PHOSPHATIDATE SYNTHESIS BY sn-GLYCEROL-3-PHOSPHATE ACYL-TRANSFERASE IN PIGEON LIVER PARTICLES

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(Received August 5th, 1969)

SUMMARY

The properties of acyl-CoA: sn-glycerol-3-phosphate acyltransferase (EC 2.3.1.15) from pigeon liver particles have been studied. The apparent $K_m$ for sn-glycerol 3-phosphate was 50 $\mu$M. Acyl-CoA inhibited the enzyme at concentrations exceeding 60 $\mu$M. This inhibition can be overcome by adding extra protein. The enzyme was inhibited by thiol-binding agents. Protection from thiol-binding agents is achieved by preincubation of the enzyme with acyl-CoA. The amount of the thiol group protected per mg enzyme protein was estimated by adding radioactive N-ethylmaleimide in the presence of glycerol phosphate. No evidence was obtained for required sulfhydryl groups other than those protected by acyl-CoA. In the presence of bovine serum albumin, several different acyl-CoA derivatives were esterified to sn-glycerol 3-phosphate at very similar rates.

INTRODUCTION

The first step in the formation of glycerides and phosphoglycerides is the acylation of sn-glycerol 3-phosphate. The enzyme that catalyzes this reaction, glycerolphosphate acyltransferase (acyl-CoA: sn-glycerol-3-phosphate acyltransferase, EC 2.3.1.15), appears to be of universal occurrence, having been found in mammalian liver particles, mammalian brain, leaf tissue of plants, yeast, and bacteria. In mammalian systems, the enzyme appears to require acyl-CoA as the acyl donor, but activity from Escherichia coli and Clostridium butyricum can also use the acyl group attached to acyl carrier protein. In mammalian systems, the enzyme appears to require acyl-CoA as the acyl donor (refs. 1, 3, 9, 11). However, the intervention of an acyl carrier protein has not been ruled out, and the nature of the thiol group that causes the loss of enzyme activity when combined with thiol-binding agents is not yet elucidated. The purification of

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the enzyme has not progressed very far. Mårtensson and Kanfer have reported a solubilization and partial purification of the enzyme from brain tissue by using a special homogenization technique. In this paper we report on the properties of the activity present in pigeon liver particles.

**Materials and Methods**

**Preparation of pigeon liver particles**

Crossbred pigeons, both male and female, were obtained from a local supplier. They were fed a standard pigeon chow. All animals were allowed free access to food and water until they were killed. The liver was rapidly removed, cooled to 0° and homogenized in 9 vol. of 0.25 M sucrose and 1 mM EDTA. The particulate fraction separating at 5000 x g for 15 min was discarded. The particles sedimenting at 78500 x g for 60 min were used as the source of the enzyme. The particles were washed once by suspending and recentrifuging in 0.25 M sucrose containing 1 mM EDTA. All operations were carried out at 0-4°. The particles were either used on the same day or were stored at -15°. Experiments showed that storage at -15° resulted in some loss of activity but this could be minimized by making the solution 10 mM in dithiothreitol.

**Chemicals**

sn-\([\beta(3)-14C]\)Glycerol was obtained from the Volk Radiochemical Company and glycerol-3-phosphate dehydrogenase from Calbiochem. sn-Glycerol 3-phosphate was prepared from glycerol and ATP as described previously. The amounts of sn-glycerol 3-phosphate formed was estimated by the method of Hohorst using sn-glycerol-3-phosphate dehydrogenase. sn-glycerol 1(3)-[32P]phosphate was prepared by the method of McMurray et al. Acyl-CoA derivatives were prepared by the method of Lands et al. from acyl chlorides obtained from the Hormel Institute. The quantity of acyl-CoA present was calculated from the extinction coefficients at 260 and 232 m\(\mu\) (ref. 16) except with the unsaturated acyl derivatives which were estimated by the extent of their reaction in the acyl-CoA:1-acylglycerophosphorylcholine acyltransferase reaction described by Lands and Hartz.

N-Ethylmaleimide was obtained from Calbiochem and ([\(1-^{14}C\)]-N-ethyl maleimide from Schwarz Bio Research, Inc. Dithiothreitol was obtained from Calbiochem.

Protein concentration was estimated using the extinction coefficients at 260 and 280 m\(\mu\) derived from the data of Warburg and Christian.

