Hydrogen Exchange in the Synthesis of Glyceryl Ether and in the Formation of Dihydroxyacetone in *Tetrahymena pyriformis*†

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ABSTRACT: We have previously shown that [1,3-^{3}H]dihydroxyacetone phosphate is incorporated enzymatically into *O*-alkyl lipids with the loss of one tritium from C-3. Further evidence for a tritium exchange has been presented in this investigation by showing, in a microsomal system from *Tetrahymena pyriformis*, that tritiated *O*-alkyl lipids are formed in the presence of tritiated water from dihydroxyacetone phosphate and hexadecanol. In another series of experiments we have shown that tritiated dihydroxyacetone phosphate yields tritiated acyldihydroxyacetone (*via* acyldihydroxyacetone phosphate) in the absence of hexadecanol and in the presence of ATP, Mg^{2+}, and CoA. Acyldihydroxyacetone is formed without a tritium exchange. Dihydroxyacetone is also formed and has lost one tritium. When hexadecanol is added to the system, the amount of dihydroxyacetone is reduced but *O*-alkyl lipids are formed instead. The *O*-alkyl lipids formed have undergone a tritium exchange. It is concluded that acyldihydroxyacetone phosphate yields acyldihydroxyacetone and dihydroxyacetone, unless hexadecanol is present in which case it yields *O*-alkyl lipids.

The known steps in *O*-alkyl-DHAP[1] synthesis include the initial formation of acyl-DHAP from fatty acid and DHAP in the presence of ATP, magnesium, and coenzyme A. Acyl-DHAP, hexadecanol, ATP, and magnesium then interact to form *O*-alkyl-DHAP (Hajra, 1970; Wykle *et al.*, 1972). In this process it is known that the oxygen of hexadecanol is retained in the product (Snyder *et al.*, 1970).

When [1,3-^{3}H]dihydroxyacetone phosphate is incorporated enzymatically into *O*-alkyl lipids, there is a loss of half of the tritium atoms from C-3[2] (Friedberg *et al.*, 1971). Since there is no net loss of hydrogen in the product, the findings point to an exchange reaction. It is also known that this reaction is specific for the same hydrogen lost in the triosephosphate isomerase reaction, but not the one lost in the fructose-1,6-diphosphate aldolase reaction (Friedberg *et al.*, 1972).

The present investigation was undertaken to obtain more information on the nature of the hydrogen loss which occurs in the formation of *O*-alkyl lipids. Evidence will be presented to show that the tritium lost from [1,3-^{3}H]dihydroxyacetone phosphate is exchanged with a hydrogen from the aqueous environment and that this exchange occurs on C-3 of the dihydroxyacetone phosphate moiety. It will also be shown that the hydrogen loss depends on the presence of coenzyme A, that acyldihydroxyacetones are formed without loss of hydrogen, and that the number of hydrogen atoms exchanged greatly exceeds the number of moles of *O*-alkyl lipid formed. The difference is in part or entirely accounted for by the formation of dihydroxyacetone. The formation of this compound also involves a hydrogen exchange. This finding will be discussed.

Materials and Methods

Materials. Dihydroxyacetone phosphate, 3-phosphoglyceraldehyde, triosephosphate isomerase (10 mg/ml), triethanolamine, glycerokinase (2 mg/ml), α-glycerophosphate dehydrogenase (10 mg/ml), dihydroxyacetone (DHA), NADPH, NADH, and ATP were obtained from the Sigma Chemical Co.

[1-^{14}C]Hexadecanol (specific activity 25.5 Ci/mol) and [2-^{14}C]dihydroxyacetone (specific activity 60 Ci/mol) were obtained from Tracerlab. Tritiated water (100 mCi/g) was obtained from the New England Nuclear Corp.

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

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

Acyldihydroxyacetone was prepared by the method of Hajra and Agranoff (1968).

PREPARATION OF MICROSOMES AND INCUBATIONS. The culturing of *Tetrahymena pyriformis* and the incubations were carried out as previously described (Friedberg *et al.*, 1971). Microsomes were prepared as previously described except that sucrose was omitted from the last wash in order to facilitate chromatography of water-soluble materials.

CHROMATOGRAPHIC AND ELECTROPHORETIC PROCEDURES. Glyceryl ethers were isolated by thin-layer chromatography on silica gel G (Brinkmann) by development in ligroin–ethyl ether–acetic acid (20:80:1, v/v).

Triosephosphates and dihydroxyacetone were separated on cellulose layers (Brinkmann) 0.1-mm thick, developed in *tert*-butyl alcohol–water–*p*-toluenesulfonic acid (60:30:2, v/v/w), *tert*-butyl alcohol–water–formic acid (60:15:2, v/v), or isopropyl alcohol–acetic acid–water (3:1:1, v/v).

Phospholipids were developed in chloroform–methanol–acetic acid–water (50:25:7:3, v/v) and neutral lipids were developed in ligroin–ethyl ether–acetic acid (20:80:1, v/v) or (90:10:1, v/v).

Neutral lipids were eluted from silica gel with ethyl ether and phospholipids with chloroform–methanol (1:1, v/v).

Radioactive profiles of labeled materials separated by thin-layer chromatography were obtained by scraping narrow

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[1] Abbreviations used are: DHAP, dihydroxyacetone phosphate; DHA, dihydroxyacetone; CAP, 1-hydroxy-3-chloro-2-propanone phosphate (chloroacetol phosphate).

[2] The unphosphorylated primary carbinol carbon is designated as C-3 of dihydroxyacetone 1-phosphate. C-3 of dihydroxyacetone 1-phosphate corresponds to C-1 of the precursor *sn*-glyceryl 3-phosphate and C-1 of 3-phospho-D-glyceraldehyde.

