Hydrogen Exchange in the Formation of Dihydroxyacetone Phosphate from Acyl Dihydroxyacetone Phosphate in O-Alkyl Lipid Synthesis in Ehrlich Ascites Tumor Cell Microsomes

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We have previously presented evidence that acyl dihydroxyacetone phosphate is converted to O-alkyl dihydroxyacetone phosphate via an endiol intermediate which then accepts a fatty alcohol to form O-alkyl dihydroxyacetone phosphate. We have further proposed that, in the absence of fatty alcohol, the endiol derivative of acyl dihydroxyacetone phosphate reacts with water to form dihydroxyacetone phosphate. In support of this hypothesis, we have shown, in an O-alkyl generating system, that the amount of hydrogen released from acyl dihydroxyacetone phosphate in the formation of the endiol is greater than the amount of hydrogen lost from the total lipid present at the end of incubation. The discrepancy is greater in the absence of added hexadecanol. The balance of the hydrogen loss can be accounted for by the formation of a non-lipid substance which was identified as dihydroxyacetone phosphate.

It is currently accepted that O-alkyl lipids are synthesized enzymatically from fatty alcohol and dihydroxyacetone-P (1–8). This is a process which involves a number of intermediate reactions. Several of these intermediate reactions are currently understood while others await elucidation. The known steps in this pathway include the formation of acyl dihydroxyacetone-P from dihydroxyacetone-P and fatty acyl coenzyme A (9, 10). Acyl dihydroxyacetone-P and fatty alcohol then react to form O-alkyl dihydroxyacetone-P (11, 12). O-Alkyl dihydroxyacetone-P is subsequently reduced in the presence of NADPH to form O-alkyl glycerol phosphate (7, 8). In addition, another aspect of the enzymatic synthesis of O-alkyl dihydroxyacetone-P is central to the mechanism; it is known that, in the conversion of acyl dihydroxyacetone-P to O-alkyl dihydroxyacetone-P, the pro-R hydrogen of the C-1 of the dihydroxyacetone-P moiety is exchanged with hydrogen from the medium with retention of configuration (13–19). It is also known that the oxygen of the fatty alcohol is retained (20).

We have recently shown that the enzyme-catalyzed hydrogen exchange which occurs at C-1 of acyl dihydroxyacetone-P in the formation of O-alkyl dihydroxyacetone phosphate does not require the presence of fatty alcohol (21). This observation has led us to postulate the occurrence of a deprotonated intermediate between acyl dihydroxyacetone-P and O-alkyl dihydroxyacetone-P. The overall reaction may be envisioned as shown in Fig. 1. According to this scheme, the deprotonated intermediate reacts with fatty alcohol to produce O-alkyl dihydroxyacetone-P. Moreover, in the absence of fatty alcohol, the deprotonated derivative of acyl dihydroxyacetone-P accepts a hydroxyl ion from the medium instead of fatty alcohol. In either case, the fatty acid is liberated. This reaction, leading to the formation of free dihydroxyacetone-P which has exchanged the C-1 pro-R hydrogen, forms the subject of this report. The liberation of dihydroxyacetone-P with this exchanged hydrogen was previously suspected from two observations. First, when Tetrahymena pyriformis is the source of enzyme, coenzyme A-dependent [3H]H2O release greatly exceeds O-alkyl lipid synthesis with the difference seemingly accounted for by coenzyme A-dependent synthesis of unphosphorylated dihydroxyacetone which has exchanged 1 hydrogen (10). Second, in the Ehrlich ascites tumor cell system, [3H]H2O release from either acyl [1-(R)-3H]dihydroxyacetone-P, or from [1-(R)-3H]dihydroxyacetone-P plus coenzyme A, ATP, and magnesium, exceeds O-alkyl lipid synthesis (21). In both cases, the discrepancy is greater when fatty alcohol is not added to the system and O-alkyl lipid synthesis is decreased.

