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# The Formation of Tritiated *O*-Alkyl Lipid from Acyldihydroxyacetone Phosphate in the Presence of Tritiated Water†

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ABSTRACT: Previous studies from this laboratory on the mechanism of *O*-alkyl bond formation using a microsomal system from *Tetrahymena pyriformis* have shown that *O*-alkyl lipid synthesized from dihydroxyacetone phosphate has exchanged one hydrogen stereospecifically from the 1-sn position of the glycerol moiety. Indirect evidence suggested that acyldihydroxyacetone phosphate, an intermediate in *O*-alkyl lipid synthesis, is probably not the locus of the exchange. In the present study it was shown that stable acyldihydroxyacetone phosphate incubated in the presence of tritiated water and *Tetrahymena* microsomes does not become tritiated. When hexadecanol is added to the system *O*-alkyl lipid is produced which has incorporated one atom of hydrogen for each mole of hexadecanol at all time periods examined. Experiments in Ehrlich ascites tumor cells have shown that the hydrogen exchange also occurs in a mammalian system. The results indicate that the mechanism of *O*-alkyl lipid ether bond formation involves a hydrogen exchange and that this exchange occurs after the formation of acyldihydroxyacetone phosphate.

The reaction sequence leading to the formation of alkyl glycerolipids involves the formation of 1-acyldihydroxyacetone phosphate and replacement of the fatty acid by a long chain fatty alcohol with the formation of *O*-alkyldihydroxyacetone phosphate (Hajra, 1970; Wykle *et al.,* 1972). The keto group is reduced with NADPH followed by acylation. For further details on the biochemistry of ether lipids, the reader is referred to a recent extensive review (Snyder, 1972).

We have previously shown that when dihydroxyacetone phosphate (DHAP[1]) is used in the enzymatic formation of *O*-alkyl lipids, there is a hydrogen exchange from the carbon that acquires the *O*-alkyl moiety (Friedberg *et al.,* 1971; Friedberg and Heifetz, 1973). This exchange is specific for the same hydrogen labilized in the triosephosphate isomerase reaction (Friedberg *et al.,* 1972). The exchange was demonstrated through the loss of one tritium from [1,3-$^3H_2$]DHAP and by the acquisition of one tritium atom by *O*-alkyl lipids formed from DHAP in the presence of tritiated water. In the microsomal system from *Tetrahymena pyriformis,* a concomitant reaction is the coenzyme A dependent formation of dihydroxyacetone from dihydroxyacetone phosphate (Friedberg and Heifetz, 1973). The dihydroxyacetone formed also appears to have undergone a hydrogen exchange, and the coenzyme A requirement was interpreted to indicate that acyldihydroxyacetone phosphate (acyl-DHAP) is a precursor of dihydroxyacetone. In our earlier studies using the *Tetrahymena* system we were not able to isolate labeled acyl-DHAP from incubations which utilized [1,3-$^3H_2$]DHAP. We were, however, able to isolate acyldihydroxyacetone (acyl-DHA) which had not lost tritium. Thus the results suggested that the tritium exchange occurs after the formation of acyl-DHAP. However, we and others (Schroepfer and Bloch, 1965; Friedberg and Greene, 1967, Wood *et al.,* 1970) have encountered unexplained relative tritium enrichment in reactions which utilized mixtures of a substrate labeled with both $^{14}C$ and $^3H$. Thus an actual tritium loss from acyl-DHA could have been masked by an artifactual enrichment or by a mechanism involving an isotope effect. We also considered that acyl-DHAP might have undergone a hydrogen exchange *via* an independent enzyme reaction similar to an isomerase or aldolase reaction. This might have accounted for the production of tritiated glyceryl ethers in our previous studies. Proof that a hydrogen exchange occurs at some point in an enzymatic reaction between acyl-DHAP and fatty alcohol is critical in discovering the mechanism of *O*-alkyl lipid