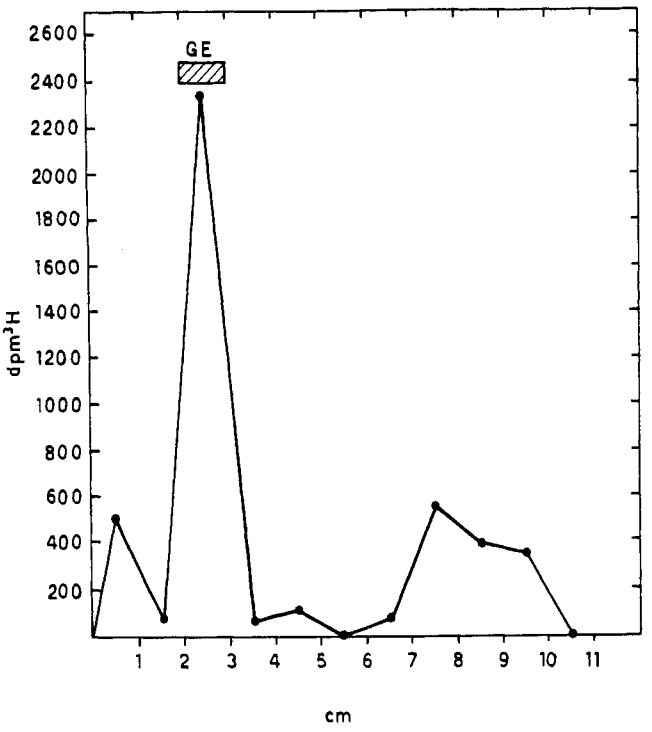

FIGURE 1: Radioactive profile of thin-layer chromatogram of lipids obtained after incubation of microsomes in tritiated water. Incubation and chromatography were carried out as described in the text. NADPH was added after 3 hr, followed by lipid extraction, and thin-layer chromatography: GE, glyceryl ether.

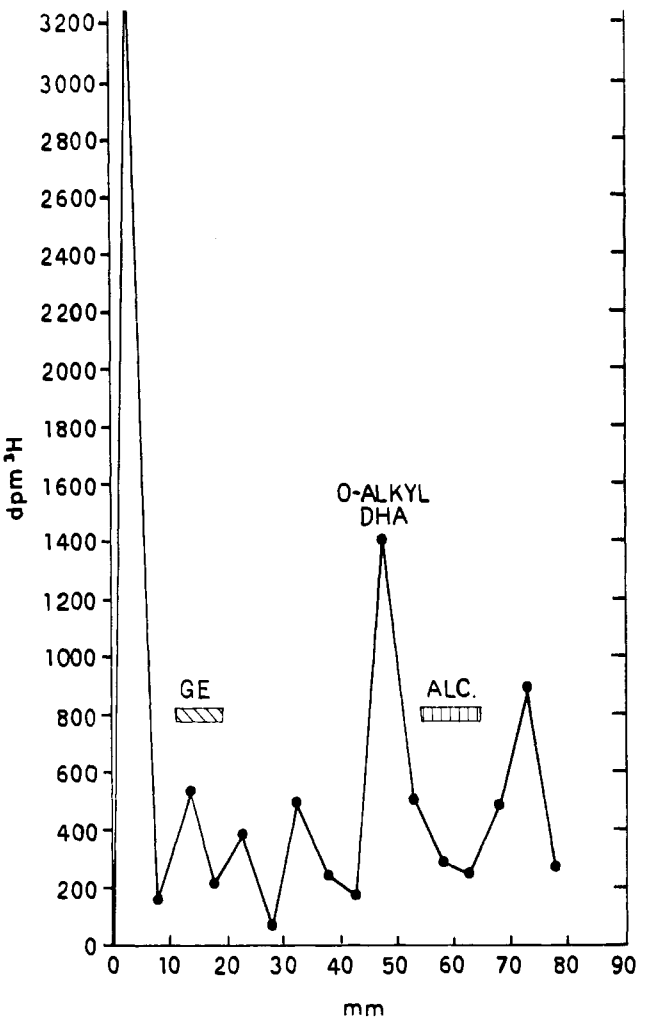

FIGURE 2: Radioactive profile of thin-layer chromatogram of lipids obtained after incubation of microsomes in tritiated water. Incubation and chromatography were carried out as described in the text. No NAPDH was added: GE, glyceryl ether; DHA, dihydroxyacetone; ALC, long-chain alcohol.

bands into counting vials. Silica gel was counted in 10 ml of phosphor–triton (2:1, v/v) and cellulose was counted in 15 ml of phosphor–triton–water (10:5:1.5, v/v).

High-voltage electrophoresis was carried out in 8% formic acid.

ENZYMATIC PROCEDURES. Triosephosphate isomerase activity was measured with α-glycerophosphate dehydrogenase and NADH as described in Results.

Dihydroxyacetone was assayed in a reaction which coupled glycerokinase and α-glycerophosphate dehydrogenase with NADH. Dihydroxyacetone was added to a cuvet which contained (in a final volume of 3 ml) 0.18 M glycine buffer (pH 9.8), 0.003 M $MgCl_2$, 2.2 mg of ATP, 0.3 mg of α-glycerophosphate dehydrogenase, 0.04 mg of glycerokinase, and 1.0 mg of NADH. Decrease in optical density was read at 340 nm.

