Loss of Hydrogen from Dihydroxyacetone Phosphate during Glycerol Ether Synthesis*

Samuel J. Friedberg,* Aaron Heifetz, and Ronald C. Greene

From the Department of Physiology and Medicine, University of Texas Medical School at San Antonio, San Antonio, Texas 78229, and the Department of Biochemistry, Duke University Medical Center, and the Durham Veterans Administration Hospital, Durham, North Carolina 27705

SUMMARY

In order to obtain information on the mechanism of ether bond formation in the synthesis of O-alkyl lipids, a study was carried out to determine if there is a loss of hydrogen from carbon 3 of dihydroxyacetone phosphate (DHAP) (non-phosphorus-linked carbon) in the course of its incorporation into glyceryl ethers. Accordingly, a mixture of [1,3-\(^3\)H\(_2\)]DHAP and [1,3-\(^14\)C]DHAP was prepared. In a microsomal enzyme system from *Tetrahymena pyriformis* which synthesizes O-alkyl lipids, it was found that there was a loss of hydrogen from carbon 3 of glyceryl ethers synthesized from double-labeled DHAP. The loss was quantitatively consistent with the labeling of one of the hydrogens linked to carbon 3 of DHAP and was not due to isomerase activity.

Previous work has shown that the alkyl side chain of glyceryl ethers is derived from long chain alcohols (1-3). Subsequently, we found that glycerol used in glyceryl ether synthesis loses hydrogen from the carbon which forms the ether bond (4). This loss of hydrogen did not occur when glycerol was used in glycereide synthesis. It was concluded that either S-phosphoglyceraldehyde or dihydroxyacetone phosphate was the source of glycerol in glyceryl ether synthesis. This view was supported by work in cell-free systems in other laboratories (5-8). Since the rapid equilibrium reaction between these triose phosphates would produce rapid loss of some of the hydrogen from carbon 3, the obligatory intermediate for the glycerol source was uncertain.

Subsequently, studies by Hajra (9) pointed to dihydroxyacetone phosphate as an obligatory intermediate. There is now good evidence that glyceryl ethers are synthesized from dihydroxyacetone phosphate and long chain alcohols and that there is a requirement for ATP, coenzyme A, magnesium, and NADPH (10-12). It has also been shown that the oxygen of \(^18\)O-labeled hexadecanol is incorporated into O-alkyl lipids (13). In addition, evidence has been presented which indicates that cetyl alcohol and acyl dihydroxyacetone phosphate may form glyceryl ethers in the absence of coenzyme A (14).

Because it has been suggested that glyceryl ether synthesis might occur from an enolized form of dihydroxyacetone phosphate or from a hemiacetal, it would be important to know if the hydrogens of carbon 3 of DHAP are labilized in the process of O-alkyl ether bond formation. The present study indicates that there is in fact a loss of hydrogen and that this loss is not the result of triose phosphate isomerase activity.

MATERIALS AND METHODS

Materials—Dihydroxyacetone phosphate, 3-phosphoglyceraldehyde, NADPH, ATP, NADH, NAD; triose phosphate isomerase (10 mg per ml), \(\alpha\)-glycerophosphate dehydrogenase (10 mg per ml), glyceraldehyde 3-phosphate dehydrogenase (2 mg per ml), and triethanolamine dihydrogen phosphate; CAP, 1-hydroxy-3-chloro-2-propanone phosphate (chloroacetol phosphate).

Preparation of Mixture of [1,3-\(^3\)H\(_2\)]Dihydroxyacetone Phosphate and [1,3-\(^14\)C]Dihydroxyacetone Phosphate—The method is a modification of the procedure described by Hajra (16) for the synthesis of \(^32\)P-dihydroxyacetone phosphate.

[1,3-\(^3\)H\(_2\)]Glycerol (2.1 mCi) and 0.22 mCi of [1,3-\(^14\)C]glycerol (total, 14.4 \(\mu\)moles) were dried in a stream of nitrogen at room temperature.

