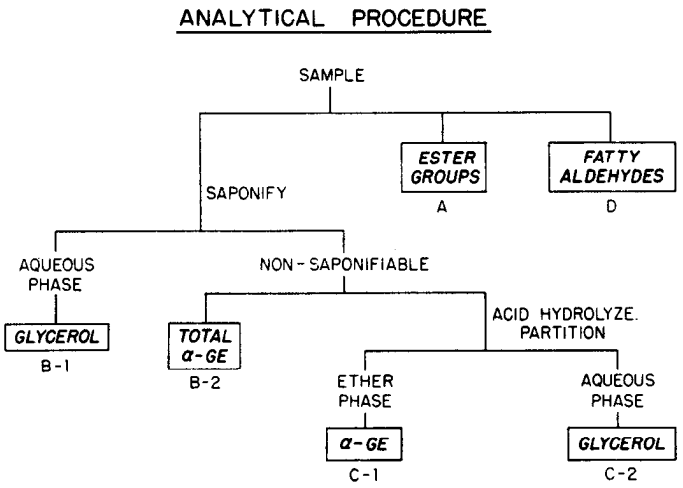

FIG. 1. Flow sheet of the analytical procedures used. The individual determinations are discussed in the text. *A*, *B-1*, *B-2*, *C-1*, *C-2*, and *D* correspond to the vertical columns of Table I. Aldehyde determinations (alkenyl ethers) (*D*) have also been performed in conjunction with the final acid hydrolysis of the nonsaponifiable fraction. *α-GE* refers to α-glycerol ethers.

EXPERIMENTAL PROCEDURE

Extraction of Lipids—Chloroform-methanol (2:1) was used to extract the lipids from all of the tissues (15) mentioned in this paper. Solvents were always removed from lipid extracts under vacuum at 30–35°. Wherever possible, the lipid was blanketed with nitrogen.

Preparation of Neutral Lipids—Solutions of lipid in chloroform were adjusted to give a concentration of 2 mg of total lipid per ml. Phosphatides were flocculated in the cold by adding twice the volume of acetone and a small amount of magnesium chloride (16). The flocculation procedure was repeated twice more, and the neutral lipids were dried. When only small amounts of lipid were available, as in the case of those extracted from human serum β-lipoprotein and chylomicrons,[2] the total lipids were applied to a silicic acid column. The neutral lipids were then freed of phosphatides by elution with chloroform (18).

Silicic Acid Chromatography of Starfish Neutral Lipid—The neutral lipids were chromatographed on silicic acid columns with *n*-heptane containing increasing amounts of diethyl ether (19, 20), as indicated in Table I. Each fraction was evaporated and taken up in *n*-heptane. Aliquots were analyzed as below.

Analytical Scheme—A flow sheet showing the general analytical procedure is given in Fig. 1. The information provided by each analytical method is designated in that figure by a capital letter (*A*; *B-1*; etc.). To facilitate matters these designations are cited, where relevant, in the paragraphs below and in Table I.

[2] Human plasma β-lipoprotein and chylomicron fractions were provided by Dr. Nancy R. Harvie and Dr. J. L. Oncley and were prepared as follows. Pooled human plasma was used. The plasma density was adjusted to 1.063 by the addition of dry NaBr; EDTA was added to a final concentration of 0.01%, and the preparation was centrifuged for 18 hours at 105,000 × *g*. The top layer containing the desired protein fractions was removed with a tube cutter. The β-lipoprotein was separated from the chylomicron fraction by centrifuging this top layer at 11,000 × *g* for 1 hour. The turbid top layer containing the chylomicrons was removed by use of the tube cutter (17).