CHEMICAL TREATMENTS AND OTHER METHODS. Lipids were extracted by the method of Folch *et al.* (1957) or Bligh and Dyer (1959). Treatment with $LiAlH_4$ was carried out at room temperature as described by Thompson (1965). Glyceryl ethers were cleaved at the ether bond with hydrogen iodide and the alkyl iodides were separated by thin-layer chromatography as previously described (Friedberg and Greene, 1967). Periodate cleavage of glyceryl ethers between vicinal hydroxyl groups was performed by the method of Thompson and Hanahan (1963) modified as previously described (Friedberg *et al.*, 1971). Protein was measured by the method of Lowry *et al.* (1951) and reduction with sodium borohydride was performed by the method of Hajra and Agranoff (1968). Phosphorus was determined by the method of Bartlett (1959).

Tritiated water was measured after incubation of [1,3-$^3H_2$,-1,3-$^{14}C_2$]dihydroxyacetone phosphate by lyophilization of an aliquot of the reaction mixture. The water evolved was recovered in a small Dry Ice–acetone trap. An aliquot was used for counting.

Liquid scintillation counting of aqueous solutions was done by the method of Patterson and Greene (1965) using toluene-phosphor–Triton X-100 (2:1). The amount of aqueous material was kept at 10% of the total volume. When necessary, [^{14}C]- and [^3H]toluene were used as an internal standard to determine absolute activity. Lipids were counted in toluene–2,5-diphenyloxazole–1,4-bis[2-(5-phenyloxazolyl)]benzene.

Results

Studies with Tritiated Water. In order to obtain further evidence that one hydrogen is labilized from C-3 of DHAP in the course of glyceryl ether synthesis, it would be of interest to know if one atom of hydrogen from the aqueous environment is incorporated into each mole of glyceryl ether synthesized and if this hydrogen becomes bound to the ether-linked carbon of glyceryl ether glycerol.

The feasibility of the present study was based on the fact that 1-ml of microsomes (2 mg of protein) is capable of forming between 10 and 20 nmol of glycerol ethers in the presence of optimal concentrations of DHAP and hexadecanol

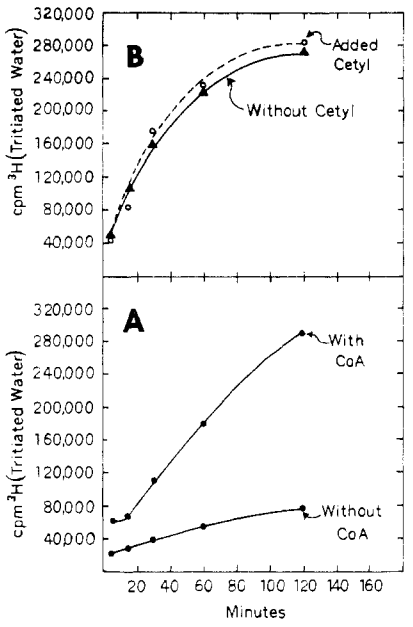

FIGURE 3: The effect of CoA and hexadecanol on the detritiation of [1,3-³H]dihydroxyacetone phosphate. In the complete system four ml of CAP-treated microsomes (8 mg of protein) were incubated with [1,3-³H₂]dihydroxyacetone phosphate (9.4 μCi, 1.0 mol), 11.7 mM ATP, 3.13 mM Mg^{2+}, 0.078 mM hexadecanol in 1% Tween 80, and 0.39 mM CoA at 30°. Hexadecanol and CoA were omitted as indicated. Aliquots (1 ml) were removed at intervals. One-half of each aliquot was reincubated with NADPH (5 mM). The other half was immediately frozen in Dry Ice–acetone and lyophilized to recover tritiated water. Lipids were extracted from the samples incubated with added NADPH and the glyceryl ethers iolated by thin-layer chromatography as described: (A) formation of tritiated water with and without coenzyme A; (B) formation of tritiated water with CoA, with and without hexadecanol.

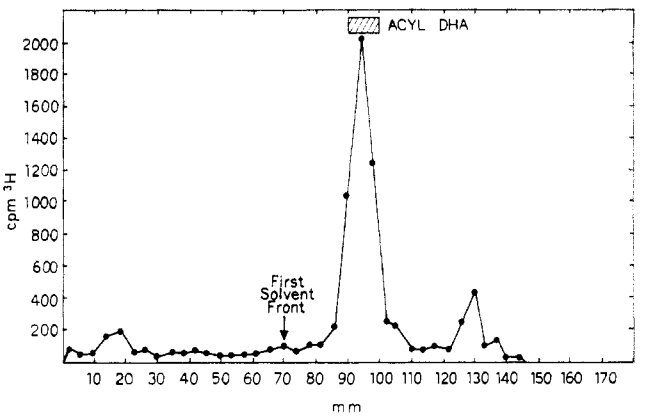

FIGURE 4: Lipids formed during the incubation of dihydroxyacetone phosphate, ATP, Mg^{2+}, CoA, and *Tetrahymena* microsomes. [1,3-³H₂]DHAP (5.2×10^6 dpm, 0.12 μmol) was incubated with 1 ml of microsomes free of isomerase activity (2 mg of protein), 10 mM ATP, 2.7 mM Mg^{2+}, 0.67 mM CoA, and 0.1 mM potassium palmitate in a final volume of 1.5 ml, for 1 hr at 30°. Lipids were extracted and separated by thin-layer chromatography on 0.25-mm prepared plates of silica gel G (Brinkmann). The plates were developed first in chloroform–methanol–acetic acid–water (50:25:7:3, v/v) for 7 cm and then redeveloped in the same direction for 15 cm in ligroin–ethyl ether–acetic acid (20:80:1, v/v).

