Glyceryl Ether Synthesis from Long Chain Alcohols in Elasmobranch Stomach*

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SAMUEL J. FRIEDBERG† AND RONALD C. GREENE

From the Department of Medicine and Biochemistry of the Duke University Medical Center, Durham, North Carolina, 27706; the Cooperative Lipid Laboratory and Radioisotope Unit of the Durham Veterans Administration Hospital, Durham, North Carolina 27705; and the Department of Zoology, Duke University Marine Laboratories, Beaufort, South Carolina 28516

SUMMARY

1. The isolated stomachs of the dogfish, Squalus acanthias, and the skate, Raja erinacea, were found to incorporate long chain alcohols and acetate, but not long chain fatty acids, into glyceryl ethers.

2. The criterion for evaluation of incorporation of radioactive substrates into glyceryl ethers was the measurement of activity of individual isopropylidene derivatives separated by means of gas-liquid chromatography.

3. A pathway for incorporation of long chain alcohols involving aldehydes and fatty acids as intermediates was ruled out by means of studies which utilized mixtures of cetyl alcohol-1-14C and cetyl alcohol-1-3H.

4. An unexpected finding was a relatively greater enrichment in tritium as compared with 14C in the process of glyceryl ether synthesis from mixtures of cetyl alcohol-1-3H and cetyl alcohol-1-14C.

Many workers in the area of glyceryl ether and plasmalogen biosynthesis have speculated on the role of fatty acids, and especially of long chain aldehydes, as precursors. The possibility that fatty alcohols may be necessary intermediates has also been considered. In relation to the present investigation Keenan, Brown, and Marks found that stearyl alcohol was a better precursor for plasmalogen synthesis than stearic acid in the perfused dog heart (1). More recently, Thompson, in his work with the terrestrial slug Arion ater, found that fed palmitic acid-1-14C and long chain 14C-alcohols served equally well as substrates for glyceryl ether synthesis (2). Further studies by the same worker revealed that incorporation of 14C from palmitate into alkyl glyceryl ethers was initially more rapid than into alkenyl glyceryl ethers, and, when labeled alkyl glyceryl ether was consumed, there was a progressive appearance of labeled alkenyl glyceryl ether, which suggested that the alkenyl bond was formed from the slow dehydrogenation of the alkyl bond (3). Although these studies may indicate that alkenyl ether bonds are formed from the alkyl glyceryl ether, the pathway leading to the formation of the saturated ether bond is far from clear.

The present study deals with the biosynthesis of glyceryl ethers in the isolated stomach of the dogfish, Squalus acanthias, and of the skate, Raja erinacea. Evidence will be presented to show that, in these species, long chain alcohols are incorporated into alkyl glyceryl ethers and constitute a more direct precursor than long chain fatty acids.

METHODS AND MATERIALS

SOURCE OF EXPERIMENTAL ANIMALS—Live dogfish were obtained during the winter months, and skates during the summer through the facilities of the Duke University Marine Laboratories at Beaufort, North Carolina. The animals were maintained in a large outdoor tank for periods up to about 10 days, during which time they did not feed. No qualitative or quantitative differences were noted between experiments performed on freshly caught animals and those with animals kept for longer periods.

