The Relationship between Palmitoyl-Coenzyme A Synthetase Activity and Esterification of *sn*-Glycerol 3-Phosphate in Rat Liver Mitochondria

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1. The specific activities for palmitoyl-CoA synthetase and for *sn*-glycerol 3-phosphate esterification, with palmitoyl-CoA generated either by the endogenous synthetase or from palmitoyl(–)-carnitine, CoA and excess of carnitine palmitoyltransferase, were measured with rat liver mitochondria. 2. The mean specific activity of palmitoyl-CoA synthetase was approximately five- and seven-fold the rates of *sn*-glycerol 3-phosphate esterification from palmitate and palmitoyl(–)-carnitine respectively. No significant correlation was found in different rats between the activities of palmitoyl-CoA synthetase and *sn*-glycerol 3-phosphate esterification from either acyl precursor. However, there was a significant correlation ($r = 0.83$, $P < 0.001$) between the rates of glycerolipid synthesis from palmitate and palmitoyl(–)-carnitine. 3. The mean molar composition of the glycerolipid synthesized from palmitate was 58% lysophosphatidate, 31% phosphatidate and 11% neutral lipid. With palmitoyl(–)-carnitine the equivalent values were 70, 23 and 7%, which were significantly different. 4. When palmitoyl-CoA synthetase had been inactivated by 60–70% after preincubation of mitochondria at 37°C, it became rate-limiting in glycerolipid biosynthesis. Additions of 1–5 mM-ATP prevented inactivation of palmitoyl-CoA synthetase. 5. Preincubation also inhibited the oxidation of palmitate, palmitoyl-CoA, palmitoyl(–)-carnitine and malate plus glutamate. These inhibitions could not be prevented by addition of ATP. 6. Diversion of palmitoyl-CoA to form palmitoyl(–)-carnitine did not inhibit *sn*-glycerol 3-phosphate esterification. 7. The palmitoyl-CoA pool synthesized by the palmitoyl-CoA synthetase was augmented by adding partially purified synthetase or carnitine palmitoyltransferase and palmitoyl(–)-carnitine. No stimulation of palmitate incorporation into glycerolipids occurred. 8. At low concentrations of Mg^{2+} , palmitate, ATP and CoA the velocity with palmitoyl-CoA synthetase decreased more than that of glycerolipid synthesis from palmitate. 9. It is concluded that in the presence of optimum substrate concentrations the activity of *sn*-glycerol 3-phosphate acyltransferase and not of palmitoyl-CoA synthetase is rate-limiting in the synthesis of phosphatidate and lysophosphatidate in isolated rat liver mitochondria.

The purpose of this investigation was to examine the relationship between palmitoyl-CoA synthetase (palmitate–CoA ligase, EC 6.2.1.3) activity and the subsequent esterification of palmitoyl-CoA to *sn*-glycerol 3-phosphate. With rat liver mitochondrial fractions we find the major products of *sn*-glycerol 3-phosphate esterification to be lysophosphatidate and phosphatidate. This esterification process was therefore catalysed by acyl-CoA synthetase, *sn*-glycerol 3-phosphate acyltransferase (EC 2.3.1.15) and monoacyl-*sn*-glycerol 3-phosphate acyltransferase.

The mean specific activities of the mitochondrial enzymes involved in phosphatidate and lysophosphatidate biosynthesis have been compared in a series of male rats to find which activity might be rate-limiting. However, it was considered that this com-

parison alone might be insufficient to decide which reaction was the rate-controlling step. Therefore a series of experiments was designed in which the concentrations of palmitoyl-CoA available to the *sn*-glycerol 3-phosphate acyltransferase were altered. The effect of this on glycerolipid synthesis was then examined.

Experimental

Materials

Animals. Male Wistar rats (160–190 g) were obtained from the Joint Animal Breeding Unit, Sutton Bonington, Leics., U.K., and allowed free access to food and water before the experiments.

Chemicals and enzymes. ATP, CoA and glycerol kinase (EC 2.7.1.30) were purchased from Boehringer Corp. Ltd., London W.5, U.K. Palmitic acid, (–)-carnitine, trifluoroacetic anhydride, trifluoroacetic

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acid and dithiothreitol were from Koch-Light Laboratories Ltd., Colnbrook, Bucks., U.K. *rac*-Glycerol 3-phosphate, ($\pm$)-4-amino-3-hydroxybutyric acid, oligomycin, rotenone and Dowex resins were from Sigma (London) Chemical Co., Kingston-upon-Thames, Surrey, U.K. Fatty acid-poor bovine serum albumin was from Pentex Inc., Kankakee, Ill., U.S.A. Aluminium oxide (Brockmann type I, alkaline) was from Hopkin and Williams Ltd., Chadwell Heath, Essex, U.K. Silica gel G was from Macherey, Nagel and Co., Düren, West Germany. All radiochemicals were obtained from The Radiochemical Centre, Amersham, Bucks., U.K.