Standard Chemical Determinations—Lipid phosphorus was determined by the method of King (21). Saponification, extraction, and determination of the unsaponifiable fraction were carried out by the method of the Society of Public Analysts (22) with suitable adjustments in scale. In the case of the column fractions, 2 to 8 μmoles of ester groups provided a suitable aliquot. The unsaponifiable fraction was finally taken up in 95% ethanol. The alkaline aqueous phase, after extraction of the unsaponifiable fraction, was neutralized with hydrochloric acid to a phenolphthalein end point, and made to a known volume. Fatty acid ester groups (*A*) were determined as described by Rapport and Alonzo (23). The method of Wittenberg, Korey, and Swenson (24) was used to estimate alkenyl ethers of glycerol. It depends upon the measurement of fatty aldehydes resulting from the cleavage of alkenyl ethers with dilute acid (*D*).

Determination of Glycerol and Total Glycerol Ethers—The method of Karnovsky and Brumm, adjusted in scale where necessary, was used to estimate the glycerol ethers (25) (*B-2*); it was also employed to estimate water-soluble glycols (*B-1*). This method depends upon periodate oxidation, followed by determination of formaldehyde according to the MacFadyen technique (26).

Differentiation of Acid-labile Glycerol Ethers (Alkenyl Ethers) from Acid-stable Glycerol Ethers (Alkyl Ethers)—The acid-stable and acid-labile glycerol ethers were determined on aliquots from the nonsaponifiable fraction after mild acid hydrolysis. The hydrolysis procedure of Wittenberg, Korey, and Swenson (24), reduced in scale by a factor of 8, was used. Optimally approximately 4 μmoles of 1,2-glycol were analyzed.

Water was added to the alcoholic hydrolysis system, and the mixture was extracted three times with diethyl ether to remove acid-stable glycerol ethers (22). The ethyl ether extracts were washed with small volumes of distilled water. The total aqueous phase remaining after ether extraction was neutralized with 1 M sodium hydroxide to a phenolphthalein end point. This solution then was made to a known volume, and aliquots were taken to determine glycerol (25) liberated from the acid-labile vinyl ethers (*C-2*). The dried residue obtained from the ethereal extract was taken up in a known volume of 95% ethanol, and an aliquot was analyzed for acid-stable glycerol ethers (25) (*C-1*).

An attempt was made to define the precision of this procedure. Replicate analyses were performed on seven samples of a solution of alkyl and alkenyl glycerol ethers. The average number of micromoles of alkenyl ether (± standard error) was 0.70 ± 0.04 per ml of original sample. The average value for alkyl ether was 1.46 ± 0.03 μmoles per ml of original sample. With respect to the accuracy of this procedure, one might refer to the agreement with determinations by independent methods, *i.e.* for fatty acid esters (23) and fatty aldehydes (24) (released from alkenyl ethers) as shown in Table I.[3]

[3] Upon completion of this report, an iodimetric method specific for the estimation of enol ethers was published (27). The accuracy of the method for alkenyl ethers used in our work was assessed by a comparison with the new procedure of Williams, Anderson, and Josik (27). Aliquots from six solutions containing alkenyl ethers of glycerol were examined by both techniques. The ratio of the values obtained by the procedure of these authors to those obtained by our procedure had a mean value of 0.94, with a range of 0.90 to 0.96. Thus the methods appear to give comparable

RESULTS AND DISCUSSION

Fractionation of Neutral Lipids of Starfish—When silicic acid column chromatography was used to fractionate the starfish neutral lipids, the following results were obtained. The first developing solvent, *n*-heptane, eluted hydrocarbons; sterol esters were eluted with 0.6% ethyl ether. The completeness of elution of the latter fraction was monitored by the Liebermann-Burchard reaction.

The elution pattern for acid-stable and acid-labile ether diesters (*C-1* and *C-2*, respectively) and glycerol triesters (*B-1*) is presented in Table I. Columns A, B, C, and D correspond to the designations in Fig. 1.

The following points emerge from Table I.

1. If the 1,2-glycols in Columns B-1 and B-2 represent glycerol from ester triglycerides and glycerol ether from ether diesters of glycerol, respectively, then the total number of micromoles of ester groups in Column A should equal the sum of (3 × B-1) + (2 × B-2). This is so, within reasonable limits of error.

