The Mechanism of Ether Bond Formation in O-Alkyl Lipid Synthesis in Ehrlich Ascites Tumor

UNUSUAL CLEAVAGE OF THE FATTY ACID MOIETY OF ACYL DHYDROXYACETONE PHOSPHATE*

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We have previously presented evidence for the formation of 1-O-alkyl dihydroxyacetone-P from acyl dihydroxyacetone-P via the initial formation of an intermediate 1-O-alkyl endiol of acyl dihydroxyacetone-P. This reaction involves a stereospecific exchange of the pro-R hydrogen of the acyl dihydroxyacetone-P moiety without change in configuration. The fatty acid is replaced by a long chain fatty alcohol which retains the oxygen of the primary carbinol. In the absence of fatty alcohol, water substitutes and the product is dihydroxyacetone-P which has also exchanged the pro-R hydro- 

with retention of the primary carbinol oxygen (10). The results of these studies suggested that O-alkyl DHAP might be formed by a reaction in which fatty alcohol was substituted directly for the fatty acid of acyl DHAP. Further experiments demonstrated, however, that a mechanism more complex than a simple substitution was involved. It was first shown that incubation of [1,3-3H,1,3-14C2]DHAP in an O-alkyl lipid-synthesizing system resulted in the formation of O-alkyl glycerols which had exchanged one of the hydrogens of the C-1 of the DHAP moiety of acyl DHAP (1). It was further shown by separate treatment of the labeled substrate, DHAP, with fructose 1,5-diphosphate aldolase and glyceraldehyde 3-P isomerase that the hydrogen which was removed had the same stereospecificity as the hydrogen removed by isomerase (pro-R hydrogen) (2). In addition, the hydrogen lost during O-alkyl lipid synthesis was replaced by another from the medium without inversion of configuration of the substituents of C-1 of the DHAP moiety (4, 5, 11, 12). These findings are consistent with an endiol intermediate (6). A precedent for this mechanism may be found in the action of isomerase in the reversible reaction between DHAP and glyceraldehyde 3-P which proceeds via an endiol mechanism with labilization of the pro-R hydrogen and without inversion of configuration of the hydrogens of C-1 of DHAP (13, 14). The aldolase reaction, on the other hand, in which the pro-S hydrogen of C-1 of DHAP is labilized, proceeds via the initial formation of a Schiff base between substrate and enzyme (15-19). Such a Schiff base mechanism involving acyl DHAP could neither be demonstrated in our laboratory nor could it be demonstrated by other investigators (6, 20). Furthermore, the lack of inversion of configuration and the stereospecificity of the reaction with retention of configuration militated against various kinds of direct substitution reactions. Additional studies showed that the presence of the fatty alcohol in the reaction mixture is not required for the hydrogen exchange and that, in the absence of fatty alcohol, water substitutes instead (reaction III) with the result that the product of the reaction is DHAP which has also stereospecifically exchanged the pro-R hydrogen (7).

The experimental observations detailed above led us to postulate the endiol mechanism shown in Fig. 1. In the ordinary hydrolysis of a carboxyl ester bond it is known that the carboxyl oxygen is retained by the fatty acid moiety and that the other oxygen is retained in the primary carbinol of the alcohol (21). As can be seen in reaction IV, however, an absolute requirement for the endiol mechanism is that the fatty acid which is removed from acyl DHAP retain both carboxyl oxygens. Therefore, demonstration of an unusual hydrolysis involving the release of fatty acid containing both
carboxyl oxygens would support the postulated mechanism. The present investigation addresses this problem.

EXPERIMENTAL PROCEDURES

Materials

Lithium aluminum hydride and N-methyl-N'-nitro-N-nitrosoguanidine were obtained from Aldrich. Glycolic acid and oxalyl chloride were from Eastman; chloroacetic acid was obtained from Sigma. Palmitoyl chloride was obtained from Fisher; BSTFA, trimethylchlorosilane, and OV 17, n-dimethyl 4-methyl-3-pentanone were obtained from Aldrich. Glycolic acid and oxalyl chloride were prepared by reacting 2.5 g of N-methyl-N'-nitro-N-nitrosoguanidine and was used later for the synthesis of [14C]palmitoyl chloride.

