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Identity between palmitoyl-CoA synthetase and arachidonoyl-CoA synthetase in human platelets?

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Apparent K_m values have been determined for the substrates ATP, CoA and fatty acids for the long-chain acyl-CoA synthetase (EC 6.2.1.3) reaction in lysates of human blood platelets. The apparent K_m for ATP was higher for saturated fatty acids ($C_{12:0}$ to $C_{18:0}$) than for unsaturated acids ($C_{18:1}$ to $C_{22:6}$). Other apparent K_m values were very similar for all long-chain fatty acids tested. Palmitic acid inhibited the formation of [^{14}C]arachidonoyl-CoA, and arachidonic acid inhibited the formation of [^{14}C]palmitoyl-CoA, with [^{14}C]arachidonate or [^{14}C]palmitate respectively as substrate. After chromatography of Triton X-100-extracted platelet protein in several systems (hydroxyapatite, DEAE-Sepharose, Sephacryl S-200 HR, CoA–Sepharose, Sephadex G-100 and AcA 34), both arachidonoyl-CoA synthetase and palmitoyl-CoA synthetase activities were eluted together in the various protein peaks, and with approximately the same ratio of activities in all peaks. After some purification steps (DEAE-Sepharose and Sephacryl S-200 HR), the acyl-CoA synthetase activity was up to 37 nmol/min per mg of protein with [^{14}C]palmitate as substrate, and up to 116 nmol/min per mg of protein with [^{14}C]arachidonate as substrate. The purification was respectively about 8- and 10-fold. The results indicate that palmitoyl-CoA (or unspecific) synthetase and arachidonoyl-CoA (or specific) synthetase are in fact the same enzyme, in agreement with previously reported results from this laboratory.

INTRODUCTION

The long-chain fatty acyl-CoA synthetase (EC 6.2.1.3) reaction is the first step in most metabolic conversions of fatty acids. Although this enzyme has been studied in platelets (Farstad & Sander, 1971; Farstad *et al.*, 1973; Berge *et al.*, 1980; Wilson *et al.*, 1982; Neufeld *et al.*, 1984; Laposata *et al.*, 1985; Bakken & Farstad, 1989), little is known about whether its specificity for fatty acids is of importance for their incorporation into phospholipids. It has been suggested that platelets possess an acyl-CoA synthetase which is specific for arachidonic acid and other prostanoid precursors (Wilson *et al.*, 1982; Neufeld *et al.*, 1984; Laposata *et al.*, 1985). We found that palmitoyl-CoA and arachidonoyl-CoA synthetase activities in human platelet were identically distributed in subcellular fractions and were heat-inactivated to the same extent (Bakken & Farstad, 1989), in spite of the observation that arachidonic acid was selectively incorporated into the dense tubular system/open canalicular system (Laposata *et al.*, 1987). It is therefore of special interest to study the conditions for the formation of acyl-CoA by the arachidonoyl-CoA ('specific') synthetase and by the palmitoyl-CoA ('unspecific') synthetase.

The present work reports similar K_m values of the synthetase in platelet lysates for three substrates of the synthetase reaction (CoA, ATP and fatty acids), using ten different fatty acids including arachidonic acid and palmitic acid. Furthermore, attempts to separate the alleged arachidonoyl- and palmitoyl-CoA synthetases using several chromatographic methods were unsuccessful. We conclude that there is only one long-chain acyl-CoA synthetase in human platelets.

MATERIALS AND METHODS

[1-^{14}C]Lauric acid ($C_{12:0}$) (specific radioactivity 36.3 mCi/mmol), [1-^{14}C]palmitic acid ($C_{16:0}$) (58 mCi/mmol), [1-^{14}C]-stearic acid ($C_{18:0}$) (57 mCi/mmol), [1-^{14}C]oleic acid ($C_{18:1,n-9}$) (56.7 mCi/mmol), [1-^{14}C]linoleic acid ($C_{18:2,n-6}$) (58 mCi/mmol), [1-^{14}C](9,12,15)-linolenic acid ($C_{18:3,n-3}$) (56.2 mCi/mmol), [1-^{14}C]arachidonic acid ($C_{20:4,n-6}$) (60.1 mCi/mmol), and [1-^{14}C]eicosa-5,8,11,14,17-pentaenoic acid (EPA, $C_{20:5,n-3}$) (55 mCi/mmol) were purchased from Amersham International (Amersham, Bucks., U.K.). [1-^{14}C]Docosa-4,7,10,13,16,19-hexaenoic acid (DHA, $C_{22:6,n-3}$) (55 mCi/mmol) was purchased from New England Nuclear (Boston, MA, U.S.A.), and [1-^{14}C]-myristic acid ($C_{14:0}$) (55 mCi/mmol) was from Commissariat a l'Energie Atomique (CEA, Saclay, France). Scint-Hei 3 from Koch–Light Laboratories (Colnbrook, Slough, Berks., U.K.) was used as scintillator. ATP, CoA, fatty acids (of the same length and saturation as their radioactive counterparts), dithiothreitol (DTT), Triton X-100, inorganic pyrophosphatase, EDTA and Hepes were purchased from Sigma (St. Louis, MO, U.S.A.). Unlabelled DHA was from Aldrich, Steinheim, Germany. All other chemicals were of the highest purity commercially available. T.l.c. plates (silica gel 60; Merck, Darmstadt, Germany) were used for the t.l.c. studies. Hydroxyapatite 'High Resolution' was from Fluka AG, Buchs, Switzerland. DEAE-Sepharose Fast Flow, Sephacryl S-200 HR, activated thiol–Sepharose 4B and Sephadex G-100 were from Pharmacia LKB Biotechnology, Uppsala, Sweden. Ultrogel AcA 34 was from Reactifs IBF, Villeneuve-la-Garenne, France.

Preparation of platelet lysates

Blood platelet lysates and subcellular fractions of platelets were prepared from samples from healthy blood donors, as described previously (Holmsen *et al.*, 1977; Bakken & Farstad, 1989).

For the chromatography experiments, approx. 60×10^9 platelets were isolated and washed as described. The platelets were resuspended in 9 ml of a 20 mM-potassium phosphate buffer (pH 7.45) containing 5 mM-DTT and 50 μM-ATP. Triton X-100 was added to a final concentration of 2 mM to lyse the platelets

Abbreviations used: EPA, eicosapentaenoic acid ($C_{20:5,n-3}$); DHA, docosahexaenoic acid ($C_{22:6,n-3}$); DTT, dithiothreitol; CMC, critical micelle concentration.