(1 mM DHAP and 0.1 mM hexadecanol). If the enzymatic system which synthesizes glyceryl ethers labilizes one hydrogen atom, as indicated by our previous study, it can be calculated that the formation of 10–20 nmol of glyceryl ethers should be sufficient to permit the demonstration of tritium labeling in the presence of tritiated water which has a specific activity of at least 100 μCi/mmol.

Accordingly, two 1-ml aliquots of microsomes (2 mg of protein/ml) were each first incubated with 0.2 ml of 0.05 M CAP for 30 min at 30°. One aliquot was incubated with 9.4 mM ATP, 2.5 mM magnesium, 0.31 mM CoA, 0.625 mM DHAP, 0.125 mM hexadecanol in 1% Tween 80, and 10 mCi of tritiated water (0.1 g). The total volume was 1.6 ml. The final specific activity of the tritiated water was 6.25 mCi/g. The incubation was carried out for 3 hr at 30°. NADPH (5 μmol) was then added and the reaction was continued for another hour to convert *O*-alkyl-DHA to glyceryl ether. The lipids were extracted by the method of Folch *et al.* (1957). The upper phase was removed and the lower phase was washed once with chloroform–methanol–water (3:48:47) (Folch *et al.*, 1957). The lower phase was then thoroughly dried under a stream of nitrogen. The glyceryl ethers were separated by thin-layer chromatography on silica gel G. The developing solvent was ligroin–ethyl ether–acetic acid (20:80:1, v/v). The silica gel was scraped in 1-cm bands and counted in phosphor–Triton–water (2:1:0.05). Absolute activity was calculated by preparing appropriate blanks with silica gel and tritiated toluene. The results (Figure 1) show tritium labeling of glyceryl ethers. The other aliquot of microsomes was used to measure the amount of glyceryl ethers formed and was therefore incubated simultaneously and under the same conditions except that an equimolar amount of [1-¹⁴C]hexadecanol was substituted for the nonradioactive hexadecanol and tritiated water was omitted. From the specific activity of the hexadecanol it was determined that 19.4 nmol of glyceryl ether was formed. The total tritium activity of the glyceryl ethers obtained in the experiment with tritiated water was 2210 dpm. This number is very close to the value calculated for the incorporation of one hydrogen atom per mole of glyceryl ethers formed; in the presence of tritiated water with specific activity of 6.25 mCi/g (124 dpm/mμatom of hydrogen), the glyceryl ethers formed should be labeled to the extent of 124 dpm/nmol, or 2410 dpm for 19.4 nmol. Since the amount of glyceryl ether formed from labeled DHAP in the absence of added hexadecanol is negligible, it is assured that the amount of endogenous fatty alcohol present in the preparation can be ignored.

The experiment with tritiated water was then repeated and the glyceryl ethers obtained were eluted with ethyl ether from the silica gel G plate. One half was treated with hydrogen iodide and the other half with periodate. The alkyl iodides obtained from the hydrogen iodide treatment were isolated by thin-layer chromatography on silica gel G in ligroin–ethyl ether–acetic acid (90:10:1, v/v). There was no radioactivity present in the alkyl iodides. All of the radioactivity was present in the long-chain ether aldehydes obtained from periodate treatment which cleaves the glyceryl ether molecule between the vicinal hydroxyls of the glycerol moiety (corresponding to C-1 and -2 of DHAP). The formaldehyde (containing C-1 of DHAP) liberated by periodate treatment was precipitated as formaldehydodimedon. This fraction also contained no radioactivity. It was therefore apparent that either C-2, -3, or both, of DHAP had become labeled. In our previous studies with [1,3-³H₂,1,3-¹⁴C₂]DHAP, loss of tritium took place on C-3 and not C-2. Since the hydrogen on C-2 comes from NADPH, the tritium must be bound to C-3. This point was verified by repeating the experiment with tritiated water without the later

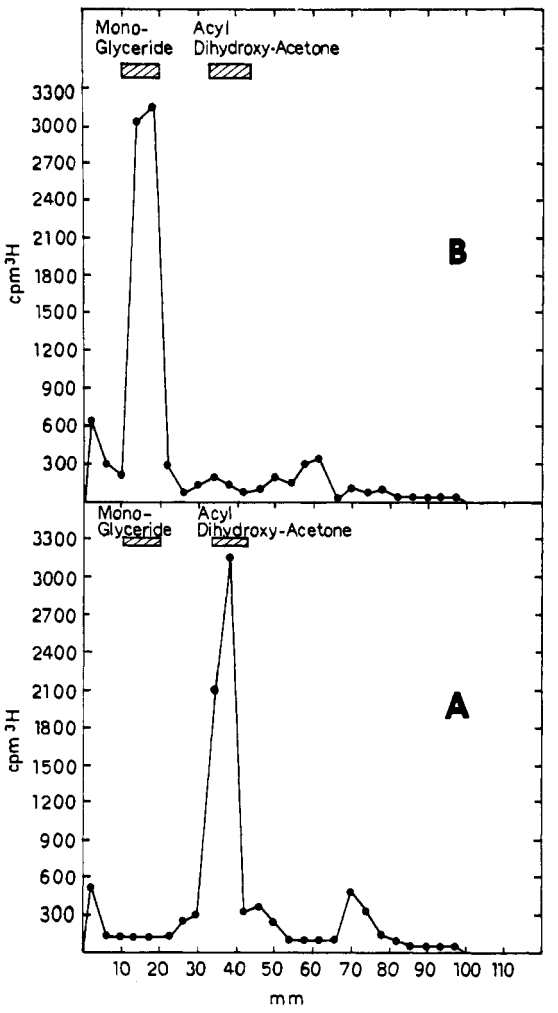

FIGURE 5: Identification of acyldihydroxyacetone by thin-layer chromatography. Lipid obtained from the experiment described in Figure 4 was separated by thin-layer chromatography on silica gel G together with authentic acyldihydroxyacetone. The developing solvent was ligroin–ethyl ether–acetic acid (20:80:1). Another aliquot of the lipid was treated with sodium borohydride and separated in the same system. (A) Thin-layer chromatogram of lipids before; (B) after treatment with sodium borohydride.

