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Palmitate Activation and Esterification in Microsomal Fractions of Rat Liver

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1. Palmitoyl-CoA synthetase activity in the microsomal fraction of rat liver was measured directly by palmitoyl-CoA production, and indirectly by converting the palmitoyl-CoA into palmitoylcarnitine under optimum conditions. Even in the latter system, palmitoyl-CoA accumulated. The rate of palmitoyl-CoA hydrolysis and the inhibition of palmitoyl-CoA synthetase by palmitoyl-CoA were each estimated to be less than 10% of the maximum rate of palmitoyl-CoA production. 2. The concentration of palmitoyl-CoA present in the assay systems used for measuring palmitate esterification to glycerol phosphate and the activity of palmitoyl-CoA synthetase by using the carnitine-linked determination were measured. These concentrations were not altered by the addition of glycerol phosphate, or of carnitine plus carnitine palmitoyltransferase. 3. The relationship between the activity of palmitoyl-CoA synthetase and the rate of glycerolipid synthesis was investigated. The latter activity was measured by using palmitoyl-CoA generated from palmitate, palmitoyl-AMP or palmitoylcarnitine. 4. It is concluded that, at optimum substrate concentrations, the activity of glycerol phosphate acyltransferase is rate-limiting in the synthesis of phosphatidate by rat liver microsomal fractions. 5. The implications of these results in the measurement of palmitoyl-CoA synthetase and in the control of glycerolipid synthesis are discussed.

The experiments described in the present paper were designed to investigate the relationship between the enzymes involved in phosphatidate synthesis. This involved comparing the relative activities of palmitoyl-CoA synthetase (EC 6.2.1.3) and *sn*-glycerol 3-phosphate esterification in the microsomal fractions obtained from the livers of male rats. In addition, conditions were chosen such that it was possible to alter the net synthesis of palmitoyl-CoA by these fractions. The effect of this alteration on the synthesis of phosphatidate was examined.

During the course of earlier experiments (Sánchez *et al.*, 1973; Lloyd-Davies & Brindley, 1973) it became evident that decreasing the activity of palmitoyl-CoA synthetase, as measured by a carnitine-linked assay, could affect the rate of glycerolipid synthesis much more than would have been expected from a comparison of the specific activities of the enzymes involved. Experiments were therefore designed to investigate the suitability of this carnitine-linked assay for measuring the activity of acyl-CoA synthetase and for relating this to the availability of palmitoyl-CoA for glycerolipid synthesis.

This involved comparing the acyl-CoA synthetase activity as determined by the carnitine-linked assay with that which is measured when palmitoyl-CoA is allowed to accumulate. The size of the palmitoyl-CoA pool which is present during glycerolipid synthesis was also studied, and the effects of the further metabolism of palmitoyl-CoA on this pool were investigated.

Materials and Methods

Animals

Male Wistar rats (170–340g) were obtained from the Nottingham University Joint Animal Breeding Unit, Sutton Bonington, Leics., U.K. They were allowed free access to food and water before the experiments.

Chemicals and enzymes

Unless stated to the contrary these were synthesized or purchased as described previously (Sánchez *et al.*, 1973; Brindley & Bowley, 1975). AMP was purchased from Sigma (London) Chemical Co., Kingston-upon-Thames, Surrey KT2 7BH, U.K., and DEAE-cellulose DE 23 was obtained from Whatman Biochemicals, Springfield Mill, Maidstone, Kent ME14 2LE, U.K.

Preparation of palmitoyl-AMP

The method was modified from that of Vignais & Zabin (1958) by using a mixed anhydride of palmitate (Sánchez *et al.*, 1973) instead of palmitic anhydride.

Preparation of carnitine palmitoyltransferase (*EC* 2.3.1.21)

This enzyme was prepared from bovine liver as described by Sánchez *et al.* (1973). In the experiments reported in Figs. 2 and 3, the enzyme was subjected

to a further purification step by Dr. M. Bowley. In this step the partially purified preparation was adjusted to 10mM with respect to potassium phosphate buffer (pH7.4), and was then adsorbed on to DEAE-cellulose. The mixture was left with intermittent shaking for 2h at 4°C and then centrifuged.

The cellulose was eluted in a batchwise manner with increasing concentrations of phosphate buffer. The protein recovered from the cellulose at a potassium phosphate buffer concentration of between 200 and 250mM was used as a source of enzyme. This procedure increased the specific activity of the carnitine palmitoyltransferase by approx. fourfold to 8.3nmol of palmitoylcarnitine formed/min per mg of protein (as determined by the method of Norum, 1964).

Preparation of microsomal fractions from rat liver

The method was that described by Mangiapane *et al.* (1973), except that the livers were rinsed in 0.3M-sucrose instead of 0.9% NaCl.

Determination of protein

Protein was measured by a biuret method (Brindley & Hübscher, 1965).

Determination of palmitoyl-CoA synthetase

Method 1: *conversion of the palmitoyl-CoA formed into palmitoyl-L-carnitine.* The method is described by Mangiapane *et al.* (1973)

Method 2: *direct measurement of palmitoyl-CoA synthetase by the formation of* [1-^{14}*C*]*palmitoyl-CoA.* The assay system was designed to resemble that used for the assay of palmitoyl-CoA synthetase by Method 1 as closely as possible. The reaction rate was found to be constant for 7min and proportional to protein concentration up to 20μg of microsomal protein in the system. Each assay mixture contained, in a final volume of 0.25ml: 25mM-Tris (adjusted to pH7.4 with HCl), 5mM-dithiothreitol, 55μM-CoA, 2.5mM-ATP, 2.5mM-$MgCl_2$, 0.8mM-potassium [1-^{14}C]palmitate (0.25μCi/μmol) and 1.5mg (approx. 90μM) of fatty acid-poor bovine serum albumin. Reactions were started with 5–20μg of microsomal protein, and after a 7min incubation at 37°C were stopped by the addition of 1ml of Dole reagent (Dole, 1956), 0.35ml of water and 0.6ml of *n*-heptane. Extraction of the [^{14}C]palmitoyl-CoA was carried out as described by Bar-Tana *et al.* (1971), except that the lower phase was washed three times with 0.6ml portions of *n*-heptane. The radioactivity in a 0.5ml sample of the lower phase was measured by liquid-scintillation counting after the addition of 0.1ml of water and 5ml of Triton X-100–xylene (1:2, v/v) containing 5.5g of 2,5-diphenyloxazole/litre and 0.1g of 1,4-bis-(5-phenyloxazol-2-yl)-benzene/litre. The ^{14}C d.p.m. obtained in the absence of microsomal protein were subtracted as a blank. More than 85% of the product was identified as palmitoyl-CoA by co-chromatography with authentic [^{3}H]palmitoyl-CoA, which had been subjected to the same extraction procedure, in the following systems: (a) t.l.c. on cellulose plates in butan-1-ol–acetic acid–water (5:2:3, by vol.) as solvent (Pullman, 1973); (b) t.l.c. on silica gel F-254 pre-coated plates in chloroform–methanol–water (3:3:1, by vol.) as solvent (Ullman & Radin, 1972). In both cases the specific radioactivity of the potassium [1-^{14}C]-palmitate was increased to 4μCi/μmol, and 100–200μl portions of the lower phase were used for chromatography.