MATERIALS AND METHODS

[1-(R)-3H]Dihydroxyacetone-P, [1,3-14C]dihydroxyacetone-P, and their acylated derivatives were prepared as previously described (21). Chloroacetol phosphate was prepared by the method of Hartman and coworkers (22). Incubation procedures and trapping of [3H]H2O were carried out as previously described (17, 21). Lipids were extracted by the method of Bligh and Dyer (23) except that 0.5 M formic acid was used instead of water. Vitride, sodium bis[2-methoxyethoxy]alumnum hydride was obtained from Eastman (Rochester, NY) and used as described by Snyder et al. (24) to reduce various lipids to O-alkyl glycerol. Alkaline methanolation was carried out by the method of Hauri (4). ATP, coenzyme A, NADPH, NAD, fructose-1,6-diphosphate aldolase (rabbit), sn-glycero-3-phosphate dehydrogenase (type I, rabbit), and glycero kinase (175 units/mg of Candida mycoderma) were obtained from Sigma Chemical Co., St. Louis, MO. All other materials and methods were as previously described (17, 21).

In determining H and 14C activities of the aqueous fractions of the Bligh and Dyer (23) extracts of incubations, a 0.360-ml aliquot was counted in 10 ml of Biofluor (New England Nuclear Corp., Boston, MA). The activity of the substrate was determined by adding an aliquot to a standard incubation which was immediately extracted. Absolute activities were determined by means of internal standards.

RESULTS

Comparison of Detertritated Lipid Production Versus Coenzyme A-dependent [3H]H2O Production from [1-(R)-3H, 1,3-14C]Dihydroxyacetone-P. The purpose of the present investigation is to determine whether or not an enolized derivative of acyl dihydroxyacetone-P yields dihydroxyacetone-P which has exchanged the pro-R hydrogen. This sequence is
shown in Fig. 1, where the postulated enolized intermediate reacts with water in the absence of fatty alcohol to form dihydroxyacetone phosphate.

Starting with a mixture of \([1-(R)]-\text{H}\text{dihydroxyacetone-P}\) and \([1,3,\text{H}]\text{dihydroxyacetone-P}\) of known ratio, coenzyme A, ATP, magnesium, and Ehrlich ascites cell tumor microsomes in an O-alkyl lipid-synthesizing system, we have measured the enzymatic cofactor-dependent production of tritiated water, total lipids, alkali-stable lipids, O-alkyl glycerol, and the tritiated water produced from nonenzymatic spontaneous enolization of the substrate. Five separate experiments, each involving two to four replicate determinations, were performed in which different enzyme preparations or slightly different substrate mixtures were used for a total of 16 determinations. The total dihydroxyacetone-P (expressed as \(^{14}\text{C}\) activity) which eventually exchanged hydrogen with the medium was calculated from the net enzymatic \([\text{H}]\text{H}_2\text{O}\) yield divided by the \(^{3}\text{H}/^{14}\text{C}\) substrate ratio (Table I). No correction was made for the expected isotope effect. As shown previously (21), and again in the present investigation, the coenzyme A-promoted detritiation of dihydroxyacetone-P occurs independently of the presence of fatty alcohol. Both with and without fatty alcohol, the total detritiated dihydroxyacetone-P exceeds the quantity of detritiated lipid.

Recovery of label in these experiments was complete. The mean \([\text{H}]\text{H}_2\text{O}\) released plus the \(^{3}\text{H}\) remaining in the water phase plus the \(^{3}\text{H}\) of the lipid phase showed the following recoveries: \(+\text{ROH}, 104\%\); \(-\text{ROH}, 100\%\); coenzyme A, 99\%. The mean \(^{14}\text{C}\) in the water phase plus the mean \(^{14}\text{C}\) of the lipid phase showed the following recoveries: \(+\text{ROH}, 100\%\); \(-\text{ROH}, 97\%\); coenzyme A, 99\%.

In Column B of Table I, it is seen that the mean \(^{14}\text{C}\) activity of coenzyme A-dependent detritiated dihydroxyacetone-P is 10,993 dpm with and 10,516 dpm without added fatty alcohol. These numbers were corrected for tritium loss due to spontaneous enolization of the substrate. The spontaneous enolization plus other non-coenzyme A tritium release was determined from simultaneous incubations from which all cofactors were omitted. Spontaneous enolization averaged 7.3\% in these studies, while the total \([\text{H}]\text{H}_2\text{O}\) release averaged 19.1\%.