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[1] Abbreviations used are: DHAP, dihydroxyacetone phosphate; DHA, dihydroxyacetone; acyl-DHA, acyldihydroxyacetone; acyl-DHAP, acyldihydroxyacetone phosphate; CAP, Hydroxy-3-chloro-2-propanone phosphate.

bond formation. Accordingly we sought more direct evidence for a hydrogen exchange in the formation of *O*-alkyl lipids after the formation of acyl-DHAP. In the present study by using stable acyl-DHAP and tritiated water as the starting substrates rather than DHAP we have obtained evidence (1) that 1 g-atom of hydrogen is exchanged for each mole of hexadecanol used in *O*-alkyl lipid formation and (2) that the exchange occurs after the formation of acyl-DHAP. Using acyl-DHAP, it was possible to rule out completely the possibility that residual isomerase activity, resulting from incomplete inhibition of this enzyme by CAP, might have been responsible for the observed hydrogen exchange. In addition, we have included data to show that the hydrogen exchange also occurs in a mammalian system using Ehrlich ascites tumor cells.

## Materials and Methods

Dihydroxyacetone phosphate, dihydroxyacetone, NADPH, ATP, coenzyme A, glycerokinase (2 mg/ml), and type II acid phosphatase (potato) were obtained from the Sigma Chemical Company. Palmitoyl chloride, glycolic acid, oxalyl chloride, and ligroine (bp 63–75°) were obtained from Eastman and were used without further purification. Diazald was obtained from Aldrich.

[1-$^{14}$C]Hexadecanol (specific activity 25.5 Ci/mol) was obtained from Tracerlab. Tritiated water (specific activity 5.0 Ci/g) was obtained from the New England Nuclear Corp.

[1,3-$^{3}H_2$]- and [1,3-$^{14}C_2$]dihydroxyacetone phosphate were prepared as previously described (Friedberg *et al.*, 1972).

1-Hydroxy-3-chloro-2-propanone phosphate (CAP), an inhibitor of triosephosphate isomerase, was prepared as described by Hartman (1970).

Acyl-DHAP was synthesized according to Hajra and Agranoff (1968) utilizing the 1-palmitoxy-3-diazoacetone intermediate as described by Schlenk *et al.* (1952). The nature of the product was verified by treatment with alkalinehydroxylamine as described by Hajra and Agranoff (1968), followed by identification of DHAP by thin-layer chromatography on cellulose.

Ehrlich ascites tumors were maintained in Swiss albino mice (HA/ICR), transplanted and harvested by the method of Levinson and Gordon (1971). Microsomes were obtained essentially by the method of Wykle *et al.* (1972) except that the crude homogenate after sonic oscillation was centrifuged at 12,800*g* for 20 min and then again at 20,000*g* for 20 min. The microsomes were prepared from the supernatant, washed three times, and stored at −80° (≃6 mg of protein/ml). The high protein concentration was chosen to allow for the subsequent dilution by tritiated water prior to incubation.

The preparation of isopropylidene derivatives of glyceryl ethers, chromatographic procedures, preparation of *Tetrahymena* microsomes, enzymatic procedures, and chemical treatments were carried out as previously described (Friedberg *et al.*, 1971, 1972; Friedberg and Heifetz, 1973).

The *O*-alkyl glycerol generating system consisted of 1 ml of CAP treated Ehrlich ascites microsomes incubated with [1,3-$^{3}H_2$,1,3-$^{14}C_2$]dihydroxyacetone phosphate (0.125 mM, 6 × $10^6$ dpm of $^3$H and 7.8 × $10^5$ dpm of $^{14}$C), ATP (10 mM), coenzyme A (0.1 mM), $MgCl_2$ (4.33 mM), and hexadecanol (62.5 μM) in 1% Tween-80. Incubations were carried out for 2 hr at 37° followed by solvent extraction and treatment with $LiAlH_4$, or continued for another hour following the addition of 5 μmol of NADPH (Snyder *et al.*, 1969).