Methods

Synthesis of [14C]Glycolic Acid—The method was derived from the work of Eichloff (24). To a solution of 1.125 g of monochloroacetic acid in 6 ml of [14C]water, 0.4 g of metallic sodium was cautiously added at room temperature under a stream of nitrogen. The resultant pH was approximately 7.0. Assay of the reaction mixture for glycolic acid (25) revealed a yield of 56.9%. The remaining metal tube. The bottom portion of the tube containing the solution was immersed in a sand bath and kept at 100 °C for 48 h. At the end of this period, the pH was approximately 7.0. Assay of the reaction mixture for glycolic acid (25) revealed a yield of 56.9%. The remaining residue was dried at 100 °C in an oven and passed through a column of 36 g of Dowex 50-X2-H⁺, wet weight. Additional water was passed through the column until the effluent was pH 6.5. The resulting solution of glycolic acid was dried at reduced pressure at 45 °C and then under high vacuum overnight. A yellow residue remained which was shaken with 200 ml of ethyl ether. Some insoluble material in the ethyl ether solution was removed by filtration. Appropriate aliquots of the product were dried under nitrogen and then lyophilized under high vacuum for 10 min, and the trimethylsilyl ether derivative was prepared by adding 0.2 ml of anhydrous pyridine and 0.2 ml of BSTFA followed by heating for 1 h at 60 °C. The product was analyzed by gas-liquid chromatography and gave a major peak with a retention time coinciding with that of authentic glycolic acid-TMS. The fragmentation pattern of the mass spectrum of the [14C]TMS derivative was identical with that of authentic glycolic acid-TMS except for a two-atomic mass unit difference for appropriate ions.

Synthesis of [14C]Hexadecanoyl DHAP—The [14C]glycolic acid was converted to [14C]palmitoyl acetyl chloride by the method of Shlenk et al. (26). The product was analyzed by direct probe insertion mass spectrometry; qualitative analysis of the ions at m/z 239 \([\text{CH}_3(\text{CH}_2)_8\text{C}=\text{O}]^+\) and at m/z 241 \([\text{CH}_3(\text{CH}_2)_8\text{C}(\text{C}^13\text{O})]^+\) indicated that the carbonyl oxygen of the hexadecanoic acid moiety contained negligible quantities of [13C]. Millimolar acetyl chloride was converted to 1-palmitoyl-3-diazooctadecanoic acid (26) which was in turn converted to hexadecanoyl DHAP by the method of Hafija (23). Preliminary identification and purity of the product were assessed by thin layer chromatography on silica gel. The developing solvent was chloroform/methanol/water (85:35:5, v/v/v). A single spot was detected which had the mobility of authentic hexadecanoyl DHAP and which gave a positive reaction for phosphate with the spray of Dittmer and Lester (27).

Conversion of [14C]Hexadecanoyl DHAP to the Methoxime-Trimethylsilyl Ether Derivative of Hexadecanoyl DHA—Hexadecanoyl DHAP, 410 mg, was dissolved in 1 ml of 0.05 M borate buffer, pH 8.2, together with 0.5 mg (20 units) of bacterial alkaline phosphatase. The reaction mixture was incubated overnight at 37 °C and extracted by the method of Bligh and Dyer (28). The lower phase was removed, and the upper phase was washed once with blank lower phase. The combined lower phases were filtered through Whatman No. 42 paper, dried under nitrogen, and subjected to high vacuum for 10 min. To the dried sample, 200 μl of a 10% solution of methoxamine HCl in anhydrous pyridine was added. The solution was heated for 30 min at 60 °C, dried under a stream of nitrogen, and then under high vacuum for 10 min. To the methoxime derivative of [14C] and [3H]hexadecanoyl DHAP, 200 μl of BSTFA containing 1% trimethylchlorosilane were added. The mixture was then reacted at 60 °C for 30 min and the product was used directly for analysis by GC-MS.