The \(^{14}\text{C}\) activity of detritiated lipids formed from labeled dihydroxyacetone-P is shown in Column C. These data were obtained by first dividing the total lipid phase \(^{13}\text{C}\) activity by the \(^{14}\text{C}/^{13}\text{C}\) of the substrate. This quotient, which gives the nondetritiated lipid \(^{14}\text{C}\) activity (i.e., acyl dihydroxyacetone-P), was subtracted from the total lipid \(^{14}\text{C}\) activity to give the \(^{14}\text{C}\) activity of all compounds which had lost the \(1-(R)^{-}\text{H}\). In the presence of hexadecanol, this activity averaged 7,136 dpm for 16 determinations and 2,124 dpm in the absence of added hexadecanol (\(p < 0.005\)). It is clear that, in the absence of fatty alcohol, most of the detritiated products of the incubation of 1-(\(R\))-\text{H} dihydroxyacetone-P which depend for their formation on coenzyme A, ATP, and magnesium are not present in the lipid phase and therefore can be present only in the aqueous phase of the extract. The quantity of detritiated compound or compounds present in the aqueous phase of the extract is the total detritiated dihydroxyacetone-P minus the detritiated lipid activity either in the presence or absence of hexadecanol, i.e., Column B minus Column C. In the situation where hexadecanol was not added, the mean \(^{14}\text{C}\) activity of detritiated compounds in the aqueous phase is 10,616 dpm - 2,124 dpm = 8,492 dpm.

Column D of Table I shows the activities in alkali-stable lipids. These data represent a more direct measurement of the relationship between total detritiated dihydroxyacetone-P activity and detritiated lipid activity. The mean activities of alkali-stable lipids in the presence and absence of hexadecanol were 4,975 and 1,400 dpm, respectively. The reason for the differences between these activities and those observed in the lipid extracts themselves is not clear.

Column E gives O-alkyl glycerol activity generated by the addition of NADPH to the incubation. These values are smaller than those obtained by alkaline methanolysis or by alkaline methanolysis of total lipid activity. The mean O-alkyl glycerol activities in the presence and absence of hexadecanol were 2,430 and 446 dpm, respectively. The discrepancy between these data and those obtained by alkaline methanolysis is, in part, due to incomplete hydrolysis of phosphate from O-alkyl glycerol-3-P or from O-alkyl dihydroxyacetone-P in the enzyme system. We conclude that the data are consistent with the hypothesis (Fig. 1) that a deprotonated derivative of acyl dihydroxyacetone-P is converted to a non-lipid substance if condensation with fatty alcohol to form O-alkyl dihydroxyacetone-P does not occur.

Based on the value for the net difference or increment in \(^{14}\text{C}\)-lipid dependent on added fatty alcohol, we have calculated that there would be approximately a 5\% decrease in the \(^{13}\text{H}/^{14}\text{C}\) ratio of the substrate \([\text{H},^{14}\text{C}]\text{dihydroxyacetone-P}\) at the end of incubation caused by a return to the water phase of detritiated \([\text{H}]\text{dihydroxyacetone-P}\) produced in the absence versus the presence of fatty alcohol. This again does not take into account a possible isotope effect which would favor the utilization of \(^{14}\text{C}\) species over \(^{13}\text{H}\) species and might magnify the fall in \(^{14}\text{C}\) ratio observed in the dihydroxyacetone-P in the water-soluble products of incubation. This expected \(^{13}\text{H}/^{14}\text{C}\) ratio of dihydroxyacetone-P in the water-soluble products at the end of incubation was calculated as follows:

\[
\text{Ratio} = \frac{\text{Total } ^{13}\text{H} \text{incubated} - [2]^{13}\text{H} \text{O} \text{released}}{\text{Total } ^{14}\text{C} \text{incubated} - \text{total lipids } ^{13}\text{H}}
\]

