Effect of Different Fatty Acids on Glycerolipid Synthesis in Isolated Rat Hepatocytes*

(Received for publication, December 26, 1973)

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SUMMARY

Glycerolipid synthesis from [3H]glycerol and [14C]dihydroxyacetone operated in enzymically isolated rat hepatocytes at a rate similar to that in the intact organ. Addition of albumin-bound fatty acids with more than 12 carbon atoms to the incubation medium markedly stimulated triacylglycerol synthesis from [3H]glycerol whereas the effects on phospholipid synthesis were smaller and more dependent on fatty acid structure. Capric acid, lauric acid, and erucic acid inhibited both phosphatidylycerolamine and phosphatidylethanolamine synthesis whereas fatty acids with 16 to 18 carbon atoms were stimulatory or without effect. Since these findings might be explained by the substrate specificities of cholinephosphotransferase, ethanolaminephosphotransferase, and diacylglycerol acyltransferase, the effects of different fatty acids on the formation of molecular species of diacylglycerols and their utilization were investigated. High proportions of labeled diacylglycerols containing 2 molecules of the added fatty acid were formed for all fatty acids. Unsaturated diacylglycerols were well utilized for phospholipid synthesis, while saturated ones were utilized to a lesser degree, especially for phosphatidylycerolamine synthesis. The low utilization of saturated diacylglycerols may represent one mechanism whereby the formation of phospholipid molecules with unsuitably high transition temperatures is avoided.

Fatty acids utilized for glycerolipid synthesis in the liver may originate from several sources, such as plasma free fatty acids, fatty acid synthesis, and degradation of plasma lipoprotein glycerolipids or hepatic glycerolipids (1). In most metabolic situations plasma free fatty acids are the dominant precursors of lipoprotein triacylglycerols secreted by the liver (2, 3). The present study concerns the quantitative and qualitative effects of different free fatty acids on the synthesis of individual glycerolipids in enzymically isolated rat hepatocytes. Recent studies indicate that glycerolipid synthesis in such cells retains many of its characteristics in vivo (4, 5). By adding different albumin-bound fatty acids to the cells the fatty acid composition of diacylglycerols, common precursors of triacylglycerols, phosphatidylycerolines, and phosphatidylethanolamines—could be significantly changed. It was thereby possible to study the utilization of different diacylglycerols for glycerolipid synthesis in a situation where they are presented to the enzymes in the form occurring in vivo.

MATERIALS AND METHODS

L-[1,2-3H]Glycerol was obtained from the Radiochemical Centre (Amersham) and [U-14C]dihydroxyacetone from International Chemical and Nuclear Corporation (Irvine, California). Bovine serum albumin (Cohn Fraction V), purchased from Serva (Heidelberg), was defatted (6) and dialyzed against Hanks' solution (7) buffered with 4.2 mM bicarbonate or 10 mM phosphate. Unsaturated fatty acids were obtained from the Hormel Institute (Austin) and saturated fatty acids from Fluka (Buchs, Switzerland). Fatty acid-albumin solutions were prepared according to Spector and Hoak (8) and the concentration of fatty acids was determined by gas-liquid chromatography of the methyl esters with pentadecanoic acid as internal standard (9). Fatty acids with 12 carbon atoms and less were determined as benzyl esters with undecanoic acid as internal standard (10). Trioleoylglycerol, diarachidonoylglycerol, didecanoylglycerol, dipalmitoleoylglycerol, dilinolenoylglycerol, and diarachidonoylglycerol were obtained from Nu-Chek-Prep (Elysian, Minn.).