The abbreviations used are: DHAP, dihydroxyacetone phosphate; CAP, 1-hydroxy-3-chloro-2-propanone phosphate (chloroacetol phosphate).
Fig. 1. Inhibition of triose phosphate isomerase by 1-hydroxy-3-chloro-2-propanone phosphate. One milliliter of Tetrahymena microsomes or commercial triose phosphate isomerase was incubated for 20 min at 30°C with and without 10 μmoles of CAP. The reaction mixture in a final volume of 3 ml contained 0.24 mM triethanolamine buffer, pH 7.6 (0.05 mg of α-glycerophosphate dehydrogenase, 0.167 mM NADH, and 1 mM 3-phosphoglyceric aldehyde). The reaction was initiated by the addition of 0.05 ml of microsomes or commercial triose phosphate isomerase. Decrease in optical density was read at 340 nm. After 15 min DHAP (2 mM) was added to the cuvette containing the CAP-treated commercial triose phosphate isomerase.

The mixture was then treated with two 0.5-g portions of charcoal. The charcoal was washed with a small amount of water. The incubation mixture and water were combined and filtered. The hydrazine was removed by extracting the incubation mixture three times with benzaldehyde and then three times with ethyl ether. The extracts were combined and re-extracted once with a small amount of water. The water was added to the incubation mixture and extracted once more with ethyl ether. One drop of 5% human serum albumin was added, and the proteins were precipitated with 0.5 ml of 50% trichloroacetic acid and removed by centrifugation. The mixture was then treated with two 0.5-g portions of charcoal. The charcoal was washed with a small amount of water. The incubation mixture and water were combined and filtered. The hydrazine was removed by extracting the incubation mixture three times with benzaldehyde and then three times with ethyl ether. The extracts were combined and re-extracted once with a small amount of water. The water was added to the incubation mixture and extracted once more with ethyl ether. One drop of 5% human serum albumin was added, and the proteins were precipitated with 0.5 ml of 50% trichloroacetic acid and removed by centrifugation. The mixture was then adjusted to pH 6 with the use of phenol red as an indicator, and 0.25 ml of 1 M CaCl₂ was added. A small amount of precipitate which formed immediately was removed by centrifugation. Ten volumes of acetone-ethanol (1:1) were added, and the mixture was kept at -20°C overnight. The precipitate was removed by centrifugation, dried with nitrogen, and mixed with 1 ml of a slurry of Dowex AG 50W-X8 (100–200 mesh, hydrogen form). The mixture was treated with two 0.5-g portions of charcoal. The charcoal was washed with a small amount of water. The yield was approximately 40%. The composition of the mixture was examined by thin layer chromatography on cellulose layers 0.1 mm thick, developed in alcohol, water, 30 ml; and p-toluene sulfonic acid, 2 g. In this system, there was good separation of α-glycerophosphate and DHAP from 3-phosphoglyceric aldehyde. High voltage electrophoresis at pH 1.5 with either 0.1 M oxalate buffer (18) or 8% aqueous formic acid gave good separation of α-glycerophosphate and DHAP, but 3-phosphoglyceric aldehyde migrated at the same rate as α-glycerophosphate. With the use of these procedures, it was found that the double labeled DHAP contained about 25% α glycerophosphate. Further isolation and purification of labeled DHAP were accomplished by high voltage electrophoresis in 8% formic acid. As shown in Fig. 2 (top), the purified material gave a single peak on thin layer chromatography. A single peak was also obtained on high voltage electrophoresis at pH 1.5 in 0.1 M oxalate buffer and at pH 6 in 0.1 M sodium bisulfite.

It was also observed that the 3H:14C ratio of the purified DHAP was 7.6, whereas the starting glycerol had a ratio of 9.6. This suggested that there was a loss of tritium from DHAP during its synthesis. The problem was investigated by incubating a mixture of [1,3-3H₂, 1,3-14C]glycerol and [1,3-14C]glycerol in the glycine hydrazine buffer used to synthesize DHAP. A tritium loss was indicated by a decrease in 3H:14C ratio in an aliquot of the dried mixture after incubation. The tritium which was lost was recovered in water distilled from the incubation mixture. A smaller loss of tritium was noted in the formation of α-glycerophosphate in triethanolamine in the absence of hydrazine hydride.

The tritium distribution and 3H:14C ratios of the substrate double-labeled DHAP were measured by periodate cleavage, precipitation of carbon atom 3 with dimedon, and determination of the activity in formaldehydedomedon and residual water. The results are shown in Table I. There appeared to be somewhat less tritium on carbon 1 than on carbon 3 in several determinations.

[1,3-3H₂, 1,3-14C]DHAP was treated with α-glycerophosphate...
ducibility, the amount of aqueous material to be counted was
maintained at 0.5% by volume of the total counting solution.