Table I: Tritium Distribution in Glyceryl Ethers Formed from [1,3-$^{3}H_2$,1,3-$^{14}C_2$]DHAP in Ehrlich Ascites Cell Microsomes.[a]

| Expt | $^3$H:$^{14}$C DHAP Substrate | $^3$H:$^{14}$C Glyceryl Ethers | $^3$H:$^{14}$C C-1 (Long Chain Aldehydes) | $^3$H:$^{14}$C C-3 (Formaldehydodimedon) |
|---|---|---|---|---|
| 1 | 7.6 | 5.3 | 4.5 | 7.6 |
| 2 | 7.6 | 5.3 | 3.8 | 7.4 |
| 3 | 15.1 | 9.7 | 4.8 | 14.6 |
| 4 | 15.1 | 9.6 | 5.6 | 14.9 |
| Av | 12.6 | 8.2 | 4.7 | 11.1 |

[a] Following incubation, glyceryl ethers were isolated by thin-layer chromatography. $^3$H:$^{14}$C of C-1 and C-3 of the glycerol moiety were determined after treatment of the *O*-alkyl glycerol with periodate and precipitation of C-1 as formaldehydodimedon.

A mixture of acyl-[1,3-$^{3}H_2$]DHA and acyl-[1,3-$^{14}C_2$]DHA of known ratio was prepared by the method of Hajra and Agranoff (1968) from a mixture of [1,3-$^{3}H_2$]DHA and [1,3-$^{14}C_2$]DHA. The labeled DHA was synthesized from a mixture of 102 μCi of [1,3-$^{3}H_2$]DHAP (0.041 μmol) and 6.9 μCi of [1,3-$^{14}C_2$]DHAP (0.414 μmol) by means of acid phosphatase. The labeled substrates were incubated for 100 min at 37° with 2 mg of acid phosphatase in a final volume of 1.25 ml. The pH of the DHAP was adjusted initially to pH 5.5 from a bicarbonate solution with 0.1 N HCl. The yield (88%) was determined by direct thin-layer chromatography on cellulose of an aliquot of the incubation mixture. The developing solvent was 2-propanol–acetic acid–water (3:1:1). Following incubation the reaction was stopped and the protein was precipitated with an equal volume of ethanol. The precipitate was removed by centrifugation and the supernatant was dried under nitrogen at 40°. The residue containing DHA and the unreacted DHAP was dissolved in 0.3 ml of anhydrous pyridine. Palmitoyl chloride (1 μl) in 50 μl of chloroform was added. The reaction was carried out at 0°. Another microliter of palmitoyl chloride was added after 10 min. The remainder of the procedure was carried out according to Hajra and Agranoff (1968). The final product was purified by thin-layer chromatography on silica gel. The developing solvent was ethyl ether–acetic acid–water (20:80:1). Authentic acyl-DHA, previously prepared and analyzed, was used as a standard. The $^3$H:$^{14}$C ratios of the starting DHAP and product acyl-DHA were the same.

## Results

*Loss of Tritium from [1,3-$^{3}H_2$,1,3-$^{14}$C]DHAP in O-Alkyl Lipid Formation in Ehrlich Ascites Cell Microsomes.* In order to determine if a hydrogen exchange also occurs in mammalian *O*-alkyl ether synthesis, Ehrlich ascites cell microsomes, pretreated with CAP, were incubated with [1,3-$^{3}H_2$,1,3-$^{14}C_2$]dihydroxyacetone phosphate and hexadecanol in an *O*-alkyl lipid generating system as described above and extracted by the method of Bligh and Dyer (1959). Lipid extracts from incubations were treated