MATERIALS—Cetyl alcohol-1-14C was obtained from the Nuclear-Chicago Corporation and was purified by thin layer chromatography on silicic gel. The plates were developed in ligoear-ethyl ether-acetic acid, 80:20:1. The purified product was eluted from silicic gel with ethyl ether. Palmitic acid-1-14C was obtained from the New England Nuclear Corporation and was chromatographically pure by thin layer and gas chromatography. Oleoyl alcohol-1-14C, cetyl alcohol-16-14C, and cetyl alcohol-9, 10-3H were prepared from oleic acid-1-14C, palmitic acid-16-14C, and palmitic acid-9, 10-3H, respectively (New England Nuclear), by reduction with lithium aluminum hydride (4, 5). The final products were isolated and purified by thin layer chromatography. Purity was checked by gas-liquid chromatography.
The cetyl alcohol-9,10-3H was found to contain about 30% impurity in the form of shorter chain alcohols, mostly myristic. The starting material, palmitic acid-9,10-3H, was examined by gas-liquid chromatography and was also found to contain impurities in the form of shorter chain fatty acids. Sodium acetate-1-14C was obtained from New England Nuclear. Cetyl alcohol with tritium on the 1st carbon atom was prepared by reduction of palmitoyl chloride (Eastman) with tritiated sodium borohydride obtained from Volk (specific activity, 1 C per mmole). The method used was that of Chaikin and Brown (6). The total lipid recovered from the reaction with tritiated sodium borohydride was methylated with boron trifluoride methanol (7) to recover cetyl alcohol from cetyl palmitate, one of the reaction products. The tritiated cetyl alcohol was purified by thin layer chromatography on silica gel with the use of ligroin-ethyl ether-acetic acid, 70:30:1, as developing solvent. The approximate specific activity of the product was calculated from the specific activity of tritiated sodium borohydride. Cetyl alcohol-1-14C, 0.25 C per mmole, was mixed with cetyl alcohol-1-2H so that there was a large tritium excess. Several mixtures of this sort were prepared, each with different specific activities and with the use of different batches of 2H-cetyl alcohol. The mixtures were then repurified by thin layer chromatography. The activities of the resulting solutions were determined on a Packard Tri-Carb liquid scintillation spectrometer. The radioactive mixtures were then examined by gas-liquid chromatography as previously described (8), together with carrier cetyl alcohol. The cetyl alcohol peak was collected, and the ratio of tritium to 14C was measured. With the exception of cetyl alcohol-9,10-3H, the ratio in each mixture was found to be the same as that of the original material recovered by thin layer chromatography. All radioactive materials were made up as micellar solutions in 1% Tween 20 (Atlas Chemical Industries) or 1% Triton X-100 (Rohm and Haas). Standard fatty acid methyl esters, alcohols, and fatty acids were obtained from Applied Science Laboratories.

Incubations were carried out with dogfish and skate stomachs. The animals were killed by a blow on the head, and the stomach was excised, opened lengthwise, emptied, blotted, weighed, and placed in 100-ml round bottom boiling flasks containing 1 ml of Krebs-Ringer phosphate buffer per 1.5 g of tissue with 0.5% sodium taurocholate and the appropriate radioactive substrate. Sodium taurocholate was used as an additional emulsifying agent. The appropriate lipid fractions with boron trifluoride methanol (7), and separated by gas liquid chromatography as described by Hanahan, Ekholm, and Jackson (10). A 6-foot column (internal diameter, 0.1 inch) packed with 15% ethylene glycol succinate on Anakrom A/B was used. Individual fractions were collected with a Packard model 830 gas chromatography fraction collector. The cartridges were packed with a 1-cm column of 60 mesh glass beads and counted as previously described (8).

The isopropylidene derivatives of the glyceryl ethers were cleaved at the ether bond by heating in a sealed tube at 120° for 3 hours in 1 ml of 57% hydrogen iodide and glacial acetic acid, (1:1 by volume). An equal amount of water was added, and the resulting alkyl iodides were extracted with petroleum ether. The procedure was first tested with standard butyl alcohol and cetyl alcohol, which were converted to octadecyl and hexadecyl iodide, respectively. On thin layer chromatography the alkyl iodides moved to the solvent front on silica gel with the use of ligoine-ethyl ether-acetic acid, 90:10:1, as developing solvent. The identity of the unknown saturated long chain alkyl iodides from incubation products was confirmed by gas-liquid chromatography with the use of the system described above. The standards used for this identification were prepared from long chain alcohols treated with hydrogen iodide. Hexadecyl iodide prepared in this manner was in turn compared with hexadecyl iodide obtained from Eastman. Although alkyl iodides could be separated from other alcohol derivatives of the same number of carbon atoms and from isopropylidene derivatives by thin layer chromatography on silica gel (ligroin-ethyl ether-acetic acid, 90:10:1), their retention time by gas-liquid chromatography was the same as that of acetylated alcohols.