Results were obtained from two different samples of DHAP.

|          | H     | C    | H3C  |
|----------|-------|------|------|
| [1,3-3H2, 1,3-14C]DHAP | 1.39 x 10^4 | 1.81 x 10^4 | 7.6  |
| aliquot  | 1.38 x 10^4 | 1.88 x 10^4 | 7.3  |
| Formaldehyde | 8.95 x 10^4 | 8.80 x 10^4 | 10.2 |
| (carbon 3)   | 7.24 x 10^4 | 8.07 x 10^4 | 9.0  |
| Water remaining (carbon 1) | 6.00 x 10^4 | 8.33 x 10^4 | 7.2  |
|          | 6.67 x 10^4 | 9.74 x 10^4 | 6.9  |

**Fig. 3.** High voltage electrophoresis of labeled DHAP and derivatives. A, [3H, 14C]DHAP and [3H, 14C]-glycerophosphate were separated by high voltage electrophoresis in 0.1 M oxalate buffer, pH 1.5. The radioactive peaks were located with a strip scanner and with appropriate standards. The radioactive areas were then cut into 4-mm strips and counted in 2:1 phosphor-Triton. B, [3H, 14C]DHAP and [3H, 14C]-glycerophosphate were treated with a-glycerophosphate dehydrogenase and NADH in triethanolamine buffer 0.3 M, pH 7.6. The reaction was stopped with tri-chloroacetic acid and the products were separated as in A. C, the procedure which was used in B was repeated. After incubation with a-glycerophosphate dehydrogenase and precipitation of enzyme with trichloroacetic acid, an aliquot was separated by electrophoresis with added [3H, 14C]DHAP.

dehydrogenase and NADH to convert the DHAP to L-a-glycerophosphate. The products of the incubation were separated by high voltage electrophoresis in 0.1 M oxalate buffer. The disappearance of the DHAP peak and the appearance of an a-glycerophosphate peak established with certainty the identity of the former (Fig. 3).

Liquid scintillation counting of aqueous solutions was done by the method of Patterson and Greene (19) with 2 parts of toluene phosphor and 1 part of Triton X-100. In order to insure reproducibility, the amount of aqueous material to be counted was maintained at 0.5% by volume of the total counting solution. Tritiated and 14C-labeled toluene were used as internal standards to determine absolute activities when necessary. Lipids were counted in toluene 2,5-diphenyloxazole-1,4-bis[2-(5-phenyloxazoly)]benzene.

**Preparation of Microsomes—**Tetrahymena pyriformis, strain HSM, was grown in 6 liters of medium with aeration at room temperature. The medium contained 1% Difco proteose peptone, 0.05% Difco liver extract, 0.1% glucose, and 0.75 ml per liter of Dow Corning Antifoam B in 0.02 M phosphate buffer, pH 6.8.

After 4 days of growth, the cells were harvested by centrifugation at 1,000 to 1,500 rpm for 2 min at 10° in a 3-liter rotor. The cells were washed once with distilled water and concentrated again. The volume of the cells was measured after centrifugation in graduated centrifuge tubes and 5 volumes of ice-cold buffer, containing 0.10 M phosphate buffer, pH 7.2; 0.04 M sodium fluoride, 0.25 M sucrose, and 0.005 M mercaptoethanol were added. The cells were then disrupted by sonicating at 2° in an ice bath by three bursts of 15-sec duration each, applied to about 50 ml of solution at a time. Between bursts the mixture was allowed to cool and the extent of cell disruption was examined microscopically. The material was then centrifuged at 8,000 rpm at 2° for 20 min. The resulting supernatant was centrifuged at 100,000 X g for 1 hour to sediment microsomes. The microsomes were washed three times in the same buffer, made up to a final volume of one-half of the volume of the original sonicated mixture, and stored at -70°. At the end of 6 weeks of storage there was little loss of activity.

**Incubations—** For investigating the synthesis of O-alkyl glycerolipids from [1,3-3H2, 1,3-14C]DHAP or from [1-14C]hexadecanol, 1 ml of microsomes (2 mg of protein) was first incubated with 0.2 ml of 0.05 M CAP for 20 min with shaking at 30°. Isomerase inhibition was established as indicated in Fig. 1. ATP, 15 μmoles; CoA, 1 μmole; Mg++, 4 μmoles, and appropriate substrates or other cofactors were then added. Incubation was stopped by extraction by the method of Bligh and Dyer (20) or Fohch, Lees, and Sloane Stanley (21).

