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The Biosynthesis of Ether-Containing Phospholipids in the Slug, *Arion ater*. II. The Role of Glyceryl Ether Lipids as Plasmalogen Precursors*

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ABSTRACT: Further studies of *in vivo* plasmalogen formation in *Arion ater* have been carried out. Under a variety of environmental conditions, incorporation of [1-^{14}C]palmitic acid into plasmalogens is much lower than incorporation into diacyl and glyceryl ether phospholipids. However, this depressed incorporation into plasmalogens is observed only in experiments of relatively short duration. After periods of 2 days or longer, the vinylic ether side chain of plasmalogens does become labeled from fed radioactive palmitic acid or glyceryl ethers. Evidence is presented that the ether-linked side chain of glyceryl ether phospholipids is the direct precursor of the plasmalogen vinylic ether side chain.

Studies carried out over the past few years (Rapport and Norton, 1962; Hartree, 1964) have demonstrated plasmalogens to be major lipid constituents in many animal tissues. Even though these lipids have a wide distribution in nature, the mechanism of their biosynthesis has not been determined.

During the course of a recent study on the structurally similar glyceryl ether phospholipids of *Arion ater*, we observed that the plasmalogens appeared to be metabolically quite inert (Thompson, 1965). The glyceryl ether phospholipids and the diacyl phospholipids rapidly incorporated radioactivity from fed [1-^{14}C]-palmitic acid and [6-^{14}C]glucose. However, over the time intervals studied (1–16 hr) the plasmalogens showed a low uptake of ^{14}C, with the specific radioactivity of their aldehydogenic side chains being only 10–20% of that found for side chains of the other phospholipids.

These observations are reminiscent of the results obtained by other investigators who have studied plasmalogen biosynthesis. When fatty acid incorporation into brain lipids was examined (Debuch, 1964; Carr *et al.*, 1963) and when the uptake of possible lipid precursors by heart was measured (Keenan *et al.*, 1961), esterified fatty acids invariably appeared to be formed much more rapidly than the ether-bound plasmalogen side chain. Bauman *et al.* (1965) have reported unusually active plasmalogen biosynthesis by rapidly growing cultures of *Clostridium butyricum*. Although the final rate of plasmalogen formation was high, there was a lag period before synthesis was detected.

In the experiments reported here plasmalogen synthesis in *A. ater* has been reexamined with particular attention to possible explanations for its low rate of formation.

Experimental Section

Lipid Analyses. Methods have previously been described for isolating and analyzing the *Arion* lipids (Thompson and Hanahan, 1963; Thompson, 1965). Briefly, the lipids were extracted with chloroform–methanol 1:1 (v/v) and, after removal of nonlipid contaminants, were chromatographed on silicic acid columns at 4°. The glyceryl ethers and glyceryl vinylic ethers[1] were prepared by $LiAlH_4$ hydrogenolysis (Thompson, 1965). The $LiAlH_4$ products were purified by silicic acid column chromatography (Thompson and

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[1] The term glyceryl vinylic ether denotes those 1-*O*-ethers of glycerol in which there is unsaturation between the α- and β-carbon atoms of the hydrocarbon chain.

Lee, 1965). Phosphorus was determined by the method of King (1932), plasmalogen by the method of Gottfried and Rapport (1962), and glyceryl ethers and glyceryl vinylic ethers by direct weighing.

The chemical and radio purity of the glyceryl vinylic ethers isolated from *Arion* tissues were confirmed by two or more of the following procedures. The compounds were purified by column chromatography (Thompson and Lee, 1965), and the fractions were examined by infrared analysis and thin layer chromatography (followed by radioassay of the eluted zones). The bulk of the radioactivity (90%) was routinely associated with the glyceryl vinylic ether, and the remaining 10% represented small levels distributed fairly uniformly over the remaining area of the plate. Aliquots of the radioactive glyceryl vinylic ethers were converted to: (1) cyclic acetals by a 0.5-hr reflux in glacial acetic acid, (2) long-chain aldehydes by a 2-hr reflux in aqueous 2 N HCl, or (3) dimethyl acetals by a 2-hr reflux in anhydrous methanol saturated with HCl gas. In all cases radioactivity of the products was found almost exclusively (92–96%) in the appropriate fraction by column or thin layer chromatography.