In addition, assays were stopped by the addition of 0.94ml of chloroform–methanol (1:2, v/v) and 15μl of 0.2M-HCl, giving a one-phase system. Samples (200μl) were used for two-dimensional t.l.c. on Kieselgel 60–Kieselgur 254 pre-coated t.l.c. plates (Merck, Darmstadt, Germany). These were developed in one direction by using chloroform–methanol–acetic acid–acetone–water (10:2:2:4:1, by vol.) (Hajra & Agranoff, 1968). This separated the phospholipid from the palmitoyl-CoA, which remained near the origin. After drying, the plates were re-developed in the same direction for the same distance in *n*-hexane–diethyl ether–acetic acid (60:40:1, by vol.) as solvent, to ensure that all the unesterified fatty acid moved to the solvent front. They were again dried, and developed in the second direction with chloroform–methanol–water (3:3:1, by vol.) (Ullman & Radin, 1972). The ^{14}C-labelled product co-chromatographed with [^{3}H]palmitoyl-CoA which had been subjected to the same initial extraction procedure. The product which co-chromatographed with palmitoyl-CoA was also degraded by hydroxylamine at pH6.7 (Vignais & Zabin, 1958), and 86% of the ^{14}C-labelled product was extractable into diethyl ether.

Separation of palmitoyl-CoA from palmitoylcarnitine

In the experiments described in Figs. 2 and 3 palmitoyl-CoA synthesis was determined as described in Method 2 above and both palmitoyl-CoA and palmitoylcarnitine were recovered in the bottom phase of the extraction system. These were separated from one another by applying 0.2ml samples of the bottom phase to columns (2.3cm×1cm) of Dowex 50W resin (H^+ form) which had been equilibrated with the bottom phase. Palmitoyl-CoA was eluted with a total volume of 5ml of synthetic bottom phase (Dole, 1956), and 2ml samples of the bottom phase were added to 0.2ml of water and 10ml of Triton X-100–xylene (1:2, v/v) containing 5.5g of 2,5-diphenyloxazole/litre and 0.1g of 1,4-bis-(5-phenyloxazol-2-yl)benzene/litre. Radioactivity was deter-

mined by liquid-scintillation counting. The recoveries of palmitoyl-CoA and palmitoylcarnitine from the columns were checked by using authentic samples, and were approx. 100% and 4% respectively. The results quoted in Figs. 2 and 3 have been corrected for the small leakage of palmitoylcarnitine from the columns.

Determination of sn-glycerol 3-phosphate esterification by using palmitoyl-CoA generated from palmitate

The assay system contained, in a final volume of 0.25ml: 25mM-Tris (adjusted to pH7.4 with HCl), 5mM-dithiothreitol, 66μM-CoA, 2.4mM-ATP, 2.4mM-$MgCl_2$, 0.8mM-potassium palmitate, 1.5mg (approx. 90μM) of fatty acid-poor bovine serum albumin and 15.6mM-*sn*-[1,3-^{3}H]glycerol 3-phosphate (1μCi/μmol). The assays were started by the addition of 25–50μg of microsomal protein, incubated for 7min at 37°C and stopped by the addition of 1.88ml of chloroform–methanol (1:2, v/v). Lipids were extracted as described by Brindley (1973) and characterized by t.l.c. as described by Sánchez *et al.* (1973).

Determination of sn-glycerol 3-phosphate esterification by using palmitoyl-CoA generated from palmitoyl-L-carnitine

The method used was that described by Mangiapane *et al.* (1973).

Determination of sn-glycerol 3-phosphate esterification by using palmitoyl-CoA generated from palmitoyl-AMP

Palmitoyl-CoA was generated from palmitoyl-AMP by using the endogenous acyl-CoA synthetase of the microsomal fraction. The assay system contained in a final volume of 0.25ml: 25mM-Tris (adjusted to pH7.4 with HCl), 5mM-dithiothreitol, 55μM-CoA, 10mM-$MgCl_2$, 0.25mM-palmitoyl-AMP and 15.6mM-*sn*-[1,3-^{3}H]glycerol 3-phosphate. Albumin was not included, since it inhibited the reaction. The reactions were started with 25–50μg of microsomal protein and stopped after a 2.5min incubation at 37°C by adding 1.88ml of chlorbform–methanol (1:2, v/v). Lipids were extracted and assayed as described for the other esterification reactions.

Determination of the amount of palmitoyl-CoA present at the end of an assay for sn-glycerol 3-phosphate esterification by using palmitoyl-CoA generated from palmitate

The assay system was that described for the determination of *sn*-glycerol 3-phosphate esterification by using palmitoyl-CoA generated from palmitate, except that the palmitate was labelled at C-1 with ^{14}C (0.25μCi/μmol), and the *sn*-glycerol 3-phosphate was unlabelled.

Reactions were stopped by the addition of 1ml of Dole reagent (Dole, 1956), 0.35ml of water and 0.6ml of *n*-heptane. Extractions and determinations of radioactivity were the same as those used for the assay of palmitoyl-CoA synthetase (Method 2). The lower phase contained, in addition to the palmitoyl-CoA, approx. 70% of the lysophosphatidate produced. The analysis of the lipids synthesized from *sn*-glycerol 3-phosphate (Table 1) allowed the determination of palmitoyl-CoA concentration to be corrected for lysophosphatidate content.