**Assay of enzyme activity**

The assay mixture had a final volume of 0.35 ml. It contained 20 mmoles of Tris chloride buffer (pH 8.0), 100 mmoles of \(sn-[^{14}C]\)glycerol 3-phosphate, 10 mmoles of stearoyl-CoA and 1 mg of particulate protein. The reaction was allowed to proceed for 2 min and then 7 ml of chloroform-methanol (2:1 v/v) was added, followed by 1.4 ml of 0.03 M HCl containing 2% acetic acid. The mixture was shaken, the two phases allowed to separate, and the upper phase removed along with the denatured protein at the interface. The lower phase was washed twice with the upper phase of a mixture of chloroform-methanol 0.03 M HCl containing acetic acid (20:10:7.5

PHOSPHATIDATE SYNTHESIS

by vol.). The washings were removed and the lower phase evaporated prior to counting in the dioxane scintillation counting system of Snyder.

RESULTS

The enzyme esterified sn-glycerol 3-phosphate at a rate that was linear with time for about 4 min (Fig. 1A) when the esterification rate then fell off. At this time only 60% of the acyl-CoA had been used up in the reaction so the decrease in rate is unlikely to be due to lack of substrate. The amount of acyl-CoA hydrolyzed by acyl-CoA hydrolase was determined by the spectrophotometric technique using 5,5'-dithio-bis-(2-nitrobenzoic acid). The rate of hydrolysis catalyzed by these preparations was very low.

Fig. 1. A. Time course of the reaction yielding phosphatidate. The standard assay mixture was used. The final volume, 0.35 ml, contained 20 μmoles of Tris chloride buffer (pH 8.0), 1 mg of enzyme protein, 100 nmoles of sn-[14C]glycerol 3-phosphate and 13.3 nmoles of palmitoyl-CoA, which was added last. B. Effect of microsomal protein. The reaction mixtures were similar to those in A with protein varied as shown. A --- A, results after 4-min incubation. O --- O, results after 2-min incubation.

The amount of product formed with varying levels of enzyme is shown in Fig. 1B. Linearity was maintained only over a narrow range of protein concentrations from 1 to 2 mg. Addition of more protein caused a non-linear increase in the rate of acylation. This may be due either to the binding of palmitoyl-CoA to the extra protein added or to the exhaustion of the acyl-CoA substrate. With a 4-min incubation and 3 mg of enzyme protein, 90% of the palmitoyl-CoA had been used up, calculating that diacyl-sn-glycerol 3-phosphate was the product.

Identification of the product as diacyl-sn-glycerol 3-phosphate

The product from the standard reaction mixture described above was extracted and the pH adjusted to 7.0–8.0, as indicated by pH paper, by addition of triethylamine. The triethylamine salts of the reaction products were chromatographed on glass fibre paper impregnated with silicic acid (Gelman Inc., Ann Arbor, Mich.) with a developing solvent of chloroform–methanol–9.73 M NH₄OH (80:18:2.4, by vol.). 90% of the isotope in the product applied to the paper chromatographed as one spot. This radioactive spot cochromatographed with authentic diacyl-glycerol phosphate. A small amount of isotope was present at the solvent front (1–5%). At the origin, where glycerol phosphate is found, 6% of the isotope was detected. The only evidence for the presence of monoacyl-sn-glycerol 3-phosphate was a slight tailing of the diacyl spot, but this tailing contained no more than 4% of the total isotope

present in the product. Further evidence for the formation of the diacyl derivative was obtained by submitting the triethylamine salt of the product obtained above to phosphatidate phosphohydrolase (EC 3.1.3.4). The enzyme was prepared from fresh chicken livers by the procedure described by Smith et al.\textsuperscript{19}. The triethylamine salts of the products of the enzyme reaction were evaporated to dryness under a stream of N\textsubscript{2} in screw-capped vials and 1.0 ml of 0.02 M Tris chloride (pH 8.0) was added. The pH was adjusted to 6.3-6.7 with 1.0 M maleic acid. Crude phosphatidate phosphohydrolase and 1.0 ml of diethyl ether were added and the reaction mixture incubated at 37\textdegree C for 2 h with shaking. The reaction products were isolated by addition of 15 ml of chloroform–methanol (2:1, v/v) and 3 ml of water. The lower phase was taken to dryness and applied to a silicic acid column. The neutral lipids were eluted with benzene–ether (1:1, v/v) and ether, and the phospholipids with methanol. Between 93 and 97\% of the counts were recovered as neutral lipid and 3–6\% were eluted as phospholipids. The neutral lipid was fractionated by thin-layer chromatography as described by Hill et al.\textsuperscript{12}. The monoglyceride fraction contained 6–16\% of the recovered isotope, the diglyceride fraction 84–94\%, and the triglyceride fraction less than 3\%. The diglyceride fraction was shown to cochromatograph with 1,2-diaclylglycerol in a small number of samples examined to check this point. These experiments identifying the products were done with phosphatidate prepared by incubating stearoyl-CoA, oleoyl-CoA, linoleoyl-CoA and a mixture of stearoyl and linoleoyl-CoA with sn-[\textsuperscript{14}C]glycerol 3-phosphate\textsuperscript{12}. From these data we conclude that the major product of the reaction is diacyl-sn-glycerol 3-phosphate.