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(Tanaka *et al.*, 1979). The suspension was kept on ice for 75 min and then centrifuged at 100000 $g_{max.}$ at 4 °C in a Beckman L8 ultracentrifuge for 60 min. The supernatant (Triton X-100 extract) was used in the different chromatography systems. All chromatography was done at 4 °C.

Hydroxyapatite chromatography

A 7 ml portion of the Triton X-100 extract was applied to a 1.36 cm × 5.2 cm hydroxyapatite column, equilibrated with buffer A-20 (containing 20 mM-potassium phosphate, 5 mM-DTT, 2 mM-Triton X-100 and 50 μM-ATP, pH 7.45). The column was then eluted with 30 ml of the same buffer, and thereafter with 50 ml of A-80 (as above but with 80 mM-potassium phosphate), and then with a 10 ml gradient buffer from 80 to 300 mM-potassium phosphate, and finally with 40 ml of buffer A-300 (i.e. containing 300 mM-potassium phosphate). The flow rate was 30 ml/h; fractions of 2 ml were collected.

DEAE-Sepharose Fast Flow chromatography

A 7.3 ml portion of the Triton X-100 extract was applied to a 94 ml column (2.6 cm × 17.7 cm) of DEAE-Sepharose Fast Flow, equilibrated with buffer A-20. The column was eluted with 150 ml of the same buffer, and thereafter with 160 ml of buffer A-80 followed by a gradient buffer increasing from A-80 to A-300. Finally the column was eluted with 175 ml of buffer A-300. The flow rate was 86 ml/h; fractions of 4.3 ml were collected. Fractions containing enzyme activity were combined and concentrated (from 22.5 to 6.5 ml) by ultrafiltration using a Diaflo membrane filter PM-10 (Amicon).

Sephacryl S-200 HR chromatography

The above concentrated solution was applied to a 136 ml Sephacryl S-200 HR column (1.6 cm × 68 cm) which was eluted with buffer A-80. The flow rate was 32 ml/h; fractions of 4.0 ml were collected. The fractions containing enzyme activity were combined and dialysed for 3.5 h against 25 mM-potassium phosphate/2 mM-Triton X-100 (pH 7.45). The buffer was changed nine or ten times.

CoA–Sepharose 4B chromatography

CoA–Sepharose 4B was prepared from activated thiol–Sepharose 4B according to Kameda *et al.* (1985). A 4 ml portion of the Triton X-100 extract (omitting ATP and DTT) was applied to a 14.5 ml CoA–Sepharose 4B column (1.6 cm × 7.3 cm) equilibrated with a 25 mM-potassium phosphate buffer (pH 7.45) containing 2 mM-Triton X-100. The column was eluted with 65 ml of the same buffer, followed by a 60 ml gradient of 0–0.7 M-KCl in the equilibration buffer. The flow rate was 15 ml/h; fractions of 2 ml were collected.

Sephadex G-100 chromatography

A 3 ml portion of the Triton X-100 extract was applied to a 115 ml (1.36 cm × 79.2 cm) Sephadex G-100 column. Elution was with buffer B-20 [20 mM-potassium phosphate (pH 7.45) containing 1 mM-ATP, 5 mM-DTT and 2 mM-Triton X-100], followed by buffer B-80 (as above but containing 80 mM-potassium phosphate). The flow rate was 10.8 ml/h; fractions of 3.6 ml were collected. Recovery of activity was 67% for both [^{14}C]palmitate and [^{14}C]arachidonate as substrates. The two fractions containing acyl-CoA synthetase activity were combined and concentrated to half volume by ultrafiltration with a Diaflo membrane filter PM-10.

AcA 34 chromatography

A 2.5 ml portion of the concentrate from the Sephadex G-100 column was applied to a 117 ml (1.36 cm × 81 cm) AcA 34 column. The elution buffer was 100 mM-potassium phosphate (pH 7.45) containing 1 mM-ATP and 5 mM-DTT. The flow rate was 9.9 ml/h; fractions of 3.3 ml were collected.

Estimation of the number of platelets

The number of platelets was determined in a Thrombocounter (Coulter Electronics Ltd., Harpenden, Herts., U.K.) with a counting error of less than ±5%; or in a Coulter Counter (Model S-plus III; Coulter Electronics, Hialeah, FL, U.S.A.).

Protein determination

Protein concentration was determined by the method of Bradford (1976), with BSA as standard.

Assay of acyl-CoA synthetase activity in lysates of human blood platelets

The acyl-CoA synthetase activity was determined by measuring the formation of [1-^{14}C]acyl-CoA from 1-^{14}C-labelled fatty acid, CoA and ATP (Bar-Tana *et al.*, 1971). The standard incubation medium contained 50 mM-Hepes buffer, pH 7.4, 5 mM-DTT, 10 mM-$MgCl_2$, 2 mM-ATP, 70 μM-CoA, 0.1% Triton X-100 (1.6 mM), 150 μM-[1-^{14}C]fatty acid and platelet lysate corresponding to (50–100) × 10^6 platelets in a final volume of 250 μl. Since the critical micelle concentration (CMC) of Triton X-100 under the present conditions was about 80 μM (Berge *et al.*, 1981), all fatty acid substrates were presented in a mixed micellar form. This ensured identical assay conditions. Incubations were carried out at 37 °C for 3 min, which is within the range giving a constant reaction rate (Wilson *et al.*, 1982; Bakken, 1986). The assay relies on heptane extraction of unreacted fatty acids and the insolubility of acyl-CoAs in heptane (Bar-Tana *et al.*, 1971). Usually five extractions were performed.

For each extraction the volume of the aqueous phase was reduced. This was taken into account when calculating the activity of the enzyme (Bakken, 1986). Using lysate, some activity was found in the absence of added ATP or CoA due to the endogenous content of these components, in agreement with previous findings (Farstad & Sander, 1971).

T.l.c. was performed to ensure that [^{14}C]acyl-CoA formed had not been incorporated into [^{14}C]phospholipids during the incubation. Collected heptane phase from experiments with [^{14}C]palmitic acid or [^{14}C]arachidonic acid were evaporated and developed on t.l.c. (Hauser & Eichberg, 1975) to separate unreacted ^{14}C-labelled fatty acids from phospholipids. (Radioactivity was measured with RITA, and a radio-t.l.c. program from Nuclear Interface.) The heptane-washed aqueous phase was thereafter extracted by chloroform/methanol (2:1, v/v) (Bligh & Dyer, 1959), and developed on t.l.c. as described above.

Working solutions of 1.5 mM fatty acids were prepared. The saturated fatty acids were dissolved in 5–25 mM-KOH, except for stearic acid, which was dissolved in 5.6 M-ethanol and 25 mM-KOH. All six unsaturated fatty acids were dissolved in 450 mM-ethanol in 5 mM-KOH. The polyunsaturated fatty acids were kept at −80 °C in the dark in an atmosphere of N_2 for not more than 1 month. Addition of antioxidants to the fatty acid suspensions was omitted to avoid any possible unwanted reactions and inhibitions during acyl-CoA formation.