A second set of assays, incubated in parallel, were stopped by the addition of 0.94ml of chloroform–methanol (1:2, v/v), 15μl of 0.2M-HCl (to adjust the pH to approx. 3) and 10μl of a carrier consisting of a total phospholipid extract of rat liver (approx. 80μg of phospholipid). After mixing and centrifugation, 200μl portions were applied to t.l.c. plates of silica gel MN (made up in 0.01M-Na_2CO_3). The plates were developed for two-thirds of their length with chloroform–methanol–acetic acid–acetone–water (10:2:2:4:1, by vol.; Hajra & Agranoff, 1968). After drying, they were redeveloped for their full length with *n*-hexane–diethyl ether–acetic acid (60:40:1, by vol.), in order to separate glycerides. Palmitoyl-CoA remained near the origin and could thus be separated from the esterification products and from unchanged palmitate. The products were assayed by liquid-scintillation counting as described by Sánchez *et al.* (1973). Good agreement was obtained for the analysis of the products of *sn*-glycerol 3-phosphate esterification by the present t.l.c. system and the one used previously (described by Sánchez *et al.*, 1973).

Determination of palmitoyl-CoA hydrolase activity

The assay was specifically designed to measure palmitoyl-CoA hydrolase activity (EC 3.1.2.2) under conditions which were as close as possible to those used for measuring the activity of palmitoyl–CoA synthetase.

The assay system contained, in a final volume of 0.25ml: 25mM-Tris (adjusted to pH7.4 with HCl), 5mM-dithiothreitol, 2.5mM-$MgCl_2$, 1.5mg of fatty-acid-poor bovine serum albumin, and 26.3μM-[9,10-^{3}H]palmitoyl-CoA (1μCi/μmol). Incubations at 37°C were started by the addition of 50μg of microsomal protein, and stopped after 7min by the addition of 1ml of Dole reagent (Dole, 1956), 0.35ml of water and 0.6ml of *n*-heptane. Extractions were performed as described above for the determination of palmitoyl-CoA synthetase (Method 2). Palmitoyl-CoA hydrolase activity was measured by the decrease in [^{3}H]palmitoyl-CoA in the aqueous phase.

In some experiments, microsomal fractions were preincubated with an inhibitor before palmitoyl-CoA hydrolase activity was measured. Preincubation was for 15min at 0°C; the microsomal fraction was in 0.3M-sucrose (adjusted to pH7.4 with $KHCO_3$), and the final concentration in the preincubation mixture was 1mg of microsomal protein/ml. In other experiments, inhibitors were added directly to the assay system.

Results and Discussion

Investigation of palmitoyl-CoA accumulation during glycerolipid synthesis and in the assay of palmitoyl-CoA synthetase by the carnitine-linked assay (Method 1)

The relationship between the activity of palmitoyl-CoA synthetase, the palmitoyl-CoA pool present during glycerolipid synthesis and the rate of palmitate esterification to glycerolipid was investigated in the experiments described in Fig. 1.

Steady-state concentrations of palmitoyl-CoA were achieved in about 4min in the assay system used to measure palmitoyl-CoA synthetase in which palmitoyl-CoA was measured directly (Method 2). This steady-state concentration was equivalent to approx. 11nmol of palmitoyl-CoA/50μg of microsomal protein and represented the utilization of about 80% of the added CoA. When glycerol phosphate was added to the system, palmitoyl-CoA was incorporated into glycerolipids, and the total flux of palmitate into palmitoyl-CoA therefore increased (Fig. 1). Since the steady-state concentration of palmitoyl-CoA was maintained, the palmitoyl-CoA synthetase had sufficient activity to sustain maximum rates of glycerolipid synthesis and at the same time to convert most of the available CoA into palmitoyl-CoA.

In the system which contained glycerol phosphate it was expected that the synthesis of palmitoyl-CoA might bear a precursor–product relation to the formation of glycerolipids, and that the esterification of palmitate would have shown a time-lag; this was not observed. Maximum rates of glycerolipid synthesis were obtained within 40s of starting the reaction, when palmitoyl-CoA was generated either from palmitate or from palmitoylcarnitine.

The ability of the palmitoyl-CoA synthetase to replenish the palmitoyl-CoA pool was investigated further by adding 2.4mM-L-carnitine and carnitine palmitoyltransferase to the basic assay system (Method 2). This incubation was effectively the same as that used to assay acyl-CoA synthetase by Method 1. These additions produced little change in the pool of palmitoyl-CoA, which again represented the conversion of almost all of the free CoA into palmitoyl-CoA (Fig. 2). However, there was a marked stimulation in the synthesis of palmitoylcarnitine such that the total flux of palmitate through the palmitoyl-CoA pool was increased approximately 4-fold after 7min of incubation. The concentration of carnitine palmitoyltransferase and of L-carnitine used in these experiments was sufficient to give maximum rates of palmitoylcarnitine synthesis (Fig. 3, and other experiments not shown). Also, increasing the concentration of carnitine had little effect on the steady-state concentration of palmitoyl-CoA. The palmitoyl-CoA synthetase therefore once

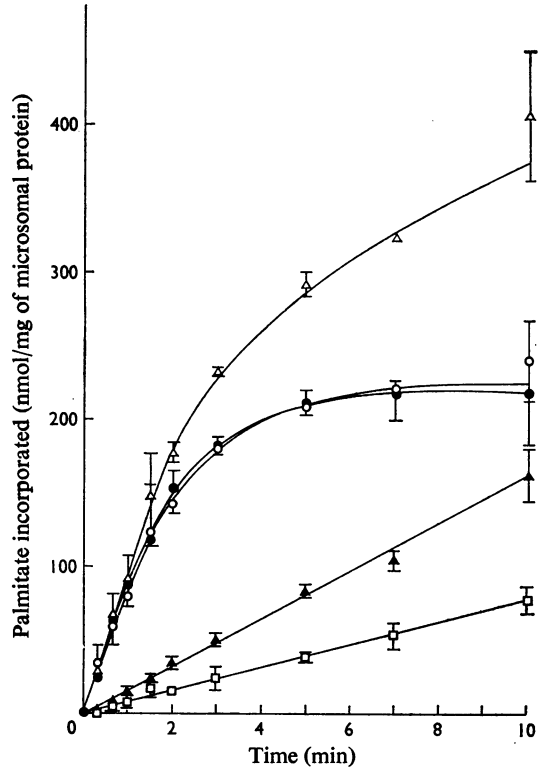

Fig. 1. *Relationship between the rate of palmitoyl-CoA synthesis and the rate of glycerolipid synthesis*