The effect of substrate concentration on the reaction rate

The substrate concentration curve for sn-glycerol 3-phosphate shows a typical saturation phenomenon (Fig. 2) indicating an apparent $K_m$ for sn-glycerol 3-phosphate of 50 \( \mu \text{M} \). This is similar to the value calculated from the results of Izur et al.\textsuperscript{20} for rat liver but is lower than that for either the yeast enzyme\textsuperscript{6} or the E. coli enzyme\textsuperscript{7}.

![Fig. 2. Effect of sn-glycerol 3-phosphate concentration on the rate of formation of phosphatidic acid. The final volume (0.35 ml) contained 20 \( \mu \text{moles} \) of Tris chloride buffer (pH 8.0), 1 mg of protein with either 50 \( \mu \text{moles} \) of palmitoyl-CoA and varied amounts of glycerol phosphate added last (□—□), or varied amounts of glycerol phosphate and 10 \( \mu \text{moles} \) of palmitoyl-CoA added last (○—○).](image)

![Fig. 3. The effect of palmitoyl-CoA content on the amount of phosphatidic acid formed by the particulate enzyme with and without added bovine serum albumin. ○—○, results when a normal reaction mixture (0.35 ml) was used with the 1.0 mg of enzyme added last; △—△, results when 5 mg of bovine serum albumin were present, and glycerol phosphate was added last.](image)

The value given by Fallon and Lamb\(^4\) for rat liver is 6.7 M, indicating a much lower affinity for sn-glycerol 3-phosphate with their enzyme preparation. The results in Fig. 2 show that the \(K_m\) value is independent of the inhibition caused by higher levels of acyl-CoA.

The substrate concentration curve for acyl-CoA shows a marked inhibition at concentrations above 60 \(\mu\)M (Fig. 3). This is similar to the inhibition of other sn-glycerol-3-phosphate acyltransferases studied. The inhibition can be overcome by adding more protein (Table I). Brandes et al.\(^{11}\) observed a similar phenomenon with the rat liver enzyme as did Martensson and Kanfer\(^8\) with the enzyme from rat brain. We found that bovine serum albumin and N-ethylmaleimide-treated microsomes were effective in relieving the inhibition, but boiled microsomes were less effective.

**TABLE I**

THE ABILITY OF ADDED PROTEIN TO OVERCOME THE INHIBITION CAUSED BY PREINCUBATION OF THE ENZYME WITH PALMITOYL-COA

Enzymatically active particles (1.8 mg protein) were preincubated with the various protein additions in the presence of 20 \(\mu\)moles of phosphate buffer (pH 6.5), 5 \(\mu\)moles of dithiothreitol and 42 \(\mu\)moles of palmitoyl-CoA in a final volume of 0.65 ml. After 5 min, the reaction was initiated by adding 100 \(\mu\)moles of sn-glycerol 3-phosphate. After 10 min of reaction, 12 ml of chloroform–methanol (2:1, v/v) were added and the radioactive products were determined by the standard procedure.

<table>
<thead>
<tr>
<th>Extra protein added</th>
<th>sn-[(^{14})C]Glycerol 3-phosphate converted to phosphatidate ((\mu)moles)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>0.068</td>
</tr>
<tr>
<td>3.6 mg boiled microsomes</td>
<td>0.16</td>
</tr>
<tr>
<td>(per 1.8 mg enzyme protein)</td>
<td>0.51</td>
</tr>
<tr>
<td>3.6 mg bovine serum albumin</td>
<td>0.51</td>
</tr>
<tr>
<td>3.6 mg N-ethylmaleimide-treated microsomes</td>
<td>0.51</td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**TABLE II**

EFFECT OF THE ORDER OF ADDITION OF SUBSTRATES ON THE AMOUNT OF PHOSPHATIDIC ACID FORMED IN 5 MIN

Final volume of assay tubes was 0.35 ml, containing 20 \(\mu\)moles of Tris chloride (pH 8.0), 100 \(\mu\)moles of sn-[\(^{14}\)C]glycerol 3-phosphate, 1.0 mg enzyme protein, and various amounts of palmitoyl-CoA. The reaction was stopped after 5 min by the addition of 7 ml of chloroform–methanol (2:1, v/v).

