

Enzymic Studies on Phosphatidic Acid Synthesis in Human Platelets

By Minoru Okuma, Satoshi Yamashita, and Shosaku Numa

Particulate preparations of human platelets were capable of catalyzing acylation of *sn*-glycerol 3-phosphate by long-chain fatty acyl-CoA thioesters. The principal lipid product formed was identified as phosphatidic acid. The highest specific activity was found in the particulate fraction that sedimented between 12,000 *g* and 105,000 *g*. The reaction exhibited a broad pH optimum around 7.4–8.5. The apparent Michaelis constant for *sn*-glycerol 3-phosphate was 0.48 mM when 28.5 μ M palmityl-CoA was used as acyl donor. Palmityl- and oleyl-CoA were better substrates than stearyl-

linoleyl-, and arachidonyl-CoA, as far as the maximal velocity was concerned. Particulate preparations of platelets from normal subjects catalyzed the incorporation of 0.271 ± 0.048 nmole of *sn*-glycerol 3-phosphate/min per mg of protein. The capacity of human platelets to acylate *sn*-glycerol 3-phosphate was approximately 40% that of human liver, as compared on the basis of the specific activity of the microsomal fraction. These results suggest that the glycerophosphate pathway makes an essential contribution to the de novo synthesis of phospholipids in human platelets.

PHOSPHOLIPIDS of human platelets comprise about 80% of their total lipids¹ and play an important role in the structure and function of these blood cells. The lipids required in the intrinsic pathway of blood coagulation are derived in vivo solely from phospholipids, which constitute an integral part of the platelet membrane.²

In certain mammalian tissues, PA has been shown to be a key intermediate in the metabolic pathway leading to the de novo synthesis of phosphoglycerides.³ It was originally demonstrated that the formation of PA in the liver proceeds through acylation of GP by long-chain fatty acyl-CoA deriva-

From the Department of Medical Chemistry, Kyoto University Faculty of Medicine, Kyoto, Japan, and the Department of Internal Medicine, Kitano Hospital, Osaka, Japan.

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Minoru Okuma, M.D.: Physician and Clinical Investigator, Division of Hematology, Department of Internal Medicine, Kitano Hospital, Osaka, Japan; Associate, Department of Medical Chemistry, Kyoto University Faculty of Medicine, Kyoto, Japan. Satoshi Yamashita, M.D.: Associate Professor, Department of Medical Chemistry, Kyoto University Faculty of Medicine, Kyoto, Japan. Shosaku Numa, M.D.: Professor, Department of Medical Chemistry, Kyoto University Faculty of Medicine, Kyoto, Japan.

Abbreviations used in text: PA, phosphatidic acid; GP, sn-glycerol 3-phosphate; ACD, acid-citrate-dextrose anticoagulant, USP formula "A;" TLC, thin-layer chromatography; LPA, lysophosphatidic acid; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PS, phosphatidylserine; SM, sphingomyelin; CL, cardiolipin; NL, neutral lipids; CoA, coenzyme A.

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tives.⁴ Recently, the presence of an alternate pathway for PA synthesis from dihydroxyacetone phosphate has been found in guinea pig liver.⁵

In studies with intact platelets, radioactive precursors (such as phosphate, acetate, glycerol, and fatty acids) were shown to be incorporated into phospholipids.⁶⁻¹⁴ Concerning the enzymic mechanism of this incorporation, several studies have been reported on phosphatidate cytidyltransferase,¹⁵ myoinositol phosphatidyltransferase,¹⁶ phosphatidate phosphatase,¹⁷ and diglyceride kinase¹⁷ in homogenates of human platelets.

The present investigation was designed to characterize the enzyme activity responsible for PA synthesis from GP in human platelets. We have demonstrated that particulate preparations from human platelets are capable of catalyzing acylation of GP by long-chain fatty acyl-CoA thioesters. The characteristics of this enzymic reaction are described. The level of the enzyme activity in human platelets, relative to that in human liver, is also reported.