Incubation Procedures—The standard incubation mixture was made by combining 150 nmol of [14C]hexadecanoyl DHAP in chloroform/methanol (9:1, v/v) with 50 nmol of [3H]hexadecanoyl DHAP in chloroform/methanol (9:1, v/v) and 0.04 M NaF and Ehrlich ascites tumor cell microsomes (1.5 mg of protein, prepared as previously described (11)) were added to give a final volume of 5 ml. It was noted that protein concentrations higher than 300 μg/ml were inhibitory. Incubation was carried out with agitation at 37 °C for 2 h. At the conclusion of the incubation, an internal standard consisting of either 10 or 20 μg of heptadecanoic acid was added. The incubation was stopped by extraction according to the method of Bligh and Dyer (28) using 1 N HCl instead of water. Assay of O-alkyl lipid synthesis was carried out by counting the activity of the total lipid extract followed by thin layer chromatography of an aliquot on 0.25-mm silica gel layers using chloroform/methanol/ethyl acetate/acetic acid (85:35:5:2, v/v/v/v) as the developing solvent (29). Areas corresponding to O-alkyl DHAP and fatty alcohol were scraped into scintillation vials. One-half ml of water was added and then 10 ml of Biofluor. Each sample was dispersed by sonication and the radioactivity was measured by liquid scintillation. Corrections for quenching and self-absorption due to silica gel were made by comparing the total counts recovered by thin layer chromatography with those obtained from the total lipid extract. In later...
experiments O-alkyl DHAP was assayed by the method of Davis and Hais (20). Similar results were obtained by both methods.

For determination of the $^{18}$O content of the free fatty acid fractions, an aliquot containing 80% of the total lipid extract was dried and dissolved in 6 ml of hexane. To isolate fatty acids, the hexane solution of the lipids was extracted with 6 ml of 0.1 N sodium hydroxide in 50% methanol containing bromphenol blue indicator. The upper phase was completely removed with several hexane washes and discarded. The bottom phase was acidified to pH 3 with 1 N HCl and the fatty acids were isolated by means of three hexane extractions. The combined extracts were dried and the fatty acids were methylated with an ethereal solution of diazomethane.

Alkaline methanolysis was carried out by the method of Hajra (30).

**Mass Spectrometry**—These studies were performed on a Hewlett-Packard model 5982 mass spectrometer in combination with a Hewlett-Packard model 5933 data system using either gas chromatography or direct probe insertion for introduction of the sample for analysis in the electron impact mode. Quantitative measurements were carried out by selected ion monitoring of specific ions in which the area under each peak was compared to the area from a corresponding peak in the internal standard. In the mass spectrometer the electron energy was 70 eV and the source temperature was 180 °C. For gas chromatography, separation of the methoxime-TMS derivative of O-alkyl DHA was carried out on a glass column (4 feet × 4 mm, inner diameter) packed with 3% OV1 on 100/200 mesh Gas-chrom Q using a column oven temperature of 240 °C. For analysis of the fatty acid methyl esters a glass column (6 feet × 4 mm, inner diameter) packed with 10% SP2330 on 100/200 mesh Chromosorb W/AW was employed at a temperature of 200 °C. In both cases the injector temperature was 250 °C, the all-glass jet separator and interface lines were 250 °C, and the helium flow rate was 30 ml/min.