<table>
<thead>
<tr>
<th>Palmitoyl-CoA ((\mu)moles)</th>
<th>Reagent added last ((\mu)moles/5 min)</th>
<th>Glycerol phosphate</th>
<th>Enzyme</th>
<th>Palmitoyl-CoA</th>
</tr>
</thead>
<tbody>
<tr>
<td>(a) Enzyme alone</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>0.03</td>
<td>0.01</td>
<td>1.4</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>0.08</td>
<td>2.05</td>
<td>4.6</td>
<td></td>
</tr>
<tr>
<td>20</td>
<td>0.92</td>
<td>6.6</td>
<td>7.1</td>
<td></td>
</tr>
<tr>
<td>50</td>
<td>3.3</td>
<td>4.77</td>
<td>2.87</td>
<td></td>
</tr>
<tr>
<td>100</td>
<td>0.64</td>
<td>0.23</td>
<td>0.21</td>
<td></td>
</tr>
<tr>
<td>(b) Enzyme plus albumin</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>0.45</td>
<td>0.49</td>
<td>0.55</td>
<td></td>
</tr>
<tr>
<td>20</td>
<td>1.45</td>
<td>1.05</td>
<td>1.09</td>
<td></td>
</tr>
<tr>
<td>50</td>
<td>3.01</td>
<td>2.38</td>
<td>2.25</td>
<td></td>
</tr>
<tr>
<td>100</td>
<td>3.66</td>
<td>3.30</td>
<td>4.92</td>
<td></td>
</tr>
</tbody>
</table>
The order of addition of the substrates was important in determining the rate of the reaction (Table II). Addition of sn-glycerol 3-phosphate followed by acyl-CoA resulted in faster initial reaction rates than if the enzyme was allowed to interact with acyl-CoA before sn-glycerol 3-phosphate was added. This effect of the order of addition of different substrates was altered when bovine serum albumin was present with the enzyme particles. In the presence of bovine serum albumin, the initial rate of reaction of acyl-CoA and sn-glycerol 3-phosphate was slower than when bovine serum albumin was not present.

**Inhibition of the enzyme by N-ethylmaleimide**

The enzyme is inhibited by incubation with N-ethylmaleimide. The inhibition can be overcome by prior incubation of enzyme with acyl-CoA (Table III). Palmitoyl-CoA, stearoyl-CoA and linoleoyl-CoA were all found to be effective in protecting the enzyme from N-ethylmaleimide inhibition. Concentrations of 0.1 mM N-ethylmaleimide gave about 90% inhibition of enzyme activity and prior incubation with acyl-CoA gave protection of about 90% of the enzyme activity.

**TABLE III**

<table>
<thead>
<tr>
<th>N-Ethylmaleimide concn. (M)</th>
<th>Reaction (% of control)</th>
<th>Preincubation conditions:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>No palmitoyl-CoA</td>
</tr>
<tr>
<td>Expt. 1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5 x 10^-2</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>3 x 10^-4</td>
<td>5</td>
<td>69</td>
</tr>
<tr>
<td>5 x 10^-5</td>
<td>14</td>
<td>100</td>
</tr>
<tr>
<td>5 x 10^-6</td>
<td>37</td>
<td>139</td>
</tr>
<tr>
<td>Control phosphatidate</td>
<td></td>
<td>1.2 nmoles</td>
</tr>
<tr>
<td>formed in 5 min</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Expt. 2</td>
<td></td>
<td>No stearoyl-CoA</td>
</tr>
<tr>
<td>1 x 10^-4</td>
<td>18</td>
<td>83</td>
</tr>
<tr>
<td>Control phosphatidate</td>
<td></td>
<td>0.81 nmole</td>
</tr>
<tr>
<td>formed in 2 min</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**[14C]N-Ethylmaleimide measurement of “protected” thiols**