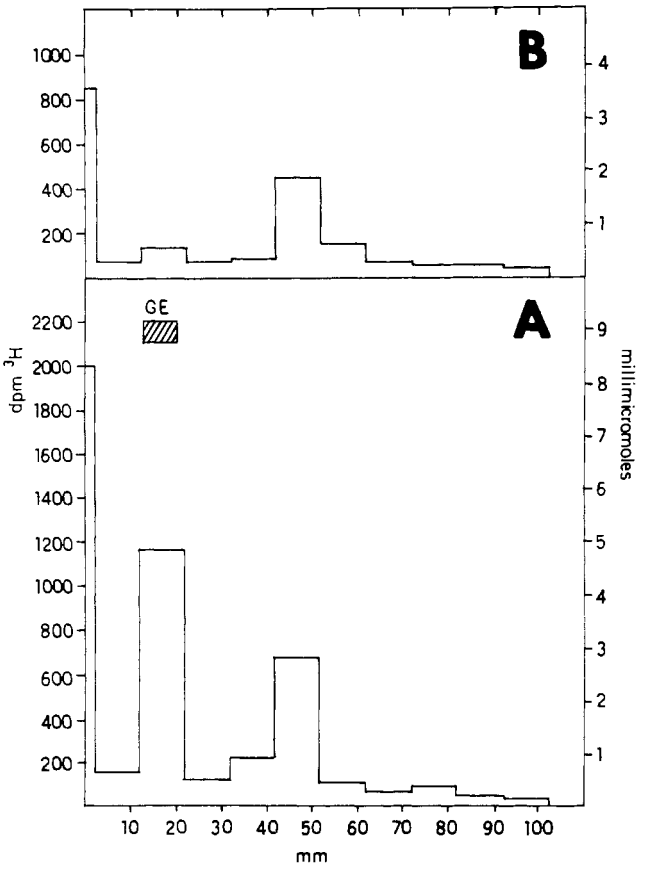


FIGURE 1: Radioactive profile of thin-layer chromatogram of lipids following incubation of acyl-DHAP in tritiated water in *Tetrahymena* microsomes. (A) One milliliter of *Tetrahymena* microsomes (2 mg protein/ml) was incubated 2.5 h at 30° in the presence of 11.5 mM ATP, 3.1 mM $MgCl_2$, 77 μM hexadecanol, 0.123 mM chimyl alcohol, 20 mCi of tritiated water (0.1 g), and 67 μM acyl-DHAP (total volume 1.3 ml). NADPH (5 μmol) was then added and the incubation was continued for 2 more hr. Glyceryl ethers were separated as described in the text. The location of the glyceryl ether peak is indicated by the position of the standard (G.E.). (B) Same as (A) without acyl-DHAP.

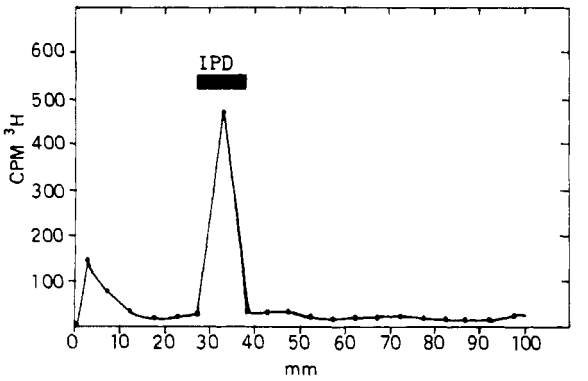


FIGURE 2: Radioactive profile of thin-layer chromatogram of isopropylidene derivatives of glyceryl ether synthesized by *Tetrahymena* microsomes from acyl-DHAP in the presence of tritiated water as described in the text.

with $LiAlH_4$ and the glyceryl ethers were isolated by thin-layer chromatography. Aliquots of the glyceryl ethers were counted and other aliquots were treated with periodate and dimedon as previously described (Friedberg *et al.*, 1971). The $^3H$:$^{14}C$ ratios of the long chain ether aldehydes (the carbon that possesses the *O*-alkyl moiety) are consistent with the loss of one tritium from the ether-linked carbon. The $^3H$:$^{14}C$ ratio of the glycerol moiety precipitated as formaldehydodimedon (carbon 3 of *sn*-glyceryl 1-phosphate) indicated that no loss had occurred from this carbon.