Radioactive Substrates. [1-^{14}C]Palmitic acid (10 mc/mmole) was obtained from New England Nuclear Corp. and was freed from trace contamination by column chromatography. [6-^{14}C]Glucose (25 mc/mmole) was purchased from Nuclear-Chicago Corp. Its purity was checked by radioassay of thin layer chromatograms. The preparation of chimyl alcohol (10^3 mc/mmole) labeled in the side chain with ^{3}H has been described previously (Thompson, 1965). This compound was repurified by column chromatography before the feeding experiments were begun. The [^{14}C]-glyceryl ethers (0.06 mc/mmole) used in expt No. 2, Table II, were a gift from Dr. W. G. Haigh and were repurified before use. The [^{14}C]glyceryl ethers used in expt No. 4, Table II, were isolated from *Tetrahymena pyriformis* grown on [1-^{14}C]acetate (Thompson and Winquist, unpublished observations). Practically all of the radioactivity was contained in the hydrocarbon side chain. The specific radioactivity of this latter material was not determined quantitatively, but was estimated to be approximately 0.5 μc/μmole. Isotopically labeled samples were assayed in a Packard Tri-Carb scintillation spectrometer with efficiencies of 49% for ^{3}H and 89% for ^{14}C.

Experimental Conditions. A high oxygen atmosphere was obtained by passing a water-saturated mixture of 95% O_2 and 5% CO_2 through the experimental enclosure. Nearly anaerobic conditions were attained by using water-saturated cylinder nitrogen gas without rigorous treatment to remove traces of oxygen.

Isotope administration was achieved by evaporating an ethanol solution of the substrate on an approximately 2-cm^2 piece of lettuce and feeding this to animals fasted overnight. Samples of slug blood were assayed for radioactivity by suspending a 6-μ laliquot in Bray's solution (Bray, 1960) for measurement in the scintillation counter. Quenching was approximately 25%.

Results

Ether Synthesis under Various Experimental Conditions. For the sake of uniformity all of our early feeding studies had been carried out under a standardized set of conditions, namely at room temperature in moist air. The purpose of the first series of experiments described below was to determine if the relative rate of plasmalogen synthesis might be higher under the influence of varied environmental or hormonal conditions.

A comparison of specific radioactivities of glyceryl ethers and the plasmalogen-derived glyceryl vinylic ethers was made in 6 of the 8 months (from March through October) in which the slugs are active. All of these [^{14}C]palmitate feeding experiments, involving *in vivo* incubation periods of 1–16 hr, showed little incorporation into glyceryl vinylic ethers. The results were in all cases similar to those already published (Thompson, 1965). There were variations in plasmalogen content between 4 and 10 mole % of total lipid phosphorus from experiment to experiment with an average in 11 experiments of 7.7 mole %. The variations bore no obvious relationship to the time of year.

The slug is known to become more active in foraging for food as the temperature drops. Although a poikilotherm, it will feed during the night at temperatures not far above freezing. Plasmalogen synthesis was studied in slugs which had been maintained for 14 days at 4° except for occasional short intervals when food was added. The animals were fed palmitate and, after 4 hr at 15°, were analyzed. Again the glyceryl vinylic ethers had incorporated only a relatively small amount of radioactivity.