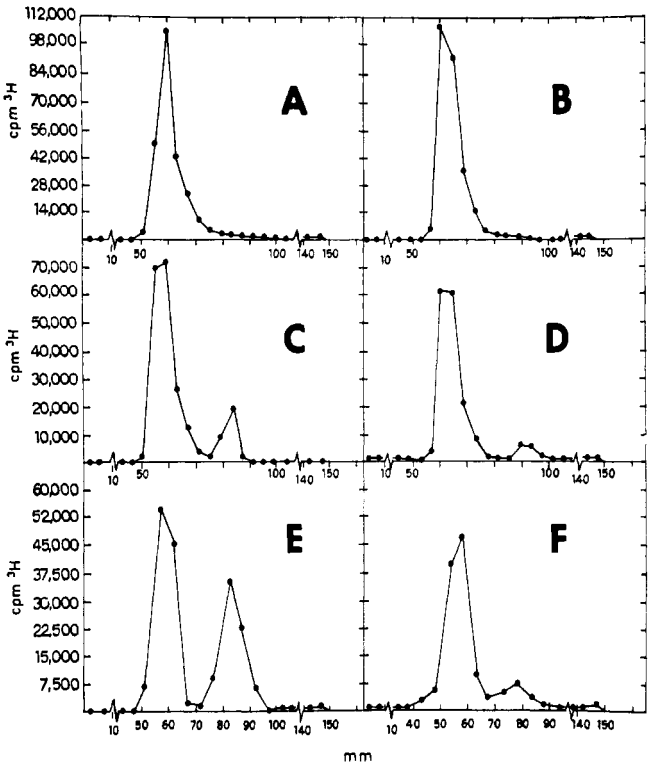

FIGURE 6: Detection of a water-soluble product of dihydroxyacetone phosphate requiring CoA for its formation. The whole system contained [1,3-$^3H_2$]dihydroxyacetone phosphate (9×10^5 dpm, 0.028 μmol), 0.5 ml of microsomes free of isomerase activity, ATP, Mg^{2+}, and CoA in the same concentrations indicated in Figure 4, in a final volume of 0.8 ml. Incubation was carried out for 30 min at 30°. The reaction was stopped by lipid extraction. The water phases were separated by thin-layer chromatography on 0.1-mm layers of cellulose (Brinkmann, MN-300) and developed as indicated in the text. (A) Whole system minus ATP, Mg^{2+}, and CoA; (B) whole system minus CoA; (C) whole system; (D) whole system plus hexadecanol (0.067 mM); (E) same as C, incubated 150 min; (F) same as D, incubated 150 min.

addition of NADPH. The thin-layer chromatography of the lipids (Figure 2) on silica gel G using ligroin–ethyl ether–acetic acid (20:80:1) for development reveals a total of 1917 dpm in the zone with a mobility corresponding to *O*-alkyldihydroxyacetone. Other lesser unidentified peaks are also present. These data, together with those presented in the previous paragraph, indicate that the glyceryl ether became tritiated on the ether-linked carbon of glycerol.

Although CAP inhibited microsomal isomerase activity in an enzymatic assay, because of the possibility that residual microsomal triosephosphate isomerase might have been responsible for tritiation of C-3 of DHAP incorporated into *O*-alkylglycerol, further investigation of the efficacy of CAP as an inhibitor of isomerase was considered essential.

Accordingly, 240 μmolar units of triosephosphate isomerase was first incubated with 0.2 ml of 0.05 M CAP for 20 min at 30°. The mixture was then added to a cuvet which contained, in a final volume of 3 ml: 0.275 M triethanolamine buffer (pH 7.38), 0.05 mg of α-glycerophosphate dehydrogenase, and 0.167 mM NADH. The reaction was initiated by the addition of 3 μmol of 3-phosphoglyceraldehyde. Decrease in optical density was read at 340 nm. With the high concentration of isomerase present, trace enzyme activity remained to the extent of 0.00105 μmolar unit, representing 1/240,000th of the uninhibited isomerase activity. However, CAP completely inhibited 24 μmolar units of isomerase.

Next, the experiment was repeated using 0.05 ml of microsomes, untreated with CAP, as a source of isomerase. From the results, it was calculated that 1 ml of microsomes contained 12.3 μmolar units of triosephosphate isomerase, an enzymatic activity less than that shown to be completely inhibited by 0.05 M CAP.

Finally, it can be calculated that the residual isomerase activity remaining after CAP treatment of even 240 μmolar units of isomerase (0.00105 μmolar unit) is not sufficient to exchange, over a period of 3 hr, more than 0.198 μmol of the 1 μmol of DHAP used in the experiments with tritiated water. From these quantities it can be calculated that a small amount of surviving isomerase activity could not have led to the results obtained in these studies.