MATERIALS AND METHODS

Collection and Procurement of Blood

Since smaller amounts of blood were available for the present studies, the platelet procurement differed in the following manner from that previously described.¹⁴ Twenty to 200 ml of whole blood were obtained by a plastic syringe or bag containing 3 volumes ACD/20 volumes blood and were centrifuged in 20- or 50-ml glass centrifuge tubes at 500 g for 10 min. The upper three quarters of the supernatant platelet-rich plasma was removed, and the same volume of 0.15 M NaCl containing 0.05 M Tris-maleate buffer, pH 6.5, (buffered saline) was mixed with the rest of blood. The suspension was recentrifuged at 280 g for 10 min. The resulting supernatant was combined with the first one to which one-seventh volume of ACD had been added. In some experiments, only the first supernatant was used. The supernatant fluid thus obtained was centrifuged at 1100 g for 30 sec to eliminate contaminated red and white cells and was then centrifuged at 1700 g for 30 min. The platelet pellet was washed twice with the buffered saline and was suspended in 1.5-8 ml of 0.44 M chilled sucrose solution ($1-4 \times 10^9$ platelets/ml); the red cell and white cell contamination was always less than 1 RBC/ 4×10^4 platelets and 1 WBC/ 2×10^4 platelets ("pure" platelet preparation). To examine the effect of contaminated red and white cells on the enzyme activity of platelet preparations, platelets contaminated with these cells in various degrees were separated from buffy coat and "bottom red cells" obtained by the 500 g centrifugation. The contaminated platelets were washed and suspended in the same manner as the pure platelet preparation, and counts were made.¹⁸ All procedures were carried out at 4°C with either plastic or siliconized equipment.

Enzyme Preparation

Unless otherwise stated, platelets, which were suspended in 1.5-3 ml of 0.44 M sucrose, were disrupted sonically for a total period of 60 sec (30 sec at a time) at minimum output with a Branson W185D sonifier. The homogenate thus prepared was centrifuged at 105,000 g for 60 min. The pellet was rinsed once with 0.25 M sucrose and was suspended in it to yield a protein concentration of 2.5-5 mg/ml. This preparation ("whole particles") was used as enzyme in most of the experiments.

Subcellular Fractionation of Platelets

The platelet homogenate prepared by sonication as described above was fractionated by sequential centrifugation at 1000 g for 20 min, 12,000 g for 12 min, and 105,000 g for 60 min. Each pellet was suspended in 0.25 M sucrose to give a protein concentration of approximately 2.5 mg/ml.

Preparation of Microsomal Fraction of Human Liver

Specimens of histologically normal human liver were obtained from surgical biopsy materials. Fresh samples were homogenized in 4 volumes of cold 0.25 M sucrose by a Teflon pestle. The homogenate was centrifuged at 12,000 g for 12 min. The microsomal fraction was prepared by centrifuging this supernatant at 105,000 g for 60 min, by rinsing the pellet with 0.25 M sucrose, and by suspending it in the same solution as described above.

Enzyme Assay

GP-acylating activity was measured by determining the incorporation of GP-1(3)-¹⁴C into lipid. Unless otherwise specified, the incubation tube contained 250 nmoles of GP-1(3)-¹⁴C (1100–5700 cpm/nmole), 10 nmoles of palmityl-CoA, 20 μmoles of Tris-HCl buffer, pH 7.6, and about 0.3 mg of protein in a final volume of 0.35 ml. The reaction was initiated by the addition of enzyme, and the incubation was performed at 20°C for 5 min. The reaction was terminated by the addition of 2 ml of methanol saturated with GP. Then 4 ml of chloroform and 1 ml of 0.2 N hydrochloric acid were added, and the tube was shaken on a Vortex mixer. The resultant lower phase was washed three times with 4 ml of methanol-0.2 N hydrochloric acid (1:1, v/v). The chloroform layer was transferred into a counting vial and was evaporated to dryness under an infrared lamp. Radioactivity was assayed in 5 ml of Bray's solution¹⁹ with a Packard 3320 scintillation spectrometer.

Analysis of Reaction Product

A sample of the reaction product extracted into the chloroform layer was concentrated under a stream of nitrogen and was subjected to TLC on an oxalate-impregnated silica gel G plate with chloroform-methanol-10 N HCl (87:13:0.5, v/v) as described by Cohen and Derksen.²⁰ In some experiments, the product was resolved on a carbonate-impregnated silica gel H plate with chloroform-methanol-acetone-acetic acid-water (200:40:80:40:20, v/v) according to the method of Hajra and Agranoff.²¹ The lipids were visualized by iodine vapor, and their identity was ascertained by cochromatography with authentic standards. The radioactive product was located by scraping equal segments of the entire chromatogram and by counting them in 5 ml of Bray's solution containing 3.5% thioxo-gel (Cab-O-Sil).