Samples of the microsomal fraction of rat liver (50μg of protein) were incubated in three different assay media (see the Materials and Methods section) for the time indicated. In the first incubation, the accumulation of palmitoyl-CoA was measured in the absence of glycerol phosphate (●). Glycerol phosphate was added to the second incubation and the accumulation of [^{14}C]palmitate in palmitoyl-CoA (○), glycerolipid (▲) and the sum of these two (△) is shown. In the third incubation, the rate of glycerolipid synthesis by using palmitoyl-CoA generated from palmitoylcarnitine was measured (□). The results are expressed as means ± 1 S.D. for three different microsomal preparations.

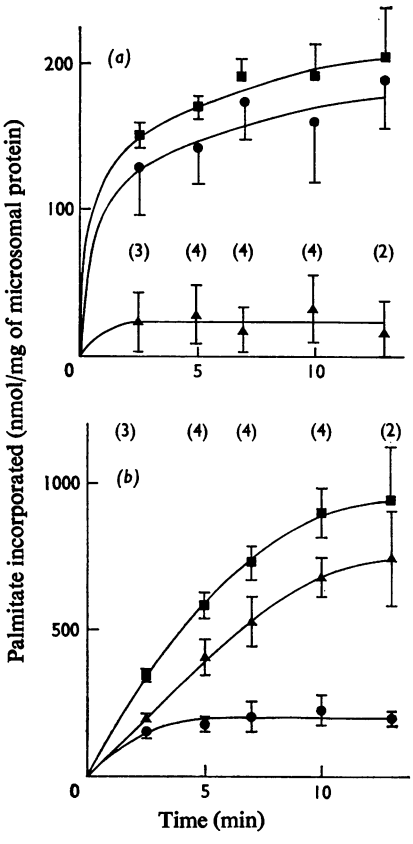

Fig. 2. *Effect of carnitine and carnitine palmitoyltransferase on the production of palmitoyl-CoA by rat liver microsomal fractions*

The assay systems used in these experiments resembled those used for the assay of palmitoyl-CoA synthetase with 50μg of microsomal protein (Method 1; see the Materials and Methods section) except that [^{14}C]palmitate (0.2μCi/μmol) was used and the carnitine was not labelled. The reactions were stopped with Dole (1956) reagent instead of butanol and the products extracted as described in the Materials and Methods section. In the experiments summarized in Fig. 2(*b*), 2.4mM-L-carnitine and carnitine palmitoyltransferase (32μg of protein) were added, whereas in Fig. 2(*a*) these additions were not made. The synthesis of palmitoyl-CoA (●), palmitoylcarnitine (▲) and palmitoyl-CoA plus palmitoylcarnitine (■) is indicated as the mean ± 1 S.D. The numbers of different microsomal preparations are indicated in parentheses.

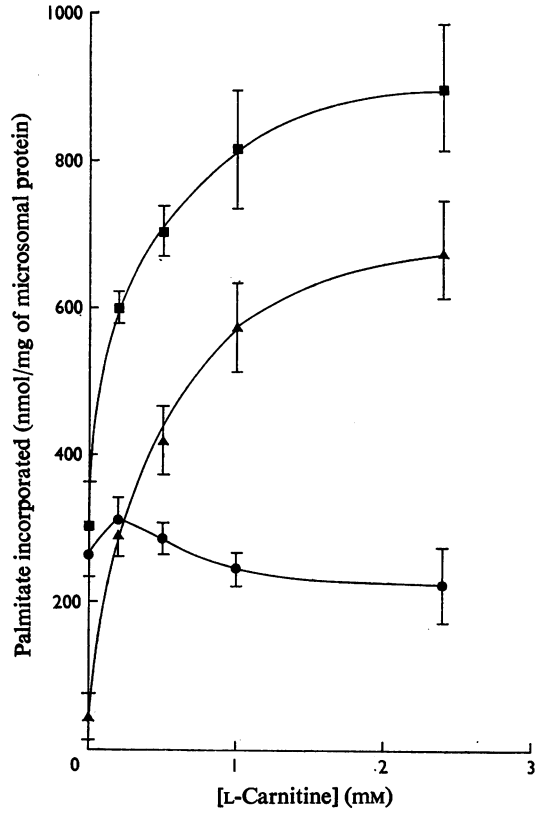

Fig. 3. *Effect of carnitine and carnitine palmitoyltransferase on the synthesis of palmitoyl-CoA by palmitoyl-CoA synthetase*

The assay conditions and the extraction procedures are those described in Fig. 2. Carnitine palmitoyltransferase (32μg of protein) was added to all incubations and the concentration of carnitine was varied as shown. The synthesis of palmitoyl-CoA (●), palmitoylcarnitine (▲) and palmitoyl-CoA plus palmitoylcarnitine (■) is indicated as the mean ± 1 S.D. obtained from four different microsomal preparations.

more had the capacity to convert most of the free CoA into palmitoyl-CoA, even when attempts were made to convert all of this palmitoyl-CoA into palmitoylcarnitine.

The likely reason for the stability of the palmitoyl-CoA pool in the presence of excess of carnitine and carnitine palmitoyltransferase is that the palmitoyl-CoA would be bound to the microsomal membranes and to the 22nmol of bovine serum albumin present in the assay medium. From the results of Lamb & Fallon (1972) and considering the relative proportion of microsomal protein (50μg) and albumin (1.5mg), it would be expected that most of the palmitoyl-CoA would be associated with the albumin.