Using data obtained in the current experiments, the estimates are as follows. The mean substrate activity and \(^{13}\text{H}/^{14}\text{C}\) ratio for 16 experiments was 485,000 \(^{13}\text{H}\) and 84,600 \(^{14}\text{C}\) (ratio = 5.76). The mean \([\text{H}]\text{H}_2\text{O} \text{releases} = 93,760 (+\text{ROH}) \text{ and } 91,260 (-\text{ROH}). The total lipid phase mean \(^{13}\text{H}\) activities were 12,726 (+ROH) and 15,328 (-ROH). The total lipid phase \(^{14}\text{C}\) activities were 9,392 (+ROH) and 4,836 (-ROH). Therefore, the expected water phase ratios are 1) plus ROH (485,000 - 93,760 - 12,726) / (84,600 - 9,342) = 5.03, and 2) minus ROH (485,000 - 93,760 - 12,726) / (84,600 - 9,342) = 4.74. The percentage change in ratio is (5.03 - 4.74)(100)/5.03 = 5.8\%.

Change in \(^{13}\text{H}/^{14}\text{C}\) Ratio of Water-soluble Products of Incubation Due to the Reaction of a Postulated Endiol Intermediate with Water—As indicated above, a consequence of the reaction between a deprotonated intermediate of acyl dihydroxyacetone-P and water, as illustrated in Fig. 1, is the
The data were obtained from five groups of experiments. Three of these were carried out in quadruplicate, and two were carried out in duplicate. Four different substrate mixtures were used. The exact \(^{1}H/^{13}C\) activity ratios of these mixtures were 4.58, 6.49, 6.25, and 6.41. In each experiment, Ehrlich ascites tumor cell microsomes were preincubated for 15 min with chloroacetol phosphate (final concentration, 38 \(\mu\)M). Each incubation mixture contained, in a final volume of 0.620 ml, 0.5 ml of microsome in 0.1 M phosphate buffer, pH 7.2, 8 mM ATP, 4 mM magnesium chloride, 0.16 mM coenzyme A, 0.065 mM hexadecanol added in 1% Tween 80, and approximately 7.5 \(nmol\) of \([1,3-^{14}C]\) dihydroxyacetone-P. An equal number of simultaneous incubations was carried out in which hexadecanol was omitted, and another set was incubated in which all cofactors were omitted. Incubation was carried out for 2 h at 37\(^{\circ}\)C, after which NADPH, 2 \(\mu\)mol was added. Incubation was continued for another hour. The reactions were stopped by freezing in a dry ice-acetone bath, and the samples were lyophilized. The water evolved was trapped in a dry ice-acetone bath and two 0.36-ml aliquots were counted in 10 ml Biofluor. The freeze-dried reaction mixtures were extracted as described in the previous section, and 0.36 ml of the aqueous phase was counted. One aliquot of the lipid phase was counted. Another aliquot was treated by alkaline methanolysis as described above. A third aliquot was used to isolate O-alkyl glycerol by thin layer chromatography in hexane/ethyl ether/acetic acid (20:80:1, v/v/v). The O-alkyl glycerol band was eluted from the silica gel with ethyl gel with ethyl ether prior to liquid scintillation counting.

<table>
<thead>
<tr>
<th>A. Experiment</th>
<th>B. Total detrinitiated(^{d}DHA) as (^{14}C) activity</th>
<th>C. Detritiated(^{d}) compounds of DHAP in lipid phase as (^{14}C) activity</th>
<th>D. Alkali-stable lipid activity</th>
<th>E. O-Alkyl lipid activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. a + ROH (4)(^{c})</td>
<td>10,234</td>
<td>10,886</td>
<td>7,969</td>
<td>8,360</td>
</tr>
<tr>
<td>2. a - ROH (4)</td>
<td>12,810</td>
<td>3,090</td>
<td>2,870</td>
<td>469</td>
</tr>
<tr>
<td>3. a + ROH (3)</td>
<td>10,781</td>
<td>6,368</td>
<td>4,403</td>
<td>3,160</td>
</tr>
<tr>
<td>4. a - ROH (3)</td>
<td>9,375</td>
<td>1,955</td>
<td>670</td>
<td>480</td>
</tr>
<tr>
<td>5. a + ROH (2)</td>
<td>10,641</td>
<td>5,818</td>
<td>5,197</td>
<td>2,570</td>
</tr>
<tr>
<td>6. a - ROH (2)</td>
<td>8,859</td>
<td>621</td>
<td>619</td>
<td>290</td>
</tr>
<tr>
<td>7. a + ROH (4)</td>
<td>12,341</td>
<td>8,440</td>
<td>5,532</td>
<td>5,810</td>
</tr>
<tr>
<td>8. a - ROH (4)</td>
<td>11,019</td>
<td>2,908</td>
<td>1,450</td>
<td>610</td>
</tr>
<tr>
<td>Mean a + ROH (16)</td>
<td>10,993 (\pm) 911(^{d})</td>
<td>7,136 (\pm) 2,592</td>
<td>5,011 (\pm) 2,046</td>
<td>4,975 (\pm) 2,690</td>
</tr>
<tr>
<td>9. a - ROH (16)</td>
<td>10,516 (\pm) 1,785</td>
<td>2,124 (\pm) 980</td>
<td>1,400 (\pm) 1,052</td>
<td>446 (\pm) 118</td>
</tr>
</tbody>
</table>