In the course of these studies it became necessary to demonstrate that tritium found in glyceryl ethers was confined to the 1st carbon atom of the alkyl ether side chain, i.e. that it remained on the 1st carbon atom of substrate cetyl alcohol. This was done by liberating the tritium on the 1st carbon atom through conversion of biosynthesized chimyl alcohol to palmitic acid. Accordingly, isopropylidene derivatives of glyceryl ethers, obtained from the incubation of the skate stomach with doubly labeled cetyl alcohol, were first treated with hydrogen iodide, as described above, with 7 mg of added carrier cetyl alcohol. The alkyl iodides were then refluxed for 3 hours in 30 ml of glacial acetic acid and 1 ml of acetyl anhydride containing an excess of silver acetate (11). After addition of an equal volume of water, the product (cetyl acetate) was extracted several times with petroleum ether and refluxed for 3 hours in 2 n methanolic KOH. After an equal amount of water was added, the product was again extracted with petroleum ether. The lipids were dried, and 0.3 ml of glacial acetic acid and 0.12 ml of a solution of 100 mg of CrO 3 in 80% acetic acid were added (12, 13). The reaction was allowed to proceed at room temperature for 18 hours. Water was added, and the lipids were extracted and isolated by thin layer chromatography on silica gel. The developing solvent was ligroin-ethyl ether-acetic acid, 60:40:1. The fatty acids obtained were recovered from the thin layer plates, methylated (7), and separated by gas liquid chromatography for assay of residual radioactivity.

Aldehydes were isolated as the dimethyl acetals by treating the appropriate lipid fractions with boron trifluoride methanol (7). The dimethyl acetals were isolated by thin layer chromatography on Silica Gel H (14), followed by gas-liquid chromatography with the system described above.
RESULTS

In the course of these studies it was found that glyceryl ether fractions, recovered from thin layer chromatograms after hydrolysis of total lipids, contained large amounts of radioactive contaminants. However, reisolation of these glyceryl ethers as the isopropylidene derivatives by thin layer chromatography was effective in removing most of the contaminating radioactive products. Final verification of the identity and purity of the isopropylidene derivatives, when necessary, was made by gas-liquid chromatography.

In a preliminary study, 100 μC of sodium palmitate-1-14C were injected intraperitoneally into a dogfish in order to evaluate the activity of different tissues in glyceryl ether synthesis. The animal was killed 2 hours later. The stomach was considerably more active than other intraperitoneal or extraperitoneal tissues in the incorporation of this isotope into diacyl glyceryl ethers. By thin layer chromatography it was found that 10 times as much palmitate-1-14C was incorporated into triglycerides as into diacyl glyceryl ethers, even though approximately equal quantities of these lipids were present on thin layer chromatograms of Squalus acanthias lipids. However, isolation of glyceryl ethers as the isopropylidene derivatives, by means of gas chromatography, revealed an extremely low and uncertain incorporation of palmitate into either chimyl alcohol or other glyceryl ethers.

Synthesis of Glyceryl Ethers from Acetate-1-14C—A dogfish stomach weighing 15 g was incubated for 8 hours with 2.5 μC of acetate-1-14C. The lipids were then extracted with chloroform-methanol, 2:1 (15). A small fraction of the lipids was separated by thin layer chromatography on silica gel with ligroin-ethyl ether-acetic acid, 90:10:1. Radioactivity was recorded as previously described (8). Of the incubated radioactivity, 9% was found in triglycerides, and 2.2% in the diacyl glyceryl ether fraction. In another study, isopropylidene derivatives were prepared and examined by gas-liquid chromatography. Fig. 1 shows the pattern of incorporation of acetate-1-14C (35 μC, 17.5 μmoles) into the isopropylidene derivatives of the glyceryl ethers. Most of the activity was found in the two saturated glyceryl ethers, chimyl and batyl alcohol. These results suggest that the acetate was incorporated into the side chain rather than into glycerol, as would have been indicated by more uniform distribution of activity. Similar observations on the lack of incorporation of acetate into glyceryl ether glycerol were first made by Karnovsky and Brumm (16). The total amount of activity incorporated into glyceryl ethers was very small and represented about 0.2% of the starting material (35 μmoles). Free alcohols were isolated by thin layer and gas-liquid chromatography, as previously described (8). A small but definite amount of radioactivity (5200 cpm) was found in a number of alcohols, mostly in stearyl and cetyl alcohol.