*$^3H$:$^{14}C$ Ratio of Acyl-DHAP Formed Enzymatically from [1,3-$^{14}C_2$]DHAP.* In the *Tetrahymena* system we were not able to demonstrate the formation of acyl-DHAP (Friedberg and Heifetz, 1973). Using the tumor cell system, we demonstrate here the formation of acyl-DHAP and show that no tritium loss occurs. [1,3-$^3H_2$,1,3-$^{14}C_2$]DHAP and CAP treated Ehrlich ascites cell microsomes were incubated in an acyl-DHAP generating system (Hajra and Agranoff, 1968; Wykle *et al.*, 1972). Acyl-DHAP and acyl-DHA were identified by thin-layer chromatography using authentic acyl-DHAP and acyl-DHA as standards. For the identification of acyl-DHAP two solvent systems were used, chloroform–methanol–7N $NH_4OH$ (60:35:5) and chloroform–methanol–acetic acid–water (50:25:7:3). After treatment with alkaline hydroxylamine, the water-soluble products were separated by thin-layer chromatography on 0.1 mm of cellulose MN 300 plates in *tert*-butyl alcohol–water–formic acid (60:20:2, v/v). A radioactive peak was found which corresponded in mobility to authentic DHAP.

Acyl-DHAP and acyl-DHA were then eluted from thin-layer chromatograms and the $^3H$:$^{14}C$ ratios were determined. The substrate DHAP had a $^3H$:$^{14}C$ ratio of 7.7. The ratios for DHAP recovered after incubation, acyl-DHAP, and acyl-DHA were 7.3, 7.3, and 7.5, respectively. The results are consistent with the conclusion that no tritium exchange occurs in the formation of acyl-DHAP from DHAP and confirm the conclusions reached from experiments in the *Tetrahymena* system in which acyl-DHAP was not detected.

More recently, however, we have been able to demonstrate acyl-DHAP formation in the *Tetrahymena* system by increasing the concentration of ATP and magnesium from 10 and 4.33 mM to 16.7 and 10 mM, respectively.

*The Formation of Tritiated O-Alkyl Lipids from Acyldihydroxyacetone Phosphate in the Presence of Tritiated Water.* In order to provide a more direct demonstration for a hydrogen exchange involving acyldihydroxyacetone phosphate, a series of experiments was performed using tritiated water, an approach which also eliminated vitiation of the results by isomerase or aldolase. This was carried out initially in the *Tetrahymena* system. Two 1-ml aliquots of *Tetrahymena* microsomes were incubated in the presence of ATP, $MgCl_2$, hexadecanol, chimyl alcohol, and 20 mCi of tritiated water (0.1 g). We have found that the addition of chimyl alcohol increases the yield of labeled glyceryl ethers probably by decreasing cleavage of the labeled product. One sample was incubated with, and one without 67 μM palmitoyl-DHAP (Wykle *et al.*, 1972). The total volume in each tube was 1.3 ml. NADPH (5 μmol) was then added and the reaction was continued for an additional 120 min; 1 μmol of additional carrier chimyl alcohol was added to each tube. The lipids were extracted and isolated as previously described (Friedberg and Heifetz, 1973).

The results reveal the formation of a radioactive substance with the mobility of *O*-alkyl glycerol (Figure 1). Another radioactive peak was also frequently present. This material, less polar than *O*-alkyl glycerol, could not be identified and is either formed or becomes tritiated even in the absence of added acyl-DHAP and, therefore, appeared not to be related to *O*-alkyl lipid synthesis. Tritiated *O*-alkyl glycerol, however, was formed only in the presence of acyl-DHAP and hexadecanol. The identity of the tritiated *O*-

