THE EFFECT OF ACYL-GROUP COMPOSITION ON THE RATE OF ACYLTRANSFERASE-CATALYZED SYNTHESIS OF LECITHIN

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SUMMARY

The acyltransferase rates for 66 combinations of substrates (6 acylglycerol-phosphorylcholines and 11 acylcoenzyme A derivatives) were measured using an enzyme preparation from pig liver. The results support the earlier findings with rat-liver enzymes showing that the position to be acylated was more significant than the composition of fatty acids in the 1-acylglycerolphosphorylcholine.

INTRODUCTION

In 1963, LANDS AND MERKLi showed that 1-acylglycerol-3-phosphorylcholine containing either saturated or unsaturated acids would be preferentially esterified with linoleic rather than stearic acid. Considering this and subsequent work the specificity of the acyl-CoA: acylglycerol-3-phosphorylcholine acyltransferases in rat liver appears particularly sensitive to the character of the acyl radical in the thio ester2-4, but not to the acyl portion of the acylglycerol-3-phosphorylcholine. The two acylglycerol-3-phosphorylcholine preparations tested contained quite different contents of saturated and unsaturated acids, but were nevertheless mixtures as so often occurs in nature. Furthermore, the overall reaction included the action of acid: CoA ligase under conditions that hopefully would not influence the observed specificity. We have therefore prepared synthetic 1-acylglycerol-3-phosphorylcholine derivatives with different acyl chains to test the specificity of the acyltransferases under defined conditions more appropriate to a kinetic analysis. The results confirm that the principal specificity is for the acid being transferred rather than the acid present in the phospholipid substrate.

EXPERIMENTAL

**Preparation of lecithin from egg yolk**

Egg yolk (8 kg obtained from University of Michigan Food Service) was homogenized with 48 l of acetone (technical grade). The homogenate was filtered and the

Abbreviation: DTNB, 5,5'-dithio-bis-(2-nitrobenzoate).
precipitate was placed in a large jar (45 cm x 30 cm) and was dissolved in 19.2 l chloroform–methanol (2:1, v/v). The entire solution was stirred with a piece of glass tubing connected to a stream of N₂. The solution was washed with 9.6 l of distilled water and was allowed to stand until the layers separated (there was also a layer of protein at the interface). The aqueous supernatant was removed and the chloroform layer was siphoned from beneath the protein. Lipids were washed from the protein layer with chloroform and combined with the first chloroform solution. A chromatographic column (9.5 cm x 90 cm) was prepared by slurrying 12 lbs of alumina in chloroform. The solution of egg-yolk lipids was placed directly onto the column which was eluted with chloroform–methanol (9:1, v/v). The fractions collected from the column were checked by thin-layer chromatography to determine the components eluted. Plates were prepared with Silica Gel G (Brinkman) at a thickness of 0.33 mm, and developed in a solvent of chloroform methanol acetic acid water (65:25:8:4, by vol.). The fractions containing the lecithin were partially evaporated, and the lecithin was precipitated by pouring the resulting syrup into 4 gallons of reagent grade acetone. The supernatant acetone was removed and the lecithin precipitate was washed by resuspending it in acetone. The lecithin precipitate was removed, dissolved in chloroform–methanol (9:1, v/v) and stored at 4°C. Yield: 34 g phospholipid/kg of which 23 g/kg of starting egg yolk was lecithin. The lecithin fraction gave only one spot on thin-layer chromatography plates in 2 different solvent systems (chloroform–methanol–acetic acid–water (65:25:8:4, by vol.); chloroform–methanol–water (65:25:4, by vol.) indicating the absence of ethanolamine phosphoglyceride, sphingomyelin and monoacylglycerol-3-phosphorylcholine.