2. The total number of micromoles of fatty aldehyde recorded in Column D should be the same as the figures of Column C-2, if the different methods used give comparable results. This is also so. Thus, aldehyde released from alkenyl ethers by mild acid treatment, measured by a colorimetric method that relies on the formation of the *p*-nitrophenylhydrazones, is equal to glycerol released from the unsaponifiable fraction by the same acid hydrolysis and measured by periodate oxidation and determination of formaldehyde generated.

3. No 1,2-glycol was detected in the aqueous phase after saponification of the lipid eluted by 1% ethyl ether in *n*-heptane (Column B-1). This fraction thus contained no triesters of glycerol.

Preparation of Concentrate of Alkenyl Ether Diesters of Glycerol—The order of elution of neutral lipid components suggests that a separation of the alkyl and alkenyl ether diesters could be achieved by collecting the initial volumes of less polar eluting solvent and combining these. This was attempted by chromatographing a number of samples of the starfish neutral lipid with 0.8% ethyl ether in *n*-heptane and collecting small volumes of the column effluent. An aliquot from each column fraction was saponified and then extracted with ethyl ether, and 1,2-glycols were determined in the aqueous and ether phases as described before. Also, an aliquot of the nonsaponifiable extract was acid-hydrolyzed, and the acid-stable glycerol ethers were determined and compared with the total unsaponifiable 1,2-glycol values. Those fractions in which acid-stable 1,2-glycols were minimal were combined and analyzed as before for ester groups, fatty aldehydes, and total as well as acid-stable and -labile glycerol ethers. The results of analysis of the pooled early fractions are as follows (in micromoles): total ester groups, 43.7; total glycerol ethers, 23.6; alkyl glycerol ethers, 2.8; alkenyl glycerol ethers, 18.3; fatty aldehyde released, 18.8. The data indicate that a concentration had been achieved so that the fraction contained only glycerol ether diesters of which approximately 85% were of the vinyl type.

Confirmation of Existence of Glycerol Alkenyl Ethers as Diesters—If the alkenyl glycerol ethers exist as diesters, then mild acetic acid hydrolysis, a procedure that has been shown to be capable of cleaving the vinyl link without hydrolyzing the ester linkages in a plasmalogen (12), should give rise to a diglyceride and a fatty aldehyde, separable by silicic acid column chromatography.

TABLE I

Chemical analysis of fractions obtained by silicic acid chromatography of starfish neutral lipid

Column A represents total fatty acid esters; B-1 shows the water-soluble 1,2-glycols (glycerol) released during saponification; B-2 indicates the total glycerol ethers of the unsaponifiable fraction; C-1 shows alkyl ether, and C-2, alkenyl ethers, of glycerol; D indicates alkenyl ethers measured as fatty aldehydes (24).

Chromatography fractions (ethyl ether in *n*-heptane)	A	B-1	B-2	C-1	C-2	D
%	*μmoles*					
1.0	48.4	0.0	24.2	14.2	11.3	10.2
1.5	133.4	29.6	25.2	20.6	5.1	5.1
2.0	186.6	56.7	7.4	5.8	1.7	1.5
2.5	130.5	41.8	0.0	0.0	0.0	0.0
4.0	147.3	53.1	0.0	0.0	0.0	0.0
Total	646.2	181.2	56.8	40.6	18.1	16.8

An experiment of this type was reported in our preliminary communication (1). A lipid fraction did appear that had the expected chromatographic characteristics, and a fatty acid ester to glycerol ratio of 2.4 was obtained (expected, 2.0). At that time only very small amounts of alkenyl ether diester were available (1). After the chromatographic concentration of alkenyl ether diesters of glycerol in early fractions (above), it was hoped that more conclusive results could be obtained through the use of a larger sample.

The mild acid hydrolysis procedure (12) was carried out on the concentrate of alkenyl ether diesters and the lipid extract chromatographed on silicic acid. The column was developed with 250 ml each of 0.6 and 4.0% ethyl ether in *n*-heptane and with 250 ml of ethyl ether.