**RESULTS**

I. Validation of the Structure of $[^{18}\text{O}]$Hexadecanoyl DHAP

A. Quantitation and Localization of $^{18}$O Bound to Hexadecanoyl DHAP by Use of the Methoxime-TMS Derivative of Hexadecanoyl DHA—Both $[^{18}\text{O}]$ and $[^{15}\text{O}]$hexadecanoyl DHAP were converted to their methoxime-TMS derivatives as detailed in the previous section. Analysis of both substances by GC-MS revealed identical fragmentation patterns with the exception that fragments containing $^{18}$O were shifted upward by two mass units. For evaluation of the overall $^{18}$O content, a peak representing loss of one methyl group from the molecular ion (M-15) was used in both cases. Quantitation of these peaks (m/z 414 for the $^{18}$O and m/z 416 for the $^{15}$O derivatives) indicated that the $[^{18}\text{O}]$hexadecanoyl DHAP contained 85.4% $^{18}$O after correction for the natural abundances of carbon, nitrogen, oxygen, and silicon isotopes. The location of the $^{18}$O was determined by examination of the ions at m/z 101, 103, and 105. By reference to Fig. 2 it can be seen that the m/z 101 ion contains the oxygen at C-1 of DHA which is putatively labeled with $^{18}$O. If $^{18}$O is incorporated at C-1 of DHA, the m/z 101 ion is shifted to m/z 103; thus reducing the size of the peak at m/z 101 and increasing the size of the peak at m/z 103 by an amount proportional to the $^{18}$O present at C-1. Any $^{18}$O bound to C-3 of the DHAP moiety likewise would have increased the peak at m/z 103 (CH$_3$O-TMS) to m/z 105. The results were compatible with $^{18}$O being present only at C-1 and not at C-3. Furthermore, evaluation of the intensity of the ion at m/z 239 ([CH$_3$(CH$_2$)$_7$]$_2$C=O) indicated that no significant amount of $^{18}$O was incorporated into the carbonyl oxygen of the hexadecanoic acid moiety. Additional measurement of the $^{18}$O content of this carbonyl oxygen was obtained as indicated below by alkaline methanolysis of hexadecanoyl DHAP.

B. Evaluation of the $^{18}$O Content of the Carbonyl Oxygen in the Carboxylic Ester Group of Synthetic $[^{18}\text{O}]$Hexadecano- 

DHA Following Alkaline Methanolysis—As shown in Table I, cleavage of $[^{18}\text{O}]$hexadecanoyl DHAP by means of alkaline methanolysis yielded methyl hexadecanoate which contained essentially no $^{18}$O. This indicates that the carbonyl oxygen of $[^{18}\text{O}]$hexadecanoyl DHAP did not contain $^{18}$O and that breakage of the substrate occurred between the acyl to oxygen bond. In order to demonstrate that these results did not occur because of exchange of the carbonyl oxygens of the hexadecanoic acid with the medium, additional experiments were performed. Hexadecanoic acid containing approximately 50% $^{18}$O in the carboxyl group was methylated with diazomethane, a procedure which leaves the carbonyl oxygens intact. The results are shown in Table II. Examination of the
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Table I

| Experiment | 18O | 16O |
|------------|-----|-----|
| 1. ['8O]Hexadecanoyl DHAP before alkaline methanolysis | 85.4 | 14.6 |
| 2. Hexadecanoic acid derived from alkaline methanolysis of ['8O]Hexadecanoyl DHAP | 0 | 100 |
| 3. Hexadecanoic acid derived from alkaline methanolysis of ['8O]Hexadecanoyl DHAP | 0.2 | 99.8 |

*The values were obtained as described in the text.

**This determination was made to measure the amount of 18O present from unknown sources in addition to the natural abundance.

**As detailed in the text, hexadecanoyl DHAP was treated by alkaline methanolysis which breaks the carboxylic ester group at the acyl bond and yields methyl hexadecanoate which retains only the carbonyl oxygen.

Table II

Determination of the stability of the 18O in [18O]hexadecanoic acid

| Experiment | Per cent with no 18O atoms (18O only) | Per cent with one 18O atom | Per cent with two 18O atoms |
|------------|-------------------------------------|--------------------------|---------------------------|
| 1. Untreated ['18O]hexadecanoic acid methyl ester | 56.7 | 37.2 | 6.0 |
| 2. Same as 1 after alkaline methanolysis | 74.4 | 25.2 | 0.4 |
| 3. Calculated value from experiment 1 if one carboxyl oxygen is lost by alkaline methanolysis | 75.8 | 24.6 | 0 |
| 4. ['18O]hexadecanoic acid after a 2-h incubation in microsomes | 61.5 | 38.5 | 0.0 |

[18O]Hexadecanoic acid was prepared by reacting palmitoyl chloride with [18O]H2O. The methyl ester was made by reaction with diazomethane which does not alter the oxygen content of the product.