Preparation of (glycerol-3-phosphorylcholine)₂(CdCl₅)₅ (ref. 5)

A solution containing 110 g of egg lecithin was taken to dryness and the lipid was dissolved in 550 ml of methanol. Sodium methoxide (200 ml, 2 M) was added to the solution which was constantly stirred. The reaction was allowed to proceed for 5 min after which the reaction mixture was poured over 27.5 g of IRC-50 (H⁺) resin premoistened with 100 ml of water. The resin was stirred and then removed by centrifugation. The resin was washed twice with 50 ml of methanol, and the washes were combined with the first methanol solution. The solvent was removed and the residue was dissolved in 2 l of chloroform–methanol (9:1, v/v) and washed twice with 55 ml of water. The combined aqueous layers were diluted with 4.4 l of absolute ethanol. A saturated solution of CdCl₅·2.5 H₂O in 95% ethanol was added until no further precipitation occurred. The CdCl₅ derivative of glycerol-3-phosphorylcholine, (glycerol-3-phosphorylcholine)₂(CdCl₅)₅, was stored over P₂O₅. Yield: 78.5 g (50% on a molar basis).

Acid chlorides

Lauroyl, myristoyl, palmitoyl, and stearoyl chlorides were obtained from Eastman Kodak. Linoleoyl chloride was prepared in the following manner: 50 g of linoleic acid (Hormel Institute, 99% purity) was placed in a round-bottom flask fitted with a reflux condensor. The complete system was flushed thoroughly with N₂. Oxalyl chloride (25 ml) was added and the system was evacuated several times with a mild vacuum to remove the HCl produced. After HCl was no longer evolved, 25 ml of oxalyl chloride were added and the mixture was heated to reflux gently. The mixture was
allowed to stand 3 h at room temperature. Unreacted oxalyl chloride and HCl were removed on a rotary evaporator under vacuum at 50°. The linoleoyl chloride was dissolved in hexane, and ice was added. When the ice melted, the aqueous layer was removed and the hexane layer was washed again with ice water. The hexane was placed over anhydrous Na₂SO₄ and allowed to stand for 0.5 h. The hexane solution was decanted and stored over fresh anhydrous Na₂SO₄ under N₂ at -20°. Hexane was removed (using a rotary evaporator) immediately before the acid chloride was used to prepare dilinoleoylglycerolphosphorylcholine. The yield was 100% based on quantitative gas chromatography using an internal standard of methyl pentadecanoate.

**Preparation of specific lecithins**

The method employed was a modification of the procedure developed by Baer and Buchnea.*

1,2-Dilauroylglycerol-3-phosphorylcholine

Superbrite glass beads (50 ml) and 11 g of dry (glycerol-3-phosphorylcholine)₂₆(CdCl₄)₂ were placed in a dry 250-ml round-bottom flask; 20 ml of lauroyl chloride in 20 ml of carbon tetrachloride (reagent grade) were added and washed in with 20 ml of carbon tetrachloride, and then 11 ml of pyridine in 30 ml of carbon tetrachloride was added. The round-bottom flask was fitted with a ground glass fitting equipped with a stopcock. The reaction mixture was shaken vigorously at 37° for 24 h with occasional venting. The reaction mixture was transferred to a thick-walled centrifuge bottle and centrifuged; the supernatant was decanted and the precipitate was washed 3 times with 50 ml of chloroform; the combined supernatant solutions were taken to a syrupy residue and dissolved in 50 ml of chloroform. The chloroform solution was centrifuged, and the precipitate was washed twice with 5 ml of chloroform. The chloroform washes were taken to dryness and placed under vacuum (0.5 mm of Hg) for 12 h. The residue was dissolved in 50 ml of chloroform–methanol (2:1, v/v) and was washed with water until the washes gave no precipitate upon addition of AgNO₃. The solvent was removed from the chloroform layer and the residue was taken up in diethyl ether–benzene (1:1, v/v). This solution was placed on a column of 60 g of silicic acid. The column was successively washed with 120 ml of diethyl ether–benzene (1:1, v/v), 200 ml of diethyl ether–ethanol (1:1, v/v), 200 ml of ethanol–methanol (1:1, v/v), and methanol until all 1 lauroylglycerol 3 phosphorylcholine was removed from the column. Thin-layer chromatography was used to check fractions for phospholipid content. Those fractions containing only dilauroylglycerol-3-phosphorylcholine were combined; the remaining material was a mixture of mono- and dilauroylglycerol-3-phosphorylcholine. Yield: pure dilauroylglycerol-3-phosphorylcholine, 10.7 mmoles; mixed mono- and dilauroylglycerol-3-phosphorylcholine, 2.24 mmoles; total (based on phosphate) 65%.