Experiments with model mixtures had shown previously that fatty aldehydes could be eluted with 0.6% ethyl ether in *n*-heptane. When the silicic acid column was developed with this solvent, a positive test for aldehydes was obtained on the eluted lipid with fuchsin-sulfurous acid reagent. The step employing 4% ethyl ether in *n*-heptane was included to elute alkyl ether diesters, and this column fraction was not analyzed further. The ethyl ether fraction that was expected to contain the diglyceride liberated by acid hydrolysis of the vinyl ether diester was dried as before and taken up in a known volume of *n*-heptane; aliquots were analyzed for ester groups and water-soluble 1,2-glycols liberated by saponification. The ester group analysis indicated a total of 10.5 μmoles of ester groups, whereas the analysis for water-soluble 1,2-glycols after saponification indicated a total of 5.3 μmoles of glycerol in this fraction. This gives a molar ratio of ester groups to glycerol of 2.03.

In the past some question has existed as to the position of the vinyl ether linkage in the plasmalogens (28, 29), but now it is understood that the vinyl ether link in the phosphatide plasmalogens is on a primary carbinol group (30, 31). This is apparently also true for neutral plasmalogens, because of the following observations.

1. Glycolaldehyde, as previously reported (1), was found to be present after periodate oxidation followed by acid hydrolysis of the nonsaponifiable extract of the starfish neutral lipid.

results for the estimation of alkenyl ethers, although the method of Williams, Anderson, and Josik is more sensitive than ours.

TABLE II

Glycerol ethers in nonsaponifiable fraction of neutral lipids from several mammalian tissues

	Total lipid[a]	Neutral lipid[b]	Phosphatide[c]	Total glycerol ethers[d]	Vinyl ethers[e]
	%	%	%	%	%
Diverticulum (starfish)	5.7	72.50	0.19	16.1	31.9
Brain (beef)	7.4	43.95	0.10	1.9	63.1
Bone marrow (beef)	88.9	99.97	0.00	2.4	61.2
Heart (calf)	5.1	62.61	0.42	2.4	31.3
Adipose tissue (rat)	76.8	98.91	0.01	1.3	82.0
Brown fat (rat)	43.1	78.40	0.04	0.4	54.3
Leukocytes (polymorphonuclear, guinea pig)		51.92	0.00	2.9	65.8
Chylomicrons (human)		85.9		0.1	78.6
β-Lipoprotein (human)				0.6	29.3

[a] Per cent of fresh weight.

[b] As per cent of total lipid, determined by weight.

[c] Residual lipid phosphorus was measured in the neutral lipid, and a factor of 25 was assumed. Expressed as per cent of neutral lipid fraction.

[d] As per cent of neutral lipid, expressed as the diesters; a molecular weight of 850 was assumed.

[e] Molar percentage of *total* glycerol ethers.

2. If the vinyl link were on the secondary carbinol group of glycerol, the sum of the acid-stable and acid-labile glycerol ethers would be greater than the value obtained for the total glycerol ethers. This would be so because in the total glycerol ether analysis a vinyl ether linkage in the 2-position of glycerol would not permit periodate oxidation, and consequently no formaldehyde would be liberated. On the other hand, after acid hydrolysis to determine acid-labile glycerol ethers, 2-linked vinyl ethers would give rise to glycerol that *would* be oxidized by periodate to yield 2 moles of formaldehyde.

3. If the ether linkage were in the 2-position of glycerol, the ratio of ester groups to apparent total glycerol ethers would be greater than 2.0, since 2-linked glycerol ether would not be subject to periodate oxidation, as pointed out above.

Alkenyl and Alkyl Ethers of Glycerol in Animal Tissues—The data in Table II indicate that from 0.1 to 2.9% of the neutral lipid in the mammalian cells and tissues studied is present as glycerol ether diesters. The table also indicates that the alkenyl glycerol ethers comprise from 29 to 82% of the total nonsaponifiable 1,2-glycols.