Some species of molluscs are known to survive for long periods of time under practically anaerobic conditions (von Brand, 1944). The specific radioactivity of glyceryl vinylic ethers was compared with that of glyceryl ethers under normal conditions, nearly anaerobic conditions, and under an atmosphere of 95% O_2 and 5% CO_2. The results are shown in Table I. There is a tendency toward greater plasmalogen synthesis at high oxygen tension, although the other lipids still

TABLE I: Distribution of Radioactivity in Phospholipids under Differing Oxygen Tensions.[a]

Conditions	Total Phospholipid (cpm/μmole of phosphorus)	Glyceryl Ethers (cpm/μmole)	Glyceryl Vinylic Ethers (cpm/μmole)
4 hr in air	2,700	2,100	145
2 hr in N_2	287	90	6
4 hr in 95% O_2	1,530	750	240

[a] Immediately prior to beginning of experiment, animals (approximately 50-g fresh weight) were fed 6.2 μc of [1-^{14}C]palmitic acid in the first experiment and 4 μc in the second and third experiments.

TABLE II: Labeling Patterns of Phospholipid Components after Long-Term Substrate Metabolism.

Expt	Fed Substrate	Time for Metabolism (Days)	cpm/μmole		
			Fatty Acids[a]	Glyceryl Ethers	Glyceryl Vinylic Ethers
No. 1	[^{14}C]Palmitate	2	1,110	4,200	1,920
No. 2	[^{14}C]Glyceryl ethers	2	36	1,180	345
No. 3	[^{3}H]Glyceryl ethers	3	524	79,200	36,000
No. 4	[^{14}C]Glyceryl ethers	3	218	10,000	6,000[b]

[a] Isolated as fatty alcohols from the $LiAlH_4$ products. [b] Calculation based on analysis of isolated dimethyl acetals rather than glyceryl vinylic ethers.

contain the bulk of the administered radioactivity.

Incorporation Patterns after Long-Term Feeding Experiments. Since it appears that changing environmental conditions do not alter the basic pattern of incorporation, experiments were designed to look at the dynamics of plasmalogen biosynthesis after longer time intervals than the 16-hr maximum previously used. When lipids were isolated from animals 2 days after [^{14}C]palmitate administration, the plasmalogens were quite significantly labeled. The specific radioactivity of the glyceryl vinylic ethers was almost one-half as great as that of the glyceryl ethers, and greater than that of the fatty acids.

The delayed appearance of radioactivity in the plasmalogen side chain came many hours after the disappearance of all unesterified [^{14}C]palmitate fed (Thompson, 1965). It seemed likely then that one or more covalently bound derivatives of the fed fatty acid must serve as a slowly metabolized intermediate in plasmalogen formation. The two most promising potential precursors appeared to be an esterified fatty acid and the hydrocarbon side chain of the glyceryl ether.

Information bearing on this point was obtained by taking advantage of a previous observation concerning glyceryl ether metabolism. It has been shown (Thompson, 1965) that fed glyceryl ethers are incorporated intact into the phospholipids of *A. ater*. When radioactive glyceryl ethers are fed, some degradation to fatty acids occurs, but the glyceryl ether lipid specific radioactivity greatly exceeds that of the ester lipids. One might expect that in a long term experiment where the fed substrate is radioactive glyceryl ethers, the vinylic ether side chain should contain little radioactivity if an ester is the precursor and much if a glyceryl ether is the precursor. In Table II data from this type of experiment are compared with data from [^{14}C]palmitate-fed animals (expt 1). In expt 2–4 the specific radioactivity of the glyceryl vinylic ethers ranges from 10 to 70 times that of the fatty acids derived from phospholipids.