Incubation Conditions—Hepatocytes were isolated from male Sprague-Dawley rats (200 to 250 g) fed a balanced diet, by the method of Berry and Friend (11) with the modifications described elsewhere (12). In some experiments rats fasted for 24 hours were used. In most experiments the incubations were performed in 25-ml Erlenmeyer flasks and all substances used were dissolved in Hanks' solution buffered with 10 mM phosphate (pH 7.4). Different fatty acid-albumin solutions (final concentration of albumin 2% (w/v)) and other components were mixed with hepatocytes (approximately 2 x 10^10 cells) and the mixture was preincubated at 37° for 10 min. [1,2-3H]Glycerol (5 to 10 μCi) or [14C]dihydroxyacetone (0.2 μCi) was then added, and the vials were capped and incubated with shaking (80 to 100 oscillations per min) for 60 min unless otherwise stated. The incubations (total volume 0.5 ml) were terminated by the addition of 3 ml of chloroform-methanol (1:1, v/v). The solvent contained 0.05% BHT (2,6-di-tert-butyl- p-cresol), which was also added to individual lipid fractions at appropriate places in the following separation procedures. After 1 hour at room temperature the mixture was transferred to centrifuge tubes together with a rinse of 1 ml of chloroform-methanol (1:1). Control experiments showed that 95 to 99% of the lipid radioactivity was extracted from the flask in this way. Two phases were created by the addition of 1.5 ml of water containing 1% NaCl and 1% NaCl. After centrifugation the upper phase was aspirated and the lower phase was washed three times with 1% NaCl-methanol-chloroform (47:48:5) (13). The lipid phase was taken to dryness under a stream of nitrogen and the residue
was redissolved in chloroform. In this procedure the lipids in the cell and those secreted into the medium (14) were extracted together.

Separation of Lipids—For the determination of the total radioactivity in lipid classes an aliquot (5 µl to 14) of the lipids were separated by thin layer chromatography on 0.5-mm Silica Gel H plates in two systems, benzene-chloroform-methanol (85:12:5) and chloroform-methanol-4 M HCl (60:30:2:5) or chloroform-methanol-acetic acid-water (65:30:5:1). Radioactivity in phosphatidic acids was determined after chromatography on Silica Gel G plates made from 0.25 mM oxalic acid (15) with chloroform-methanol-formic acid-hydrochloric acid (90:11:2:0:1) as solvent. Carrier lipids were added before separation and the spots were identified after spraying with 0.2%, 2,7-dichlorofluorescein in ethanol. The zones were transferred to scintillation vials and counted by a method similar to that of Webb and Metz (16). Three drops of acetic acid, 1 ml of methanol-water (1:1), and 10 ml of Instagel (Packard)-toluene (1:1) were added to each vial. In experiments where the distribution of molecular species was studied, the lipid extract was first separated into different phospholipids and total neutral lipids by thin layer chromatography in chloroform-methanol-acetic acid-water (65:30:5:1). The lipids were then treated with chloroform-methanol-acetic acid-water (100:78:2:20). Total neutral lipids were taken to dryness and treated with 0.2 ml of pyridine-acetic anhydride (1:1) for 3 hours at room temperature. The solvents were evaporated and the lipids, dissolved in chloroform, were separated by thin layer chromatography with diethyl ether-petroleum ether (50:70) as developing solvent. Triacylglycerols and 1,2-diacyl-3-acytyl-sn-glycerols were eluted with diethyl ether.

The lipid classes were separated into molecular species by argentation chromatography on Silica Gel H plates containing 7% silver nitrate. Phosphatidylethanolamines were resolved as N-benzoyl-O methyl derivatives (17). Phosphatidylcholines were converted into diacylacylglycerols by treatment with phospholipase C (18), and subsequent acetylation. The diacylacylglycerols were purified as described above and then resolved by argentation chromatography (9). Triacylglycerols were separated into different species as described earlier (19). The different zones from the silver nitrate-containing plates were transferred directly to scintillation vials. Acetic acid (2 to 4 drops) and 0.5 ml of 20% NaCl were added together with 10 ml of Bray's scintillation fluid (30) or 10 ml of Instagel, which gave higher counting efficiency.

Other Methods—Radioactivity was measured by liquid scintillation counting as described elsewhere (21). Protein was determined according to Lowry et al. (22) with bovine serum albumin as a standard.