\(^{d}\)Net enzymatic tritium release divided by substrate \(^{1}H/^{13}C\) ratio.

Hydrogen Exchange in Dihydroxyacetone Phosphate Formation

### Table II

Ratio of \(^{3}H/^{14}C\) of water-soluble products of incubation obtained following incubation of \([1,3-^{14}C]\) dihydroxyacetone-P in an O-alkyl lipid-synthesizing system

<table>
<thead>
<tr>
<th>Experiment</th>
<th>+ROH</th>
<th>-ROH</th>
<th>-CoA</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>(4)</td>
<td>9.37 (\pm) 0.09</td>
<td>8.45 (\pm) 0.09</td>
</tr>
<tr>
<td>2</td>
<td>(2)</td>
<td>9.02 (\pm) 0.02</td>
<td>8.41 (\pm) 0.10</td>
</tr>
<tr>
<td>3</td>
<td>(2)</td>
<td>8.86 (\pm) 0.06</td>
<td>8.81 (\pm) 0.04</td>
</tr>
<tr>
<td>4</td>
<td>(4)</td>
<td>9.06 (\pm) 0.28</td>
<td>8.56 (\pm) 0.15</td>
</tr>
<tr>
<td>5</td>
<td>(4)</td>
<td>8.92 (\pm) 0.11</td>
<td>8.42 (\pm) 0.06</td>
</tr>
<tr>
<td>Mean</td>
<td>9.05 (\pm) 0.20</td>
<td>8.63 (\pm) 0.17</td>
<td>9.31 (\pm) 0.15</td>
</tr>
</tbody>
</table>

Postulated formation of detrinitiated dihydroxyacetone-P. This should result in a lowering of the \(^{1}H/^{13}C\) ratio of the substrate which is more extensive in the absence of added fatty alcohol. A large number of determinations was carried out because it was clear that the effect, as indicated above, would be a theoretical change of 5%, and it was uncertain initially that a difference of this magnitude would, in fact, be measurable.

The data are presented in Table II. Since different substrate mixtures with different ratios were used in different sets of experiments, the ratios obtained were all normalized to a starting ratio of 10 for easier comparison and analysis of data. The results show that the \(^{3}H/^{14}C\) ratio of the aqueous phase at the end of incubation without cofactors was 931 \(\pm\) 0.15. In the experiments which contained added fatty alcohol, the ratio was 9.05 \(\pm\) 0.20. In the absence of fatty alcohol, the ratio fell to 8.53 \(\pm\) 0.17. Thus, as predicted by the hypothesis, not adding fatty alcohol to the system resulted in a lowering of the \(^{1}H/^{13}C\) ratio of 6% (\(p < 0.005\)).