To make sure that the ethanol in the incubation mixture did not inhibit the acyl-CoA synthetase reaction, an experiment with palmitate as radioactive substrate and increasing amounts of ethanol in the incubation mixtures was performed. The formation of palmitoyl-CoA was not influenced by ethanol at concentrations below 130 mM, which is well above the concentration of ethanol in our assays (45 mM) investigating the unsaturated fatty acids.

The specific radioactivities of the fatty acid substrates were 800–1000 c.p.m./nmol when counted in a Packard Tri-Carb spectrometer (model 2450).

The formation of acyl-CoA from the endogenous non-esterified fatty acids in blood platelet lysates was analysed by an h.p.l.c. method (Ingebretsen *et al.*, 1982*a*). The activity was calculated by measurement of the AMP formed in the incubation mixture over 3 min, without addition of fatty acids. P^1P^5-Diadenosine 5′-pentaphosphate was added to the incubation medium to inhibit the adenylate kinase in the lysate (Lienhard & Secemski, 1973). The reaction was stopped by the addition of $HClO_4$, and after centrifugation the supernatant was neutralized with K_2HPO_4 before injection on to the chromatograph. The calculated value of acyl-CoA formation was about 3 nmol/min per 10^9 platelets, giving a concentration of 2.8 μM endogenously formed acyl-CoA in the incubation mixture within 3 min.

Calculations

The apparent K_m values of substrates for acyl-CoA synthetase were calculated from the Lineweaver–Burk plot, or were derived directly from the Michaelis–Menten saturation curves if the Lineweaver–Burk plots were non-linear. All straight lines in the different plots have been calculated by the least-squares method.

The maximum activity of acyl-CoA synthetase was measured at concentrations approx. 10 times the K_m for each of the three substrates.

RESULTS

Assay conditions of acyl-CoA synthetase activities

T.l.c. of pooled heptane phase revealed no trace of [^{14}C]-acyl-CoA or ^{14}C-labelled phospholipids (results not shown). The heptane-extracted aqueous phase was re-extracted with methanol/chloroform (2:1, v/v). T.l.c. of chloroform layer showed no spot of ^{14}C-labelled phospholipids (results not shown). Hydrolysis of newly formed acyl-CoA did not take place under the incubation conditions used, since acyl-CoA hydrolase activity is strongly inhibited in the presence of 10 mM-$MgCl_2$ (Berge & Farstad, 1978), and the presence of 1.6 mM-Triton X-100 will leave no free acyl-CoA, which is the substrate for the long-chain acyl-CoA hydrolase (Berge *et al.*, 1981).

Activity of acyl-CoA synthetase and apparent K_m values with different substrates

To study 'unspecific' and 'specific' acyl-CoA synthetase activities in human platelets (Wilson *et al.*, 1982; Neufeld *et al.*, 1984; Laposata *et al.*, 1985), determinations of apparent K_m values for all substrates of the acyl-CoA synthetase reaction were performed. Table 1 shows that apparent K_m values for ATP were higher with saturated than with unsaturated fatty acids, whereas apparent K_m values for CoA and fatty acids were in the same range for all fatty acids, except for a somewhat higher K_m value for CoA with laurate ($C_{12:0}$) as the fatty acid substrate.

The substrate curves with ATP or fatty acids as variable substrates showed simple Michaelis–Menten kinetics. With CoA as the variable substrate, slightly sigmoid curves were found with all fatty acids. Fig. 1 shows Eadie–Hofstee plots with CoA as the variable substrate and with palmitate (*a*) or arachidonate (*b*) as the fatty acid substrate. A positive co-operativity is clearly demonstrated. With ATP (*c* and *d*) or fatty acid (*e* and *f*) as the variable substrate, straight lines were produced. Hill coefficients were calculated for all three substrates with all fatty acids. Table 2 shows that the values for CoA ranged from 1.6 to 1.8 for all fatty acids investigated (stearate was an exception, possibly due to the high content of ethanol in the incubation mixture), indicating the presence of two active sites for CoA on the enzyme. For both ATP and fatty acids as variable substrate the Hill coefficient was 0.8–1.1 for all fatty acids. Thus the optimal conditions for palmitoyl-CoA and arachidonoyl-CoA synthetase activities as well as activities for other long-chain fatty acids are identical in human platelet lysates.

Table 1. Apparent K_m values of substrates for acyl-CoA synthetase

The reaction mixture contained 50 mM-Hepes, pH 7.4, 5 mM-DTT, 10 mM-$MgCl_2$, 0.1% Triton X-100, lysate from 80×10^6 platelets, ATP, CoA and [^{14}C]fatty acids in various amounts in a final volume of 250 μl. For each K_m investigated, the concentrations of the two non-varied substrates were approx. 8–10 times the K_m. The incubation temperature was 37 °C, and the incubation time was 3 min. For the apparent K_m values for ATP, the endogenous ATP has not been accounted for.

	K_m (μM)		
Fatty acid	ATP	CoA	Fatty acid
$C_{12:0}$	200	12	13
$C_{14:0}$	200	9	15
$C_{16:0}$	130	5	17
$C_{18:0}$	150	5	15
$C_{18:1,n-9}$	91	5	16
$C_{18:2,n-6}$	55	7	20
$C_{18:3,n-3}$	65	7	11
$C_{20:4,n-6}$	90	7	17
$C_{20:5,n-3}$	55	6	17
$C_{22:6,n-3}$	96	4	19

Fig. 2 shows the maximal activity of acyl-CoA synthetase with different fatty acids as substrates, given as means in lysates from 2–8 different blood donors. Also, the activities in a P fraction (dense-tubular-enriched fraction) and an M fraction (mitochondria-enriched fraction) are shown. The pattern of activity was almost identical in whole homogenate and in the M and P fractions, with one peak for palmitate and one peak for arachidonate.

Fatty acid specificity of platelet acyl-CoA synthetase

To study differences in the specificity of the synthetase reaction with palmitic acid or arachidonic acid as the fatty acid substrate, two sets of experiments were performed.