**Chromatographic Procedures—**Glycerol ethers were isolated by thin layer chromatography on Silica Gel G (Brinkmann Instruments, Inc., Westbury, New York) in ligroine-ethyl ether-acetic acid (20:80:1, v/v) or chloroform-methanol-water (90:5:0.5, v/v). Phospholipids were developed in chloroform-methanol-acetic acid-water (50:25:7.5). Triose phosphates were separated on cellulose layers (Brinkmann) 0.1 mm thick, developed in a mixture of tert-butyl alcohol, 60 ml; water, 30 ml; and p-toluene sulfonic acid, 2 g. Silicic acid column chromatography was done on a column (1 cm in diameter) containing 1 g of silicic acid. Neutral lipids were eluted with ethyl ether and phospholipids with methanol.

**Chemical Treatments and Other Methods—**Lipids were treated with LiA1H4 at room temperature as described by Thompson (22), and glycerol ethers produced were converted to their iso propylylamine derivatives by the method of Hanahan, Ekholm, and Jackson (23). Glycerol ethers were cleaved at the ether bond with hydrogen iodide, and the alkyl iodides produced were separated by thin layer chromatography as previously described (1). Reduction with sodium borohydride was carried out as described by Hjara and Agronoff (24). Less than 1 μmole of lipid was dissolved in 0.1 ml of ethanol and 0.02 ml of 0.1 M NaBH4 was added. After 15 min at room temperature an additional 0.01 ml of 0.1 M NaBH4 was added. After an additional 15 min 4 ml of chloroform-methanol-concentrated HCl (20:10:0.1,
v/v) were added followed by 1 ml of water. The upper phase was removed and the lower phase was washed with simulated upper phase. The lower phase was then dried and stored in benzene. Phosphorus was determined by the method of Bartlett (25) and proteins by the method of Lowry et al. (26).

Periodate cleavage of glyceryl ethers between vicinal hydroxyl groups (27) or of DHAP between the carbon atoms 1 and 2 was performed as follows. Glyceryl ethers in 1 ml of ethanol or DHAP in 1 ml of water were mixed with a 2-fold molar excess of 0.05 N sodium metaperiodate in 25% acetic acid and left in the dark for 30 min with occasional shaking. At times, 18 μmole (2 μl) of carrier formaldehyde were added before the periodate to minimize oxidation of labeled formaldehyde. The reaction was stopped by addition of a 10-fold excess of 0.5 N sodium arsenite and the long chain aldehydes were extracted with ethyl ether. The water phase was then brought to pH 6 with 0.1 N NaOH and 15 ml of saturated aqueous dioxane were added (approximately 0.4%). After 8 hours at 2–4°C, the formaldehydodimethane precipitate was extracted with petroleum ether and an aliquot was dried and counted in 2,5-diphenyloxazole-1,4-bis[2-(5-phenyloxazolo-lysyl)benzene toluene.

RESULTS

Incorporation of [1,3-3H2, 1,3-14C2]Dihydroxyacetone Phosphate into O-Alkyl Lipids—The criterion for the formation of O-alkyl lipids in this study was the chromatographic isolation of labeled glyceryl ethers and their conversion to isopropylidene derivatives. Thus, lipids extracted after incubation were treated with LiAlH4 and separated by thin layer chromatography. Two peaks are apparent (Fig. 4): one coincides with glyceryl ether, and another is an unknown material remaining at the origin. The identity of the glyceryl ethers was established by conversion to their isopropylidene derivatives followed by thin layer chromatography (Fig. 4).

It was also considered of importance to examine the products of [1,3-3H2, 1,3-14C2]DHAP incubation as compared with the products obtained after incubation of [14C]hexadecanol prior to further treatment with other agents. Accordingly, phospholipids and neutral lipids were first separated by silicic acid column chromatography. Material obtained after the incubation of labeled DHAP and labeled hexadecanol was then compared by thin layer chromatography. In the neutral lipid fraction, a peak corresponding to the O-alkyl dihydroxyacetone described by Snyder, Wykle, and Malone (5) was found. This material gave a peak with the mobility of glyceryl ether after treatment with sodium borohydride and rechromatography in the same system. In the phospholipid fraction, a number of peaks were found which we have not identified. It was of interest to note, however, that the several radioactive peaks derived from labeled DHAP and labeled hexadecanol corresponded, although the relative amounts differed.