1,2-Dimyristoylglycerol-3-phosphorylcholine

The preparation was the same as that used for the dilauroyl derivative except that myristoyl chloride (0.1 mole) was used and the reaction mixture was incubated for 45 h at 37°. The separation of the reaction products was accomplished in the same manner as with dilauroylglycerol-3-phosphorylcholine. Yield: dimyristoylglycerol-3-
phosphorylcholine, 6.61 mmol; mixed mono- and dimyristoylglycerol-3-phosphorylcholine, 3.64 mmol; total (based on phosphate) 51%.

1,2-Dipalmitoylglycerol-3-phosphorylcholine
This was prepared in the same manner as dilauroylglycerol-3-phosphorylcholine with the following exception: 0.084 mol palmitoyl chloride were used. The reaction mixture was incubated at 37° for 40 h. Yield: dipalmitoylglycerol-3-phosphorylcholine, 2.92 mmol; mixed mono- and dipalmitoylglycerol-3-phosphorylcholine, 3.68 mmol; total (based on phosphate) 32%.

1,2-Distearylglycerol-3-phosphorylcholine
The preparation was the same as for dilauroylglycerol-3-phosphorylcholine except 8.25 g of (glycerol-3-phosphorylcholine)_{2}(CdCl)_{3}, 90 mmol stearoyl chloride and 8.3 ml pyridine were used. The mixture was incubated at 37° for 80 h. Yield: distearoylglycerol-3-phosphorylcholine, 1.47 mmol; mixed mono- and distearoylglycerol-3-phosphorylcholine, 1.37 mmol; total (based on phosphate) 19%.

1,2-Dioleoylglycerol-3-phosphorylcholine
This was prepared in the same manner as dilauroylglycerol-3-phosphorylcholine except 10 g (glycerol-3-phosphorylcholine)_{2}(CdCl)_{3} and 86 mmol oleoyl chloride were used. The reaction mixture was incubated at 37° for 20 h. The reaction products were separated in the same manner. Yield: dioleoylglycerol-3-phosphorylcholine, 0.80 mmol; mixed mono- and dioleoylglycerol-3-phosphorylcholine, 0.02 mmol; total (based on phosphate) 4.5%.

1,2-Dilinoleoylglycerol-3-phosphorylcholine
Prepared in the same manner as dilauroylglycerol-3-phosphorylcholine except 180 mmol linoleoyl chloride was used. All reagents and containers were flushed with N_{2} to remove oxygen. The 250-ml round-bottom flask was wrapped in aluminum foil while it was incubated in the dark at room temperature for 15 h. The products of the reaction were handled in the same manner as dilauroylglycerol-3-phosphorylcholine except all solvents and containers were flushed with N_{2}. Yield: dilinoleoylglycerol-3-phosphorylcholine, 0.78 mmol; mixed mono- and dilinoleoylglycerol-3-phosphorylcholine, 2.03 mmol; total (based on phosphate) 14%.