RESULTS

Incubation Conditions for Glycerolipid Synthesis from [3H]Glycerol—To establish the adequate incubation conditions for the study of glycerolipid synthesis incubations were performed in different vials. The rate of [3H]glycerol incorporation was found to be higher in 25-ml Erlenmeyer flasks than in vials with smaller diameter (Table 1), which also applies to the rate of fatty acid and cholesterol synthesis (12). The distribution of radioactivity among lipid classes was, however, not significantly influenced. The incubations were routinely performed under air since the rate of [3H]glycerol incorporation in 25-ml flasks was not affected by substitution for oxygen. When Hanks' solution buffered with either 4.2 mM HCO₃⁻, 10 mM phosphate, or 19.4 mM N₂-hydroxyethylpiperazine-N'-2-ethanesulfonic acid was used as incubation medium, [3H]glycerol incorporation was higher in the two latter media. This difference was probably due to the different buffering capacity of the media, since the incorporation of [3H]glycerol into triacylglycerols was more rapid at lower pH values in the presence of 1 mM oleic acid, whereas phospholipid radioactivity was unchanged. Similar findings have been reported for cultured liver cells and Ehrlich ascites cells (23, 24). pH decreased approximately 0.05 unit per hour when 3.6 mg of cell protein were incubated in 0.5 ml of phosphate-buffered medium. The decrease was not influenced by the presence of 2 mM glycerol or 1 mM oleic acid. The incorporation of labeled substrate occurred almost exclusively in the glycerol portion of the molecule, since maximally 5% could be extracted with acidified petroleum ether after alkaline hydrolysis of the lipids. When fatty acids were present in the medium the proportion was still lower. The incorporation expressed as nanomoles of [3H]glycerol per 60 min was constant for all lipid classes at glycerol concentrations above 2 mM. When the cell concentration was varied, the incorporation of glycerol per mg of protein into total lipids was constant up to 7 mg of protein per incubation. The incorporation of glycerol into triacylglycerols, phosphatidylcholines, and phosphatidylethanolamines was linear for 60 to 120 min. In such experiments the phosphatidic acid radioactivity rapidly reached a constant level, while diacylglycerol radioactivity rose with time especially in the presence of oleic acid. This does not support the idea that phosphatidic acid phosphatase is the rate-limiting enzyme in glycerolipid synthesis (25-27).

Table 1

<table>
<thead>
<tr>
<th>Incubation vial</th>
<th>Addition of 0.5 ml oleic acid</th>
<th>Incubation time (min)</th>
<th>[3H]Glycerol incorporation (nmol/min/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>5-ml flat-bottomed tube</td>
<td>-</td>
<td>60</td>
<td>0.099</td>
</tr>
<tr>
<td>(12 mm²)</td>
<td>+</td>
<td>120</td>
<td>0.081</td>
</tr>
<tr>
<td>15-ml round-bottomed test tube</td>
<td>(15 mm²)</td>
<td>-</td>
<td>60</td>
</tr>
<tr>
<td>+</td>
<td>120</td>
<td>0.112</td>
<td></td>
</tr>
<tr>
<td>25-ml Erlenmeyer flask</td>
<td>+</td>
<td>120</td>
<td>0.439</td>
</tr>
<tr>
<td>(35 mm²)</td>
<td>+</td>
<td>120</td>
<td>0.136</td>
</tr>
<tr>
<td>35-ml round-bottomed test tube</td>
<td>(20 mm²)</td>
<td>-</td>
<td>60</td>
</tr>
<tr>
<td>+</td>
<td>120</td>
<td>0.465</td>
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</tbody>
</table>

* Inner diameter.

Effect of Different Fatty Acids on Glycerolipid Synthesis

Addition of albumin-bound fatty acids to the medium markedly stimulated triacylglycerol synthesis (Figs. 1A and 2). Lauric acid, myristic acid, palmitic acid, oleic acid, and linoleic acid had indistinguishable effects on triacylglycerol synthesis, whereas capric acid, stearic acid, and erucic acid consistently were less stimulatory. In several experiments palmitoleic acid was the most potent stimulator. The effects of fatty acids on phospholipid synthesis were smaller and less uniform (Figs. 1, B and C, and 2). Addition of capric acid, lauric acid, or erucic acid gave a lower phosphatidylethanolamine synthesis than the other fatty acids. These fatty acids and also myristic acid inhibited phosphatidylethanolamine synthesis while the other fatty acids tested were stimulatory or without effect. Glycerolipid synthesis from [14C]dihydroxyacetone was higher or equal to that from [14C]glycerol in a series of experiments. The addition incorporation occurred predominantly into triacylglycerols. The incorporation of [14C]dihydroxyacetone was influenced by different fatty acids very similarly to [3H]glycerol incorporation (Fig. 2). With the exception of lauric acid the fatty acids stimulated the incorporation of [3H]glycerol into phosphatidic acids and diacyl-
Synthesis of Molecular Species of Different Glycerolipids—The proportions of molecular species synthesized in the absence of added fatty acids were very similar to that observed in intact liver (9, 28, 29) and in liver slices (30, 31) after the administration of labeled glycerol. The saturated-monoenoic and saturated-dienoic species dominated among diacylglycerols and phosphatidylethanolamines while saturated-hexaenoic phosphatidylethanolamines were most abundant. Addition of increasing amounts of oleic acid gave rise to increasing proportions of the monoenoic-monoenoic species in diacylglycerols and to the trienoic species in triacylglycerols (Fig. 3). In diacylglycerols the two species containing 1 oleic acid residue per molecule (saturated-monoenoic glycerols (Figs. 1, D and E), which probably indicates enlargements of these pools. The accumulation in phosphatidic acids was relatively smaller, again indicating that the phosphatidic acid phosphatase reaction was not rate-limiting when the rate of glycerolipid synthesis was increased severalfold by the fatty acid addition. It was verified, by chromatographing the labeled diacylglycerols in the presence of a dilauroylacetylglycerol standard, that the low incorporation into diacylglycerols upon lauric acid addition was not due to experimental error. Interestingly 1.5 mM stearic acid caused a large accumulation of [3H]glycerol in diacylglycerols and phosphatidic acids, which may indicate that the capacity for the system to utilize distearoyl molecules is lower than for other molecular species.