According to generally accepted concepts regarding this type of experiment, during some period of an isotope incorporation study a precursor must have a higher specific radioactivity than its product. Therefore, unless a small pool of highly radioactive esters exists in the tissues, an ester could not be the vinylic ether precursor. Detailed analysis of the individual phospholipids from glyceryl ether fed slugs by thin layer and column chromatography failed to detect any such pool of esters which could qualify as vinylic ether precursors. Likewise, the neutral glycerides contained fatty acids of much lower specific radioactivity than the plasmalogen-derived vinylic ethers. Glyceryl ethers and glyceryl vinylic ethers also are present in the neutral lipids, where they occur as the diacyl derivatives. Analysis of these components by thin layer chromatography showed that radioactivity was present in the glyceryl ethers but not in the more plentiful glyceryl vinylic ethers. The glyceryl ethers were not present in quantities sufficient for a determination of specific radioactivity. As a result of these experiments interest was focused on the glyceryl ether side chain as a probable source of the plasmalogen vinylic ether group.

Approximately 90% of the plasmalogens of *Arion* occur as the ethanolamine derivative. The similarities between side chains of the glyceryl ethers and glyceryl vinylic ethers isolated from this phospholipid fraction are remarkable (Thompson, 1965). A comparison was made of the specific radioactivities of glyceryl ethers and glyceryl vinylic ethers derived from the purified ethanolamine phospholipids 72 hr after feeding 12 μc of [^{3}H]chimyl alcohol. The glyceryl ethers contained 40,700 cpm/μmole and the glyceryl vinylic ethers 28,800 cpm/μmole. It may be significant that the glyceryl ethers isolated from choline lipids in the same experiment contained 71,700 cpm/μmole, an activity 76% higher than the analogous derivative from the ethanolamine fraction. This is in contrast to short-term labeling experiments where, after 1 and 15 hr, the glyceryl ethers of both phosphatide fractions from [1-^{14}C]palmitate-fed animals had equal activity. One might speculate that in the ethanolamine fraction, where plasmalogens are more plentiful than glyceryl ether phospholipids, a quite significant percentage of the radioactivity may be eventually channeled through glyceryl ethers into plasmalogens. The lack of an appreciable amount of plasmalogen in the choline lipids could lead to a relative retention of radioactivity in glyceryl ethers.

TABLE III: Distribution of Lipids and Lipid Radioactivity in Tissues of *Arion ater*.[a]

	Fresh Wt (g)	Phospholipid Phosphorus (μmoles)	Plasmalogen (mole % in PL)	Glyceryl Ethers (*ca.* mole % in PL)	Radioactivity in Total Lipid (cpm)	Radioactivity in Glyceryl Ethers
Intestinal contents	1.4	...	...	..	6,900,000	15,000 (100%)[b]
Crop and intestine	2.2	38	15	10	2,500,000	58,000 (36%)
Hepatopancreas	6.0	83	9[c]	30	1,500,000	138,000 (6%)
Other organs	5.7	93	8	40	155,000	8,000 (0%)
Mantle	23.9	165	13	15	52,000	...

[a] Animals were fed 6.2 μc of [1-^{14}C]palmitate 2 hr before dissection. The separated tissues were quick-frozen at −78°. [b] Parentheses show per cent of glyceryl ether radioactivity arising from diacyl glyceryl ethers. [c] Presence of colored material may interfere, giving slightly low result.

Of the lipid components described above, only the side chain of glyceryl ethers fills the requirements of a vinylic ether precursor. Before assuming that this is the actual sequence, however, it is essential to rule out the misleading participation of water-soluble compounds. For example, if enough of fed glyceryl ethers were degraded to some nonlipid product which could serve as a specific vinylic ether precursor, the labeling patterns we have observed could result.

The recovery of radioactivity in total lipids from slugs fed radioactive glyceryl ethers was variable and averaged only about 50% of that fed. Because the animals used for long term studies were kept in a rather large terrarium, the amount of unabsorbed glyceryl ethers excreted was generally not determined. However, after one 48-hr experiment in which the recovery in lipids was particularly low, the entire terrarium was extracted with organic solvents. This procedure recovered enough unchanged glyceryl ethers to account for 100% of the administered radioactivity. Another finding is indicative of very little extensive degradation and subsequent reincorporation. None of the lipid radioactivity is recovered in the water-soluble $LiAlH_4$ products, which include glycerol, choline, ethanolamine, etc., even after an experiment lasting 10 days. In the light of these findings, the formation of a water-soluble plasmalogen precursor is extremely unlikely.