Preparation of Acyl-CoA Thioesters

Palmityl-, stearyl-, oleyl-, linoleyl-, and arachidonyl-CoA thioesters were prepared by the mixed anhydride method of Wieland and Rueff.²² The concentrations of acyl-CoA thioesters were determined spectrophotometrically by absorption at 260 mμ as described by Zahler et al.²³

Preparation of GP-1(3)-¹⁴C

GP-1(3)-¹⁴C was prepared from glycerol-1-¹⁴C and ATP by the action of glycerokinase from *Candida mycoderma*. The radioactive product was purified from the deproteinized reaction mixture by column chromatography on Dowex 1 × 8 (formate type). After washing the column with water, GP-1(3)-¹⁴C was eluted with a linear gradient of formic acid (0–5 N). Fractions containing the radioactive product were combined and lyophilized to remove formic acid.

Preparation of LPA

LPA was prepared by treating PA with *Trimeresurus flavoviridis* venom phospholipase A;²⁴ PA was obtained from egg lecithin with the use of carrot root chromoplasts.²⁵ The product was loaded on a silicic acid column and was eluted successively with 20%, 40%, and 70% methanol in chloroform. Fractions were analyzed for fatty acid ester/phosphorus ratio, and those fractions exhibiting a ratio of 0.98–1.02, which were eluted with 70% methanol, were used as standard LPA for TLC.

Chemical Analysis

Protein was determined by the method of Lowry et al.²⁶ with bovine serum albumin as the standard. Particulate protein was solubilized with 2% deoxycholate prior to the determination. Phosphate determination was carried out according to the method of Bartlett,²⁷ and optical densities were read at 660 m μ instead of 830 m μ . Total phosphorus was determined after ashing the samples.²⁸ Fatty acid ester was measured by the method of Shapiro.²⁹

Materials

Glycerol-1-¹⁴C (15.3 mCi/mmole) was purchased from the Radiochemical Centre, Amersham, England. CoA and glycerokinase were products of Boehringer, Mannheim, Germany. ATP was obtained from Sigma Chemical, St. Louis, Mo., and PA was from Mann Research Lab., New York, N. Y. Egg lecithin and *Trimeresurus flavoviridis* venom were gifts of Dr. Jun-ichi Kawanami, Shionogi Research Laboratory, Osaka, Japan. Palmitic acid, linoleic acid, and dipalmitin were purchased from Nakarai Chemical, Kyoto, Japan. Stearic acid and oleic acid were obtained from Fisher Scientific, Fairhaven, N. J. Arachidonic acid, as well as PC, PE, PS, SM, and CL, were products of Applied Science Lab., State College, Pa. Silica gels G and H were purchased from E. Merck AG, Darmstadt, Germany, and Cab-O-Sil was from Packard Instrument, Downers Grove, Ill.

RESULTS

Preliminary Studies

Experiments with platelet preparations contaminated with other blood cells in various degrees indicated that 1% or less of the GP-acylating activity present in the pure platelet preparation could be attributed to contamination by white cells. Red cell contamination was not a possible source of activity.

As shown in Table 1, no enzyme activity was demonstrable when intact platelets were assayed. Sonication yielded higher enzyme activity in "whole particles" than did freezing and thawing. The highest activity was recovered by sonication for a period of 60–90 sec. Shortened or prolonged sonication,

Table 1. Effect of Platelet Disruption on GP-Acylating Activity

Treatment	Specific Activity*	Protein† (mg)
No disruption (intact platelets)	0	4.4
Sonication‡		
30 sec	0.151	2.0
60 sec	0.239	2.0
90 sec	0.217	1.8
180 sec	0.168	1.6
Freezing and thawing		
once	0.150	1.8
3 times	0.141	2.0
6 times	0.101	1.8

*nmole of GP incorporated per minute per milligram of protein; "whole particles" were used for enzyme assay except for intact platelets.

†Derived from 1 ml of a "pure" platelet preparation; values for "whole particles" except for intact platelets.

‡The duration gives the total period (30 sec at a time).