Synthesis of Chimyl Alcohol from Cetyl Alcohol and Selachyl Alcohol from Oleyl Alcohol—Cetyl alcohol-1-14C (2 to 10 μmoles and 2 to 10 μC) was incubated, in a number of different studies, with dogfish stomach under the same conditions as for acetate. The glyceryl ethers were isolated as the isopropylidene derivatives and examined by gas-liquid chromatography. Radioactivity was detected only in chimyl alcohol (Fig. 2). When oleyl alcohol-1-14C was incubated, it was found in selachyl alcohol, with very much smaller amounts in chimyl alcohol and in the glyceryl ether considered to contain palmitoleyl alcohol (Fig. 3). When palmitate-1-14C of high specific activity (20 μC per μmole) was incubated, the amount of radioactivity detected in the isopropylidene derivatives of the glyceryl ethers was extremely small, although it was actively incorporated into acyl linkage of both glyceryl ethers and triglycerides.
Enzymatic synthesis of glyceryl ethers from mixtures of cetyl alcohol $^1$H and cetyl alcohol $^1$C in skate stomach

Each incubation contained two skate stomachs, 10 ml of Krebs-Ringer phosphate buffer, 0.5% sodium taurocholate, and substrate as indicated in the table. Incubations were carried out for 8 hours at room temperature. Activity data are from isopropylidene derivatives isolated by thin layer chromatography.

<table>
<thead>
<tr>
<th>Study</th>
<th>Cetyl alcohol</th>
<th>Ratio of $^3$H: $^1$C</th>
<th>% incorporation</th>
<th>Product</th>
<th>Isopropylidene derivatives</th>
<th>Ratio of product ($^3$H: $^1$C)</th>
<th>(substrate ($^3$H: $^1$C)</th>
<th></th>
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<tbody>
<tr>
<td>1</td>
<td>2.9</td>
<td>41.2</td>
<td>60</td>
<td>53.8</td>
<td>1.31</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>0.206</td>
<td>16.0</td>
<td>3,640</td>
<td>30.3</td>
<td>1.59</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>0.205</td>
<td>16.0</td>
<td>7,300</td>
<td>28.4</td>
<td>1.78</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>1.98</td>
<td>9.9</td>
<td>316,580</td>
<td>15.2</td>
<td>1.54</td>
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<td></td>
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<tr>
<td>5</td>
<td>1.12</td>
<td>11.9</td>
<td>188,660</td>
<td>17.5</td>
<td>1.47</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>1.12</td>
<td>11.9</td>
<td>35,040</td>
<td>16.4</td>
<td>1.38</td>
<td></td>
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</tbody>
</table>

Studies on Incorporation of Long Chain Alcohols into Glyceryl Ethers by Means of Double Isotope Experiments—The experiments described above suggest that long chain alcohols, and not long chain fatty acids, are precursors of glyceryl ethers. However, long chain alcohols are readily oxidized to aldehydes and to fatty acids with the same number of carbon atoms. In order to rule out the possibility that fatty acids or aldehydes were somehow involved in the synthesis of glyceryl ethers, experiments were done in which the substrate consisted of a mixture of (a) cetyl alcohol with tritium on the 1st carbon atom; and (b) cetyl alcohol-1-14C. As described in “Materials and Methods,” careful measurement by gas-liquid chromatography was made of the exact number of tritium and $^1$C counts in several mixtures prepared on separate occasions and with different specific activities. Micellar solutions of the isotopes were made only after the latter had been mixed to insure that there would be no difference in their physical form. The doubly labeled cetyl alcohol substrate was then incubated with stomach as described in the previous experiments, and the activity of the isopropylidene derivatives of the glyceryl ethers was determined. Lack of availability of dogfish throughout the year made it necessary to use skate stomachs for these later studies. These stomachs were satisfactory, and no differences were noted which could in any way alter the conclusions. If palmitic acid were an intermediate product in the formation of glyceryl ether, all of the tritium would be theoretically lost, and only $^1$C would remain in the product. If palmitaldehyde were an intermediate, the ratio of tritium to $^1$C would be reduced to half, since conversion to the aldehyde would require loss of only 1 tritium on the 1st carbon atom. If cetyl alcohol were incorporated unchanged, the ratio would remain the same. The results in Table I indicate that the ratio of tritium to $^1$C found in the isopropylidene derivatives did not decrease in a number of consecutive studies which utilized different preparations of the substrate. The experiment was repeated a number of times because of the unexpected finding that tritiated cetyl alcohol was incorporated better than cetyl alcohol-1-$^1$C. In all instances the results were remarkably similar, and it can be seen that only approximately 53 to 78% as much $^1$C was incorporated as tritium (Table I). The ratio of tritium to $^1$C was examined in the whole and in different portions of the chimyl alcohol peak collected from gas-liquid chromatography, and in the isopropylidene derivatives isolated by thin layer chromatography (Table I). When the unused cetyl alcohol remaining in tissues at the end of incubation was reisolated, the ratio was the same as that of the starting material. Furthermore, the fatty acid fraction derived from enzymatic dehydrogenation of cetyl alcohol has lost 90% or more of the tritium. Although these results indicated that cetyl alcohol was the precursor of glyceryl ethers, the increase in the ratio of tritium to $^1$C could not be explained. The possibility that tritium removed from cetyl alcohol in the course of dehydrogenation could have been transferred, e.g. by tritiated DPNH or by other pathways, to other portions of the glyceryl ether molecule had to be considered. The fact that gas-liquid chromatography did not reveal incorporation of this isotope into the isopropylidene derivatives of other glyceryl ethers militated against this possibility.