Methods

Synthesis of palmitoyl(-)-carnitine. Palmitoyl(-)-carnitine was prepared by a method based on that of Chase & Tubbs (1972). Palmitic acid (40mmol) was dissolved in 5ml of trifluoroacetic acid plus 5ml of trifluoroacetic anhydride and left at 20°C for 30min. Then 25.5mmol of (-)-carnitine dissolved in 10ml of trifluoroacetic acid was added. After a further 30min at room temp., the reaction was stopped with 20ml of ice-cold water and the volume was decreased to a minimum by rotary evaporation. The residue was washed with diethyl ether to remove excess of palmitic acid and trifluoroacetic acid and dissolved in butan-1-ol. This solution was washed twice with 66mM-potassium phosphate buffer, pH 5.9, and once with water. The butanol was removed by freeze-drying and the palmitoyl(-)-carnitine was crystallized from chloroform to yield 6.0mmol of product. The product was characterized by chromatography on t.l.c. plates of silica gel G developed with chloroform-methanol-0.1M-sodium acetate (4:4:1, by vol.). The palmitoyl(-)-carnitine was standardized by weighing and by carboxylic ester bond determination (Stein & Shapiro, 1953) and no significant difference was found between the two values.

Preparation of palmitoyl-CoA. Palmitoyl-CoA was prepared by treating CoA with the mixed anhydride of palmitic acid, which was prepared by the following method. To 1ml of methylene chloride was added 200 μ mol of palmitic acid and 200 μ mol of triethylamine, which had been stored over NaOH. The reaction mixture was left at room temp. for 30min. After cooling to 0°C, 200 μ mol of ethyl chloroformate was added and the mixture left for 2h at 0°C. Methylene chloride was removed with a stream of N₂, and 3.3ml of tetrahydrofuran, which had previously been passed through an alumina column to remove peroxides, was added. The mixture was shaken and centrifuged, and 1ml of the clear supernatant was transferred to a darkened tube containing 20 μ mol of CoA in 2ml of 1M-KHCO₃. The tube was flushed with N₂ and incubated with gentle shaking at room temp. for 90min, after which the pH was decreased

to 1 with 2M-HCl. The remaining tetrahydrofuran was removed with a stream of N₂ and the precipitate collected by centrifugation. This pellet was washed five times with 4ml of diethyl ether and three times with 2ml of 0.1M-HCl to remove palmitic acid, the mixed anhydride of palmitate and unchanged CoA. The remaining pellet was dissolved in water and adjusted to pH 4.5 with KHCO₃. The product was assayed by using $\epsilon_{260} = 15400$ for the adenine moiety, and by its thiol ester content, which was measured by the hydroxamate assay (Brindley & Hübscher, 1966). Good agreement was obtained between the two assays and the average value was taken to calculate yields, which were 50–70% based on CoA recovery.

Preparation of ($\pm$)-[³H]carnitine. ($\pm$)-[³H]Carnitine was prepared and extracted by the method of Carter & Bhattacharyya (1953) by using 4.6mmol of ($\pm$)-4-amino-3-hydroxybutyric acid and 27.6mmol of [³H]methyl iodide (0.86 μ Ci/ μ mol). The product was further purified by chromatography on a column (2cm $\times$ 50cm) of Dowex 50 (X8; 300–400 mesh) with 0.05M-HCl for loading and 1.5M-HCl for elution. ($\pm$)-[³H]Carnitine chloride was recovered by evaporating the excess of HCl at 50°C under reduced pressure. The residue was taken up in 10ml of water, adjusted to pH 5.5 with KHCO₃ and characterized by paper chromatography (Bregoff *et al.*, 1953). The product was radiochemically pure. It was standardized by measuring its radioactivity and assuming a specific radioactivity of 2.58 μ Ci/ μ mol. The yield was 73% from ($\pm$)-4-amino-3-hydroxybutyric acid. Before use the ($\pm$)-[³H]carnitine was diluted with non-radioactive (-)-carnitine.

Preparation of *sn*-[1,3-³H]glycerol 3-phosphate. This was prepared from [1,3-³H]glycerol by the method of Smith & Hübscher (1966). The product had a specific radioactivity of 9.72 μ Ci/ μ mol and was diluted appropriately with *rac*-glycerol 3-phosphate before use in enzyme assays.