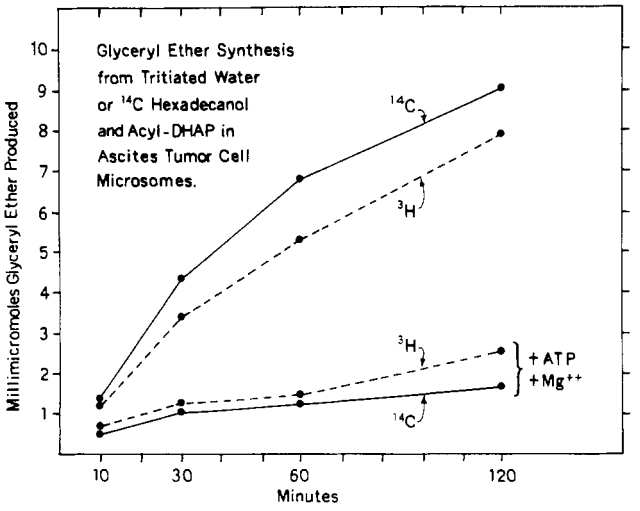


FIGURE 3: Stoichiometry of glyceryl ether synthesis from acyl-DHAP measured by tritiated water *vs.* [1-$^{14}$C]hexadecanol in ascites tumor cell microsomes; 1-ml aliquots of ascites cell microsomes were incubated at 30° for the time periods indicated in the presence of 67 $\mu$M palmitoyl-DHAP, 20 mCi of tritiated water (0.1 g, final concentration of tritium, 305 dpm per millimicroatom of hydrogen), 77 $\mu$M hexadecanol, 0.123 mM chimyl alcohol, with and without 11.5 mM ATP and 3.1 mM $MgCl_2$. Another series of tubes incubated simultaneously contained [1-$^{14}$C]hexadecanol (77 $\mu$M, 0.992 $\mu$Ci), instead of nonradioactive hexadecanol and tritiated water. The final volume was 1.3 ml. The *O*-alkyl-DHAP synthesis was terminated by the addition of 5 $\mu$mol of NADPH. The *O*-alkyl glycerol products were extracted and isolated by thin-layer chromatography as described in the text.

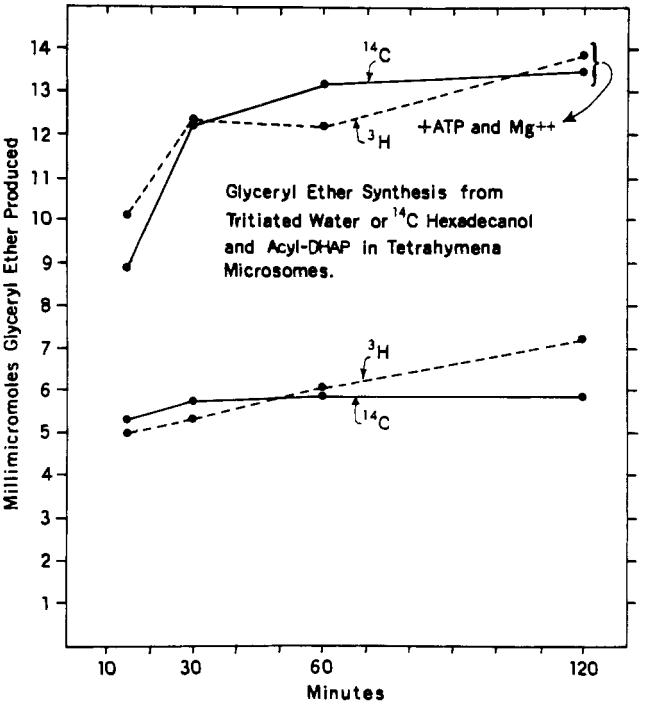


FIGURE 4: Stoichiometry of glyceryl ether synthesis from acyl-DHAP measured by tritiated water *vs.* [1-$^{14}$C]hexadecanol in *Tetrahymena* microsomes. Experimental conditions are described in Figure 3.

alkyl glycerol was established by the following procedure. The *O*-alkyl glycerol obtained from five incubations was purified by thin-layer chromatography. An aliquot was converted to the isopropylidene derivative and examined by thin-layer chromatography using an authentic isopropylidene derivative of chimyl alcohol as a standard. The results (Figure 2) establish the identity of the tritiated compound as *O*-alkyl glycerol (recovery, 94%). Tritiated glyceryl ethers were not formed when DHAP was incubated in tritiated water in the absence of coenzyme A indicating that tritiation of *O*-alkyl DHAP had not occurred by recycling *via* breakdown of acyl-DHAP to DHAP and isomerization (Rose and Rieder, 1958; Rieder and Rose, 1959). Tritiated glyceryl ethers were also formed from the incubation of acyl-DHAP in tritiated water when NADPH reduction was replaced by reduction with $LiAlH_4$.