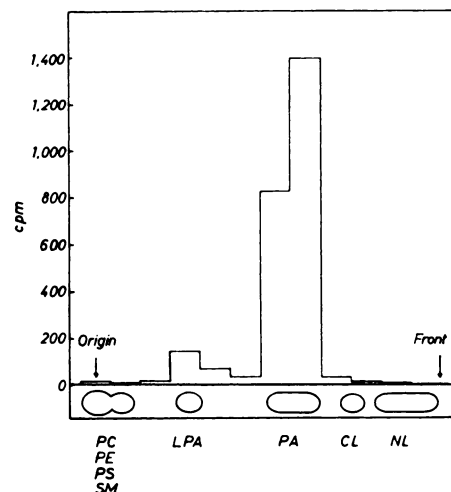


Fig. 1. TLC of the radioactive product. A reaction mixture containing 20 μ moles of Tris-HCl, pH 7.6, 250 nmoles of GP-1(3)- 14 C (5700 cpm/nmole), 10 nmoles of palmityl-CoA, and whole particles (1.0 mg of protein) was incubated at 20°C for 5 min. After lipids were extracted and separated by TLC,²⁰ the radioactivity was measured.

as well as repeated freezing and thawing, resulted in lower recoveries of activity.

Identification of Reaction Product

The lipid product, which was isolated from the reaction mixture containing whole particles as enzyme, was identified by TLC. The results of a typical experiment are depicted in Fig. 1; 86% and 9% of the total radioactivity present on the chromatogram were recovered in the areas corresponding to PA and LPA, respectively. No significant radioactivity was found in the areas corresponding to other phospholipids and NL. These results were confirmed with the use of another procedure of TLC.²¹ Similar results (PA, 86%; LPA, 10%) were also obtained when the particulate preparation that sedimented between 12,000 g and 105,000 g (microsomal fraction) was used as enzyme.

Subcellular Distribution of GP-Acylating Activity

As shown in Table 2, the sonicated platelet material sedimenting between 12,000 g and 105,000 g (Fr. 3) exhibited the highest specific activity. The

Table 2. GP-Acylating Activity and Protein Content in Platelet Subcellular Fractions

Platelet Fraction	Total Activity (%)	Specific Activity*	Protein (%)
Whole homogenate (Sonicate)	100	0.153	100
1,000 g pellet (Fr. 1)	24.8	0.195	19.4
12,000 g pellet (Fr. 2)	7.6	0.091	12.7
105,000 g pellet (Fr. 3)	28.2	0.400	10.7
105,000 g supernatant (Fr. 4)	7.6	0.025	46.5
"Whole particles"†	61.0	0.230	40.0

*nmole of GP incorporated per minute per milligram of protein.

†See Materials and Methods.

supernatant from 105,000 g centrifugation (Fr. 4) had the lowest specific activity and did not inhibit enzyme activity when mixed with other fractions. It was also found that a major portion of enzyme activity was associated with Fr. 3. Recovery of enzyme activity after fractionation of the whole homogenate was 68%, whereas that of protein was 89%. The use of 0.25 M sucrose instead of 0.44 M sucrose for suspending platelets gave similar results as to the subcellular distribution of enzyme activity and of protein. When platelets were subjected to prolonged sonication, the enzyme activity as well as the protein content of the 1000 g pellet (Fr. 1) was reduced; however, recovery of enzyme activity in the other fractions was not significantly raised.

Time Course of Reaction

Figure 2 shows that incorporation of GP-1(3)-¹⁴C into lipid increased linearly for the initial 10 min under the conditions used. Therefore, a 5-min incubation period was chosen for routine enzyme assay.

Enzyme Concentration Curve

As seen in Fig. 3, the rate of GP acylation was not completely linear with the protein concentration. On the basis of this enzyme concentration curve, assay was routinely carried out at protein concentrations around 1 mg/ml.

pH Optimum

A broad pH optimum around 7.4–8.5 was observed for the GP-acylating activity of human platelets (Fig. 4). Thus, enzyme activity was routinely assayed at pH 7.6.

Effects of Substrates

When the rate of GP acylation was plotted against the concentration of GP as variable substrate, a typical saturation curve was obtained (Fig. 5). The apparent Michaelis constant for GP at a fixed palmityl-CoA concentration of 28.5 μ M was found to be 0.48 mM.

The substrate saturation curves for palmityl-, stearyl-, oleyl-, linoleyl-, and arachidonyl-CoA are illustrated in Fig. 6. The optimal concentration varied with the kind of acyl donor; the optimal concentration for arachidonyl-CoA was the lowest, while that for linoleyl-CoA was the highest. At higher than optimal concentrations, each acyl donor exhibited an inhibitory effect. With regard to the maximal velocity, palmityl- and oleyl-CoA were better substrates than stearyl-, linoleyl-, and arachidonyl-CoA. In the absence of added acyl-CoA, no significant esterification of GP was observed. This indicates that the particulate preparation from platelets did not contain a sufficient amount of endogenous acyl-CoA to acylate GP.