First, inhibition was studied in two experiments with both the ^{14}C-labelled fatty acid substrate and the inhibitory non-labelled fatty acid at concentrations close to their apparent K_m values. Fig. 3 shows that the formation of [^{14}C]palmitoyl-CoA was inhibited by the presence of any of the unsaturated fatty acids tested, in the order $C_{18:3} > C_{22:6} > C_{18:1} > C_{18:2} > C_{20:5} > C_{20:4}$. However, the difference between the last three fatty acids was small. The inhibition was not of a clear competitive type, but rather of a mixed competitive/non-competitive type, possibly due to the content of endogenous fatty acids (see later). Fig. 4 shows a similar experiment in which formation of [^{14}C]arachidonoyl-CoA was studied in the presence of $C_{16:0}$, $C_{18:3}$, $C_{20:4}$, $C_{20:5}$ or $C_{22:6}$ as inhibitors. Under these conditions, the formation of [^{14}C]arachidonoyl-CoA was inhibited by all fatty acids added, in the order $C_{20:4} > C_{20:5} > C_{22:6} > C_{18:3} > C_{16:0}$. The difference between the last two acids was small. In this experiment also the inhibition was of a mixed competitive/non-competitive type.

Secondly, inhibition was studied in two experiments where the [^{14}C]palmitate or [^{14}C]arachidonate was kept at saturating

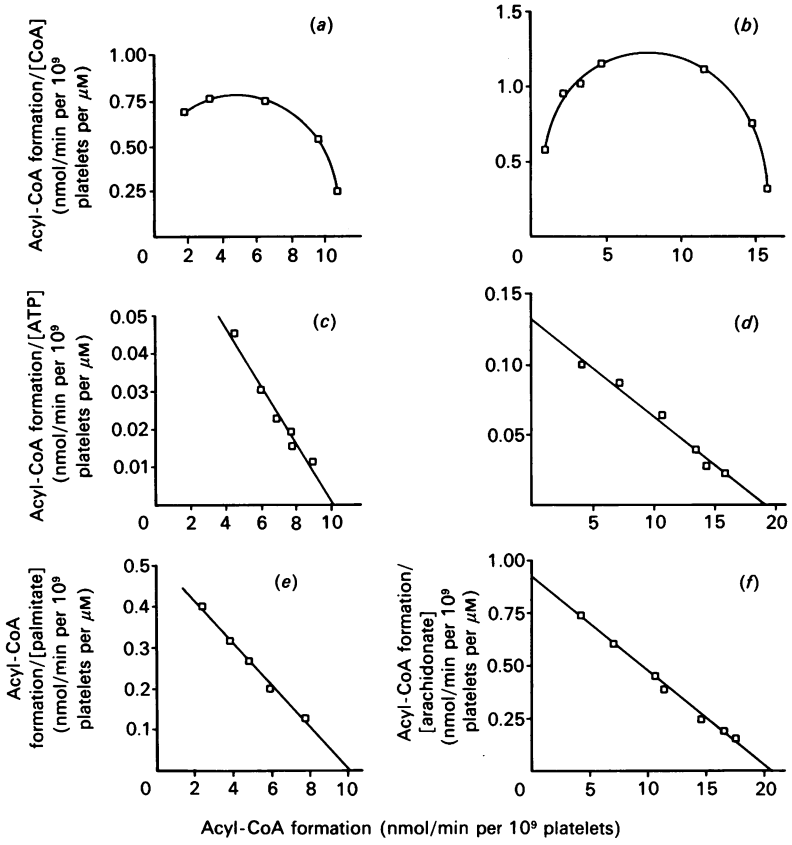

Fig. 1. Eadie–Hofstee plots of the formation of [^{14}C]acyl-CoA as a function of CoA, ATP and fatty acid concentration with [^{14}C]palmitate (*a*, *c*, *e*) or [^{14}C]arachidonate (*b*, *d*, *f*) as the fatty acid substrate

The reaction mixture contained 50 mM-Hepes (pH 7.4), 10 mM-$MgCl_2$, 5 mM-DTT, 0.1% Triton X-100, various concentrations of CoA (*a*, *b*), ATP (*c*, *d*) or ^{14}C-labelled fatty acids (*e*, *f*), and lysate from 81×10^6 platelets in a final volume of 250 μl. The incubation temperature was 37 °C and the incubation time was 3 min. The non-varied substrates were added at concentrations 10 times the apparent K_m values.

concentration. Under optimal conditions for formation of [^{14}C]palmitoyl-CoA ([^{14}C]palmitate, ATP and CoA at concentrations 10 times the respective K_m values), arachidonic acid was added (Fig. 5). On addition of arachidonate up to a concentration of about 15 μM a sharp drop in [^{14}C]palmitoyl-CoA formation was seen. At higher concentrations a further, but more moderate, inhibition was observed. When palmitate was used as an inhibitor of [^{14}C]arachidonoyl-CoA formation under similar conditions, [^{14}C]arachidonoyl-CoA formation was inhibited up to a palmitate concentration of about 30 μM. Under these conditions, $C_{20:4}$ was a more potent inhibitor than was $C_{16:0}$; otherwise the pattern of inhibition was similar.

In an additional experiment in which the [^{14}C]arachidonate concentration was varied, addition of 150 μM unlabelled palmitate resulted in a moderate inhibition of arachidonoyl-CoA synthesis. The inhibition was, however, less than 30% of a theoretical inhibition of an 'unspecific' acyl-CoA synthetase (results not shown).

Table 2. Hill coefficients for the acyl-CoA synthetase substr. tes

Assay conditions were as in Table 1.

	h								
	$C_{14:0}$	$C_{16:0}$	$C_{18:0}$	$C_{18:1}$	$C_{18:2}$	$C_{18:3}$	$C_{20:4}$	$C_{20:5}$	$C_{22:6}$
CoA	1.7	1.9	1.3	1.6	1.8	1.7	1.8	1.6	1.6
ATP	1.1	0.9	1.0	0.9	0.8	0.8	1.1	1.0	0.9
Fatty acid	1.0	1.0	–	0.9	1.0	1.0	1.1	1.1	1.0

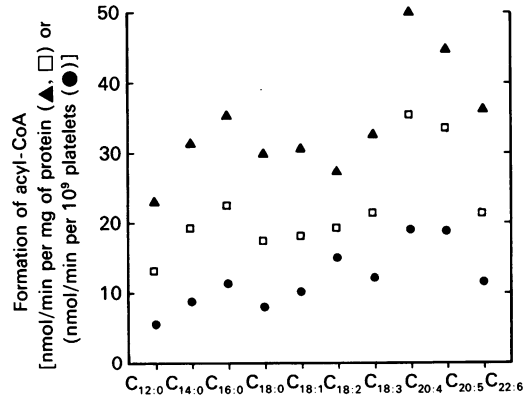

Fig. 2. Acyl-CoA synthetase activity of fatty acids measured in different lysates and in the dense-tubular-enriched (P) fraction and the mitochondria-enriched (M) fraction of human platelets

Each point is the mean value of the acyl-CoA synthetase activity in platelets from 2–8 blood donors. For each activity measured, the concentrations of the three substrates were approx. 8–10 times the K_m. The conditions were otherwise as stated in the Materials and methods section. ▲, Acyl-CoA synthetase activity in the P fraction; □, activity in the M fraction; ●, activity in the platelet lysate.