Preparation of carnitine palmitoyltransferase (EC 2.3.1.23). This enzyme was prepared from bovine liver by the method of Norum (1964), except that CaCl₂, EDTA and palmitoylcarnitine were omitted during the extraction of the freeze-dried residue. This extract was dialysed against 20vol. of 1mM-dithiothreitol and finally against 20vol. of 10mM-Tris, adjusted to pH 7.4 with HCl, and containing 1mM-dithiothreitol. The dialysis residue was centrifuged for 1h at 4°C and 130000g (r_{av} , 7.38cm) and the clear supernatant was concentrated by using a Millipore Pellicon membrane with a 1000mol.wt. cut-off. The resulting solution had a protein concentration of 21mg/ml.

Partial purification of palmitoyl-CoA synthetase. A freeze-dried microsomal fraction from rat liver was prepared and extracted with Triton X-100 by the method of Bar-Tana & Shapiro (1964), except that

1 mM-dithiothreitol and 1 mM-ATP were included in all buffers to stabilize the palmitoyl-CoA synthetase. The Triton-solubilized enzyme preparation was then added slowly to 10 vol. of acetone at -15°C and the precipitate collected by centrifugation. The pellet was washed once with water-acetone (1:10, v/v) and once with acetone-diethyl ether (1:1, v/v) at -15°C . The residue was dried with a stream of N_2 and extracted with deoxycholate (Bar-Tana *et al.*, 1971) with the inclusion of 1 mM-dithiothreitol and 0.1 mM-ATP in all buffers. The dialysed deoxycholate extract was then treated with powdered $(\text{NH}_4)_2\text{SO}_4$. The precipitate obtained between 20 and 55% saturation was dialysed against 100 vol. of 5 mM-Tris adjusted to pH 7.9 with HCl and containing 20% (v/v) glycerol, 0.025% (w/v) sodium deoxycholate, 1 mM-EDTA, 0.25 mM-dithiothreitol and 0.1 mM-ATP. The dialysis residue was stored at -20°C and showed an approx. 2.3-fold increase in specific activity of palmitoyl-CoA synthetase when compared with the freeze-dried microsomal fraction.

Preparation of rat liver mitochondria. Mitochondria were prepared and protein was determined as described previously (Manning & Brindley, 1972).

Enzyme assays. Unless otherwise stated, all enzymes were assayed under conditions of optimum substrate and cofactor concentrations such that reaction rates were constant throughout the period of incubation and were proportional to enzyme concentration. The apparent incorporation of substrate obtained in the absence of mitochondrial protein was subtracted from each determination as a blank. The accuracy of the determinations was $\pm 5\%$. The reaction conditions for different enzymes were made as similar as possible to facilitate comparison between different specific activities.

Determination of palmitoyl-CoA synthetase activity. Palmitoyl-CoA synthetase activity was determined by converting the palmitoyl-CoA produced into palmitoyl[^3H]carnitine with excess of carnitine palmitoyltransferase (Farstad *et al.*, 1967). The assay system contained, in a volume of 0.25 ml, 25 mM-Tris buffer adjusted to pH 7.4 with HCl, 5 mM-dithiothreitol, $48\text{ }\mu\text{M}$ -CoA, 2.5 mM-ATP, 4.8 mM- MgCl_2 , 0.3 mM-potassium palmitate, 1.5 mg of fatty acid-poor bovine serum albumin, 2.4 mM-(-)-[^3H]carnitine [$0.4\text{ }\mu\text{Ci}/\mu\text{mol}$ with respect to (-)-carnitine], excess of carnitine palmitoyltransferase (approx. $420\text{ }\mu\text{g}$ of protein), 80 mM-KCl, 1 mM-EDTA, $0.75\text{ }\mu\text{g}$ of oligomycin and $0.4\text{ }\mu\text{g}$ of rotenone. The reactions were started with 20–50 μg of mitochondrial protein and after 7 min at 37°C were stopped with 0.1 ml of 5.5 M-HCl. Palmitoyl[^3H]carnitine was extracted with butan-1-ol (Farstad *et al.*, 1967) and the radioactivity measured by liquid-scintillation counting. It was necessary to freeze and thaw the samples in the presence of butan-1-ol to gain a complete recovery of palmitoylcarnitine.

Determination of sn-glycerol 3-phosphate esterification from palmitate. The biosynthesis of glycerolipids was determined by measuring the incorporation of *sn*-[1,3- ^3H]glycerol 3-phosphate into lipids. The assay system contained, in a volume of 0.25 ml, 25 mM-Tris buffer adjusted to pH 7.4 with HCl, 5 mM-dithiothreitol, $48\text{ }\mu\text{M}$ -CoA, 2.5 mM-ATP, 4.8 mM- MgCl_2 , 0.3 mM-potassium palmitate, 1.5 mg of fatty acid-poor bovine serum albumin, 5 mM-*sn*-[1,3- ^3H]glycerol 3-phosphate ($1.0\text{ }\mu\text{Ci}/\mu\text{mol}$), 80 mM-KCl, 1 mM-EDTA, $0.75\text{ }\mu\text{g}$ of oligomycin and $0.4\text{ }\mu\text{g}$ of rotenone.