GP-acylating Activity in Normal Human Platelets

“Whole particles” from platelets of ten normal subjects were found to catalyze the incorporation of 0.271 ± 0.048 nmole (mean \pm 1 SD) of GP/min/mg of protein. On the other hand, Fr. 3 (microsomal fraction) prepared

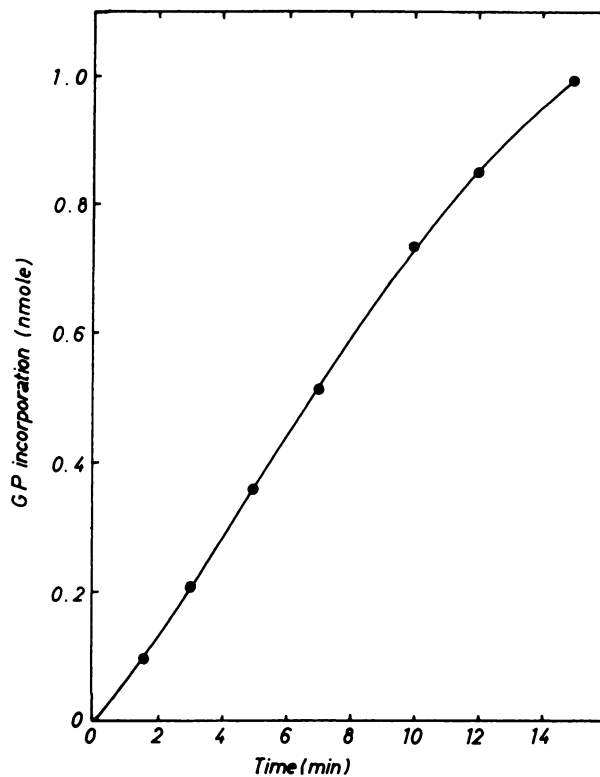


Fig. 2. GP acylation as a function of time. Incubation was carried out with whole particles (0.3 mg of protein) for varying periods of time as indicated.

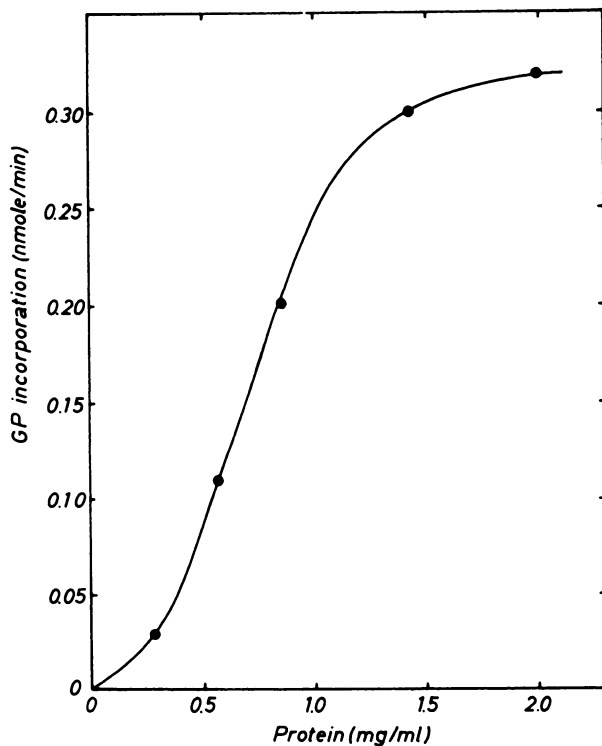


Fig. 3. Effect of protein concentration on GP acylation. Enzyme activity was assayed with varying amounts of whole particles as indicated.