The excess of carnitine palmitoyltransferase has the capacity to convert most of the palmitoyl-CoA, which the palmitoyl-CoA synthetase can produce, into palmitoylcarnitine. This therefore gives an estimate of palmitoyl-CoA synthetase activity. However, the measurement of palmitoylcarnitine syn-

Table 1. *Specific activities and products of palmitate activation and sn-glycerol 3-phosphate esterification in rat liver microsomal fractions*

Enzyme activities are expressed as nmol of palmitate activated or esterified/min per mg of microsomal protein, and product compositions in mol/100mol. All values are given as means ± S.D.; the numbers of animals are given in parentheses. The composition of the products at the end of each assay was used to express the incorporation in terms of palmitate rather than of *sn*-glycerol 3-phosphate. Palmitoyl-CoA synthetase was measured by palmitoyl-L-carnitine formation in the presence of carnitine and excess of carnitine palmitoyltransferase (Method 1), and by direct measurement of the palmitoyl-CoA produced (Method 2).

Enzyme assay	Specific activity	Composition of products (mol/100mol)		
		Lysophosphatidate	Phosphatidate	Neutral lipids
Palmitoyl-CoA synthetase				
Method 1	51.3 ± 25.3 (72)	—	—	—
Method 2	55.7 ± 16.5 (22)	—	—	—
Glycerolipid synthesis with palmitoyl-CoA generated from palmitate	17.9 ± 7.3 (38)	32 ± 9 (38)	61 ± 9 (38)	8 ± 5 (38)
Glycerolipid synthesis with palmitoyl-CoA generated from palmitoyl-L-carnitine	14.7 ± 6.8 (18)	29 ± 3 (18)	63 ± 14 (18)	7 ± 4 (18)
Glycerolipid synthesis with palmitoyl-CoA generated from palmitoyl-AMP	9.1 ± 3.7 (6)	8 ± 5 (6)	53 ± 17 (6)	40 ± 19 (6)

thesis alone would underestimate this activity by the size of the palmitoyl-CoA pool.

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

The relative specific activities of the enzymes involved in palmitate activation and esterification are shown in Table 1. The rates quoted for each activity are the maximum reaction rates which were measured with at least two concentrations of the microsomal protein. Details of the time-courses of some of these reactions can be obtained from Figs. 1 and 2.

Table 1 shows that the mean specific activity of palmitoyl-CoA synthetase, measured either by palmitoyl-L-carnitine formation or directly by palmitoyl-CoA formation, was approximately threefold greater than that of *sn*-glycerol 3-phosphate esterification. However, by using a paired *t* test, the specific activity of palmitoyl-CoA synthetase measured by the carnitine-linked assay was almost 20% higher ($P<0.001$) than when palmitoyl-CoA production was measured directly. Also by using a paired *t* test, it was found that the specific activities of *sn*-glycerol 3-phosphate esterification with palmitoyl-CoA generated from palmitate or from palmitoyl-L-carnitine did not differ significantly. The rate of glycerolipid synthesis was also measured by using palmitoyl-AMP to generate palmitoyl-CoA through the endogenous palmitoyl-CoA synthetase. The incubation time had to be decreased to 2.5min in order to obtain initial rates of reaction, and albumin was omitted (see the Materials and Methods section). The rate of glycerolipid synthesis (Table 1) was approx. 56% of that obtained in an equivalent incubation in which palmitoyl-AMP was replaced by 2.4mM-ATP and 0.8mM-potassium palmitate, and the concentration of $MgCl_2$ was decreased to 2.4mM. Omitting albumin from the latter system decreased the rate of palmitate esterification by less than 5%. The palmitoyl-AMP-linked assay is technically more difficult to perform and to reproduce than those systems in which palmitoyl-CoA is generated from palmitate or palmitoyl-L-carnitine. However, it measures the involvement in glycerolipid synthesis of that part of the total palmitoyl-CoA synthetase activity which can utilize palmitoyl-AMP and which has been claimed to respond to physiological adaptation (Brandes *et al.*, 1973).

From the incubation medium which was used to measure glycerolipid synthesis from palmitate, it was possible to measure the concentration of palmitoyl-CoA which accumulated during the assay. This concentration had reached a steady state by the end of the 7min incubation period (Fig. 1), at which time it was found to be 7.6 ± 2.0nmol of palmitoyl-CoA/50μg of microsomal protein (mean ± S.D.; 25 animals).

The specific activities of palmitoyl-CoA synthetase, measured by either method, and of glycerolipid synthesis from palmitate and palmitoylcarnitine were plotted against each other and subjected to linear regression analysis. There was a direct correlation ($r=0.83$; $P<0.001$) between palmitoyl-CoA synthetase activity assayed either by direct measurement of palmitoyl-CoA or by palmitoyl-L-carnitine formation. The intercept on the ordinate was 28.8 ± 7.5nmol of palmitate activated/min per mg of

microsomal protein (S.E.M.; 22 animals), which is significantly greater than zero ($P<0.01$). If it is valid to extrapolate to this intercept, then the carnitine-linked assay appears to measure a portion of the palmitoyl-CoA synthetase activity which is not accounted for by the direct measurement of palmitoyl-CoA formation under the conditions used.

There are a number of differences between the two methods used for determining the activity of palmitoyl-CoA synthetase. First, the carnitine-linked assay (Method 1) would underestimate the activity by the amount of palmitoyl-CoA which is not converted into palmitoylcarnitine (see above). In addition, this assay would detect the synthesis of acyl-CoA esters from acids other than palmitic. The rate obtained in the absence of palmitate in the present experiments was approx. 30% of that with optimum concentrations of palmitate. However, in the latter situation, the rate of acylcarnitine production from acids other than palmitic should have been minimized by competition with the excess of exogenous palmitate.

In the other assay (Method 2), the production of palmitoyl-CoA was measured directly and the palmitoyl-CoA was allowed to accumulate. There was little synthesis of palmitoylcarnitine (Fig. 2*a*) or glycerolipids in this system, since more than 85% of the product was identified as acyl-CoA. Even that proportion of the product which was converted into palmitoylcarnitine would have been included in the measured rate, since palmitoylcarnitine is isolated with palmitoyl-CoA by the extraction procedure. In order to obtain initial rates of reaction for the palmitoyl-CoA synthetase in this system, the concentration of microsomal protein had to be lowered to 5–20 μg compared with the 50 μg used in Method 1. This assay would be specific for the activation of palmitate, since the rate was measured by using exogenous [^{14}C]palmitate.