Experiments were conducted to determine the properties and amount of the N-ethylmaleimide-sensitive acyltransferase. This was done by examining the thiol group that could be protected from N-ethylmaleimide binding by preincubation of the enzyme with acyl-CoA. The results are shown in Table IV. Preincubation of 50 mg of enzyme with 500 nmol of stearoyl-CoA in a final volume of 5 ml of 10 mM Tris-maleate (pH 7.0) for 10 min was followed by treatment of aliquots of the protein with 0.1 M N-ethylmaleimide. Controls were not treated with N-ethylmaleimide. The particles were centrifuged (140000 x g for 30 min) through 0.25 M sucrose at 0°. This washing procedure was adopted to remove N-ethylmaleimide from the treated aliquots. In these three experiments, preincubation with acyl-CoA prior to treatment with N-ethylmaleimide resulted in the protection of 43, 42 and 31% of the enzyme activity measurable in particles that had been similarly preincubated.
TABLE IV
DETERMINATION OF THE AMOUNT OF THIOL GROUP ASSOCIATED WITH GLYCEROL PHOSPHATE ACYLTRANSFERASE

<table>
<thead>
<tr>
<th></th>
<th>[14C]N-Ethylmaleimide binding</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Supernatant</td>
<td></td>
<td></td>
</tr>
<tr>
<td>[14C] counts</td>
<td>mg protein</td>
<td></td>
</tr>
</tbody>
</table>

Expt. 1 (43%)
Incubated with glycerol phosphate
5575 0.46 5465 0.46 11040 0.92
Incubated in the absence of glycerol phosphate
3975 0.33 5720 0.48 9695 0.81
Difference 1600 0.13 -255 -0.02 1345 0.11
Difference x 100/43 0.30 -0.04 0.26

Expt. 2 (42%)
Difference between incubating with and without glycerol phosphate
1350 0.06 890 0.04 2240 0.10
Difference x 100/42 0.14 0.095

Expt. 3 (31%)
Difference between incubating with and without glycerol phosphate
5650 0.076 1667 0.02 7317 0.096
Difference x 100/31 0.25 0.06 0.31

with acyl-CoA but not treated with N-ethylmaleimide. The amount of protection by added acyl-CoA was less than that shown in Table III. This was probably due to the longer time of contact when the excess N-ethylmaleimide was removed by centrifugation in contrast to reacting it with dithiothreitol. After removal of excess unreacted N-ethylmaleimide, aliquots of the treated protein were incubated in the

TABLE V
EFFECT OF SONICATION WITH 0.01% DEOXYCHOLATE ON THE DISTRIBUTION OF ENZYME ACTIVITY

The assay system had a final volume of 0.35 ml. It contained 1 µmole of dithiothreitol, 100 nmoles of sn-[1(3)-14C] glycerol 3-phosphate, 20 µmoles of Tris chloride (pH 8.0), either 9.6 or 14.4 nmoles of stearoyl-CoA and the indicated amount of protein was added to start the reaction. After 2-min incubation at room temperature, 7 ml of chloroform-methanol 2:1, v/v was added. The N-ethylmaleimide-treated fractions were exposed at 25° and pH 7.4 to 0.2 mM N-ethylmaleimide (400 nmoles of N-ethylmaleimide). 5 µmoles of dithiothreitol were then added to combine with excess N-ethylmaleimide and the protein added to incubation mixtures as indicated. The observed rates of synthesis are expressed as a percent of the value obtained with intact microsomes.