Experiments with Acyl Dihydroxyacetone-P—Experiments in which acyl dihydroxyacetone-P was used as the starting substrate were in agreement with those obtained with dihydroxyacetone-P. However, difficulties in obtaining enough substrate precluded obtaining sufficient data for statistical analysis. The details of one such experiment are shown in Table III. The data were not corrected for spontaneous enolization and are therefore qualitative. It is shown that the yield of \([^{1}H]H_{2}O\) is the same in the presence or absence of hexadecanol, as previously reported (21). The activity of alkali-stable lipid is greater in the presence of added hexadecanol. The total detrinitiated substrate is greater than the alkali-stable lipid activity, especially in the absence of added hexadecanol, and the \(^{1}H/^{13}C\) ratio of the water-soluble products of incubation is less when hexadecanol is not added. One of the products of incubation of acyl dihydroxyacetone-P with Ehrlich ascites cell microsomes has been previously identified as dihydroxyacetone-P (21). It is clear, therefore, that the major product of the incubation of acyl \([^{1}H,^{13}C]Dihydroxyacetone-P\) with Ehrlich ascites tumor cell microsomes is dihydroxyacetone-P which is not deprotonated and which is the product of an ordinary hydrolysis (Table III). This cleavage is extensive and amounts to 60 to 80% of the starting substrate. It is quantitatively variable from experiment to experiment and decreases the reliability of measuring that portion of dihydroxyacetone-P which has been detrinitiated.

Identification of a Deprotonated Water-soluble Sub-
Comparison of the total detrinitiation of acyl \([1-(R)-^3H]\)-dihydroxyacetone-P with alkali-stable lipid activity generated in the presence and absence of added hexadecanol

Ehrlich ascites cell microsomes were preincubated with 0.5 mM chloroacetal phosphate for 45 min at 37°C. The complete system contained, in a final volume of 0.4 ml of 0.1 M phosphate buffer, pH 7.2, 0.1 mM acyl dihydroxyacetone-P (112,600 dpm of acyl \([1-(R)-^3H]\)-dihydroxyacetone-P and 11,100 dpm of acyl \([1,3,3^2C]\)-dihydroxyacetone-P) in 3.5 μl of 1% Triton X-100 and 80 nmol of hexadecanol added in 3.5 μl of 1% Triton X-100. After 2 h of incubation at 37°C, 1 mg of NADPH was added, and incubation was continued for another hour. The mixture was frozen in dry ice-acetone and lyophilized. \([^3H]H\)O was trapped in a dry ice-acetone bath. The lyophilized residue was extracted as described in Table I. An aliquot of the aqueous phase (0.36 ml out of 3.6 ml) was counted in 10 ml of Biofluor. The lipid phase was treated by alkaline methanalysis as described under "Materials and Methods" to measure activity of alkali-stable lipids.

<table>
<thead>
<tr>
<th>Substrate (acyl DHAP)</th>
<th>112,600 dpm</th>
<th>112,600 dpm</th>
<th>9,800 dpm</th>
<th>9,800 dpm</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(^3H)H(_2)O-liberated</td>
<td>(^3H)H(_2)O-liberated</td>
<td>(^3H)H(_2)O-liberated</td>
<td>(^3H)H(_2)O-liberated</td>
</tr>
<tr>
<td>Total detrinitiated DHAP as ([^14C]DHAP</td>
<td>2,473 dpm</td>
<td>2,473 dpm</td>
<td>8.78</td>
<td>8.06</td>
</tr>
<tr>
<td>Activity of water-soluble products of incubation</td>
<td>69,200 dpm</td>
<td>79,000 dpm</td>
<td>8.78</td>
<td>8.06</td>
</tr>
<tr>
<td>(^3H/14C) ratio in water-soluble products of incubation</td>
<td>69,200 dpm</td>
<td>79,000 dpm</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Alkali-stable lipids</td>
<td>1,520 dpm</td>
<td>1,520 dpm</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

\(^a\) \(([^3H]H\(_2\)O activity)/[^3H/14C of substrate].

The lipid phase was treated by alkaline methanalysis as described under "Materials and Methods" to measure activity of alkali-stable lipids.

Identification of a Water-soluble Detritiated Compound—Evidence that the peak having the chromatographic mobility of dihydroxyacetone-P does not disguise the presence of some other detrinitiated compound was obtained by isolating the peak chromatographically via cellulose thin layer chromatography, treating the material with glycerol-3-P dehydrogenase and again separating the products by thin layer chromatography on cellulose. Thus, dihydroxyacetone-P was largely, but not entirely, converted to glycerol-3-P. If the original peak is