![Figure 1](http://www.jbc.org/) (A through E). Effect of different fatty acids on synthesis of individual glycerolipids from [3H]glycerol. Hepatocytes (2.14 mg of protein) were incubated with 2 mM [3H]glycerol and different albumin-bound fatty acids. Incubation time, 60 min. Each point is the mean from two incubations. A, triacylglycerols; B, phosphatidylcholines; C, phosphatidylethanolamines; D, phosphatidic acids; and E, diacylglycerols. Added fatty acids: O, lauric acid; , myristic acid; , palmitic acid; , stearic acid; , oleic acid; and , linoleic acid.

![Figure 2](http://www.jbc.org/) Effect of different fatty acids on the synthesis of individual glycerolipids from [3H]glycerol and [14C]dihydroxyacetone. Hepatocytes (1.56 mg of protein) were incubated with 2% albumin and 2 mM [3H]glycerol or 5 mM [14C]dihydroxyacetone. Fatty acids (1 mM) were added at the bottom: number of carbon atoms to number of double bonds. Incubation time, 60 min. Data are means from two incubations. A, triacylglycerols; B, phosphatidylcholines; and C, phosphatidylethanolamines. Precursors: [3H]glycerol, open bars; [14C]dihydroxyacetone, filled bars.

![Figure 3](http://www.jbc.org/) Effect of oleic acid on the synthesis of molecular species of glycerolipids from [3H]glycerol. Hepatocytes (1.86 mg of protein) were incubated with 2% albumin, oleic acid as indicated, and 0.1 mM [3H]glycerol for 60 min. Data are expressed as per cent of [3H]glycerol in each lipid class. A, diacylglycerols; B, phosphatidylcholines; C, phosphatidylethanolamines; and D, triacylglycerols. Symbols (A through C): , saturated-monoenoic; O, monoenoic-monoenoic; , saturated-dienoic; , monoenoic-dienoic; and , species with four double bonds and more (mainly saturated-tetraenoic and saturated-hexaenoic). D, triacylglycerols with one (O), two ( ), three ( ), four ( ), and more than four ( ) double bonds.
FIG. 4. Effect of different unsaturated fatty acids on the synthesis of molecular species of diacylglycerolipids from [3H]glycerol. [3H]Glycerol (0.1 mM) was incubated with hepatocytes (0.41 to 0.66 mg of cell protein) and different fatty acids for 60 min. In one experiment 2.0 mM fatty acid-4% albumin was used (palmitoleic acid, elaidic acid (18:lt), and linoleic acid), in another 1.0 mM fatty acid-2% albumin (oleic acid (18:l), linolenic acid, and monoenoic-dienoic) were elevated at the lower oleic acid concentrations but then decreased. Several very similar experiments were performed with different unsaturated fatty acids. Addition of palmitoleic acid, elaidic acid, or linoleic acid resulted in large proportions of labeled diacyl lipids containing 2 molecules of these fatty acids (Fig. 4). A reference sample of dipalmitoylecylglycerol co-chromatographed with the saturated-dienoic fraction. After incubation with fatty acids containing 3 to 6 double bonds, almost exclusively polyenoic species were formed. The detailed composition of such species was not investigated. However, after linolenic acid addition, a large part of the phosphatidylethanolamine radioactivity after conversion to diacylglycerol co-chromatographed with dilinolenoyl-acylglycerol. After incubation with docosahexaenoic acid 43% of the radioactivity in phosphatidylcholines after hydrogenation co-chromatographed with a dibehenoyl standard upon reversed phase partition chromatography (32). Thus large proportions of molecules containing two fatty acids with 3 to 6 double bonds were formed. The similar pattern of molecular species in the three diacyl lipids after the addition of unsaturated fatty acids, at least the monoenoic ones and linoleic acid, indicates that the major labeled diacylglycerols were well utilized for phosphatidylethanolamine and phosphatidylcholine synthesis.