There was also a direct correlation ($r=0.59$; $P<0.01$) between the rate of glycerolipid synthesis measured with palmitoyl-CoA generated from palmitate or from palmitoyl-L-carnitine. In this case, the intercept was not significantly different from zero. This indicates that the measurement of glycerolipid synthesis from either precursor is effectively a measurement of glycerol phosphate acyltransferase activity. There was, however, no correlation ($r=0.15$; $P<0.5$) between the rate of glycerolipid synthesis from palmitate and the activity of palmitoyl-CoA synthetase measured by the carnitine-linked assay. A similar result was obtained with rat liver mitochondrial fractions (Sánchez *et al.*, 1973), but with the microsomal fraction of guinea-pig intestinal mucosa, a significant correlation was found (Brindley, 1973). In this intestinal system, the rate of palmitate activation was only approx. 1.3-fold the rate of esterification to glycerol phosphate. The correlation was probably found in the intestinal system (Brindley, 1973) because the rates of esterification closely approached the maximum rate of palmitoyl-CoA synthesis. In the rat liver systems this is not so (Sánchez *et al.*, 1973; Lloyd-Davies & Brindley, 1973), and alterations in the rate of palmitoyl-CoA synthesis could not be used to predict the ability to synthesize glycerolipids (Tables 2 and 3).

Products of glycerolipid synthesis

After a 7min incubation, approx. 60% of the *sn*-[^{3}H]glycerol 3-phosphate incorporated by using palmitoyl-CoA generated either from palmitate or from palmitoyl-L-carnitine was isolated as phosphatidate (Table 1). A predominant synthesis of phosphatidate by hepatic microsomal fractions has also been found by other workers (Abou-Issa & Cleland, 1969; Daae & Bremer, 1970; Daae, 1972). The molar composition of the glycerolipids formed was almost identical in both assay systems. The neutral lipid fraction consisted of 73% monoglyceride, 17% diglyceride and 10% triglyceride in the case of *sn*-glycerol 3-phosphate esterification from palmitate. This is similar to the results of Daae & Bremer (1970) and Daae (1972).

Preincubation of rat liver microsomal fractions with ATP, Mg^{2+}, EDTA or NaCl: effect on the activity of palmitoyl-CoA synthetase and glycerolipid synthesis

The experiments reported in Tables 2 and 3 were performed to investigate the effect of varying the flux of palmitate to palmitoyl-CoA on the synthesis of glycerolipids. Preincubation of microsomal fractions at 37°C leads to an inhibition of acyl-CoA synthetase (Farstad, 1968), which in turn decreases the rate of glycerolipid synthesis from palmitate and *sn*-glycerol 3-phosphate (Brindley & Ferrier, 1972; Brindley, 1973; Lloyd-Davies & Brindley, 1973).

In agreement with previous observations with microsomal preparations from guinea-pig intestinal mucosa (Brindley & Ferrier, 1972; Brindley, 1973), ATP protected palmitoyl-CoA synthetase and glycerolipid synthesis from palmitate from inhibition during preincubation (Table 2, Expt. 1). As the microsomal preparations used in these experiments probably contained some endogenous CoA, the activation which was observed when ATP was included in the preincubation medium may have been caused by the mechanism described by Bar-Tana & Shapiro (1964). Also, as shown by Farstad (1968), NaCl helped to stabilize the activity of palmitoyl-CoA synthetase; it also prevented some of the inhibition of glycerolipid synthesis from *sn*-glycerol 3-phosphate and palmitate.

However, in contrast with the results of Farstad (1968), the results given in Table 2 show that the inclusion of 2mM-EDTA in the preincubation

Table 2. *Effect of the composition of the preincubation medium on the activities of glycerolipid synthesis in rat liver microsomal fractions*

Rat liver microsomal fractions (1 mg of protein/ml) were preincubated at 37°C for 30min in 10mM-Tris buffer (adjusted to pH7.4 with HCl and containing 5mM-dithiothreitol). Other additions to the preincubation medium are indicated in the Table. After 30min, 50μl samples were taken for the analysis of palmitoyl-CoA synthetase, glycerolipid synthesis from palmitate and from palmitoylcarnitine, and the concentration of palmitoyl-CoA during glycerolipid synthesis from palmitate. The specific activity or concentration before preincubation is given as nmol of palmitate activated or esterified/min per mg of protein, or as nmol of palmitoyl-CoA produced/50μg of protein. Each activity or concentration obtained with preincubated microsomal preparations is expressed as a percentage of its own activity or concentration before preincubation. Each value is the mean ± 1 S.D. from three different preparations.

Expt. no.	Addition	Palmitoyl-CoA synthetase (Method 1)	Concn. of palmitoyl-CoA	Glycerolipid synthesis from palmitate	Glycerolipid synthesis from palmitoylcarnitine
		Specific activity or concn. before preincubation			
1	None	59±24	—	19±8	13±6
2	None	10±3	7±1	6±1	4±1
		Percentage of original activity or concn. remaining after preincubation			
1	None	33±7	—	36±14	73±22
	5mM-ATP	124±18	—	139±32	112±7
	5mM-$MgCl_2$	33±21	—	49±26	97±27
	2mM-EDTA	37±11	—	7±2	17±3
	50mM-NaCl	74±29	—	80±36	91±29
2	5mM-$MgCl_2$	39±11	57±33	62±4	99±4

medium did not stabilize palmitoyl-CoA synthetase activity significantly, nor did Mg^{2+} (5mM) promote its inhibition. Both EDTA and Mg^{2+} had a profound effect on the stability of glycerol phosphate acyltransferase during preincubation. The addition of EDTA rendered the acyltransferase extremely labile, as indicated by the decreased synthesis of glycerolipids from palmitoyl-L-carnitine; glycerolipid synthesis from palmitate was also inhibited. Preincubation of the microsomal fraction in the absence of Mg^{2+} inhibited glycerol phosphate acyltransferase activity (Table 2) and this inhibition became more marked if the 30min preincubation period was extended (results not shown). The susceptibility of glycerol phosphate acyltransferase to this inhibition varied considerably in different preparations, but the addition of 5mM-$MgCl_2$ abolished the inhibition. The effect of Mg^{2+} appeared to be concerned with the stability of the acyltransferase rather than with providing a cofactor in the acyl-transfer reaction. The addition of up to 5mM-$MgCl_2$ to the incubation medium used to measure *sn*-glycerol 3-phosphate esterification with palmitoyl-L-carnitine did not alter the activity obtained with either untreated microsomal fractions or those which had been preincubated with EDTA (Table 2). It has been reported that glycerol phosphate acyltransferase has a bivalent-cation requirement, but this is observed by using partially purified preparations rather than mitochondrial or microsomal membranes (Yamashita & Numa, 1972; Monroy *et al.*, 1973). It was suggested that bivalent cations might preserve the correct conformation of the membrane-bound glycerol phosphate acyltransferase (Yamashita & Numa, 1972).