Preparation of 1-acylglycerol-3-phosphorylcholine derivatives
Distearoylglycerol-3-phosphorylcholine (530 μmol) was suspended in 100 ml of ether, 0.25 ml of 0.01 M CaCl_{2} and 36.3 mg lyophilized Crotalus adamanteus venom in 3.3 ml of 0.1 M Tris chloride (pH 7.4) were added followed by an additional 100 ml of ether. The reaction mixture was shaken vigorously and allowed to stand 12 h during which time the 1-stearoylglycerol-3-phosphorylcholine precipitated. The supernatant ether was removed and the 1-stearoylglycerol-3-phosphorylcholine was dissolved in 10 ml of chloroform after removal of residual ether. A chromatographic column of 50 g of silicic acid was prepared in chloroform. The crude 1-stearoylglycerol-3-phosphorylcholine preparation in chloroform was placed on the column and the column was washed with 100 ml of chloroform, 400 ml of chloroform–methanol (3:1, v/v), and 700 ml of methanol. The eluted fractions were checked with thin-layer
ACYLTRANSFERASE-CATALYZED SYNTHESIS OF LECITHIN

Fig. 1. Influence of acylglycerol-3-phosphorylcholine concentration on the acyltransferase initial velocities. A: O--O, 18:0; ▲--▲, 18:1; ○--○, 18:2. B: O--O, 12:0; ▲--▲, 14:0; ○--○, 16:0.

chromatography (silicic acid <200 mesh, chloroform–methanol–water (65:25:4, by vol.). Fractions containing r-stearoylglycerol-3-phosphorylcholine were combined, evaporated to dryness and dissolved and stored at o° in chloroform–methanol (2:1, v/v). The yield, based on phosphate was 97% and the ratio of ester to phosphate in the product was 1.05.

With the exception of r-linoleoylglycerol-3-phosphorylcholine, other i-acylglycerol-3-phosphorylcholines were prepared in the same manner as i-lauroylglycerol-3-phosphorylcholine. These results are summarized in Table I. In the preparation of r-linoleoylglycerol-3-phosphorylcholine, the precipitate was washed twice with

TABLE I
PREPARATION OF I-ACYLGLYCEROL-3-PHOSPHORYLCHOLINE DERIVATIVES

<table>
<thead>
<tr>
<th>Acylglycerol-3-phosphorylcholine</th>
<th>Diacyl precursor (µmoles)</th>
<th>Yield (%)</th>
<th>Ester: phosphate</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lauroyl</td>
<td>950</td>
<td>98</td>
<td>0.82</td>
</tr>
<tr>
<td>Myristoyl</td>
<td>1200</td>
<td>95</td>
<td>0.93</td>
</tr>
<tr>
<td>Palmitoyl</td>
<td>840</td>
<td>98</td>
<td>0.97</td>
</tr>
<tr>
<td>Stearoyl</td>
<td>530</td>
<td>97</td>
<td>1.05</td>
</tr>
<tr>
<td>Oleoyl</td>
<td>170</td>
<td>97</td>
<td>0.83</td>
</tr>
<tr>
<td>Linoleoyl</td>
<td>78</td>
<td>93</td>
<td>0.96</td>
</tr>
</tbody>
</table>

5 ml of diethyl ether. The r-linoleoylglycerol-3-phosphorylcholine was taken up in chloroform–methanol (2:1, v/v) from which oxygen had been removed by bubbling N₂ through it, and the solution was stored at -20°. The yield based on phosphate was 93%, a trace of dilinoleoylglycerol-3-phosphorylcholine impurity was observed on thin-layer chromatography; ester: phosphate = 0.96.

The r-acylglycerol-3-phosphorylcholine had the following fatty acid compositions based on quantitative gas–liquid chromatography: r-lauroylglycerol-3-phosphorylcholine: 12:0, 97.8%; 14:0, 0.5%; 16:0, 1.7%; r-myristoylglycerol-3-phosphoryl-

choline: 12:0, 1.2%; 14:0, 95.7%; 16:0, 2.6%; 18:0, 0.6%; 1-palmitoyl-3-glycerol-3-phosphorylcholine: 14:0, 1.2%; 16:0, 96.2%; 18:0, 2.6%; 1-stearoyl-3-phosphorylcholine: 16:0, 4.0%; 18:0, 96.0%; 1-oleoyl-3-phosphorylcholine: 16:0, 6.7%; 16:1, 9.0%; 18:1, 84.3%; 1-linoleoyl-3-phosphorylcholine: 17:0, 0.4%; 18:2, 99.6%. Gas-liquid chromatographic analyses were performed in the presence of an internal standard as described by Lands and Hart16. Phosphorus was determined by the method of Bartlett7.