Recently a more detailed analysis of the lipids of the epididymal fat pad of the rat has been attempted. The silicic acid column chromatography was extended beyond the elution of triglycerides by using 250-ml volumes of 10% ethyl ether in *n*-heptane and 25% ethyl ether in *n*-heptane to elute free sterols and diglycerides. Analysis of the diglyceride fraction indicated the presence of an alkenyl glycerol ether monoester as well as diglycerides (glycerol diesters). This finding is supported by observations that samples of this fraction spotted on filter paper gave a positive test for vinyl ethers when treated with mercuric chloride and diphenylcarbohydrazide (5). Analysis of this diglyceride fraction for aldehydes by the method of Wittenberg Korey, and Swenson (24) indicated that a *total* of 3.4 μmoles of alkenyl ether was present. When the diglyceride fraction was subjected to a mild acid hydrolysis and the lipid was rechromatographed on silicic acid, three lipid-containing fractions were observed. The first was eluted with 0.8% ethyl ether in *n*-heptane and contained fatty aldehydes, which were detected as *p*-nitrophenylhydrazones (24). This fraction was not quantified, because free aldehydes have not been completely recoverable from silicic acid columns in our laboratory. The second fraction was eluted with 25% ethyl ether in *n*-heptane and contained glycerol diesters. A third fraction was eluted with ethyl ether and was expected to contain a monoglyceride if an alkenyl ether monoester had originally been present. When this fraction was analyzed for fatty acid ester groups, a total of 2.8 μmoles was found. After saponification of this fraction and analysis of the aqueous phase for 1,2-glycols, 2.5 μmoles of glycerol were present. Upon acidification of the saponification mixture and extraction with *n*-heptane, the presence of 2.4 μmoles of fatty acid was determined by titration according to the method of Albrink (32). This gives a molar ratio of fatty aldehyde to glycerol of 1.37, of fatty acid ester to glycerol of 1.10 and of fatty acids (extracted after saponification) to glycerol of 0.96. These data agree with those expected for an alkenyl ether monoester. The ratio of fatty aldehydes to glycerol deviated from the expected value of 1.0 because alkenyl ethers were determined as aldehydes *before* acid hydrolysis and chromatography, whereas the other chemical analyses were done subsequent to these procedures, on material recovered from the column. Some loss of material during the manipulations certainly would be expected. It is clear, however, that mild acid hydrolysis of a component of the diglyceride fraction yielded a monoglyceride and free aldehyde. The alkenyl ether monoester represents approximately 0.2% of the neutral lipid if a saturated 18 carbon chain is assumed for the fatty acid and alkenyl groups. A trace of alkyl ether monoester was also found.

The possibility that this alkenyl ether monoester of glycerol is an intermediate in the biosynthesis of glycerol ether diesters is under investigation. It has been shown to be the precursor of phosphatide plasmalogens (12), thus having a role that probably is analogous to that of D-diglycerides in the metabolic scheme.

The widespread distribution of the glycerol ethers in many mammalian tissues of widely differing type and function, as shown in this paper and by Schogt, Begemann, and Koster, who detected alkenyl ethers of glycerol in the nonsaponifiable lipids from milk fat, beef tallow, and ox heart (2), raises intriguing questions concerning the functions of this type of lipid. The fact that such a substantial proportion of the glycerol ethers is made up of alkenyl ethers of glycerol has not been previously appreciated.

SUMMARY

1. The presence of glycerol alkenyl ether diesters in the neutral lipid fraction of the lipids from the diverticulum of the starfish (*Asterias forbesi*) has been established by chromatographic concentration and analysis.

2. That the previously known glycerol alkyl ethers actually exist in nature as glycerol alkyl ether diesters is also established.

3. A number of mammalian tissue lipid extracts have been shown to contain glycerol ethers of both alkyl and alkenyl types, which together comprise 0.1 to 2.9% of the neutral lipid calculated as their fatty acid diesters. The alkenyl ethers were found to form from 29 to 82% of the total glycerol ethers in the neutral lipids of mammalian tissue.

4. The presence of a glycerol alkenyl ether monoester, in addi-

tion to the alkenyl ether diester, has been demonstrated in the lipid extracted from the epididymal fat pad of the rat.

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