The utilization decreased with increasing or decreasing chain length. Dimyristoylglycerol was utilized for phosphatidylethanolamine synthesis in the same way as unsaturated diacylglycerols, arachidonic acid, and docosahexaenoic acid). Data are expressed as per cent of total radioactivity within each lipid class. Molecular species: 1, saturated-saturated; 2, saturated-monoenoic; 3, monoenoic-monoenoic; 4, saturated-dienoic; 5, monoenoic-dienoic; 6, dienoic-dienoic; 7, saturated-tetraenoic, and 8, species with more than four double bonds (mainly saturated-hexaenoic).

FIG. 5. Effect of different saturated fatty acids on the synthesis of molecular species of diacylglycerolipids. [3H]Glycerol, (0.1 mM), 1 mM fatty acid, and 2% albumin were incubated for 60 min with hepatocytes (1.58 mg of protein) isolated from a rat fasted for 24 hours. Data are expressed as per cent of total radioactivity within each lipid class. Abbreviations as in Fig. 4.
saturated diacylglycerols irrespective of chain length. In experiments where the synthesis of molecular species was studied, a low concentration (0.1 mM) of \[^{3}H\]glycerol was used to obtain sufficient amounts of radioactivity in the products. The incorporation was linear for 30 to 60 min and then leveled off. In the presence of stearic acid the proportion of saturated phospholipids was higher at the earlier time intervals and its decrease with time was mainly compensated for by increases in the saturated tetraenoic species. In a similar experiment with oleic acid and \[^{3}H\]glycerol the proportion of the monoenoic-monoenoic species decreased with time in all diacylglycerols.

The addition of fatty acids also influenced the synthesis of molecular species of triacylglycerols (Fig. 3D). Saturated fatty acids caused a large increase in the totally saturated species (43 to 68% of total triacylglycerol radioactivity) which was most prominent when lauric acid was added. Similarly, addition of oleic acid or linoleic acid resulted in large proportions of trioleoylglycerol (Fig. 3D) and trilinoleoylglycerol, respectively. Although this indicates that different diacylglycerols were well utilized for triacylglycerol synthesis, no detailed analysis has been made due to the complexity introduced by the third fatty acid.

**DISCUSSION**

**General Characteristics of Glycerolipid Synthesis in Isolated Liver Parenchymal Cells**—The present data extend the earlier finding that enzymatically isolated rat hepatocytes retain several of the characteristic features of glycerolipid biosynthesis found in vivo (4, 5), in contrast to mechanically prepared cells (4). The rate of glycerolipid synthesis from 2 mM \[^{3}H\]glycerol and 1 mM oleic acid in isolated hepatocytes (0.30 to 0.85 μmol per min per 10 g of liver wet weight) was approximately half of that observed in the intact animals (33). The observed rates are in the same range as those estimated from the tracyglycerol accumulation in rat livers perfused with fatty acids (34), but are 10-fold higher than the rate of glycerolipid synthesis from labeled glycerol observed in rat liver slices (35, 36).