The reactions were started with 20–50 μg of mitochondrial protein, incubated for 7 min at 37°C and stopped with 1.2 ml of butan-1-ol. Lipid products were extracted by the method of Daae & Bremer (1970) and radioactivity was determined by liquid-scintillation counting. The extraction of lipids by this method was superior to the chloroform-methanol extractions of Hajra *et al.* (1968) and Garbus *et al.* (1963), which gave 64 and 35% respectively of the ^3H -labelled lipid, which was recovered in butan-1-ol. The discrepancy appeared to be due to low recoveries of lysophosphatidate, which is the major product of the mitochondrial system. With microsomal fractions of rat liver and guinea-pig intestinal mucosa, where the major product was phosphatidate, the extraction of Hajra *et al.* (1968) gave slightly higher recoveries than the other two methods (Brindley, 1973).

Determination of glycerol 3-phosphate esterification by palmitoyl-CoA generated from palmitoyl(-)-carnitine. This activity was assayed by the method of Daae & Bremer (1970). The assays contained, in a volume of 0.25 ml, 25 mM-Tris buffer adjusted to pH 7.4 with HCl, 5 mM-dithiothreitol, $50\text{ }\mu\text{M}$ -CoA, $124\text{ }\mu\text{M}$ -palmitoyl(-)-carnitine, a saturating quantity of carnitine palmitoyltransferase (approx. $130\text{ }\mu\text{g}$), 1.5 mg of fatty acid-poor bovine serum albumin, 80 mM-KCl, 5 mM-*sn*-[^3H]glycerol 3-phosphate ($1\text{ }\mu\text{Ci}/\mu\text{mol}$), $0.75\text{ }\mu\text{g}$ of oligomycin and $0.4\text{ }\mu\text{g}$ of rotenone. The reaction was started with 50 μg of mitochondrial protein, incubated at 37°C for 7 min and extracted with butan-1-ol as described by Daae & Bremer (1970).

We confirm the findings of Daae & Bremer (1970) that palmitoyl-CoA generated from palmitoyl(-)-carnitine is a better substrate for esterification than exogenous palmitoyl-CoA. A similar assay system containing $19\text{ }\mu\text{M}$ -palmitoyl-CoA but with no albumin, carnitine palmitoyltransferase or CoA, was incubated for 4 min at 37°C . Specific activities were 15–20% of those obtained with the palmitoyl-CoA-generating system.

Measurement of fatty acid oxidation. Oxidation was measured with a Clark-type oxygen electrode at 37°C . Malonate was added to prevent citrate formation. The incubation medium contained, in a volume

of 3.8 ml, 20 mM-Tris adjusted to pH 7.4 with HCl, 5 mM-dithiothreitol, 80 mM-KCl, 0.1 mM-EDTA, 3 mM-MgCl₂, 0.1 mM-2,4-dinitrophenol, 5.2 mM-sodium malonate, 10 mM-potassium phosphate buffer, pH 7.4, 20 mg of fatty acid-poor bovine serum albumin and 5.7 µg of oligomycin. Other additions are indicated in the text.

Characterization of lipids synthesized from *sn*-glycerol 3-phosphate. Neutral lipid classes were separated by t.l.c. on silica gel G by using light petroleum (b.p. 40–60°C)–diethyl ether–acetic acid (60:40:1, by vol.) for development. Phospholipids were separated on silica-gel G plates by the method of Vavrečka *et al.* (1969), or as described by Hajra & Agranoff (1968). Areas of the silica gel were scraped into counting vials and to each 1 ml of methanol, 2.5 ml of water and 10 ml of Triton X-100–xylene (1:2, v/v) containing 5 g of 2,5-diphenyloxazole/l and 0.1 g of 1,4-bis-(5-phenyloxazol-2-yl)benzene/l were added. The vials were shaken to form gels and the samples assayed by liquid-scintillation counting. Recoveries from the plate were normally 90–105%.

Statistical procedures. The results were evaluated statistically as described by Bradford-Hill (1967) and regression analysis was performed on a K.D.F.9 computer.