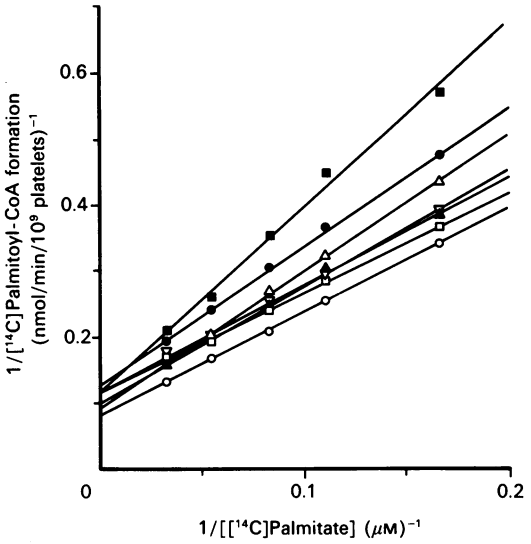

Fig. 3. Inhibition of [^{14}C]palmitoyl-CoA formation by $C_{18:1,n-9}$ (△), $C_{18:2,n-6}$ (▲), $C_{18:3,n-3}$ (■), $C_{20:4,n-6}$ (□), $C_{20:5,n-3}$ (▽) and $C_{22:6,n-3}$ (●)

The reaction mixture contained, in a final volume of 250 μl, 50 mM-Hepes, pH 7.4, 10 mM-$MgCl_2$, 2 mM-ATP, 5 mM-DTT, 70 μM-CoA, 0.1% Triton X-100, 20 μM unlabelled fatty acids, various amounts of [^{14}C]palmitate, and lysate from 63×10^6 platelets. The incubation temperature was 37 °C and the incubation time was 3 min. ○, No unlabelled fatty acid added.

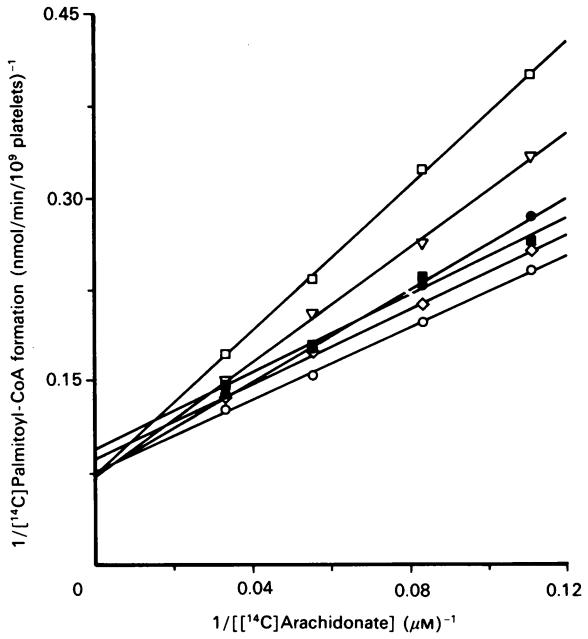

Fig. 4. Inhibition of [^{14}C]arachidonoyl-CoA formation by $C_{16:0}$ (◇), $C_{18:3,n-3}$ (■), $C_{20:4,n-6}$ (□), $C_{20:5,n-3}$ (▽) and $C_{22:6,n-3}$ (●)

The reaction mixture contained, in a final volume of 250 μl, 50 mM-Hepes, pH 7.4, 10 mM-$MgCl_2$, 2 mM-ATP, 5 mM-DTT, 70 μM-CoA, 0.1% Triton X-100, 20 μM unlabelled fatty acids, various amounts of [^{14}C]arachidonate, and lysate from 85×10^6 platelets. The incubation temperature was 37 °C and the incubation time was 3 min. ○, No unlabelled fatty acid added.

Product inhibition studies were performed using palmitate as the variable substrate in one set of experiments, and CoA as the variable substrate in another. Concentrations of 5, 10 and 15 μM-palmitoyl-CoA were added to the reaction mixtures. No product inhibition was observed in these experiments (results not shown).

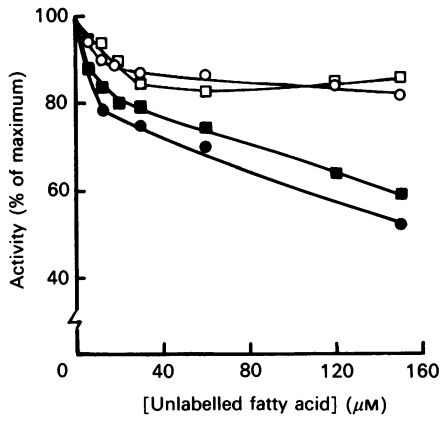

Fig. 5. Inhibition of [^{14}C]arachidonoyl-CoA formation by increasing amounts of palmitate and inhibition of [^{14}C]palmitoyl-CoA formation by increasing amounts of arachidonate

The reaction mixture contained, in a final volume of 250 μl, 50 mM-Hepes, pH 7.4, 10 mM-$MgCl_2$, 2 mM-ATP, 5 mM-DTT, 70 μM-CoA and 0.1% Triton X-100 lysate from two different platelet preparations. For ○ and □, 150 μM-[^{14}C]arachidonate was the substrate and increasing amounts of palmitic acid were added. For ● and ■, 150 μM-[^{14}C]palmitate was the substrate and increasing amounts of arachidonic acid were added. Circles represent one lysate and squares the other lysate.

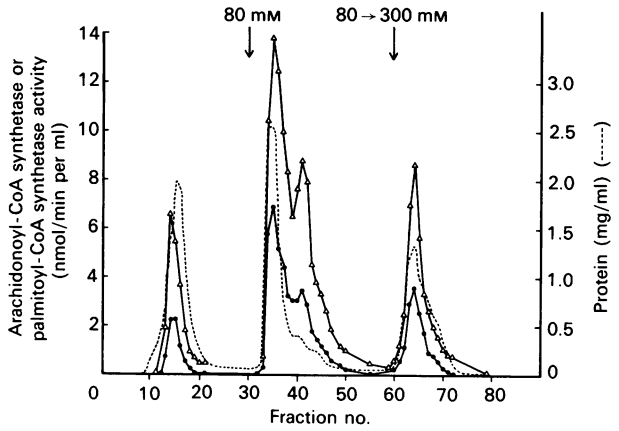

Fig. 6. Activity of palmitoyl-CoA synthetase (●) and arachidonoyl-CoA synthetase (△) in fractions of eluate from hydroxyapatite chromatography of the Triton X-100 extract

The column was eluted with a potassium phosphate gradient buffer. For details, see the Materials and methods section. Enzyme activities were assayed with [^{14}C]palmitate or [^{14}C]arachidonate as substrate, as described in the Materials and methods section.