Dependency of the Loss of Tritium of Dihydroxyacetone Phosphate on Coenzyme A. [1,3-$^3H_2$]Dihydroxyacetone phosphate, ATP, potassium palmitate, magnesium, and CAP-treated microsomes were incubated in the presence of and absence of coenzyme A. The amount of tritium released was determined by trapping water from lyophilization of the

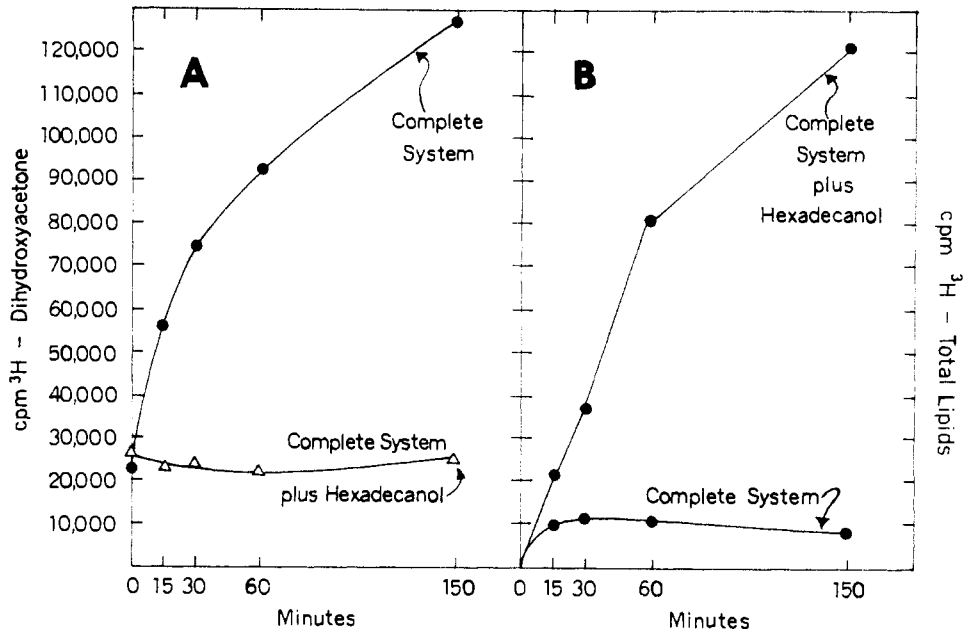

FIGURE 7: Formation of the water-soluble product of dihydroxyacetone phosphate with and without added hexadecanol. The complete system contained, in a final volume of 0.8 ml, [1,3-$^3H_2$]dihydroxyacetone phosphate (1.32×10^6 dpm, 0.076 μmol), 0.5 ml of microsomes free of isomerase activity, ATP, Mg^{2+}, CoA, with and without hexadecanol in the same concentrations indicated in Figure 4. Samples were incubated at 30° and were removed at specified times. The reaction was stopped by lipid extraction. (A) The water phases were separated by thin-layer chromatography on 0.1-mm layers of cellulose (Brinkmann MN-300) and developed in *tert*-butyl alcohol–water–*p*-toluenesulfonic acid (60:15:2, v/v/w). The appropriate areas of cellulose were scraped into vials and counted in 1.5 ml of H_2O and 15 ml of phosphor–Triton (2:1, v/v); (B) total lipids.

medium after incubation. The results (Figure 3A) indicate that some loss of tritium occurs without added coenzyme A but that loss of tritium is three to four times greater in the presence of coenzyme A. In the absence of coenzyme A there was no *O*-alkyl lipid formation. In the presence of neutral hydroxylamine, which reacts with acyl-CoA, the formation of fatty acid hydroxamates could be demonstrated and loss of tritium was completely inhibited.

Effect of Hexadecanol on Loss of Tritium. The coenzyme A dependent loss of tritium of [1,3-^{3}H]dihydroxyacetone phosphate occurs in the absence of added long-chain alcohol. When the experiments described above were repeated in the presence of CoA and hexadecanol, it was found that there was no effect on the amount of tritium lost; the amount of tritium released into the medium was the same with and without added hexadecanol (Figure 3B).

Formation of Acyldihydroxyacetone Phosphate and Acyldihydroxyacetone from [1,3-$^3H_2$,1,3-$^{14}C_2$]Dihydroxyacetone Phosphate. [1,3-$^3H_2$,1,3-$^{14}C_2$]Dihydroxyacetone phosphate was incubated with CAP-treated *Tetrahymena* microsomes, ATP, magnesium, coenzyme A, and potassium palmitate. After incubation for 2 hr at 30°, the lipids were extracted and separated by thin-layer chromatography on silica gel G (Brinkmann-precoated plates). Development was first carried out in chloroform–methanol–acetic acid–water (50:25:7:3) for 7 cm. The plates were dried and then developed in ligroin–ethyl ether–acetic acid (20:80:1) for 15 cm in the same direction. After many attempts, which included short incubation periods, and the addition of sodium fluoride, the formation of acyldihydroxyacetone phosphate was not determined with certainty in the *Tetrahymena* system (Figure 4). The small peak between 10 and 22 mm having the mobility of lysophosphatidic acid could not be identified and was the only radioactive substance having the chromatographic characteristics of a phospholipid. However, a large peak was found which was identified as acyldihydroxyacetone by cochromatography with authentic acyldihydroxyacetone (Figure 5A) and reduction of the unknown with sodium borohydride to monoglyceride (Figure 5B). Consequently, it was apparent that if acyldihydroxyacetone phosphate is an intermediate in *O*-alkyl lipid synthesis in the *Tetrahymena* system, it does not accumulate and must be converted to other products. One of these products is acyldihydroxyacetone.