In order to demonstrate that the tritium in the glyceryl ether molecule had remained on the 1st carbon atom, the isopropylidene derivatives of other glyceryl ethers were cleaved at the ether bond with hydrogen iodide. After this procedure the ratio of tritium to $^1$C in the alkyl iodides remained essentially unaltered, an indication that the glycerol did not contain significant amounts of $^1$H (Table II). Alkyl iodides were then converted to the corresponding fatty acids via the fatty alcohols, as described in “Materials and Methods.” The fatty acids were then purified by thin layer chromatography and methylated with boron trifluoride methanol. Methyl palmitate recovered by gas chromatography was then assayed for radioactivity. It was found that virtually all the tritium was lost (Table II).

When 9,10-tritiated cetyl alcohol, instead of cetyl alcohol tritiated on the 1st carbon atom, was mixed with cetyl alcohol-1-14C and the incubation was repeated, no increase in the $^3$H:$^1$C ratio was noted. As indicated in “Materials and Methods,” this substrate mixture of cetyl alcohol-9,10-$^3$H and cetyl alcohol-1-$^1$C contained a significant amount of tritiated impurity which could not be removed by the means available. The presence of impurity, i.e. chain lengths other than C-16, was circumvented by making all measurements at the substrate and product level on C-16 components. Under these circumstances, the $^3$H:$^1$C ratio of substrate cetyl alcohol, determined by gas-liquid chromatography, was 8.0. The $^3$H:$^1$C ratio in the isopropylidene derivative of chimyl alcohol, also isolated by gas-liquid chromatography, was 8.7. The hexadecyl iodide obtained from the chimyl alcohol had a ratio of 7.0. When the hexadecyl iodide was converted to methyl palmitate and again isolated by gas-liquid chromatography, the ratio was found to be 7.4. These results indicate that the increase in the $^3$H:$^1$C ratio, found in all experiments in which cetyl alcohol-1-$^1$H was used, cannot be explained by an isotope effect in which there is a relatively greater reactivity of $^1$C versus $^3$H. The results also rule out a $^1$C impurity and a counting error due to undetected quenching (Table II).

In order to evaluate the problem further, another experiment was done with a mixture of cetyl alcohol-16-14C and cetyl alcohol-1-$^1$H. If the observed increase in the $^3$H:$^1$C ratio were due to an isotope effect involving the 1st carbon atom itself, no change in ratio would be anticipated when cetyl alcohol-16-14C was used instead of cetyl alcohol-1-$^1$C. The isotope ratio of substrate in this study was 5.7. All products were examined by gas-liquid chromatography. After incubation, chimyl alcohol had