Palmitoyl-CoA and arachidonoyl-CoA synthetase activities in eluates after hydroxyapatite chromatography of platelet homogenates

Fig. 6 shows that acyl-CoA synthetase was eluted in three peaks by hydroxyapatite chromatography of Triton X-100 extracts of human platelets. The enzyme activity was eluted in the main protein peaks. In each of the three peaks the ratio of activities between palmitoyl-CoA and arachidonoyl-CoA synthetase, using [^{14}C]palmitate or [^{14}C]arachidonate as substrate, were similar. The formation of [^{14}C]arachidonoyl-CoA was about twice as high as the formation of [^{14}C]palmitoyl-CoA in the different peaks. In this experiment about 60% of the total enzyme activity was extracted by the first Triton X-100 extraction.

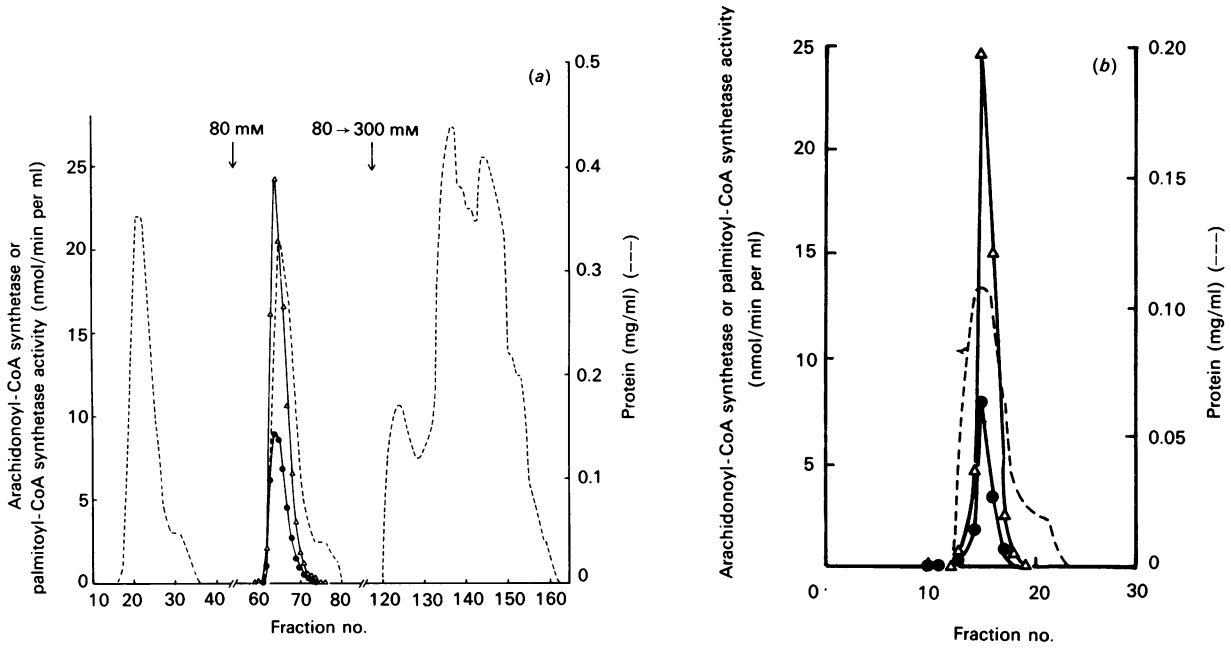

Fig. 7. Partial purification of acyl-CoA synthetase on DEAE-Sepharose and Sephacryl S-200 HR

(*a*) Activity of palmitoyl-CoA synthetase (●) and arachidonoyl-CoA synthetase (△) in fractions of eluate from DEAE-Sepharose Fast Flow chromatography of the Triton X-100 extract. The column was eluted with a 20 mM-potassium phosphate buffer containing 5 mM-DTT, 50 μM-ATP and 2 mM-Triton X-100, pH 7.45. The gradient elution is indicated by an arrow. For details, see the Materials and methods section. Enzyme activities were assayed with [^{14}C]palmitate or [^{14}C]arachidonate as substrates, as described in the Materials and methods section. (*b*) Purification of acyl-CoA synthetase by Sephacryl S-200 HR chromatography of pooled fractions from DEAE-Sepharose chromatography. The column was eluted with a 80 mM-potassium phosphate buffer containing 5 mM-DTT, 50 μM-ATP and 2 mM-Triton X-100, pH 7.45. For details, see the Materials and methods section. Enzyme activities were assayed with [^{14}C]palmitate (●) or [^{14}C]arachidonate (△) as substrate, as described in the Materials and methods section.

When the platelet precipitate was extracted once more with Triton X-100 similar results were found, except that the total activity was lower in all peaks.

Partial purification of acyl-CoA synthetase on DEAE-Sepharose and Sephacryl S-200 HR

Three protein peaks were eluted from the DEAE-Sepharose Fast Flow column. Peak II, which was eluted in 80 mM-potassium phosphate (pH 7.45)/50 μM-ATP/5 mM-DTT/2 mM-Triton X-100, contained all of the acyl-CoA synthetase activity (Fig. 7*a*). The specific activity for palmitoyl-CoA synthetase increased from 5 nmol/min per mg of protein in the Triton X-100 extract to 28 nmol/min per mg, and from 9 nmol/min per mg for arachidonoyl-CoA synthetase to 76 nmol/min per mg. The pooled concentrated fractions containing enzyme activity from the DEAE-Sepharose chromatography were applied to a Sephacryl S-200 HR column (Fig. 7*b*). Although the enzyme was eluted in the void volume, the specific activities had increased to 37 nmol/min per mg of protein with palmitate as substrate and to 116 nmol/min per mg with arachidonate as substrate. This procedure resulted in purifications of respectively 8- and 12-fold for the two enzyme activities.