Acyldihydroxyacetone was eluted from thin-layer chromatograms and the ratio of ^{3}H to ^{14}C examined. The determinations were repeated a number of times and the results are summarized in Table I. It can be seen that there was no tritium loss. It was concluded that the precursor of acyldihydroxyacetone is acyldihydroxyacetone phosphate and that the formation of the former occurs without a hydrogen loss or exchange.

Formation of Dihydroxyacetone. The results thus far indicate that there is extensive CoA-dependent loss of tritium from [1,3-$^3H_2$]dihydroxyacetone phosphate. This occurs in the absence of hexadecanol and in the absence of *O*-alkyl lipid formation. We have also shown that [1,3-$^3H_2$,1,3-$^{14}C_2$]dihydroxyacetone phosphate recovered after incubation has not lost tritium. It appeared, therefore, that the extensive tritium loss observed must be attended by the formation of substantial amounts of another compound which must be tritium poor. The most abundant lipid product present in the incubation was acyldihydroxyacetone which was not tritium poor. Another product was sought, therefore, which was water soluble and which depended for its formation on the presence of coenzyme A. Accordingly, CAP-treated *Tetrahymena* microsomes were incubated with [1,3-$^3H_2$]dihydroxyacetone phosphate, ATP, and magnesium, in the presence and absence of coenzyme A and hexadecanol. The lipids were extracted and

TABLE I: Ratio of $^3H:^{14}C$ of Substrate [1,3-$^3H_2$,1,3-$^{14}C_2$]DHAP *vs.* the Ratio of $^3H:^{14}C$ of Acyl-DHA Formed.

Expt	$^3H:^{14}C$ DHAP	$^3H:^{14}C$ Acyl-DHA
1	9.1	10.4
2	9.0	8.3
3	12.0	12.3
4	10.1	10.3
5	9.6	11.3
Average	10.0	10.5

the water phase was examined by thin-layer chromatography. The developing solvent was *tert*-butyl alcohol–water–*p*-toluenesulfonic acid (60:30:2, v/v/w).

In the presence of coenzyme A, a peak was found which was not detected in the presence of microsomes, ATP, and magnesium alone. The quantity of this substance was greatly reduced when both hexadecanol and coenzyme A were present in the incubation mixture (Figure 6). Figure 7 shows the formation of this substance with and without added hexadecanol. The possibility that the material might be dihydroxyacetone was entertained when it was found that it did not bind to Dowex 1 (Cl^-) and that it migrated poorly by high-voltage electrophoresis; however, its mobility on cellulose thin-layer chromatography was the same as that of authentic dihydroxyacetone. When it was treated with glycerokinase it was converted to a substance having the same mobility as dihydroxyacetone phosphate (Figure 8). When the eluted material was incubated in an *O*-alkyl lipid synthesizing system containing added glycerokinase, glyceryl ethers were formed.

In order to determine whether or not acyldihydroxyacetone is a precursor of dihydroxyacetone, [2-^{14}C]acyldihydroxyacetone, synthesized as described above, was incubated in the system which synthesizes both acyldihydroxyacetone and dihydroxyacetone. This experiment yielded labeled dihydroxyacetone as the only water-soluble product. When [2-^{14}C]dihydroxyacetone was used as a substrate in the same system, no acyldihydroxyacetone was recovered indicating that the reaction does not go in reverse.

These results suggested the possibility that dihydroxyacetone, since it had undergone a hydrogen loss (Table II), might be a precursor of *O*-alkyl lipids. This was ruled out by incubating [2-^{14}C]dihydroxyacetone with microsomes hexadecanol, ATP, magnesium, and coenzyme A in the presence and absence of added glycerokinase. In the absence of added glycerokinase, dihydroxyacetone was a poor precursor of glyceryl ethers. In the presence of glycerokinase, the yield of *O*-alkyl lipids was excellent.

It is now known that when NADPH is present initially in the glyceryl ether synthesizing system *O*-alkyl lipid formation does not occur (Snyder *et al.*, 1969; Wykle and Snyder, 1970). This is because acyldihydroxyacetone phosphate is converted to lysophosphatidic acid. Since our studies suggest that dihydroxyacetone arises in the *Tetrahymena* system from acyldihydroxyacetone phosphate, NADPH should inhibit dihydroxyacetone formation. Accordingly, microsomes were incubated with [1,3-$^3H_2$]dihydroxyacetone phosphate, ATP, magnesium, CoA, and NADPH. The results demonstrated that the formation of dihydroxyacetone was abolished but instead the formation of α-glycerophosphate was detected presumably *via* the NADPH reductase that reduces acyl-