In the second experiment shown in Table 2, 5mM-$MgCl_2$ was added to the preincubation medium to ensure that glycerol phosphate acyltransferase activity was not lost while palmitoyl-CoA synthetase activity was inhibited. The concentration of palmitoyl-CoA at the end of the assay of *sn*-glycerol 3-phosphate esterification from palmitate was also found to be decreased by preincubation.

Effect of rate-limiting concentrations of Mg^{2+}, palmitate, ATP and CoA on palmitoyl-CoA synthetase activity and glycerolipid synthesis

The rate of palmitoyl-CoA synthesis by microsomal fractions can be decreased by lowering the concentrations of cofactors and substrates necessary for the synthetase reaction. This also decreased the esterification of palmitate to *sn*-glycerol 3-phosphate. However, no correlation was found between the percentage decrease in the observed palmitoyl-CoA synthetase activity and the decrease in glycerolipid synthesis (Lloyd-Davies & Brindley, 1973).

This discrepancy was investigated further by

Table 3. *Effect of rate-limiting concentrations of palmitate, Mg^{2+}, ATP and CoA on the incorporation of palmitate into palmitoyl-CoA and glycerolipids*

The concentrations of palmitate, Mg^{2+}, ATP or CoA were varied while other cofactors and substrates were kept at optimum concentrations. Incubations were for 7min at 37°C with 50μg of microsomal protein. The incorporations at rate-limiting concentrations are expressed as a percentage of the appropriate maximum incorporation. Results are expressed as means ± 1 S.D. and the numbers of microsomal preparations are indicated in parentheses.

System	Maximum incorporation of palmitate (nmol)	Percentage of maximum incorporation with rate-limiting cofactors or substrate			
		Palmitate (50μM)	Mg^{2+} (125μM)	ATP (125μM)	CoA (2.5μM)
Palmitoyl-CoA synthetase (Method 1)	17±5 (6)	53±12 (6)	29±12 (6)	24±21 (6)	45±19 (6)
Glycerolipid synthesis from palmitate	7±3 (6)	54±27 (6)	34±9 (6)	79±18 (6)	29±6 (6)
Palmitoyl-CoA present during glycerolipid synthesis from palmitate	7±1 (3)	53±6 (3)	47±4 (3)	33±8 (3)	8±6 (3)
Palmitate present in glycerolipid plus that in palmitoyl-CoA	16±4 (3)	52±23 (2)	40±5 (3)	58±14 (3)	22±4 (3)

measuring the effects of cofactor and substrate limitation on the palmitoyl-CoA pool which was produced in the assay medium used for measuring the rate of glycerol phosphate esterification. As was observed in previous experiments (Lloyd-Davies & Brindley, 1973), rate-limiting concentrations of palmitate, Mg^{2+} and ATP produced greater or similar decreases in palmitoyl-CoA synthetase (measured by the carnitine-linked assay, Method 1) compared with their effects on glycerolipid synthesis (Table 3). In contrast, the rate-limiting concentrations of CoA (2.5μM) inhibited glycerolipid synthesis to a relatively greater extent than acyl-CoA synthetase ($P<0.02$, by a paired t test). The average decrease in the palmitoyl-CoA synthetase activity (Method 1) was 55%, whereas the pool of palmitoyl-CoA present during glycerolipid synthesis showed an average decrease of 91%. This was equivalent to a decrease of 78% in the total quantity of palmitate activated, as estimated by adding the palmitoyl-CoA pool to the palmitate present in glycerolipid. It therefore appears that the carnitine-linked assay (Method 1) overestimates the potential of acyl-CoA synthetase to provide activated palmitate for glycerolipid synthesis in conditions where the concentration of CoA is low, possibly in part because CoA is recycled.

In contrast with the results obtained with rate-limiting concentrations of CoA, decreasing the concentration of ATP to 125μM (Table 3) produced a much greater decrease in the apparent activity of acyl-CoA synthetase (Method 1) than in the rate of glycerolipid synthesis ($P<0.01$ by a paired t test) and in the total quantity of palmitate present in glycerolipid plus that in palmitoyl-CoA ($P<0.05$ by a paired t test).

In order to try to explain these results, a series of experiments was designed to investigate whether the activity of palmitoyl-CoA hydrolase, or a product inhibition of palmitoyl-CoA synthetase by palmitoyl-CoA, could significantly influence the observed rates of palmitate activation and esterification.

Measurement of palmitoyl-CoA hydrolase activity

The activity of palmitoyl-CoA hydrolase was measured under conditions which closely resembled those used in the synthetase assay (see the Materials and Methods section). During a 7min incubation, 2.24±0.37nmol of palmitoyl-CoA (S.E.M.; eight animals) was hydrolysed/min per mg of microsomal protein, compared with the mean synthesis of 24.8nmol of palmitoyl-CoA/min per mg of microsomal protein in the same preparations. The rate of hydrolysis is therefore approx. 10% of the rate of synthesis of palmitoyl-CoA. This represents a smaller activity than that found by previous workers (e.g. Lands & Hart, 1965; Fallon & Lamb, 1968). The low activity of palmitoyl-CoA hydrolase in the system described in the present paper was probably due to the presence of 6mg of albumin/ml in the assays; palmitoyl-CoA hydrolase has been reported to be more active towards micellar than protein-bound substrate (Barden & Cleland, 1969; Lamb *et al.*, 1973) and inhibition of the enzyme by serum albumin is known to occur in rat liver microsomal fractions (Brandes *et al.*, 1963; Fallon & Lamb, 1968; Barden & Cleland, 1969; Lamb *et al.*, 1973) as well as other tissues (Daniel & Rubinstein, 1968; Anderson & Erwin, 1971).