The fractions from Sephacryl chromatography containing enzyme activity were pooled, dialysed and applied to the CoA–Sepharose 4B column. The enzyme activity was lost during this procedure. Shortening the dialysis time to 3.5 h did not preserve the enzyme activity. Therefore a Triton X-100 extract omitting DTT and ATP (Kameda *et al.*, 1985) in the platelet suspension was centrifuged at 100000 $g_{max.}$. The supernatant was applied to the CoA–Sepharose 4B column. Two protein peaks were eluted, the first with the equilibration buffer, and the second with the KCl gradient buffer (results not shown). Acyl-CoA synthetase activity was found only in the first peak, and no separation of the palmitoyl-CoA and arachidonoyl-CoA synthetase activities was obtained.

Arachidonoyl-CoA synthetase and palmitoyl-CoA synthetase activities in eluates after chromatography on Sephadex G-100 followed by AcA 34 chromatography

Both arachidonoyl-CoA and palmitoyl-CoA synthetase activities were eluted with the void volume by Sephadex G-100 chromatography (similar results were obtained with Sephacryl S-200 HR). In the peak fractions the activities were, however, increased by a factor of about 1.3 (Fig. 8*a*). When the Diaflo-membrane-filter concentrate from the Sephadex G-100 column was eluted from an AcA 34 column, both arachidonoyl-CoA and palmitoyl-CoA synthetase activities appeared together in two peaks with approximately the same ratio of activities with the two substrates (Fig. 8*b*). The first, smaller, peak was eluted with the void volume. The third protein peak contained no activity. The recovery of activity was low, only about 25% for both substrates.

DISCUSSION

In the crude enzyme preparations used in these studies of fatty acyl-CoA synthetase(s), unspecific binding of added as well as endogenous fatty acids to platelet proteins and other cell components (Ockner & Manning, 1974) will cause a small overestimation of the measured K_m values for the fatty acids. From Table 1 we can infer that the active sites of acyl-CoA synthetase have a strong affinity for the substrates.

The measurement of the fatty acid activation in this investigation is most probably without side-reactions, for the following

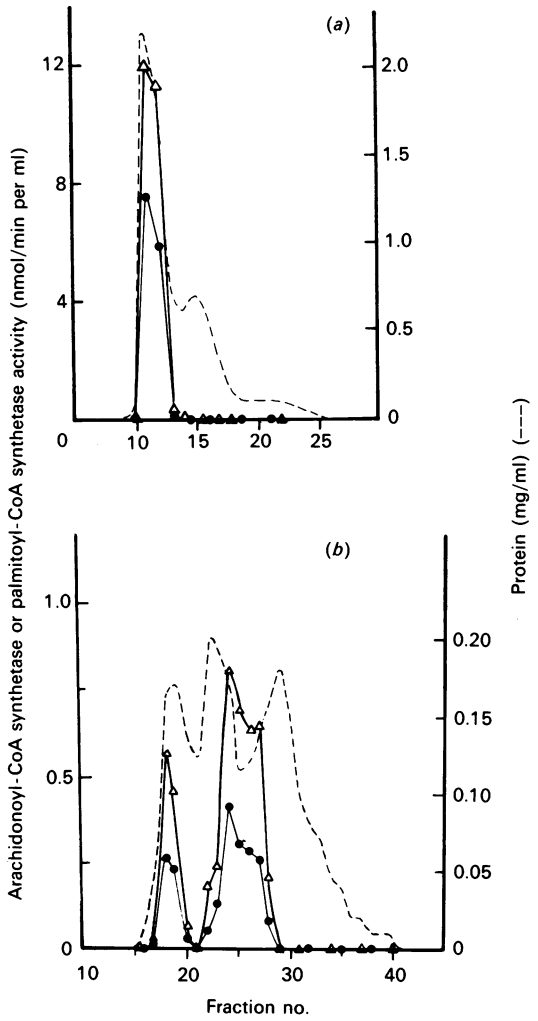

Fig. 8. Activity of palmitoyl-CoA synthetase (●) and arachidonoyl-CoA synthetase (△) (*a*) after chromatography of Triton X-100 extract on Sephadex G-100, and (*b*) in concentrated fractions from the Sephadex G-100 chromatography, chromatographed on an AcA 34 column

(*a*) The column was eluted with 20 mM-potassium phosphate buffer containing 1 mM-ATP, 5 mM-DTT and 2 mM-Triton X-100, followed by 80 mM-phosphate buffer containing ATP, DTT and Triton X-100. Enzyme activities were assayed as described in the Materials and methods section. (*b*) The AcA 34 column was eluted with 100 mM-potassium phosphate buffer, pH 7.45, containing 1 mM-ATP and 5 mM-DTT. Enzyme activities were assayed as described in the Materials and methods section.

reasons. (1) The mitochondria were disrupted when preparing the lysate, so sub-reactions such as β-oxidation are unlikely. (2) Platelet long-chain acyl-CoA hydrolase was inactive under the present conditions with 10 mM $MgCl_2$ (Berge & Farstad, 1978), and 1.6 mM-Triton X-100 would bind free acyl-CoA (Berge *et al.*, 1981), in agreement with McKean *et al.* (1982). (3) No measurable [^{14}C]phospholipids were formed during the 3 min incubation period.

In our experiments the acyl-CoA synthetase activity was almost twice as high with arachidonic acid and EPA as substrate compared with when palmitic acid or oleic acid was the substrate (Fig. 2). Also, the fatty acid activation pattern was almost the same in platelet lysates as in the dense-tubular-enriched fraction and the mitochondria-enriched fraction. Wilson *et al.* (1982) found that formation of arachidonoyl-CoA was four times faster than formation of oleoyl-CoA. These authors found an activity of 2.9 nmol/min per 10^9 platelets for arachidonic acid, whereas we found an activity of approx. almost 20 nmol/min per 10^9 platelets for arachidonic acid, and up to 50 nmol/min per mg in a dense-tubular-enriched fraction (Fig. 2). The apparent K_m values recorded by Wilson *et al.* (1982) were all higher (130 μM for CoA, 500 μM for ATP and 30 μM for arachidonic acid) than reported here. This is unexpected, since Wilson *et al.* (1982) measured the acyl-CoA synthetase activity in isolated platelet membranes, which are supposed to be a purer source of the enzyme than are platelet lysates. In a later work, however, workers in the same laboratory (Neufeld *et al.*, 1984) reported a K_m value for arachidonate of 12 μM.

Acyl-CoA synthetase in platelet lysates is stimulated by Triton X-100 with a maximal activity at 0.1 % or about 1.6 mM-Triton X-100 (Vollset & Farstad, 1979; Berge *et al.*, 1981; Bakken, 1986), which was used in the present work. As the CMC for Triton X-100 in the incubation mixture at 37 °C is probably less than 0.01 %, or about 80 μM (Berge *et al.*, 1981), the fatty acid substrates were present in mixed micelles at all concentrations tested (molar ratio of fatty acid/Triton X-100 from 1:10 to 1:250).