<table>
<thead>
<tr>
<th>Protein present (mg):</th>
<th>Protein present (mg):</th>
<th>Protein present (mg):</th>
<th>Protein present (mg):</th>
</tr>
</thead>
<tbody>
<tr>
<td>9.6 nmoles stearoyl-CoA</td>
<td>100</td>
<td>88</td>
<td>77</td>
</tr>
<tr>
<td>14.4 nmoles stearoyl-CoA</td>
<td>100</td>
<td>90</td>
<td>76</td>
</tr>
</tbody>
</table>
presence of 0.1 mM \([^{14}\text{C}]\text{N-ethylmaleimide}\) both with and without added sn-glycerol 3-phosphate. Previous protection studies showed that with this concentration of N-ethylmaleimide present in the reaction mixture only small amounts of phosphatidate were formed. After 10 min, excess unreacted N-ethylmaleimide was allowed to react with dithiothreitol and the protein was washed free of low-molecular weight \([^{14}\text{C}]\)-labeled compounds by centrifuging (14,000 \times g for 30 min) through 0.25 M sucrose. The protein pellet obtained after three such wash cycles was essentially free of low-molecular-weight \([^{14}\text{C}]\)-labeled compounds as judged by centrifugation in sucrose and chromatography on Sephadex G-200. The protein pellet was suspended in 0.25 M sucrose, 1 mM EDTA and 0.01% sodium deoxycholate and sonicated 3 times for 15 sec with a Branson sonifier (8 A). The mixture was centrifuged at 140,000 \times g for 30 min to separate the solubilized and insoluble proteins. The counts in the supernatant and pellet were recorded. The greater change in \([^{14}\text{C}]\)N-ethylmaleimide binding after glycerol phosphate treatment occurred with the supernatant protein rather than the pellet. The enzyme activity of the sonicated fractions was measured in the standard glycerol phosphate incorporation assay procedure. These studies confirmed that most of the enzyme activity moved with the active thiol groups during this procedure (Table V). Thus, the enzyme was purified 2-fold by sonication in the presence of 0.01% sodium deoxycholate. Further purification by gel filtration on Sephadex G-200 was attempted. However all the protein and enzyme activity eluted as one peak in the void volume. The activity measured in the supernatant was not enhanced by recombining it with either the N-ethylmaleimide treated or untreated protein from the pellet.

The amount of \([^{14}\text{C}]\)N-ethylmaleimide that was bound to the enzyme fraction after treating the enzyme acyl-CoA complex with sn-glycerol 3-phosphate was about 0.1 nmole per mg of enzyme protein. This same protein fraction was capable of producing 0.66 nmole of diacyl-sn-glycerol 3-phosphate in the assay mixture in 2 min. Thus, the product formed in 2 min was already 6.6 times the amount of free thiol group determined by the \([^{14}\text{C}]\)N-ethylmaleimide labeling technique.

**The non-selectivity of acylation of different acyl groups**

When offered both stearoyl CoA and linoleoyl-CoA in the incubation mixture, the enzyme did not show any selective incorporation of either acyl-CoA into the

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**Fig. 4.** Reaction rates of various acyl-CoA esters in the presence of 5 mg of bovine serum albumin. The standard 2-min assay with the addition of 5 mg of bovine serum albumin was used. The final volume, 0.35 ml, contained 20 \(\mu\)moles of Tris chloride buffer (pH 8.0), 1 mg of enzyme protein, 5 mg of bovine serum albumin, 100 nmole of sn-glycerol 3-phosphate and various amounts of acyl-CoA as indicated: ●—●, oleoyl-CoA; □—□, linoleoyl-CoA; ○—○, stearoyl-CoA; №—№, lauroyl-CoA; №—№, palmitoyl-CoA.

1- or 2-positions of the glycerol molecule. Using the system containing 1 mg of particle protein with 5 mg of bovine serum albumin in the 0.35 ml assay mixture, there was no difference in the rate of incorporation of a variety of acyl-CoA esters (Fig. 4). This indicates a lack of specificity of the enzyme for the acyl portion of the acyl-CoA under the conditions of the experiment.

**DISCUSSION**

The nature of the product formed by the acylation of sn-glycerol 3-phosphate by particles from pigeon liver was determined to be diacyl-sn-glycerol 3-phosphate. This was shown using chromatography on silicic acid impregnated glass fibre paper, by catalytic hydrolysis of the phosphate group with chicken liver phosphohydrolase, and by identifying the major product as a diglyceride with only small amounts (about 10%) present as monoglyceride. The product reported by Fallon and LAMB from rat liver microsomes was identified as monoacyl-sn-glycerol 3-phosphate on the basis of chromatographic evidence in several systems. BRANDES et al. have reported an acyl group to sn-glycerol 3-phosphate ratio of 1.3, indicating the formation of a mixture of diacyl and monoacyl derivatives using rat liver microsomes. The ratio was not dependent upon a direct measure of acyl incorporation, but rather the increased amount of 260-μm absorbance when glycerol phosphate was present. Such a measurement may give a minimal value when glycerol phosphate is present as a preferred acceptor relative to water. Possmayer et al. reported the formation of diacyl-sn-glycerol 3-phosphate by rat liver microsomes as did others. In addition, guinea pig liver microsomes produced the diacyl derivative, as did brain preparations. The presence of an enzyme in liver that is capable of acylating 1-acylglycerol 3-phosphate makes an accumulation of the monoacyl derivative hard to interpret.