In order to determine if the proposed conversion of the glyceryl ether to glyceryl vinylic ether might be reversible, the radioactive vinylic ethers obtained in expt 3, Table II, were fed back to slugs. Extraction of the lipids after 24 hr reclaimed only 30% of the fed radioactivity. Roughly 40% of the radioactivity recovered from phospholipids was in plasmalogen-bound glyceryl vinylic ethers, 50% was in fatty acids, and 2% was in glyceryl ethers. This distribution of radioactivity could reflect the breakdown of a portion of the administered vinylic ethers to fatty acids followed by an incorporation of a very small amount of these into glyceryl ethers.

In a second experiment, slugs were fed during the course of 3.5 days a total of 70 μmoles of nonradioactive glyceryl vinylic ethers isolated from beef heart lipids. Four hours before sacrificing the animals, 6 μc of [^{14}C]palmitate was also fed. An increase in plasmalogen to 13 mole % of the total phospholipid was detected, and gas chromatographic analysis indicated that the plasmalogens contained unsaturated vinylic ether elements resembling those of beef heart. No significant increase in phospholipid glyceryl ethers was found, and no trace of unsaturated glyceryl ethers was detected by gas chromatography. However, the incorporation of radioactivity into glyceryl ethers was depressed to approximately 10% of the expected value.

Ether Synthesis in Various Organs. In experiments such as described above, which involve the analysis of whole animals, the comparisons made would be invalid if the distribution and metabolism of lipids differ significantly from one part of the body to another. A number of experiments have been carried out to investigate this point. As a result it is possible to state with confidence that the differences are not sufficient to render results using whole organisms misleading.

To begin with, it should be pointed out that the compositions of phospholipids in the various parts of the slug are similar, although there are some differences in percentages of ether lipids. When animals were dissected into intestine plus crop, hepatopancreas, remaining organs (principally sexual organs), and mantle, the pattern of phospholipids as seen by thin layer chromatography was indistinguishable from one fraction to another, and all four fractions contained plasmalogens and glyceryl ether phosphatides. Triglycerides, however, were found in quantity only in the hepatopancreas and the intestinal tract. Two hours after a group of slugs was fed [1-^{14}C]palmitate, 67% of the radioactivity remained free in the intestine as palmitic acid. Of the lipid that had been absorbed and bound covalently, 58% was in the intestinal walls and 37% had been transported to the hepatopancreas. The move-

ment of radioactivity to the more outlying tissues was negligible. Table III summarizes the data from this experiment. The distribution of radioactivity among the different phosphatides was similar from organ to organ (mantle lipids were not analyzed), with the choline and ethanolamine lipids containing the highest activity. It would seem from this experiment that subsequent to the absorption of fatty acids there is no mechanism for prompt transport of the lipid beyond the digestive tract.

Special interest was aroused when radioactive glyceryl ethers were isolated from the intestinal contents. However, this was probably the result of dislodging some cells from the intestinal mucosa, since other studies of isolated intestinal contents showed no glyceryl ether synthesis by the microflora present therein.

The appearance of radioactivity in the blood can be measured by withdrawing samples from the cephalopedal blood sinus through a cannula.[2] After oral feeding of 1.45×10^6 cpm of [^{14}C]palmitate, the analysis of 6 μl. aliquots of blood collected over a period of 19 hr revealed that the peak of radioactivity (72 cpm/6μl.) is not reached until approximately 9 hr after a meal. After 19 hr the radioactivity had declined only slightly to 62 cpm/6 μl. By making a crude estimate of the total blood volume, it was reckoned that at no time during this period did the blood contain more than 0.5% of the administered radioactivity.