The mean concentration of free CoA in platelets *in vivo* is 8–10 μM (Ingebretsen *et al.*, 1982*b*), i.e. very close to the apparent K_m values for CoA (Table 1).

Majerus *et al.* (1983) found that [^{14}C]arachidonoyl-CoA formation in isolated platelet membranes was not inhibited by $C_{18:0}$, $C_{18:1}$ or $C_{18:2}$, but only by arachidonic acid and dihomo-γ-linolenic acid ($C_{20:3,n-6}$). In the present experiments, using lysate, all fatty acids tested inhibited [^{14}C]palmitoyl-CoA formation (Figs. 3 and 5) at concentrations near the apparent K_m values for the inhibitory fatty acids. Also [^{14}C]arachidonoyl-CoA formation was inhibited by all fatty acids tested under similar conditions (Figs. 4 and 5). Our assay conditions differed from those of Majerus *et al.* (1983), as the CoA concentration was 8–10 times less than in their experiments, and our incubation time was 3 min rather than 10 min. The lack of a clear competitive inhibition in our experiments is probably due to the presence of endogenous fatty acids in our platelet preparation (see the Materials and methods section).

Iritani *et al.* (1984) examined the fatty acid specificity of rat platelet acyl-CoA synthetase activity in competition experiments. They concluded, like Wilson *et al.* (1982), that platelet membranes prepared by sonication had more than one long-chain acyl-CoA synthetase. On the other hand, purified long-chain acyl-CoA synthetases from both mitochondria and microsomes of rat liver showed a broad specificity with approximately the same specific activity for palmitate and arachidonate (Tanaka *et al.*, 1979).

Laposata *et al.* (1985) reported separation of a specific arachidonoyl-CoA synthetase from an unspecific enzyme by hydroxyapatite chromatography. In our experiments both palmitoyl-CoA and arachidonoyl-CoA synthetase activities were eluted together, with a near-constant ratio of activities after chromatography on hydroxyapatite, DEAE-Sepharose followed by Sephacryl S-200 HR, CoA–Sepharose 4B, and Sephadex G-100 followed by AcA 34 (Figs. 6, 7 and 8).

Noy & Zakim (1985) presented their fatty acid substrates in phosphatidylcholine liposomes, and obtained activities twice as high as those recorded in the present work, where Triton X-100 was used. However, the addition of various fatty acids in the form of liposomes would result in co-variations of the concentrations of phosphatidylcholine liposomes with the concentrations of the fatty acid substrate. Phosphatidylcholine is a substrate for the CoA-dependent transacylation of platelet phospholipids (Kramer *et al.*, 1984). Also, variations of non-esterified fatty acids in liposomes are not straightforward in experiments for

determination of apparent K_m values. We therefore chose an approach using a constant concentration of Triton X-100, thus ensuring that all substrates were added under identical conditions.

We are aware that the presence of Triton X-100 in the elution buffers resulted in ineffective separation by gel filtration. This is most likely due to micelle-like complexes of active enzyme, resembling the effects observed with long-chain acyl-CoA hydrolase (Berge *et al.*, 1981). In spite of this, gel filtration with Sephacryl S-200 HR resulted in a significant purification (12- and 8-fold respectively) of the activity with both [^{14}C]arachidonate and [^{14}C]palmitate as substrate (see Fig. 7*b*). Some purification was also obtained by chromatography on Sephadex G-100 followed by AcA 34 when Triton was omitted from the elution buffer (see Fig. 8).

The present results differ to a great extent from the results reported from Majerus' group (Wilson *et al.*, 1982; Majerus *et al.*, 1983; Neufeld *et al.*, 1984; Laposata *et al.*, 1985). Whereas these workers have reported the existence of an acyl-CoA synthetase specific for arachidonic acid and other prostanoid precursors, the present results indicate that palmitoyl-CoA (or unspecific) synthetase and arachidonoyl-CoA (or specific) synthetase are the same enzyme. This assumption is based on the following facts. (1) There is near identity between K_m values for CoA, ATP and fatty acids ($C_{12:0}$ to $C_{22:6}$) with the two activities. (2) The activities of both arachidonoyl-CoA synthetase and palmitoyl-CoA synthetase are very much higher in our experiments. (3) There is mutual and moderate inhibition of [^{14}C]arachidonate and [^{14}C]palmitate activation, both at their respective apparent K_m values and at saturating concentrations, and there is also the moderate inhibition of [^{14}C]arachidonate activation at high concentrations of unlabelled palmitate. (4) The subcellular distributions of the activation of all fatty acids from $C_{12:0}$ to $C_{22:6}$ are identical for the two activities (Bakken & Farstad, 1989). (5) Two pools of enzyme(s) are present, one which is very rapidly inactivated by heat ($t_{\frac{1}{2}}$ 1.5 min at 45 °C), and one with a slower inactivation ($t_{\frac{1}{2}}$ 8–10 min at 45 °C). However, both pools show almost identical patterns of heat inactivation of [^{14}C]arachidonoyl-CoA and [^{14}C]palmitoyl-CoA synthetase activities (Bakken & Farstad, 1989). (6) Palmitoyl-CoA synthetase and arachidonoyl-CoA synthetase from the Triton X-100 extract of human platelet lysates were eluted in almost the same proportions and in the same protein peaks in six different chromatographic systems: hydroxyapatite, DEAE-Sepharose, Sephacryl S-200 HR, CoA–Sepharose 4B, Sephadex G-100 and AcA 34. (7) Partial purification of acyl-CoA synthetase (Fig. 7*b*) resulted in protein peaks with very high activities with both arachidonate and palmitate as substrates (purification factors of 12- and 8-fold respectively).

The reasons for different findings in the present study compared with previous data may, at least partly, be due to differences in the treatment of platelet homogenates. We extracted and assayed the acyl-CoA synthetase activity in the presence of a non-ionic detergent, Triton X-100, which stimulates acyl-CoA synthetase activity (Vollset & Farstad, 1979). Tanaka *et al.* (1979) and Suzuki *et al.* (1990) also used Triton X-100 during their purification of long-chain acyl-CoA synthetase. The results reported by Wilson *et al.* (1982), Majerus *et al.* (1983), Neufeld *et al.* (1984) and Laposata *et al.* (1985) were obtained with platelet homogenates after repeated sonication, which probably inhibited the enzyme by as much as 75% (Norum *et al.*, 1966; Bakken & Farstad, 1989; Pande *et al.*, 1990). Long-chain acyl-CoA synthetase has also been reported to be very unstable in crude preparations, and is stabilized by the presence of ATP (Tanaka *et al.*, 1979; Suzuki *et al.*, 1990).

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