The marked inhibition by acyl-CoA of the sn-glycerol 3-phosphate acyltransferase is characteristic of this activity isolated from all sources so far studied. This inhibition for the pigeon liver enzyme occurred above a palmitoyl-CoA concentration of about 60 μM when 1 mg of enzyme protein was present in the reaction mixture. That level is also fairly near the value reported for the rat liver enzyme and the yeast enzyme. Although enzyme inhibition by acyl-CoA is common, its cause is not clear, but is usually ascribed to the detergent properties of the substrate. The effect of acyl-CoA causing inhibition to several enzymes is well documented and the effect of adding extra protein in relieving this inhibition has been studied. Adding different levels of particulate protein or bovine serum albumin raised the amount of palmitoyl-CoA that had to be added to the standard assay mixture before inhibition occurred. These data suggest that the physical form of the palmitoyl-CoA is modified by the presence of the enzyme and the amount of the modification is dependent on the amount of protein present in the reaction mixture. It is likely that binding of acyl-CoA to protein reduces the effective concentration of palmitoyl-CoA below the critical micelle concentration (ref. 30). Alternatively the presence of soluble protein could change the micellar formation of palmitoyl-CoA sufficiently to prevent inhibitory micellar formation before 20 nmoles are added to the reaction mixture. Evidence to support the idea that palmitoyl-CoA interacts with the protein is found in the change in the rate of reaction with the different order of addition.
of substrates (Table II). The initial rate of product formation was much slower when
sn-glycerol 3-phosphate was added last. The possibility of hydrolysis of palmitoyl-
CoA prior to addition of sn-glycerol 3-phosphate seems precluded by the observation
that in the presence of 5 mg of bovine serum albumin the rate of formation of phos-
phatidate was very similar no matter which order of addition of reagents is adopted.

The beneficial effect of adding acyl-CoA last raises the possibility that some
functionally active site is slowly inactivated in the presence of high levels of substrate.
One possible explanation considered was that the enzyme required a free prosthetic
group essential to its transferase function, and that high concentrations of substrate
kept it in the acylated form, preventing it from continued acyl transfer from CoA
to glycerol phosphate. Presumably when glycerol phosphate is present first, the acyl
groups are transferred to many more glycerol hydroxyls before the essential site of
the enzyme becomes blocked. On the other hand, added albumin could bind substrate
and slow the rate of the inactivating protein acylation. In such a case, the CoA
thiol esters may have a greater tendency to form the “blocked” acylated transferase
than other acyl donors such as acylated acyl-carrier protein. The speed of this
reversible acyl inactivation could be considerably dependent on the acyl donor used.

The best acyl donor in experiments with Cl. butyricum was shown to be acyl
carrier protein by Goldfine et al. These workers believed that acyl-CoA is not
an intermediate in their system. In the E. coli system, acyl-carrier protein also acts
as acyl donor. Fatty acid, ATP, CoA and Mg2+ were used in the plant system and
the intervention of acyl-carrier protein was not described, although acyl-carrier
protein has been isolated from spinach leaves by Simoni et al. The acyl protein
of the yeast fatty acid synthetase was found incapable of serving as an acyl donor
unless CoA was added, presumably to serve in forming an acyl-CoA intermediate.
In animal systems, acyl-carrier protein has not been isolated and the question
whether the acyl-CoA first transfers the acyl group to a particle-bound acyl-carrier
protein is not clear. However, the results with pigeon liver particles suggest that
no appreciable reservoir of bound acyl groups are transferred to glycerol phosphate.
When stearoyl-CoA, oleoyl-CoA or linoleoyl-CoA were incubated with particles only
phosphatidates with 0, 2 and 4 double bonds respectively were formed. This use of
added acyl-CoA is in striking contrast to the situation reported for Cl. butyricum
where large amounts of endogenous acyl groups, presumably derived from acylated
acyl-carrier protein are acylated to sn-glycerol 3-phosphate. In that case, the actual
acyl donors are not known, and there may be a variety of new substrates to examine
as acyl donors.