Although only a small portion of the ingested fatty acids appears to reach the musculature, lipid synthesis from other precursors proceeds fairly actively in this tissue. Twenty-four hours after injecting [6-^{14}C]glucose into the circulatory system, phospholipid from the mantle contained 11 cpm/μmole of phosphorus as compared to 23 cpm/μmole of phosphorus for the viscera.

The question of whether ether–lipid interrelationships are similar in the mantle and the viscera was approached by the following experiment: [^{3}H]Chimyl alcohol was fed to 10 slugs. The slugs were maintained outdoors in a terrarium for 10 days and were fed during this time a diet of lettuce smeared with nonradioactive selachyl alcohol (1-*O*-octadecenylglycerol) to act as a second possible label for the plasmalogens.[3] After dissecting the slugs into 2 fractions, viscera and mantle, the lipids of each fraction were analyzed. Two of the animals were discarded after being unintentionally killed prior to the end of the experiments. Even so, the recovery of tritium from the remaining eight was 15×10^6 cpm, approximately 56% of that fed.[4] A summary of the lipid analysis is shown in Table IV. The incorporation of radioactivity into all types of lipid is more pronounced in the viscera, indicating a selective retention of the ingested substrate there. The general pattern of labeling in the two tissue pools is fairly similar and would lead one to believe that the metabolic interrelationship between the types of ethers does not differ greatly. Perhaps the most interesting result from this experiment is the finding that the uncharacteristic glyceryl ether, 1-*O*-octadecenyl glycerol, when incorporated into the phospholipids, gives rise to plasmalogens containing, again uncharacteristically, the analogous side chain. The level of the corresponding fatty acid ester (the oleoyl group) shows no increase above normal.

TABLE IV: Incorporation Pattern in *Arion ater* Phospholipids ten Days after Feeding [^{3}H]-1-*O*-Hexadecylglycerol Plus Unlabeled 1-*O*-Octadecenylglycerol.[a]

Fraction	Viscera	Mantle
Total cpm	8,360,000	2,680,000
cpm/μmole of phospholipid	40,300	10,400
cpm/μmole of fatty acid	2,900	716
cpm/μmole of glyceryl ether	54,300	26,000
% octadecenyl chain in glyceryl ether	25	7
cpm/μmole of glyceryl vinylic ether	35,200	8,800
% octadecenyl chain in glyceryl vinylic ether	12	8

[a] Dissected tissues were frozen until total pools were ready for extraction. Wet weights: viscera, 14.0 g; mantle, 29.8 g.

Discussion

Schemes for the biosynthesis of plasmalogens have been suggested from time to time during the past few years (for review, see Hartree, 1964). However, experimental evidence to support these schemes has never materialized. A few authors, impressed by the structural similarities between glyceryl ether phospholipids and plasmalogens, have speculated that some form of precursor–product relationship exists. A consideration of the few pertinent chemical and biochemical analogies has led to the suggestion that the glyceryl ether lipids may arise by the enzymatic addition of hydrogen to the α,β-double bond of plasmalogens (Gilbertson and Karnovsky, 1963). Earlier studies with *A. ater* (Thompson, 1965) indicated that this is not the case. Now further data from investigations on the same organism lead us to propose that the reverse situation holds true; *i.e.*, glyceryl ether phospholipids are precursors of plasmalogens. This idea has also been suggested previously (Korey and Orchen, 1959) but with no experimental basis. The present evidence, if not foolproof, is convincing. Numerous feeding experiments show that during the period (up to 16 hr) in which labeled fatty acids are present as such in the tissues, only traces of radioactivity become a part of the plasmalogen carbon skeleton. After feeding either fatty acids or glyceryl

[2] The procedure of cannula implantation was kindly demonstrated by Dr. Arthur Martin, Department of Zoology.

[3] Selachyl alcohol is not a normal constituent of slug lipids, but massive feedings result in considerable incorporation (Thompson, 1965).