Determination of acyltransferase reaction rates

The acyltransferase reaction was followed continuously using the internal colorimetric indicator, 5,5'-dithio-bis-(2-nitrobenzoate) (DTNB), for liberated sulfhydryl groups and measuring the absorbance at 413 μ. A typical reaction mixture contained: 0.8 ml of 0.1 M Tris chloride buffer (pH 7.4), 0.1 ml of 0.01 M DTNB, 30 μmoles acyl-CoA, 0.18 mg microsomal protein from pig liver and 200 μmoles 1-acylglycerol-3-phosphorylcholine in 0.1 ml of 0.1 M Tris chloride (pH 7.4) (400 μmoles of the lauroyl derivatives were used).

RESULTS

The low Kₘ values (<10⁻⁸ M) reported for the acyl-CoA derivatives1,2 were confirmed in preliminary experiments. All subsequent work was done with thiol ester concentrations (3·10⁻⁸ M) considered to be capable of saturating the enzyme. The initial acyltransfer velocities increased with increasing concentrations of the acylglycerol-3-phosphorylcholine derivatives up to 10⁻¹⁵·10⁻⁸ M (except the lauroyl derivative at 40·50·10⁻⁸ M). The plots of initial velocity versus substrate concentration are shown in Fig. 1. The Kₘ values estimated from these results are 13, 3, 3, 3, 2.5 and 2·10⁻⁸ M for the 12:0, 14:0, 16:0, 18:0, 18:1 and 18:2 derivatives, respectively.

Acyltransferase rates obtained with different acyl-CoA and acylglycerol-3-phosphorylcholine derivatives under maximal conditions are shown in Table II. The results with palmitoyl- and stearoyl-3-phosphorylcholine agree with the earlier observation that 1-acylglycerol-3-phosphorylcholine is acylated more rapidly with the medium-chain saturated fatty acids than with the long-chain saturated acids. This selectivity is less, however, with the lauroyl- and linoleoyl derivatives. With each acylglycerol-3-phosphorylcholine tested the overall pattern of specificity is similar to that found with the other acylglycerol-3-phosphorylcholine derivatives. In other words, the differences in acyltransfer rate observed in the horizontal rows are not as great as those seen along the vertical columns.

DISCUSSION

The difficulties encountered in earlier work of insuring both solubility of the fatty acid substrate and optimal activity of the acid: CoA ligase needed to produce the intermediate acyl-CoA are avoided in the direct spectrophotometric assay of the acyltransfer reaction. Both substrates for this reaction appear to be freely soluble at the concentrations used in this study and relatively low levels of protein are needed in each experiment. This low level (0.18 mg/ml) eliminates much of the non-specific
TABLE II

ACYLTRANSFERASE ACTIVITIES

The rates are averages of four assays with the exception of the following pairs which are averages of two assays: 1–14:0 glycerol-3-phosphorylcholine, 16:1 CoA; 1–16:0 glycerol-3-phosphorylcholine, 16:1 CoA. The values are ± 1 μmole/min per mg protein. Since each assay involved a continual recording of the product formed, each velocity reported represents a large number of recorded determinations.

<table>
<thead>
<tr>
<th>Acyl-CoA</th>
<th>Acyltransferase activities (μmole/min per mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>12:0</td>
</tr>
<tr>
<td>12:0</td>
<td>5.7</td>
</tr>
<tr>
<td>14:0</td>
<td>7.3</td>
</tr>
<tr>
<td>16:0</td>
<td>6.4</td>
</tr>
<tr>
<td>18:0</td>
<td>6.4</td>
</tr>
<tr>
<td>18:1</td>
<td>0</td>
</tr>
<tr>
<td>18:2</td>
<td>9.6</td>
</tr>
<tr>
<td>18:3</td>
<td>18</td>
</tr>
<tr>
<td>18:4</td>
<td>18</td>
</tr>
<tr>
<td>20:3</td>
<td>32</td>
</tr>
<tr>
<td>20:4</td>
<td>13</td>
</tr>
</tbody>
</table>