*If both carboxyloxgens are randomly labeled with 18O, the expected result after alkaline methanolysis would be that no molecules would contain two 18O atoms. The expected per cent containing one 18O atom in the present case after acyl bond cleavage by alkaline methanolysis would be as follows: 37.2/2 = 18.6 (see "1" and "3" above).

*After incubation, the fatty acids were extracted as described under "Methods" and methylated with diazomethane.

*The values were corrected for the endogenous microsomal fatty acid content by using the results of an incubation containing no ['18O]hexadecanoyl DHAP.

*Not measured.

18O methyl hexadecanoate by selected ion monitoring MS of m/z 74, 76, and 78 (31) revealed that 56.7% of the fatty acid contained no 18O, 37.2% contained one 18O, and 6% contained two 18O atoms. After alkaline methanolysis of the methyl ester there was loss of half of the 18O, demonstrating that the latter was distributed approximately equally between the two carboxyloxgens and that there was no loss of carboxyl 18O to the medium. It was concluded that the data in this and in the preceding section jointly indicate that all of the 18O of the synthetic [18O]hexadecanoyl DHAP was located at the C-1 of the DHAP moiety. In addition it was shown that incubation of [18O]hexadecanoic acid in Ehrlich ascites tumor cell microsomes did not result in loss of 18O (Table II).

C. Nonenzymatic Release of Hexadecanoic Acid from [18O] Hexadecanoyl DHAP in Buffer—The nonenzymatic release of hexadecanoic acid from [18O]hexadecanoyl DHAP was measured for the purpose of correcting data obtained in experiments which utilized an O-alkyl lipid-synthesizing system. To determine this, [18O]hexadecanoyl DHAP, 70 nmol, and an octadecanoic acid internal standard, 352 nmol, were added to 1 ml of phosphate buffer and incubated at 37 °C for 2 h. The sample was extracted by the method of Bligh and Dyer (28). An identical sample was extracted without incubation. Another sample contained the internal standard only. The fatty acids were isolated by thin layer chromatography and methylated with diazomethane. The fatty acid methyl esters were analyzed and quantitated by selected ion monitoring GC-MS using ions at m/z 74, 76, 270, and 272. It was determined that only 0.3 nmol was recovered as [18O]hexadecanoic acid after 2 h of incubation (0.41%). The total free hexadecanoic acid present was 4.96 nmol (7.1% of total hexadecanoyl DHAP).

II. Demonstration of an Unusual Cleavage of Acyl DHAP in O-Alkyl DHAP Synthesis

A. The Effect of NADPH—Hexadecanoyl DHAP, 150 nmol, containing 85.4% of 18O at C-1 of DHAP was incubated in an O-alkyl lipid-synthesizing system as described under "Methods." The free hexadecanoic acid present after incubation was quantitated as the methyl ester, and its 18O content was measured by GC-selected ion-monitoring MS of the ions at m/z 74 and m/z 76. These ions, as shown in Fig. 3, represent, respectively, the McLafferty rearrangement (31) of methyl hexadecanoate containing 18O only or one 18O atom and one 16O atom. The data were corrected for the natural abundances

Fig. 3. Electrospray ionization mass spectrum of methyl hexadecanoate. Mass spectrometry conditions are the same as in Fig. 2. A, methyl hexadecanoate derived from standard hexadecanoic acid after extraction and methylation as described under "Methods"; B, methyl hexadecanoate isolated from the microsomal O-alkyl lipid-synthesizing system using a substrate of hexadecanoyl-DHAP which had 85.4% of the oxygen at C-1 of the DHAP moiety of hexadecanoyl DHAP labeled with 18O.
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Data showing retention of both carboxyl oxygens by hexadecanoic acid liberated from hexadecanoyl DHAP during O-alkyl DHAP synthesis and data showing the effect of NADPH

Incubations, isolation of products, and GC-MS were carried out as described under "Methods."

| Conditions | A | B | C | D |
|-----------|---|---|---|---|
| 1. Complete system | 197.6, 196.7 | 4.5, 5.3 | 118.2, 104.3 | 4.5, 5.5 |
| 2. Complete system minus hexadecanoyl DHAP | 73.9, 88.1 | 1.0, 0.9 | N/A* | N/A |
| 3. Complete system plus NADPH (1.725 mM) | 222.8, 200.8 | 29.1, 27.6 | 138.9, 102.7 | 7.2, 8.3 |

* These values were obtained by GC-MS using selected ion monitoring of m/z 74.