In order to obtain further evidence that the isotope incorporated into glyceryl ethers came from DHAP and not from an unidentified radioactive contaminant, the effect of added unlabeled DHAP was evaluated. The addition of increasing concentrations of DHAP from 0.0133 to 1.2 mM yielded increasing amounts of glyceryl ethers. A plot of the data according to the method of Lineweaver and Burk suggested typical first order enzyme kinetics. The results are shown in Fig. 5. The addition of increasing amounts of unlabeled DHAP caused a progressive decrease in the amount of radioactivity incorporated. This is characteristic of dilution of the labeled precursor and substantiates our previous characterization of the substance as DHAP.

Analysis of Tritium Activity and Its Distribution on O-Alkyl Lipids—In accordance with the principal objective of this study, a series of experiments was carried out to determine whether or
not any of the hydrogens of the ether-linked carbon of DHAP are labilized in the process of O-alkyl lipid formation.

In a typical experiment, 5 ml of microsomes were first incubated with 1 ml of 0.05 M CAP for 20 min at 30°C. Then ATP, 90 μmoles; CoA, 6 μmoles; MgCl₂, 24 μmoles; [1,3-3H₂, 1,3-³²C]DHAP, 0.48 μ mole; [³H (141,500,000 dpm) and ¹⁴C (21,050,000 dpm) (³H:¹⁴C ratio, 7.6); and hexadecanol, 0.6 μ mole in 1% Tween 80 were added. Incubation was carried out for 3 hours at 30°C. The lipids were extracted by the method of Bligh and Dyer (20) and stored in benzene. The aqueous phase containing residual DHAP and other materials was filtered, dried on a rotary evaporator, and dissolved in 5 ml of water. An aliquot of the lipid sample was treated with LiAlH₄ and purified by thin layer chromatography after addition of 5 mg of carrier tetradecyl glyceryl ether. Rechromatography of this material gave a single peak of radioactivity. The ³H:¹⁴C ratio (dpm) of this material was 7.1, which is not significantly different from that of the starting DHAP. The results of the degradation experiments described below are therefore unexpected.

The purified, double-labeled glycerol ethers, 0.5 mg, ³H (54,300 dpm) and ¹⁴C (620 dpm), were cleaved with periodate. The formaldehyde released was precipitated with dimedon, and the long chain aldehydes extracted as described under "Materials and Methods." The long chain aldehyde activity (carbon 3) was 12,300 dpm ³H and 3,670 dpm ¹⁴C (ratio, 3.3). The formaldehydodimethoxon activity (carbon 1) was 48,000 dpm ³H and 3,670 dpm ¹⁴C (ratio, 11.8).

The procedure was repeated several times with the same and different batches of labeled DHAP and different microsomes. The results were similar in each instance. That is, there was a loss of tritium from the ether-linked carbon. There was also a substantial increase in ³H:¹⁴C ratio of carbon 1. The results of five periodate cleavages are summarized in Table II. After treatment of glyceryl ethers with hydrogen iodide, it was found that the alkyl residue contained no activity. An examination of the DHAP remaining after incubation was carried out to determine if an explanation for the increase in ³H: ¹⁴C ratio of carbon 1 in glycerolipids would become apparent. Thin layer chromatography on cellulose with tert-butyl alcohol-water-ρ-toluene sulfonic acid (90:30:2, v/v/w) revealed the appearance of several unidentified new tritium poor peaks and an increase in relative tritium activity of the remaining DHAP (Fig. 2). In addition, no activity in 3-phosphoglycerate and 3-phosphoglyceraldehyde was detected, indicating complete inhibition of triose phosphate isomerase by CAP. The peak corresponding to DHAP was eluted. This material showed a relative tritium enrichment as compared with the starting DHAP (Table II).

A fraction of the water phase remaining after incubation was also treated with periodate, and the formaldehyde containing the carbon 3 of DHAP was precipitated with dimedon. The ³H:¹⁴C ratio of formylglycerol was 10.3, indicating that there was no loss of tritium from the ether-linked carbon of residual DHAP during the course of the incubation.