Table I: Glyceryl Ether Synthesis in Elasmobranch Stomach

| Study | Substrate | Product | Isopropylidene derivatives | Ratio of product ($^3$H: $^1$C) | (substrate ($^3$H: $^1$C) |
|-------|-----------|---------|-----------------------------|----------------------------|-----------------|---|
| 1     | Cetyl alcohol | Ratio of $^3$H: $^1$C | % incorporation | Isopropylidene derivatives | Ratio of product ($^3$H: $^1$C) | (substrate ($^3$H: $^1$C) |  |
| 1     | 2.9 | 41.2 | 60 | 53.8 | 1.31 |  |  |
| 2     | 0.206 | 16.0 | 3,640 | 30.3 | 1.59 |  |  |
| 3     | 0.205 | 16.0 | 7,300 | 28.4 | 1.78 |  |  |
| 4     | 1.98 | 9.9 | 316,580 | 15.2 | 1.54 |  |  |
| 5     | 1.12 | 11.9 | 188,660 | 17.5 | 1.47 |  |  |
| 6     | 1.12 | 11.9 | 35,040 | 16.4 | 1.38 |  |  |
TABLE II

Loss of tritium following chemical conversion of isopropylidene derivatives of glyceryl ethers to fatty acids

Isopropylidene derivatives of glyceryl ethers obtained from incubation with double labeled cetyl alcohol treated as described in the text to remove 3H from the 1st carbon atom of the aliphatic chain. Ratios of counts were obtained from fractions recovered by gas-liquid chromatography. (See text for further explanation.)

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Chymyl alcohol from substrate</th>
<th>Hexadecyl iodide from chymyl alcohol</th>
<th>Methyl palmitate from hexadecyl iodide</th>
</tr>
</thead>
<tbody>
<tr>
<td>Isotope mixture</td>
<td>H:14C</td>
<td>H</td>
<td>H:14C</td>
</tr>
<tr>
<td>Cetyl alcohol-1-3H and cetyl alcohol-1-14C</td>
<td>3.3</td>
<td>348,900</td>
<td>54,370</td>
</tr>
<tr>
<td>Cetyl alcohol-1-3H and cetyl alcohol-16-14C</td>
<td>5.7</td>
<td>419,000</td>
<td>53,700</td>
</tr>
<tr>
<td>Cetyl alcohol-9,10-3H and cetyl alcohol-1-14C</td>
<td>8.0</td>
<td>268,000</td>
<td>19,240</td>
</tr>
</tbody>
</table>

A ratio of 8.3; hexadecyl iodide, 8.2; and methyl palmitate, 0.06. These data, together with the foregoing, indicate an enrichment of tritium on the 1st carbon atom of chymyl alcohol, with virtually all the tritium having been lost in the conversion to methyl palmitate.

Dimethyl acetals from aldehydes isolated from stomach lipids and recovered by gas-liquid chromatography contained ratios of tritium to 14C varying from 40 to 65% of the ratio of the substrate cetyl alcohol. The relevance of these data will be discussed.

DISCUSSION

Most investigators have sought an explanation for plasmalogen and glyceryl ether synthesis at the aldehyde or fatty acid level. However, the present work indicates clearly that glyceryl ethers can be synthesized directly from fatty alcohols. This finding may in no way relate to the formation of vinyl ether bonds in plasmalogens or in alkenyl glyceryl ethers. These could be formed via an entirely different mechanism. However, recent work by Thompson indicates that alkyl glyceryl ethers may undergo dehydrogenation to alkenyl glyceryl ethers (3). We have no explanation for the fact that the results of the present study are in conflict with those of other studies in which evidence for the utilization of fatty acid in glyceryl ether formation was obtained in vivo. It is possible that long chain alcohols could be synthesized from fatty acids, and in the present study we have demonstrated synthesis of free fatty alcohols from acetate in dogfish stomach. It is also possible that species differences may exist.

An initial series of experiments with dogfish liver, which seemed to be a more logical choice because of the large amount of glyceryl ether in this organ, was unrewarding. Although thick liver slices and mince were very active in synthesizing acyl bonds in both triglycerides and diacyl glyceryl ethers with palmitic acid-1-14C, ATP, CoA, and magnesium, incorporation of cetyl alcohol and palmitic acid into ether linkage was uncertain or inconclusive. Studies with various shark stomach homogenates, for the purpose of establishing cofactor requirements, were not successful. In intact dogfish intestine was also inactive.