![Fig. 2. Chromatographic and enzymatic identification of dihydroxyacetone phosphate](image-url)
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entirely made up of dihydroxyacetone-P which has, in part, lost tritium, both the residual dihydroxyacetone-P and the newly formed glycerol-3-P would have the same $^{3}H/^{14}C$ ratio. On the other hand, if the dihydroxyacetone-P peak masks another compound which has lost tritium, the newly formed glycerol-3-P will have a higher $^{3}H/^{14}C$ ratio, while the residual peak which would represent a suspected unknown compound would theoretically contain no tritium. The results are shown in Fig. 2. Fig. 2A shows the dihydroxyacetone-P peak isolated from the products of incubation in the microsomal system. Fig. 2B shows the results of treating the dihydroxyacetone-P with NADH and glycerol-3-P dehydrogenase. It can be seen that the larger peak is glycerol-3-P which contains the same $^{3}H/^{14}C$ ratio as the residual peak. The residual peak which migrates at the same rate as dihydroxyacetone-P has retained tritium.

DISCUSSION

The proposed mechanism of $O$-alkyl lipid synthesis depicted in Fig. 1 indicates that acyl dihydroxyacetone-P forms a positively charged enolate intermediate which may accept a long chain fatty alcohol. In the absence of fatty alcohol, a hydroxyl ion is accepted instead, the fatty acid is lost, and dihydroxyacetone-P is formed. Evidence in support of an endiol mechanism leading to the formation of $O$-alkyl lipid was recently presented (21). The observations supporting this mechanism include the following: acyl dihydroxyacetone-P stereospecifically and irreversibly loses the pro-$R$ hydrogen of the C-1 of the dihydroxyacetone-P moiety; the amount of hydrogen liberated in this reaction is independent both of the amount of fatty alcohol present in the medium and of the amount of $O$-alkyl lipid synthesized; hydrogen is regained from the medium without change in configuration of the C-1 substituents; oxygen at C-1 is derived from fatty alcohol (20).

The present investigation presents evidence to support the hypothesis that, in the absence of fatty alcohol, a hydroxyl ion is gained at C-1 with formation of dihydroxyacetone-P which has exchanged the pro-$R$ hydrogen at C-1. The evidence obtained was gathered from three independent and complementary experiments. First, we have shown that incubation of a mixture of $[1-(R)^{-3}H]$- and $[1,3^{-14}C]$dihydroxyacetone-P in an $O$-alkyl lipid-synthesizing system yields $[^{3}H]H_{2}O$ which is dependent on the prior formation of acyl dihydroxyacetone-P (i.e. it is coenzyme $A$, ATP, and Mg-dependent) but not on the presence of fatty alcohol. In this setting, we have further shown that the tritium loss cannot be accounted for by the total tritium loss measured in the lipids at the end of incubation and that this lipid tritium loss is considerably less than the loss of fatty alcohol. It is concluded that the remainder of the tritium loss must be accounted for by the presence of a tritium-poor water-soluble substance. The intermediacy of a lipid product in the total tritium loss is evidenced by the cofactor dependency of the reaction. It is of interest that the tritium loss measurable in the total lipids is always greater than that measured in the alkali-stable lipids or in the $O$-alkyl glycerol. Whether these differences represent technical losses or the presence of one or more unknown intermediates is not known. Second, if, as suggested above, a water-soluble deuteriated substance is formed, there should be a fall in $^{3}H/^{14}C$ ratio of the water-soluble materials, particularly in the absence of fatty alcohol. The data presented show that this is the case. Third, we have isolated a chromatographic peak from the water-soluble products of incubation which is tritium-poor and has the chromatographic mobility of authentic dihydroxyacetone-P. The material obtained from incubations not containing added fatty alcohol is relatively more depleted in tritium. Finally, we have documented that this tritium-poor material is dihydroxyacetone-P by treatment with $sn$-glycerol-3-phosphate dehydrogenase. Further experiments starting with acyl $[1-(R)^{-3}H]$- and $[1,3^{-14}C]$dihydroxyacetone-P lend further support to those in which labeled dihydroxyacetone-P was used.

It now remains to be shown that the fatty acid, according to the scheme presented in Fig. 1, is liberated with retention of both carboxyl oxygens. Since the primary carbinol oxygen of fatty alcohol is retained in the product, it is likely that the fatty acid would be liberated with retention of both oxygens.

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REFERENCES