binding of substrate that led to the high apparent $K_m$ value observed earlier with about 6–7 mg of protein per ml. The present $K_m$ values for acylglycerol-3-phosphorylcholine are more in agreement with those reported by Lands and Hartz for liver microsomes and by Stein, Stein and Shapiro for homogenates of rat heart. The latter workers, however, noted severe inhibition by higher concentrations of acylglycerol-3-phosphorylcholine which prevented a reliable estimate of $K_m$. In the present study, reciprocal plots of velocity versus substrate concentration show a slight upward curvature suggesting that the very low levels of acylglycerol-3-phosphorylcholine may not be fully effective. The non-specific binding of such a detergent as acylglycerol-3-phosphorylcholine to microsomal protein is probably unavoidable in this system. Fortunately, the acylglycerol-3-phosphorylcholine is not a particularly strong inhibitor in this system so that the enzyme can be effectively saturated with both substrates. The acyltransfer rates with 16:0 and 18:2 acylglycerol-3-phosphorylcholine for 18:0 and 18:2 acyl-CoA are most appropriate for comparison with the earlier experiment showing that the position to be acylated was more significant than the composition of fatty acids in the acylglycerol-3-phosphorylcholine.

One of the important relationships that we wished to investigate was whether the palmitoylglycerol-3-phosphorylcholine would react more rapidly with oleate than with arachidonate. Similarly the stearoylglycerol-3-phosphorylcholine might react more rapidly with arachidonate than oleate or linoleate. Such a correlative specificity would help explain the often observed association of the two pairs of acids in naturally-occurring phospholipids (e.g. ref. 10). The results obtained in this work indicate that the tendency for these acids to occur as pairs rather than in fully random combinations cannot be explained by acyltransferase selectivity. The common alternative explanation in such a situation would be that the two lecithins (16:0–18:1 and 18:0–20:4) may be formed from metabolically separate pools of fatty acids.

The character of the acyl group of the 6 different acylglycerol-3-phosphorylcholines had very little effect on the acyltransfer rates for the various unsaturated...
acyl-CoA derivatives. However, two rather unusual findings merit further consideration. First, the arachidonyl-CoA was essentially inert with all substrates tested. Second, the observed rates tend to decrease as the total carbon atoms in the fatty acids of the saturated lecithin formed increase from 26 to 38. These "families" of lecithins would be indicated by diagonal lines in Table II. The average rates for these types are shown below:

<table>
<thead>
<tr>
<th>Carbon atoms</th>
<th>26</th>
<th>28</th>
<th>30</th>
<th>32</th>
<th>34</th>
<th>36</th>
<th>38</th>
</tr>
</thead>
<tbody>
<tr>
<td>Average rate</td>
<td>8.6</td>
<td>7.2</td>
<td>5.1</td>
<td>2.2</td>
<td>0.6</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

Although the average rate has no exact physical significance, the trend in velocities seems correlated with the solubility of the product. In such a case, we must seriously consider whether the rate-limiting process in some acyltransferase reactions is the forming of the ester linkage from soluble precursors or the removal of relatively insoluble products from the active site. That problem, however, goes beyond the methods and purposes of this paper.

The results reported here for reactions catalyzed by microsomal preparations from pig liver agree with those obtained earlier with a mixed acylglycerol-3-phosphorylcholine preparation. In addition, they show specificities that are similar to those found for enzyme preparations from rat liver. Although the general specificity patterns for acyl-CoA:acylglycerol-3-phosphorylcholine acyltransferases are similar for different animals, some differences also exist. Further work in our laboratory is directed towards the characterization of a number of different acyltransferases that may be present.

While this manuscript was in preparation, we found that similar results were obtained by Van den Bosch et al.

ACKNOWLEDGEMENT

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