Results

*Comparison of the activities of palmitoyl-CoA synthetase and palmitate esterification with *sn*-glycerol 3-phosphate*

Table 1 shows that the mean specific activity of palmitoyl-CoA synthetase was approximately fivefold that of *sn*-glycerol 3-phosphate esterification from free palmitate. By using a paired *t* test, this latter

activity was shown to be significantly greater ($P < 0.01$) than the rate of *sn*-glycerol 3-phosphate esterification when palmitoyl-CoA generated from palmitoyl(–)-carnitine was used. Therefore the rate of *sn*-glycerol 3-phosphate acyltransferase was underestimated by about 20% when palmitoyl-CoA was generated from palmitoyl(–)-carnitine rather than from palmitate by the endogenous acyl-CoA synthetase. This was probably caused by the detergent properties of palmitoyl(–)-carnitine.

The specific activities presented in Table 1 differ from those reported by Daae & Bremer (1970), who found the mitochondrial activity of *sn*-glycerol 3-phosphate acyltransferase to be 1.4-fold that of palmitoyl-CoA synthetase.

In individual rats there was no correlation ($r = 0.007$, $P > 0.05$) between the specific activities of palmitoyl-CoA synthetase and palmitate incorporation into glycerolipids (Fig. 1*a*). However, there was a significant correlation ($r = 0.83$, $P < 0.01$ considering all points, or $r = 0.76$, $P < 0.05$ with the top three points omitted) between the activities of glycerolipid synthesis from palmitate and palmitoyl-carnitine (Fig. 1*b*). The intercept was not significantly different from zero.

The major product of *sn*-glycerol 3-phosphate esterification with both systems was lysophosphatidate (Table 1). By using a paired *t* test, significant differences were found between the percentage of phosphatidate ($P < 0.02$) and lysophosphatidate ($P < 0.01$) produced by the two acylating systems. A predominant synthesis of lysophosphatidate was also observed by Daae & Bremer (1970), Daae (1972) and Monroy & Pullman (1972), but the product isolated by Stoffel & Schiefer (1968) and Zborowski & Wojtczak (1969) was mainly phosphatidate. The

Table 1. *Specific activities of the enzymes involved in fatty acid activation and *sn*-glycerol 3-phosphate esterification in rat liver mitochondria and composition of the products of the reactions*

The mean values $\pm$ S.E.M. of the enzyme activities are given in nmol of palmitate activated or incorporated/min per mg of mitochondrial protein. The mean values $\pm$ S.E.M. for the product composition are given in mol/100 mol. The numbers of animals used are shown in parentheses.

Enzyme system	Sp. activity	Product composition (mol/100 mol)		
		Lysophosphatidate	Phosphatidate	Neutral lipid
(1) Palmitoyl-CoA synthetase	80.3 $\pm$ 6.6 (14)	—	—	—
(2) Esterification of glycerophosphate with palmitoyl-CoA generated from palmitate	16.0 $\pm$ 2.7 (17)	58 $\pm$ 6 (9)	31 $\pm$ 5 (9)	11 $\pm$ 2 (9)
(3) Esterification of glycerophosphate with palmitoyl-CoA generated from palmitoyl(–)-carnitine	12.6 $\pm$ 3.2 (12)	70 $\pm$ 7 (7)	23 $\pm$ 5 (7)	7 $\pm$ 3 (7)

Fig. 1. Relationship between the specific activity of palmitoyl-CoA synthetase and glycerolipid synthesis in the mitochondrial fraction of rat liver

The specific activities shown in Table 1 were plotted relative to each other and subjected to linear regression analysis. Specific activities are expressed as nmol of palmitate activated or incorporated into glycerolipids/min per mg of mitochondrial protein. (a) The relationship between palmitoyl-CoA synthetase and glycerolipid synthesis from palmitate; (b) the relationship between glycerolipid synthesis from palmitate and palmitoyl(-)-carnitine.

differences might be due to the fact that butan-1-ol is a more suitable solvent for the extraction of lysophosphatidate than is chloroform-methanol (Daae & Bremer, 1970; see the Experimental section). This might also explain why Davidson & Stanacev (1972) were unable to detect a mitochondrial acylation of *sn*-glycerol 3-phosphate, since chloroform-methanol extraction at neutral pH and low ionic strength, as described by Bligh & Dyer (1959), would not give good recoveries of lysophosphatidate.

The neutral lipid reported in Table 1 consisted of approx. 75% diglyceride and 25% monoglyceride. Daae & Bremer (1970) reported that 10–20% of their total incorporation was recovered as monoglyceride and suggested that this might arise through the action of a lysophosphatidate phosphohydrolase. The monoglyceride in our experiments represented 2–3% of the total glycerolipid.

Effect of preincubation on the activation of palmitate and its subsequent incorporation into glycerolipids

Rat liver mitochondria were preincubated at 37°C to inactivate palmitoyl-CoA synthetase activity (Farstad, 1968; Lippel, 1971; Brindley & Ferrier, 1972) and the effect of this inhibition on *sn*-glycerol 3-phosphate esterification was studied.