Although the activity of palmitoyl-CoA hydrolase in the present series of experiments was relatively low, attempts were made to abolish it completely by using reported inhibitors, under conditions which would still produce maximum rates of palmitoyl-CoA synthesis and glycerol phosphate esterification.

The potential inhibitors which were chosen

Table 4. *Effect of exogenous [^{3}H]palmitoyl-CoA on the activity of palmitoyl-CoA synthetase*

Palmitoyl-CoA synthetase was assayed by [^{14}C]palmitoyl-CoA formation (see Method 2 in the Materials and Methods section), by using an incubation volume of 0.25ml with the amount of microsomal protein and concentrations of CoA indicated. In addition [^{3}H]palmitoyl-CoA was added as shown. The results are expressed as mean activities ± 1 S.D. and were obtained by using three different microsomal preparations for each of the three experiments.

Expt.	Microsomal protein (μg)	CoA (μM)	Palmitoyl-CoA synthesized (nmol) in the presence of the concentration of [^{3}H]palmitoyl-CoA indicated				
			0μM	18μM	37μM	56μM	75μM
1	50	55	7.9±0.5	7.6±0.7	7.5±0.7	7.4±1.2	7.0±0.9
			0μM		30μM		60μM
2	50	5.5	1.5±0.5		2.5±0.2		—
		27.5	3.9±0.0		3.8±0.2		4.6±0.3
		55	7.5±1.0		—		—
			0μM	24μM	47μM	68μM	
3	15	55	6.1±1	5.5±0.2	5.1±0.4	4.6±0.2	

included di-isopropyl phosphorofluoridate, propan-2-ol, fluoride, *p*-chlorophenoxyisobutyrate, clofenapate, fenfluramine, *N*-(2-benzoyloxyethyl)norfenfluramine and protamine sulphate. However, none of these inhibitors gave reproducible inhibition of palmitoyl-CoA hydrolase while at the same time preserving maximum rates of palmitoyl-CoA synthesis.

Effect of added palmitoyl-CoA on the activation of palmitate by rat liver microsomal fractions

These experiments were performed to investigate whether the addition of exogenous [^{3}H]palmitoyl-CoA to the assay medium used for the direct measurement of palmitoyl-CoA synthetase activity (Method 2) would decrease either the initial rate of palmitoyl-CoA synthesis or its steady-state concentration.

In Expts. 1 and 2 (Table 4), 50μg of microsomal protein was used in the 0.25ml of assay medium, which is equivalent to that used for measuring glycerolipid synthesis. In these experiments the synthesis of palmitoyl-CoA would have reached a steady-state after the 7min incubation period (Figs. 1 and 2). In Expt. 1 (Table 4) an average of 7.9nmol of palmitoyl-CoA was synthesized from [^{14}C]palmitate, which would have consumed approx. 57% of the 13.8nmol of CoA added to the incubation. The further addition of up to 75μM-[^{3}H]palmitoyl-CoA (approx. twice the steady-state concentration of [^{14}C]-palmitoyl-CoA) only decreased the accumulation of [^{14}C]palmitoyl-CoA by approx. 11%. This relatively small inhibition may have been caused by the protection of palmitoyl-CoA synthetase by free CoA (Pande, 1973). In Expt. 2 (Table 4) the CoA concentrations were lowered and became sub-optimum for the synthesis of palmitoyl-CoA. At 5.5μM-CoA, the accumulation of [^{14}C]palmitoyl-CoA would have accounted for all the CoA that was added. Further addition of 30μM-[^{3}H]palmitoyl-CoA did not inhibit the accumulation of [^{14}C]palmitoyl-CoA but produced a significant ($P<0.05$) stimulation. Since about 10% of the [^{3}H]palmitoyl-CoA appeared to be hydrolysed during the incubation, the release of CoA would have been approximately sufficient to account for the increased synthesis of [^{14}C]palmitoyl-CoA. A similar situation was observed at 27.5μM-CoA, where approx. 56% of the CoA was consumed for palmitoyl-CoA synthesis. Addition of 60μM-[^{3}H]-palmitoyl-CoA stimulated the synthesis of palmitoyl-CoA *de novo*.

In Expt. 3 (Table 4) the amount of microsomal fraction used for the assay was decreased to 15μg so that the initial rate of [^{14}C]palmitoyl-CoA synthesis could be measured. The addition of up to 68μM-[^{3}H]palmitoyl-CoA (approx. 3 times the maximum concentration of [^{14}C]palmitoyl-CoA synthesized) did inhibit palmitoyl-CoA synthesis, but only by approx. 25%.

The extent of product inhibition in this system therefore appears to be small. In all experiments quoted in Table 4, the recovery of [^{3}H]palmitoyl-CoA at the end of the incubation was 90–95%, confirming that the rate of palmitoyl-CoA hydrolysis is also low.

Conclusions

The results described in the present paper emphasize the complexity of studying the sequence of reactions involved in glycerolipid synthesis. It is evident that the assay of acyl-CoA synthetase activity and the relationship of this activity to the subsequent

esterification of fatty acids is more involved than has hitherto been appreciated. The main conclusion is that the palmitoyl-CoA synthetase of rat liver microsomal fractions has ample reserve capacity in most circumstances to maintain optimum rates of glycerolipid synthesis. This can be seen in Fig. 1. Maximum rates of glycerol phosphate esterification were obtained after an incubation of 40s, when the concentration of palmitoyl-CoA in the system was approx. 12μM. These results also show that the microsomal glycerol phosphate acyltransferase has a high affinity for palmitoyl-CoA even in the presence of 6mg of bovine serum albumin/ml. The conclusion that glycerol phosphate acyltransferase is rate-limiting in phosphatidate synthesis is confirmed by the lack of correlation between the activities of palmitoyl-CoA synthetase and the rate of palmitate esterification to glycerol phosphate. The results in Tables 2 and 3 also indicate that the rate of palmitoyl-CoA synthesis or the concentration of palmitoyl-CoA in the system gave a poor prediction of the rate of glycerolipid synthesis. The capacity to activate palmitate was also greater than its rate of esterification to glycerol phosphate in mitochondrial fractions of rat liver (Sánchez *et al.*, 1973). Although these types of experiments cannot exclude the possibility that changes in the activity of palmitoyl-CoA synthetase might control hepatic glycerolipid synthesis *in vivo*, it seems more likely that more effective control could be exerted later in the pathway.

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