We have shown that the thiol group essential for enzyme activity in the pigeon
liver particulate fraction is not separated from the enzyme activity in sonication of
the particles in 0.01% sodium deoxycholate (Tables IV and V). The amount of thiol
group protected from N-ethylmaleimide binding by prior incubation of the enzyme
with acyl-CoA has been estimated. N-ethylmaleimide combines with thiol groups
on proteins but also reacts slowly with other groups. This slow reaction with
other groups could be partially responsible for the high blank values when [14C]N-
ethylmaleimide is incubated with particles that had been treated earlier with non-
isotopic N-ethylmaleimide without any prior protecting incubation with acyl-CoA.
The amount of thiol groups present in the pigeon liver particles associated with 42%
of the enzyme activity is, at maximum, 0.1 nmole per mg protein and could be less
than this. The synthesis of phosphatidate in amounts 6.6 times greater than
detectable bound sulphydryl groups leads us to believe that the enzyme is capable
of a turnover of acyl groups on the thiol group according to the following scheme.

\[
\begin{align*}
\text{acyl-S-CoA} & \quad \text{enzyme-SH} & \quad \text{acyl-sn-glycerol 3-phosphate} \\
\text{HSCoA} & \quad \text{enzyme-S-acyl} & \quad \text{sn-glycerol 3-phosphate}
\end{align*}
\]

The need for rather high levels of acyl-CoA to protect the activity from N-ethyl-
maleimide may reflect the position of the equilibrium between the two thiol esters.

The turnover of acyl groups to the N-ethylmaleimide treated preparation would
suggest that no additional catalytic thiol groups are needed other than those protected
by the acyl-CoA. Thus, if an acyl-carrier protein did serve as the immediate acyl
donor to glycerol phosphate, its ability to be recharged by acyl-CoA indicates that
additional catalytic transferase thiol groups must also be protected for such turnover
to occur. Those thiol groups are apparently concerned with only the first acylation
of glycerol 3-phosphate, since the enzyme(s) catalyzing the second acylation func-
tion(s) adequately in the presence of N-ethylmaleimide and 5,5'-dithio-bis-(2-nitro-
benzoic acid). Added protein from the insoluble (pellet) fraction that normally
contains the other acyltransferase did not restore activity to the N-ethylmaleimide
treated preparation (Table V). Further experiments with more highly purified
fractions are needed to interpret the slight enhancement noted.

A low specificity for the yeast enzyme can be inferred from the finding of
Kuhn and Lynen\(^8\) that oleoyl-CoA protected the palmitoyl-CoA dependent incor-
poration of glycerol phosphate from N-ethylmaleimide poisoning and vice versa.

The enzyme activity of pigeon liver as isolated \textit{in vitro} showed no specificity
in its reaction with a variety of acyl-CoA derivatives under the conditions reported
in this paper and reported by Hill \textit{et al.}\(^1\). Possmayer \textit{et al.}\(^2\) have concluded that
phosphatidate synthesis in rat liver microsomes \textit{in vitro} occurred with positional
specificity. Their system differed from that used in our experiments in that the acyl
groups were presented as radioactive fatty acids. The labeled acids in the phospha-
tidate could arise by an acylation of some endogenous precursors and the different
acids could be diluted by endogenous pools of different sizes making comparisons
difficult. In our experiments the newly-formed phosphatidates were identified by the
\(^{14}\text{C}\)glycerol which could only have arisen by acylation of both hydroxy groups of
\(sn\)-glycerol 3-phosphate. The lack of a specificity for acyl groups when studied \textit{in vitro}
was also reported in another laboratory recently\(^4\), and all these data reflect
the original demonstrations of no acyl\(^1\) or positional\(^2\) specificity. If the enzyme
system measured \textit{in vitro} is responsible for the specific placement of saturated or
unsaturated acyl groups at the 1- and 2-positions in phosphatidates formed in the
intact cell, the breakdown of the microenvironment with the disruption of the cell
must remarkably change the properties of the enzyme system. This change may be
due to the different protein concentrations and solvent conditions used when the
enzyme is assayed \textit{in vitro}. The presence of protein does markedly affect the properties
of the enzyme particularly in regard to its behavior to varying acyl-CoA levels. The
failure to observe specific placement of different acyl groups on the 1- and 2-positions
of \(sn\)-glycerol 3-phosphate may be due to the presentation of the acyl donors in the
wrong form. The detergent effects of micellar acyl-CoA may alter the properties of

the enzyme when assayed *in vitro*. Such effects would not be expected with physiological levels of protein or with acyl-carrier protein serving as the substrate. Alternatively, the provocative possibility remains that the enzyme system that is involved in phosphatidate synthesis in the intact cell may not be the same as that studied *in vitro*.

**REFERENCES**