Preincubation led to a striking inactivation of palmitoyl-CoA synthetase and a much smaller inhibition of *sn*-glycerol 3-phosphate acyltransferase activity (Fig. 2). In one experiment, the inhibition of *sn*-glycerol 3-phosphate esterification from free palmitate became greater than that from palmitoyl(-)-carnitine, but only after a 20 min preincubation (rat no. 1). With the three rats examined, the synthesis of palmitoyl-CoA became rate-limiting when 60–70% of the synthetase activity had been lost. These results indicate that the activity of the synthetase in the original mitochondrial fraction was in an approximately threefold excess over the rate of palmitate esterification.

It was reported that the inhibition of palmitoyl-CoA synthetase produced by preincubation could be reversed by incubating in the presence of a heat-labile soluble protein, ATP and Mg^{2+} (Farstad *et al.*, 1967; Farstad, 1967). However, in agreement with Bar-Tana *et al.* (1971) and Lippel (1971), we were unable to obtain such a stimulation. It was therefore not possible to investigate the effect of an increase in synthetase activity on glycerolipid synthesis by this method.

Effect of preincubation on mitochondrial oxidation

The same mitochondrial preparation as was used in the experiments described in Fig. 2 was also

assayed for its ability to oxidize palmitate, palmitoyl-CoA and palmitoyl(-)-carnitine (Table 2). The oxidation of these substrates was strongly inhibited by preincubation. To find whether this inhibition was limited to β -oxidation, the rate of oxidation of malate plus glutamate was also determined. As the latter system was inhibited to a similar extent, a general inhibition of mitochondrial electron transport had probably occurred.

In agreement with the observations of Farstad (1968), addition of 1–5 mM-ATP to the preincubation medium prevented inhibition of palmitoyl-CoA synthetase. However, no such protection was observed with the oxidation of any of the substrates used. The omission of dithiothreitol from the preincubation medium did not affect the extent of inhibition.

From these findings it follows that the nature of

Fig. 2. Effect of preincubation on palmitate activation and esterification

Rat liver mitochondria (30 mg of protein/ml) were preincubated at 37°C in 0.25 M-sucrose containing 4 mM-Tris adjusted to pH 7.4 with HCl, 1 mM-ethyleneglycol-bis-(β -amino-ethylether) *NN'*-tetra-acetate and 5 mM-dithiothreitol. At the times indicated 50 μ l samples of mitochondria were diluted with 1.45 ml of the same medium containing 1 mM-ATP and at 0°C to prevent further inhibition. Samples of diluted mitochondria (50 μ g of protein) were taken for measurement of palmitoyl-CoA synthetase (●, rat no. 1; ○, rat no. 2; △, rat no. 3) and *sn*-glycerol 3-phosphate esterification from either palmitate (▲, rat no. 1; ▤, rat no. 2; ▤, rat no. 3) or palmitoyl(-)-carnitine (■, rat no. 1; □, rat no. 2; ▤, rat no. 3). The enzyme activities at the various time-intervals are expressed as a percentage of their own activities before incubation.

Table 2. Effect of preincubation on mitochondrial oxidation

Preincubation time (min)	A Palmitate		B Palmitoyl-CoA		C Palmitoylcarnitine		D Malate + glutamate	
	Oxygen consumed	Inhibition (%)	Oxygen consumed	Inhibition (%)	Oxygen consumed	Inhibition (%)	Oxygen consumed	Inhibition (%)
0	103	0	60	0	112	0	122	0
10	48	54	43	26	—	—	56	54
20	37	64	26	57	35	69	43	65
30	23	78	26	57	29	74	—	—
50	23	78	24	60	—	—	40	67

Rat-liver mitochondria were preincubated at 37°C as described in Fig. 2. At various time-intervals 0.1 ml of mitochondrial suspension was pipetted into the oxygen-electrode chamber, which contained the incubation medium (see the Experimental section) with the following additions: A, 36 μ M-CoA, 2.5 mM-ATP, 1.8 mM-(±)-carnitine and 0.3 mM-potassium palmitate; B, 1.8 mM-(±)-carnitine and 14 μ M-palmitoyl-CoA; C, 54 μ M-palmitoyl(-)-carnitine; D, 5 mM-potassium malate and 5 mM-potassium glutamate. The results are expressed as ng-atoms of oxygen consumed/min per mg of mitochondrial protein.