*Stoichiometry of the Incorporation of Tritiated Water and Hexadecanol into O-Alkyl Lipids.* In order to demonstrate the stoichiometry of the reaction used in the formation of *O*-alkyl lipid the production of *O*-alkyl lipid was measured simultaneously over time with [1-$^{14}$C]hexadecanol and acyl-DHAP and with tritiated water and acyl-DHAP in the Erhlich ascites cell system. Tritiated water and hexadecanol were used in one set of tubes and [1-$^{14}$C]hexadecanol in another set of tubes. At stated time periods, 5 $\mu$mol of NADPH was added and the reaction continued for an additional hour. The addition of NADPH reduced further *O*-alkyl lipid formation rapidly enough so that the time course formation of *O*-alkyl lipids was not obscured (Wykle and Snyder, 1970). The experiment was repeated in the *Tetrahymena* system.

The results obtained with ascites cell microsomes (Figure 3) show that the rates of formation of tritiated *O*-alkyl glycerol and [1-$^{14}$C]-*O*-alkyl glycerol are in good agreement. ATP and magnesium were inhibitory in the ascites cell system. On the other hand, in the *Tetrahymena* system (Figure 4) ATP and magnesium were not required but appeared to be stimulatory.

In the *Tetrahymena* system the formation of *O*-alkyl lipid reaches a maximum in 15 min. That this is the result of rapid conversion or breakdown of acyl-DHAP to other compounds was previously shown indirectly (Friedberg and Heifetz, 1973) and here by the fact that the formation of *O*-alkyl lipid resumes upon further addition of acyldihydroxyacetone phosphate (Figure 5).

The cause of the inhibitory effect of ATP and magnesium in the tumor cell system and the opposite effect in the *Tetrahymena* system are unknown. Changing the concentrations of both ATP and magnesium has not provided an explanation. However, our findings in the *Tetrahymena* system resemble those found in the guinea pig liver mitochondrial and rat brain microsome systems of Hajra (1970) in that ATP and magnesium had a stimulatory effect. In the tumor system our findings are in agreement with those of Wykle *et al.* (1972) who found no magnesium requirement. Our data also show that ATP alone, without added magnesium, increased the synthesis of *O*-alkyl bonds from acyldihydroxyacetone phosphate although this effect was minimal.

*The Effect of Tritiated Water on Acyl-DHAP Incubated in Ehrlich Ascites Cell Microsomes in the Absence of Hexadecanol.* We have shown a stoichiometric relationship between the incorporation of tritium and hexadecanol in *O*-alkyl lipid synthesis. One explanation is that acyl-DHAP might become rapidly tritiated before hexadecanol is linked. The present study indicates that this is not the case. Thus, in the ascites cell system, when 100 nmol of acyl-[1,3-$^{3}H_2$]DHAP obtained enzymatically were incubated for 2 hr, approximately 30–50 nmol remained. Assuming complete tritiation, in a parallel incubation of stable acyl-DHAP in tritiated water (125 dpm of $^{3}$H/nmol of water), residual acyl-DHAP should have become tritiated to the ex-