At present, we have no explanation for the fact that cetyl alcohol-1-3H was incorporated better than 14C-cetyl alcohol. A number of possibilities were considered. Counting errors were ruled out, first, by careful evaluation of quenching by means of internal standards consisting of toluene-14C and toluene-3H and second, by the fact that no shift in tritium or 14C spectra on the liquid scintillation spectrometer was noted.

Another possibility was considered, namely, an isotope effect produced by the cleaving of the 14C to oxygen bond of cetyl alcohol. The expected result of such an effect would have been a relative decrease in incorporation of 14C versus 3H. That the increase in tritium to 14C ratio was a tritium rather than a 14C effect was shown by the observation that when cetyl alcohol-1-14C was mixed with cetyl alcohol-9,10-3H instead of cetyl alcohol-1-3H, no change in ratio occurred. In addition, the use of the cetyl alcohol-16-14C-cetyl alcohol-1-3H mixture again pointed to the absence of a 14C isotope effect and to a relative enrichment in cetyl alcohol-1-3H. The possibility of a 14C impurity in the substrate was ruled out by careful analysis of substrates and products by gas-liquid chromatography and by the study which utilized the 9,10-tritiated 1-14C-cetyl alcohol mixture.

It was also considered that tritium enrichment might take place by a process in which tritium was removed from the 1st carbon atom, e.g. by pyridine nucleotide. The tritium might then be transferred to other glyceryl ethers or might form tritiated alcohols or aldehydes from fatty acids which might then enter into the formation of glyceryl ethers. This possibility was ruled out by the fact that no tritium was found in any of the other glyceryl ethers, such as butyl or selachyl alcohols.

The available information, then, seems to point to an unexplained tritium enrichment. Evidence that this observation is a real biological phenomenon and not a methodological error has recently come from another source. Preliminary studies in this laboratory with Tetrahymena pyriformis (3), rather than shark stomach, have confirmed the essential observations described in this paper, with the single exception that the tritium enrichment was of a much smaller magnitude. These observations were made under conditions in which the same substrate mixture was used in the experiments with Tetrahymena and shark stomach. In Tetrahymena, tritium enrichment by only a factor of 1.2 was noted, and it was estimated that glyceryl ether synthesis was 10 to 20 times more active than in shark stomach.

Among the explanations which could account for a tritium enrichment in the glyceryl ether fraction, the one which we believe may have the greatest merit is the occurrence of a selective depletion of nontritiated cetyl alcohol from a critical intracellular pool from which glyceryl ethers were synthesized.
Loss of cetyl alcohol through dehydrogenation or other pathways, coupled with an isotope effect, would leave a cetyl alcohol pool enriched in tritium because enzymatic systems that transport hydrogen are extremely sensitive to small gradations in activation energy of C–H bonds (17). Since dehydrogenation of cetyl alcohol in the studies described is active, it is likely that this mechanism is pertinent. Unfortunately, we have thus far been unable to demonstrate tritium enrichment in substrate cetyl alcohol isolated from tissues after incubation, possibly because the appropriate pool was not sampled.

The demonstration of the proposed mechanism may be possible by the use of kinetic studies and pulse labeling experiments. It is also possible that the effect may occur after the formation of glyceryl ethers through a selective depletion of nontritiated species.

Although the preferential utilization of tritiated cetyl alcohol in glyceryl ether synthesis remains a puzzling phenomenon, the results of these studies are consistent with the basic conclusion that cetyl alcohol is an intermediate. In order to give further support to these findings, chimyl alcohol obtained from incubations was cleaved to hexadecyl iodide and then oxidized to palmitic acid. This procedure left no doubt that all the tritium was confined to the 1st carbon atom of the aliphatic chain, and that this tritium was present in amounts which ruled out an aldehyde pathway.

In summary, the evidence from these studies, which indicates that long chain alcohols are more direct precursors of glyceryl ethers than fatty acids or aldehydes, comes from several directions. First, fatty acids were not incorporated. Second, long chain alcohols did serve as substrates. Third, although the tritium enrichment could not be satisfactorily explained, the studies with doubly labeled alcohols indicated clearly that aldehydes were also not precursors, especially since aldehydes isolated from the experiments showed the anticipated reduction in the tritium to $^{14}$C ratio.

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**REFERENCES**

Glyceryl Ether Synthesis from Long Chain Alcohols in Elasmobranch Stomach
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