The numbers in column B, which were calculated from those in column A-1, show the net quantities of [18O]hexadecanoic acid derived from [18O]hexadecanoyl DHAP. These results reveal without ambiguity that magnesium did not inhibit lipolysis of [18O]hexadecanoyl DHAP which proceeds by the usual mechanism, i.e. breakage of the acyl to oxygen bond.

The release of hexadecanoic acid which retained both carboxyl oxygens was substantial and was, furthermore, consonant with the yield of O-alkyl DHAP (column D). The release of this 18O-containing species of hexadecanoic acid was almost completely inhibited by magnesium. Hence, both O-alkyl lipid synthesis and the release of hexadecanoic acid which was cleaved at the DHAP C-1 to oxygen bond were simultaneously inhibited by magnesium. It may also be noted that the ordinary hydrolysis at the acyl to oxygen bond exceeded the unusual hydrolysis which occurs at the DHAP C-1 to oxygen bond.

In column D, it can be seen that the yield of O-alkyl DHAP was 16.1 ± 1.7 nmol in the absence of magnesium. These amounts, as expected, are somewhat less than the amounts of hexadecanoic released by the unusual cleavage (column C). In the presence of magnesium, the yield of O-alkyl DHAP was negligible.

C. Experiments With and Without Hexadecanol—According to the mechanism which we have postulated for O-alkyl DHAP synthesis (Fig. 1), the unusual fatty acid cleavage of acyl DHAP does not require the presence of long chain fatty alcohol. This aspect of the mechanism was tested by measuring the yield of [18O]hexadecanoic acid from [18O]acyl DHAP in the presence and absence of added hexadecanol. In three experiments in which [18O]hexadecanoyl DHAP was incubated in an O-alkyl DHAP-synthesizing system containing hexadecanol, 18.9 ± 1.5 nmol of O-alkyl DHAP was formed and 22.5 ± 2.1 nmol of hexadecanoic acid was recovered which had retained both carboxyl oxygens. When hexadecanol was omitted, 16.1 ± 1.3 nmol of hexadecanoic acid was recovered which had retained both carboxyl oxygens. Thus the results showed that the unusual cleavage occurred in the absence of added hexadecanol. The reason for the slightly lower release of [18O]hexadecanoic acid in the absence of added hexadecanol is not clear. One could postulate that the unusual cleavage
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Data showing retention of both carboxyl oxygens by hexadecanoic acid liberated from hexadecanoyl DHAP during O-alkyl DHAP synthesis and data showing the effect of magnesium ion

Incubations and isolation of products by GC-MS were carried out as described under "Methods."

| Experiment | Conditions | A | B | C | D |
|------------|------------|---|---|---|---|
|            |            | 1 \[^{18}O\] | 2 \[^{18}O\] | Hexadecanoic acid from breakage of acyl to oxygen bond of hexadecanoyl DHAP | Hexadecanoic acid from breakage of DHAP C-1 to oxygen bond of hexadecanoyl DHAP | Yield of O-alkyl DHAP |
| 1 (7)      | Complete system            | 106.6 ± 12.5 | 17.5 ± 3.8 | 33.3 ± 16.7 | 19.7 ± 4.5 | 16.1 ± 1.7 |
| 2 (3)      | Complete system plus 40 mM Mg\(^{2+}\) | 117.0 ± 23.6 | 2.2 | 34.1 ± 8.6 | 1.7 | 0.33 |
| 3 (3)      | Complete system minus \[^{18}O\]hexadecanoyl DHAP | 66.3 ± 12.2 | 0.2 | N/A | N/A | 0.10 |
| 4 (3)      | Complete system minus \[^{18}O\]hexadecanoyl DHAP plus 40 mM Mg\(^{2+}\) | 55.1 ± 12.0 | 0.4 | N/A | N/A | 0.13 |

* The number of experiments is given in parenthesis.