[4] Intestinal absorption of fed glyceryl ethers varies depending upon the amount fed, but the uptake is seldom complete.

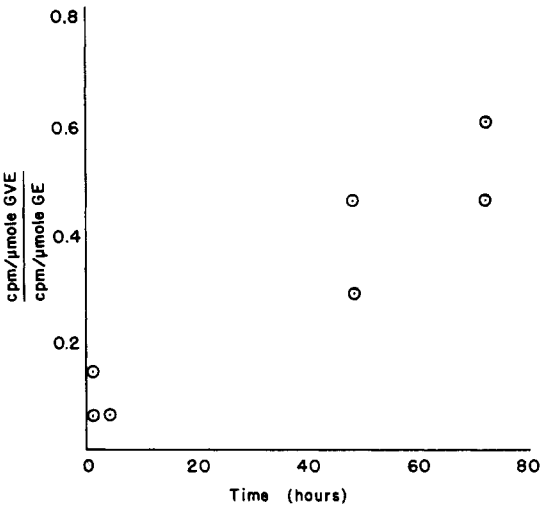

FIGURE 1: Comparison of specific radioactivities of glyceryl vinylic ethers (GVE) and glyceryl ethers (GE) isolated from total phospholipids after various time intervals. See text for description.

ethers, many hours must elapse before significant incorporation of their carbon atoms into plasmalogens occurs. With either substrate essentially all radioactivity can be accounted for in the form of glyceryl ethers, esterified fatty acids, and glyceryl vinylic ethers from the plasmalogens, thus ruling out the presence in substantial amounts of some unidentified precursor. The glyceryl vinylic ethers eventually approach the glyceryl ethers in specific radioactivity. When radioactive glyceryl ethers have been fed, neither the total fatty acids nor the fatty acids of individual lipid species ever achieve a high enough specific radioactivity to be considered as the plasmalogen precursor. Only the glyceryl ether molecule fulfills this requirement. A comparison can be made (Figure 1) of the specific radioactivity ratios of glyceryl vinylic ethers and glyceryl ethers from the experiments in Table II and the purified products of short term experiments (Thompson, 1965) performed under identical conditions. Semiquantitative data described in the earlier publication indicated that the ratios after 8 and 16 hr are not appreciably different from those obtained at 1 and 4 hr. The ratios at all time intervals agree with the proposed slow transformation of glyceryl ether phospholipids into plasmalogens.

Final evidence supporting the precursor role of glyceryl ethers arises from the finding that introduction of the octadecenyl chain into the glyceryl ether pool leads to an appearance of the analogous side chain in glyceryl vinylic ethers.

On the basis of the data collected thus far, a tentative pathway for ether biosynthesis can be formulated (Figure 2). The first step is purely speculative. The initial ether compound is a diacyl glyceryl ether (IV) in accordance with the observation (Thompson, 1965) that this compound is formed extremely rapidly in tissues of *Arion*. It was further observed that radio-