The possibility that either LiAlH₄ or NaBH₄ reduction of O-alkyl lipids might have resulted in tritium loss was considered. This was ruled out by the fact that there was no change in ³H:¹⁴C ratio when the dihydroxyacetone ether formed in the course of incubation was treated with these agents and converted to glyceryl ether.

**Table II**

| Periodate cleavages | ³H | ¹⁴C | ³H:¹⁴C ratio |
|---------------------|----|-----|-------------|
|                     | dpm | dpm |            |
| [1,3-³²H₂, 1,3-¹⁴C]DHAP substrate | 6.44 | 8.32 | 7.60 |
| Glyceryl ethers, aliquot for periodate cleavage | 4.52 | 6.25 | 7.54 ± 0.45 (S.D.) |
| Long chain ether aldehydes from periodate cleavage of glyceryl ethers | 5.52 | 7.25 | 3.50 ± 0.21 (S.D.) |
| Formaldehydodimethoxon from periodate cleavage of glyceryl ethers | 4.58 | 3.95 | 11.80 ± 0.42 (S.D.) |
| [1,3-³²H₂, 1,3-¹⁴C]DHAP re-isolated after incubation | 9.70 | 1.26 | 8.32 |

**DISCUSSION**

We have previously indicated that we could visualize the possibility of a mechanism whereby glyceryl ether synthesis proceeds via the formation of an alkyl glycoside (4). This implies the synthesis of an enediol. However, several other mechanisms can be envisioned, and the data are not sufficient to draw firm conclusions at this time. Nevertheless, any explanation of the mechanism of O-alkyl bond formation may have to take three facts into consideration. These are the retention of the oxygen of hexadecanol, the preferential utilization of acyl dihydroxyacetone phosphate, and the loss of hydrogen from the ether-linked carbon of DHAP.

The results indicate that somewhat more than half of the tritium of carbon 3 of DHAP is lost in the process of O-alkyl lipid synthesis. If 3-phosphoglycerate and 3-phosphoglyceraldehyde were an intermediate and had appeared as a result of isomerase activity, a tritium loss would also have occurred. However, CAP completely inhibited the isomerase present in the microsomal system, and labeled 3-phosphoglycerate did not appear in the course of incubation as determined by thin layer chromatography.

Although the loss of tritium from carbon 3 of the glycerol moiety of glyceryl ethers can be mechanistically explained, the reason for enrichment of tritium in carbon 1 of the glyceryl ethers and in the residual DHAP is not so clear cut. An artifact which
would tend to give falsely high $\text{H}^{3}:^{14}\text{C}$ ratios of the formaldehydodimedon isolated after periodate cleavage has been described by Von Simon and Heubach (28). These workers reported that in the course of periodate oxidation a part of the formaldehyde produced is oxidized by excess periodate. Due to the tritium isotope effect (29), nontritiated formaldehyde is oxidized more rapidly than tritiated formaldehyde, resulting in a relative tritium enrichment of the residual formaldehyde. To minimize loss of $[^1\text{H}]$formaldehyde with a resultant increase in the $3\text{H}:^{14}\text{C}$ ratio, periodate oxidations were carried out in the presence of excess unlabeled formaldehyde. Although this effect could cause overestimates of the $3\text{H}:^{14}\text{C}$ ratio of formaldehyde, we believe they are small since we generally obtained good recoveries of $^{14}\text{C}$ in formaldehydodimedon. Another explanation for tritium enrichment is selective metabolism of nontritiated DHAP due to a tritium isotope effect in reactions occurring in microsomal preparations which utilize DHAP. In the present instance, there was a tritium enrichment of the residual DHAP remaining after incubation. It is possible, therefore, that this enrichment would result in enrichment of carbon 1 of the glycerol moiety of glyceryl ethers. We have previously encountered tritium enrichment of $[^1\text{H},^3\text{H}]$hexadecanol of equal magnitude in the course of glyceryl ether synthesis in elasmobranch stomach (1) and similar effects have been reported by Wood et al. (30) and by Schroepfer and Bloch (31).

Regardless of the reasons for tritium enrichment in the various compounds, it is clear that carbon 3 of the glycerol moiety of glyceryl ethers is deficient in tritium, whereas the corresponding carbon of DHAP is not. This finding indicates that cleavage of a carbon-hydrogen bond at carbon 3 is a step in the conversion of DHAP to glyceryl ether.

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