Fig. 3. Effect of rate-limiting concentrations of Mg^{2+} , palmitate, ATP and CoA on palmitoyl-CoA synthetase and glycerolipid biosynthesis

The concentrations of (a) Mg^{2+} , (b) palmitate, (c) ATP and (d) CoA were varied separately while the concentrations of other cofactors and substrates were kept at optimum concentrations. Palmitoyl-CoA synthetase (●, rat no. 4; ○, rat no. 5) and glycerolipid synthesis from palmitoyl-CoA generated from palmitate (▲, rat no. 4; △, rat no. 5) were measured and expressed relative to the maximum velocity for the appropriate activity. Maximum velocities were 48.6 and 83.4 nmol of palmitate activated/min per mg of mitochondrial protein and 7.1 and 6.6 nmol of palmitate incorporated into glycerolipids/min per mg of mitochondrial protein for rats nos. 4 and 5 respectively.

the inhibition of β -oxidation caused by preincubation of mitochondria at 37°C is different from that encountered when glycerolipid biosynthesis is being

measured. The decrease in palmitate oxidation is unlikely to have been caused by an inhibition of palmitoyl-CoA synthetase.

Effect of carnitine palmitoyltransferase and (–)-carnitine or palmitoyl(–)-carnitine on the incorporation of palmitate into glycerolipids

Excess of carnitine palmitoyltransferase (420 µg of protein) was added to the assay system in which glycerolipid biosynthesis from free palmitate was measured. This did not significantly alter the rate of *sn*-glycerol 3-phosphate esterification. By further adding palmitoyl(–)-carnitine or (–)-carnitine it was possible to augment or decrease the pool of palmitoyl-CoA produced by the endogenous acyl-CoA synthetase.

The concentrations of (–)-carnitine used were 0.4–1.8 mM. At 1.8 mM (–)-carnitine, which was almost an optimum concentration, the rate of palmitoyl(–)-carnitine synthesis was 61 nmol/min per mg of mitochondrial protein. As the maximum rate of palmitoyl-CoA synthesis measured with the synthetase assay (see the Experimental section) was 79 nmol/min per mg of mitochondrial protein, this represented a diversion of 77% of the total capacity to synthesize palmitoyl-CoA but did not result in an inhibition of *sn*-glycerol 3-phosphate esterification. The result of this experiment differs from that of Zborowski & Wojtczak (1969), who found that 5 mM (±)-carnitine inhibited *sn*-glycerol 3-[³²P]phosphate incorporation into lipids by 50–70%. As palmitoyl(–)-carnitine alone was unable to serve as a direct acyl donor in our experiments it is concluded that the activity of the palmitoyl-CoA synthetase was not rate-limiting in glycerolipid biosynthesis.

The rate of palmitoyl-CoA production in the assay system was increased by adding 30–60 µM-palmitoyl(–)-carnitine in the presence of excess of CoA (50 µM). No change in the rate of glycerolipid synthesis was observed. Higher concentrations of palmitoyl(–)-carnitine (60–350 µM) inhibited the esterification of *sn*-glycerol 3-phosphate. This was probably caused by the detergent properties of the palmitoyl(–)-carnitine and the increased palmitoyl-CoA pool. The results indicated that the rate of glycerolipid synthesis was independent of the rate of palmitoyl-CoA production and was limited by the activity of *sn*-glycerol 3-phosphate acyltransferase.

Effect of adding partially purified palmitoyl-CoA synthetase on glycerolipid biosynthesis

Increasing quantities of partially purified palmitoyl-CoA synthetase were added to rat liver mitochondria to discover whether increasing palmitoyl-CoA synthesis would alter the rates of glycerolipid production.

Although palmitate activation was increased by as much as 64% the incorporation of palmitate into glycerolipids was not affected. These results again indicate that the rate-limiting step in the synthesis

of lysophosphatide is not at the palmitoyl-CoA synthetase reaction but, by inference, is at the step catalysed by *sn*-glycerol 3-phosphate acyltransferase.

Effect of rate-limiting concentrations of Mg²⁺, palmitate, ATP and CoA on palmitoyl-CoA synthetase activity and glycerolipid biosynthesis

The effect of rate-limiting concentrations of substrates and cofactors on the activity of palmitoyl-CoA synthetase and on glycerolipid biosynthesis from palmitate was studied. Glycerolipid biosynthesis in the latter system involves both palmitoyl-CoA synthetase and *sn*-glycerol 3-phosphate acyltransferase activities. Therefore if the synthetase were rate-limiting and palmitoyl-CoA synthesis were decreased by lowering substrate and cofactor concentrations, then glycerolipid synthesis should be decreased to a similar extent.