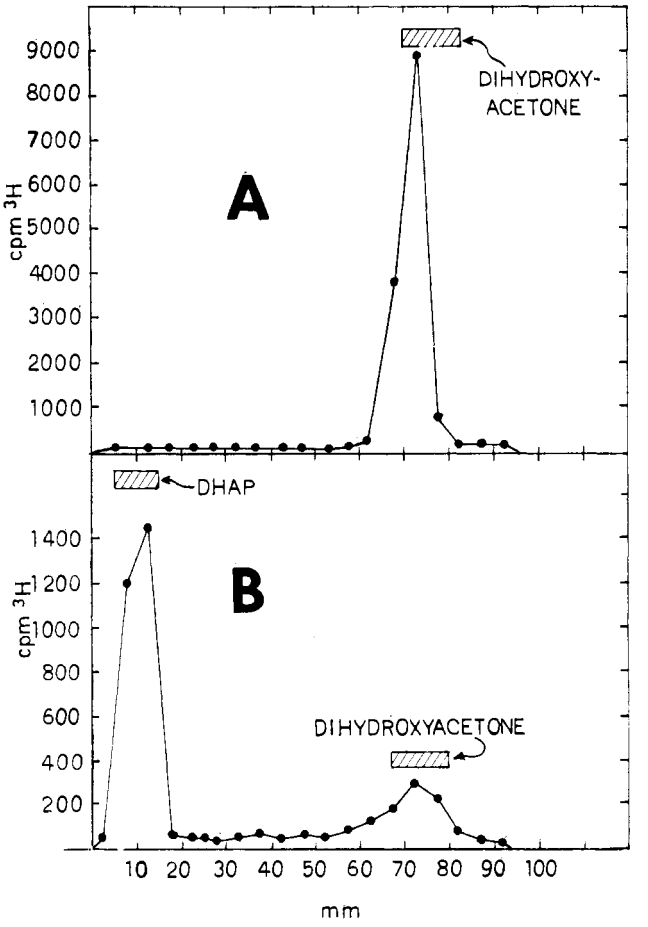

FIGURE 8: Identification of dihydroxyacetone. The unknown material from the incubation of [1,3-$^3H_2$]DHAP was purified by thin-layer chromatography on cellulose as described. An aliquot (0.1 ml, 120,000 cpm of ^3H), 0.025 ml of 0.1 M ATP, 0.005 ml of 0.1 M Mg^{2+}, and 10 μl of glycerokinase (2 mg/ml) were incubated in 0.2 M glycine buffer (pH 9.8) (total volume, 0.3 ml) for 30 min at 30°. An aliquot was separated by thin-layer chromatography as described.

TABLE II: Ratio of Activity of $^3H:^{14}C$ of Substrate [1,3-$^3H_2$,1,3-$^{14}C_2$]Dihydroxyacetone Phosphate *vs.* the Ratio of Dihydroxyacetone Formed.[a]

Expt	$^3H:^{14}C$ DHAP after Incubation	$^3H:^{14}C$ DHA	DHA: DHAP
1	12.6	9.7	0.77
2	12.4	9.7	0.78
3	12.1	9.3	0.77
4	12.2	9.5	0.78
Average	12.33	9.55	0.775

[a] [1,3-$^3H_2$,1,3-$^{14}C_2$]Dihydroxyacetone phosphate (8 μCi total; $^3H:^{14}C$ ratio, 12.3) was incubated as described in Figure 3. After incubation the reaction was stopped by lipid extraction. An aliquot of the aqueous phase was applied to a 0.1-mm cellulose thin-layer plate (Brinkmann 300 mn). The developing solvent was *tert*-butyl alcohol–water–formic acid (60:15:2, v/v). Dihydroxyacetone and dihydroxyacetone phosphate were used as standards and the areas corresponding to these were scraped and eluted with water; 1.5 ml of the eluate was counted with 15 ml of phosphor–Triton (2:1, v/v).

dihydroxyacetone-P to 1-alkyl-*sn*-glycero-3-P which is subsequently deacylated.

Discussion

In order to provide corroborative evidence indicating an exchange of hydrogen on DHAP during glyceryl ether synthesis, it would be essential to demonstrate that hydrogen from the aqueous environment becomes bound to the glyceryl ether molecule at C-3 of the DHAP moiety. The data presented here indicate that each molecule of *O*-alkyl lipid formed in the presence of tritiated water acquires tritium activity consistent with the addition of 1 H/mol of *O*-alkyl lipid formed. The data also indicate that the tritium is gained at C-3 of DHAP of *O*-alkyl-DHAP.

In addition to being consistent with the findings of others that acyl-DHAP is a precursor of *O*-alkyl lipid, other facets of this study can be summarized as follows. In the *Tetrahymena* system, acyl-DHAP leads to the formation of acyl-DHA and to DHA; the formation of DHA proceeds *via* a hydrogen exchange and is CoA dependent; in the presence of hexadecanol, acyl-DHAP is preferentially used in *O*-alkyl lipid formation, a reaction, or a series of reactions, which involve a hydrogen exchange; the preferential utilization of acyl-DHAP in *O*-alkyl lipid formation is reflected in reduced DHA formation. We do not consider the data presented to be evidence that acyl-DHA is necessarily the only precursor of DHA.

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4-Sphingenine Derivatives in Wheat Flour Lipids†

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ABSTRACT: *erythro*-4-Sphingenine and *erythro*-sphinganine were isolated from hydrolysates of wheat flour cerebrosides. Derivatives of both these bases, containing cis or trans double bonds at C_8, were also isolated. Argentation chromatography, gas–liquid chromatography, infrared spectroscopy, partial hydrazine reduction, ozonolysis and hydrogenolysis were used for identification of the new bases. The presence of a trans double bond at C_4 is typical of animal long-chain bases, but has not previously been described in plant systems.

In sphingoglycolipids of plants the known long-chain bases (LCB)[1] are derivatives of sphinganine and 4*D*-hydroxysphinganine (Carter *et al.*, 1961a). On the other hand, the LCB typical of animal systems, 4-sphingenine, has not been found in higher plants. The present report shows that wheat flour cerebrosides contain 4-sphingenine and its derivatives.

Materials and Methods

Isolation of Wheat Long-Chain Bases. Two samples of wheat flour were studied: 2.3 kg of Manitoba II (1969) and 1.5 kg of White Rose (1970), both of which were obtained from the State Granary (Helsinki). They had not been subjected to any previous chemical treatment.

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[1] Abbreviation used is: LCB, long-chain bases. The nomenclature used for the LCB's is that proposed by the IUB Commission (1967).