** The numbers represent the difference between the hexadecanoic acid content of the complete system and the complete system without hexadecanoyl DHAP. Mean corrected values were used.

** All values were corrected for nonenzymatic cleavage of \[^{18}O\]hexadecanoyl DHAP. The final values were calculated using a value of 85.4% for the \[^{18}O\] content of hexadecanoyl DHAP.

** N/A, not applicable.

The endiol mechanism which we have proposed has gained support elsewhere. Davis and Hajra (20) have postulated that an electrophilic enol ester may be the intermediate which is attacked by the nucleophilic alcoholate ion. Presumably, this mechanism (20) is similar, if not identical, with the one which we proposed. These authors also suggest, and we concur, that the enol ester intermediate might be 1,2-cyclic, a possibility which is also in accord with the available data and which represents a slight variation of the basic mechanism shown in Fig. 1.

The present investigation augments previous experimental evidence supporting the endiol concept of O-alkyl lipid ether bond synthesis. We have shown here that loss of the fatty acid of acyl DHAP in the process of O-alkyl DHAP synthesis occurs via an unusual cleavage of the fatty acid at the DHAP C-1 to oxygen bond, i.e. the C-1 oxygen of DHAP remains with the fatty acid. This loss of the DHAP C-1 oxygen is in accord with the endiol mechanism for O-alkyl lipid bond formation which we have proposed (5, 6) and is also in accord with the previously demonstrated fact that the oxygen of the fatty alcohol is retained by the product, O-alkyl DHAP (10). In addition to this unusual cleavage, acyl DHAP also undergoes an ordinary hydrolysis at the acyl to oxygen bond in the crude microsomal O-alkyl lipid-synthesizing system used in these studies. This ordinary hydrolysis is presumably mediated by a phospholipase A-1. When O-alkyl DHAP synthesis is inhibited by NADPH or magnesium, only the ordinary hydrolysis occurs. Further support for the endiol mechanism derives from the demonstration in the present study that the fatty alcohol is not necessary for the unusual cleavage of the fatty acid of acyl DHAP. Neither is the fatty alcohol obligatory, as demonstrated in a previous study (6), for the DHAP C-1 pro-R hydrogen exchange.

Davis and Hajra (32) have demonstrated that Ehrlich ascites cell microsomes can catalyze the exchange of the acyl group of acyl DHAP with free fatty acids and have postulated that this reaction is mediated by the same enzyme which is responsible for the synthesis of O-alkyl DHAP. The role which this reaction may play in O-alkyl DHAP synthesis has not been determined at this time.

In earlier investigations, we have shown that the number of hydrogen atoms lost from C-1 of acyl DHAP substantially exceeds the number of molecules of O-alkyl DHAP formed (7). The difference is accounted for by the formation of DHAP which has exchanged the C-1 pro-R hydrogen. By inference, the amount of fatty acid liberated with retention of both carboxyl oxygens should also exceed the amount of O-alkyl DHAP formed. However, the incubation conditions used in the present investigation produced much higher yields of O-alkyl DHAP than we have obtained under previously used conditions. This may explain the closer stoichiometric equivalence of O-alkyl DHAP production and \[^{18}O\]hexadecanoic acid release seen in the present experiments.

In the proposed mechanism, the step in which the hydrogen is lost is irreversible, as evidenced by the fact that acyl DHAP incubated in \[^{18}OH_2O\] does not become labeled (4). Therefore, the putative endiol intermediate is left to react with either a molecule of fatty alcohol or a molecule of water (7). The extra hydrogen in this reaction is accounted for by the formation of DHAP which has exchanged the pro-R hydrogen (7). Consequently, if the intermediary endiol does not accumulate, a necessary condition for the proposed mechanism is that the total amount of \[^{18}O\]hexadecanoic acid liberated should equal to the amount of pro-R hydrogen exchanged. Demonstrating this stoichiometric relationship, however, was beyond the scope of the present investigation and could not be addressed at the present time.

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