The results from such an experiment are shown in Fig. 3. In all assays the activity of palmitoyl-CoA synthetase was decreased by a greater percentage than that of *sn*-glycerol 3-phosphate esterification. However, the absolute values of palmitate activation were three- to five-fold the rate of palmitate incorporation into glycerolipids. From the results shown in Fig. 2, and after diversion of palmitoyl-CoA to palmitoyl(–)-carnitine, it would have been expected that palmitoyl-CoA synthesis at most substrate and cofactor concentrations would have been adequate to sustain the maximum rates of glycerolipid synthesis. No appreciable changes were observed in the composition of glycerolipids synthesized and so these discrepancies could not have resulted from a shift from monoacyl- to diacyl-phosphatide. Such a shift might have resulted in underestimation of the rate of palmitate incorporation into glycerolipids. It is also unlikely that the low rate of glycerolipid biosynthesis resulted because the limiting cofactors and substrates were required for *sn*-glycerol 3-phosphate acyltransferase activity. The rates shown in Table 1, enzyme system (3), were obtained in assay systems to which ATP, Mg²⁺ and free palmitate were not added. Increasing the concentration of Mg²⁺ did not stimulate acyltransferase activity.

With rate-limiting concentrations of palmitate and CoA the percentage reduction in velocity for the synthetase was 10–40% lower than for *sn*-glycerol 3-phosphate esterification. However, at 0.5 mM-ATP, palmitoyl-CoA production was decreased by 50–60%, whereas glycerolipid synthesis was not affected at all. We cannot satisfactorily explain why the relative percentage decrease in glycerolipid synthesis and palmitoyl-CoA synthetase activity should differ when different substrates and cofactors were made rate-limiting. It is also surprising that glycerolipid synthesis should have been decreased, as palmitoyl-CoA synthesis was much greater than palmitate

esterification to *sn*-glycerol 3-phosphate at most points. These results indicate that the maximum rates of palmitoyl-CoA production, as measured by the synthetase assay, do not necessarily reflect the availability of palmitoyl-CoA to *sn*-glycerol 3-phosphate acyltransferase. This problem will be discussed in the next paper (Brindley, 1973).

Despite these difficulties of interpretation the results shown in Fig. 3 do indicate that when palmitate activation proceeds at maximum rates, palmitoyl-CoA production is not rate-limiting in glycerolipid biosynthesis.

Discussion

The results of all experiments undertaken indicated that *sn*-glycerol 3-phosphate acyltransferase and monoacyl-*sn*-glycerol 3-phosphate acyltransferase, and not palmitoyl-CoA synthetase, were rate-limiting in biosynthesis of lysophosphatidate and phosphatidate. Fatty acid activation also appeared not to be a rate-determining step of triglyceride synthesis in the adipose tissue of man (Galton & Wilson, 1970) and rat (Lippel *et al.*, 1971). No correlation between adipose-tissue concentrations of long-chain acyl-CoA esters or of *sn*-glycerol 3-phosphate and the rate of triglyceride biosynthesis was observed (Denton & Halperin, 1968; Saggerson & Greenbaum, 1970*a,b*). In contrast to the results obtained with rat liver mitochondria in the present paper, acyl-CoA synthetase may become rate-limiting in phosphatidate biosynthesis in the microsomal fraction of guinea-pig intestinal mucosa (Brindley & Ferrier, 1972; Brindley, 1973) and rat liver (Lloyd-Davies & Brindley, 1973).

As palmitoyl-CoA synthetase is inactivated by preincubation of mitochondria at 37°C it was decided to see whether this treatment caused it to become rate-limiting in β -oxidation. Palmitate oxidation was inhibited (Table 2), but this could not have been caused by the decreased formation of palmitoyl-CoA. A more likely explanation is that the inhibition of respiration is caused by a loss of ions from the mitochondrial matrix, which results in a decrease in the sucrose-impermeable space (Nicholls & Lindberg, 1972). However, there is evidence to suggest that fatty acid activation is not rate-limiting in β -oxidation in rat liver (Shepherd *et al.*, 1965; Van Tol & Hülsmann, 1969; Lippel & Beattie, 1970; Pande, 1971; De Jong, 1971).

The possibility that there may be multiple forms of palmitoyl-CoA synthetase, which generate metabolically different pools of palmitoyl-CoA, is discussed in the next paper (Brindley, 1973).

However, assuming that there is competition for common pools of acyl-CoA, the excess of acyl-CoA production over *sn*-glycerol 3-phosphate esterification obtained *in vitro* might be much decreased *in vivo* owing to the consumption of acyl-CoA by the di-

hydroxyacetone phosphate pathway, β -oxidation and fatty acid elongation. Also, the esterification of *sn*-glycerol 3-phosphate would proceed to a diacyl phosphatide whereas *in vitro* the major product was lysophosphatidate.

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