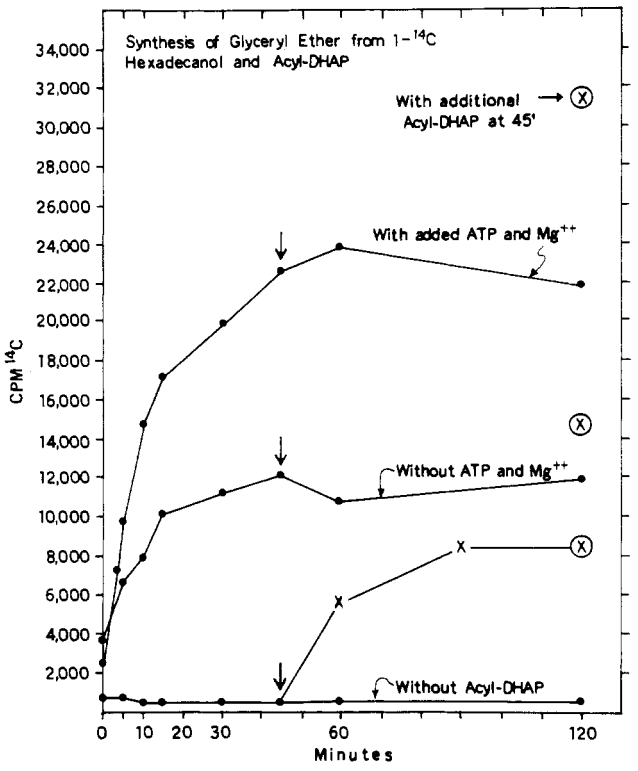


FIGURE 5: Time course of formation of glyceryl ether from [1-$^{14}$C]hexadecanol and acyl-DHAP in *Tetrahymena* microsomes and the effect of additional acyl-DHAP at 45 min. The experimental conditions are described in Figure 3. At 45 min additional acyl-DHAP (0.1 μmol) was added to another simultaneously incubated series of tubes (arrow). One set contained ATP and magnesium, one set contained no ATP or magnesium, and a third set contained no acyl-DHAP. The final activities in glyceryl ethers for each of the three experimental conditions with later addition of acyl-DHAP are indicated by ⊗.

tent of 3750–6260 dpm's. The results showed that tritiated acyl-DHAP was not formed.

*The Synthesis of Dihydroxyacetone from Acyldihydroxyacetone in Relation to Hydrogen Exchange.* We have previously shown that [1,3-$^{3}H_2$,1,3-$^{14}C_2$]DHAP incubated with CAP treated *Tetrahymena* microsomes yields DHA which has exchanged one tritium. Acyl-DHA is also produced. The production of DHA from DHAP requires ATP, magnesium, and coenzyme A (Friedberg and Heifetz, 1973). In order to determine whether or not acyl-DHA is the source of the hydrogen exchanged DHA we incubated acyl[1,3-$^{3}H_2$,1,3-$^{14}C_2$]DHA with *Tetrahymena* microsomes at 30° for 3 hr. The results showed that DHA recovered from the incubation of 1 μmol of acyl-DHA had not lost tritium. The yield of DHA was 69%. When 2 μmol of nonisotopic acyl-DHA was incubated in the presence of 20 mCi of tritiated water, tritiated DHA was not formed.

## Conclusions

In the present investigation we have shown directly that acyl-DHAP formed from [1,3-$^{3}H_2$,1,3-$^{14}C_2$]DHAP had not lost a tritium and that *O*-alkyl lipid formed from stable acyl-DHAP acquires one tritium in the presence of tritiated water, thereby confirming previous indirect studies with DHAP. This was shown to occur in both mammalian and protozoan systems and again indicates a tritium exchange in the formation of *O*-alkyl-DHAP.

That acyl-DHAP itself does not exchange a tritium, either through an independent enzyme reaction or as part of the mechanism of *O*-alkyl bond formation, was demonstrated in experiments with tritiated water and stable acyl-DHAP.

In addition, although acyl-DHA breaks down to DHA, we have shown in the present investigation that a tritium exchange does not occur at this step. Therefore our previous data showing a tritium exchange when DHA is produced suggest that DHA is not produced from acyl-DHA but from some other unidentified intermediate.

## Acknowledgments

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