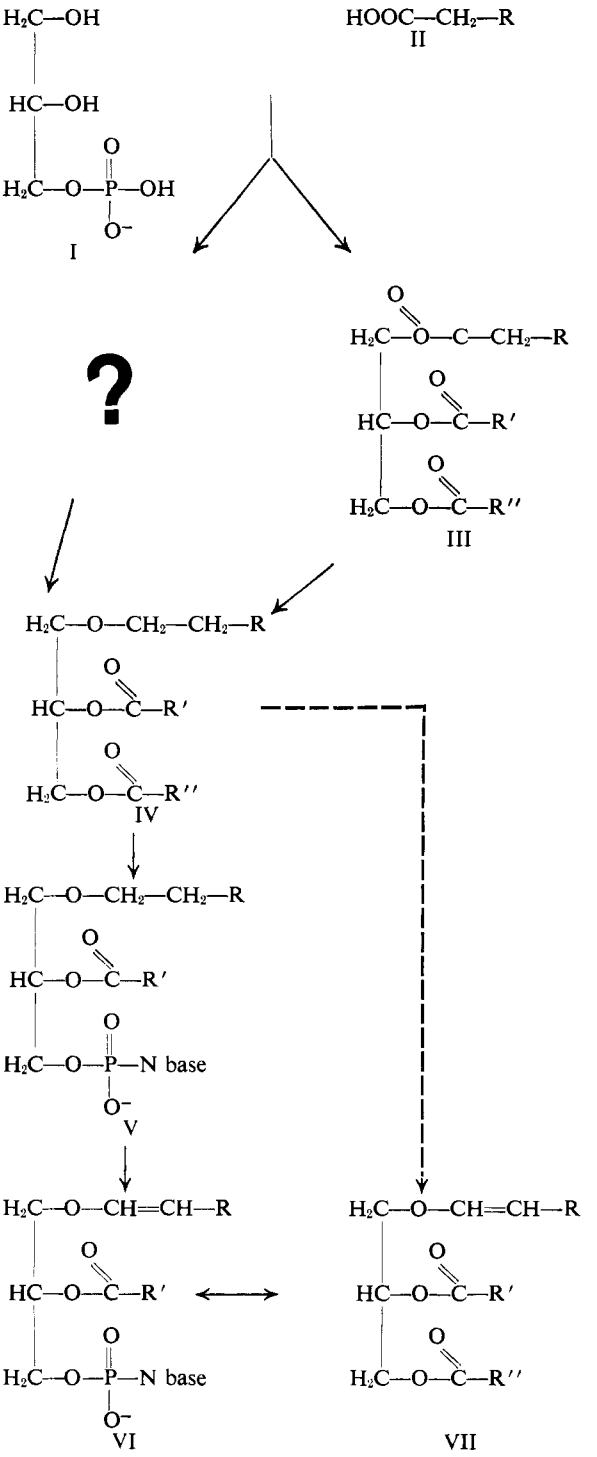

FIGURE 2: Proposed pathway of ether-lipid biosynthesis.

activity in the diacyl derivative quickly declines as that in the glyceryl ether phospholipids (V) increases. Over a much longer period, the glyceryl ether phospholipids have now been demonstrated to yield the vinylic side chain of plasmalogens (VI). The failure to find appreciable radioactivity in neutral plasmalogens (VII),

even after prolonged metabolism of labeled substrates, has led us to propose that this compound is a plasmalogen degradation product. Alternatively, VII could arise by the slow dehydrogenation of IV. Results from the glyceryl vinylic ether feeding experiments indicate that the step from VI to VII is reversible.

The evidence which has been accumulated deals solely with the ester- and ether-linked side chains. The question of whether the entire glyceryl vinylic ether moiety of plasmalogens is formed from a glyceryl ether without cleavage of the ether bond could perhaps be answered by radioactive isotope feeding experiments using glyceryl-labeled glyceryl ethers.

One must conclude that the rate of plasmalogen biosynthesis in *A. ater* is very much lower than that of the other major lipid components. The precursors employed here must first be incorporated into a large pool of phospholipid, presumably serving a structural function. The dilution of radioactivity in this pool undoubtedly contributes to the slow incorporation into plasmalogens, causing the rate of synthesis to appear even lower than it actually is. Due to the lengthy time intervals required for the uptake of radioactivity by plasmalogens, *in vitro* experiments may have limited usefulness in studying *Arion* and higher organisms. *In vivo* studies seem to provide the best initial approach, particularly if degradative activity is low, as it is in the case of *Arion*.

Because the techniques for the isolation and purification of relatively small amounts of glyceryl ethers have only recently been developed, few tissues have been analyzed for these compounds. In view of the evidence reported here, it is important that their role as a probable plasmalogen precursor be tested in other biosynthetically active systems.

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