**Effects of Fatty Acids on Glycerolipid Synthesis**—Our study shows that in isolated hepatocytes the addition of fatty acids stimulates triacylglycerol synthesis, whereas phospholipid synthesis is influenced to a lesser degree, which confirms earlier studies with hepatocytes (5) and liver slices (37, 38). The effects of different fatty acids may reflect differences in the uptake, activation, or distribution of individual fatty acids between phosphatidic acid synthesis, fatty acid oxidation, or monoacylglycerolipid acylation. It is noteworthy that a lower \(V_{\text{max}}\) has been observed with stearic acid or decanoic acid as substrates in fatty acid activation than with other long chain fatty acids (39, 40). Palmitoyl-CoA is a more active substrate than stearyl-CoA for glycerol 3-phosphate acyltransferase in liver microsomes (41–43), which may explain the difference between the two fatty acids in their capacity to stimulate glycerolipid synthesis found by us and by Goodridge (44). Other acyl-CoA esters have been found to be different (43, 45–47) or similar (41, 48) as substrates for this enzyme, but no exact parallelism with our data from the microsomal or mitochondrial enzyme (41, 45, 46, 49, 50).

In the intact liver (32, 51) and in liver slices (30) phosphatidic acids and therefore also diacylglycerols are synthesized with high positional specificity. Saturated fatty acids occupy mainly position 1 and unsaturated fatty acids, position 2. The present study shows that this specificity in intact cells is not absolute, since large proportions of diacylglycerols containing either two saturated or two unsaturated fatty acids were formed upon the addition of such fatty acids (Figs. 4 and 5). In a few experiments the distribution of molecular species among phosphatidic acids was found to be similar to that among diacylglycerols.

The utilization of diacylglycerols for the synthesis of triacylglycerols, phosphatidylethanolamines, and phosphatidylethanolamine synthesis varied with the structure of the added fatty acid (Figs. 4 and 5). We suggest that the substrate specificities of cholinephosphotransferase (EC 2.7.8.1), ethanolaminephosphotransferase (EC 2.7.8.1), and diacylglycerolacyltransferase (EC 2.3.1.20) were responsible for such effects to a large extent. Earlier studies have indicated that different unsaturated diacylglycerols are utilized randomly for phosphatidylethanolamine synthesis in liver in vivo (9, 28, 29) and in cell-free systems (52–55), whereas the hexaenoic species is preferentially used for phosphatidylethanolamine synthesis (9, 28, 29, 54), although the latter specificity is much less pronounced in vitro (54). This specificity also operates on diacylglycerols formed from phosphatidylethanolamines (56). The present results confirm that unsaturated diacylglycerols are well utilized for the synthesis of both phospholipids. The proportion of the hexaenoic species was higher in phosphatidylethanolamines than in the other lipids irrespective of fatty acid addition. Fully saturated diacylglycerols, which occur at low concentrations in liver tissue (19), are insignificantly utilized for phosphatidylethanolamine synthesis, whereas their utilization for phosphatidylethanolamine synthesis varies with the chain length. Also in liver microsomes unsaturated diacylglycerols are the superior substrates for cholinephosphotransferase although the lack of reactivity of dilaurin and dipalmitin (57), probably due to insufficient solubilization, is not consistent with our findings. Studies on substrate specificities toward lipid substrates are certainly more valid when the utilization of different substrates formed within the cell is measured.

Although the low utilization of saturated diacylglycerols for phosphatidylethanolamine synthesis may be explained by the substrate specificity of ethanolaminephosphotransferase, it is noticeable that different saturated fatty acids influence the rate of phosphatidylethanolamine synthesis differently (Figs. 1 and 2). This may indicate that the fatty acid effects on phospholipid synthesis are not entirely exerted via the substrate specificities of ethanolaminephosphotransferase and cholinephosphotransferase. The rate of phospholipid synthesis varies within narrow limits in the presence of fatty acids with 16 to 18 carbon atoms, in spite of the wide variation in the fatty acid composition of the synthesized molecules and the variation in the proportion of participating endogenous fatty acids. Whether this depends on a constant rate of formation of CDP-choline and CDP-ethanolamine or on the enzymes being relatively insensitive to variations in the concentration of substrates remains to be elucidated.

It has been suggested (58) that one of the functions of the distribution of acyl residues in membrane phospholipids is to provide the correct fluidity for optimal metabolic function in the tissue. Transition temperatures of the phospholipid measured by thermal analysis (58) or x-ray diffraction (59) decrease with decreasing chain length and increasing unsaturation of the fatty acids. The low utilization of saturated diacylglycerols may represent one mechanism whereby the formation of phospholipid molecules with unsuitably high transition temperature is avoided.

**Acknowledgments**—Miss Birgitta Mårtensson and Miss Gertrud Ohlsson gave excellent technical assistance.
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