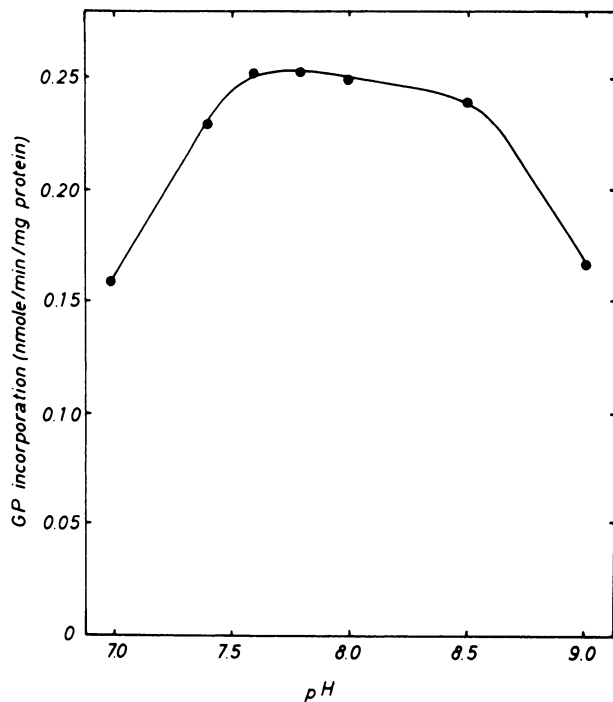


Fig. 4. pH-dependence of GP acylation. Enzyme activity was assayed with whole particles as described, except that Tris-HCl buffers of various pH values were used.

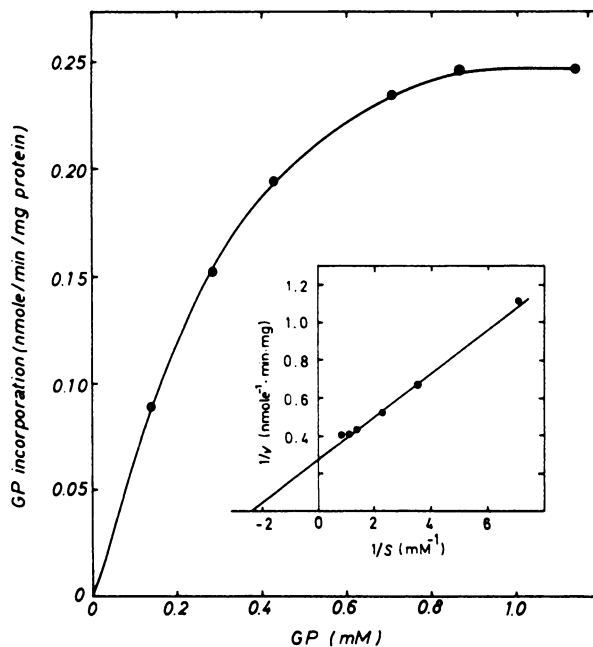


Fig. 5. Effect of GP concentration on reaction rate. Enzyme activity was assayed with whole particles as described, except that GP concentration was varied as indicated. The inserted figure represents the Lineweaver-Burk plot;³⁰ S and v denote the GP concentration and initial reaction velocity, respectively.

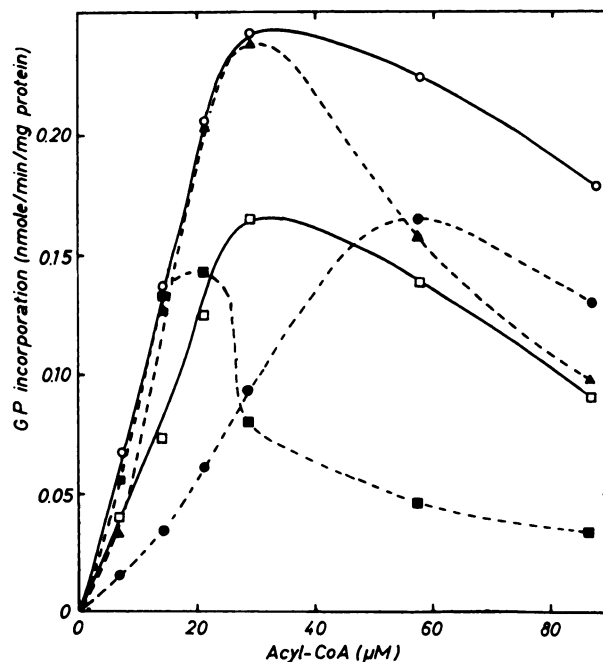


Fig. 6. Acyl donor specificity. Enzyme activity was assayed with whole particles as described, except that palmitoyl-CoA (open circles), steryl-CoA (open squares), oleyl-CoA (black triangles), linoleyl-CoA (black circles), or arachidonyl-CoA (black squares) was added in varying concentrations as indicated.

from platelets of five normal subjects exhibited a specific activity of 0.357 ± 0.052 nmole/min/mg.

In three subjects, the levels of GP-acylating activity both in platelets and in a sample of the liver, which was obtained during surgery, were determined (Table 3). The level of enzyme activity in platelets was approximately 40% that in the liver. T.M. was a patient who was subjected to gastrectomy for stomach cancer; K.A. had adenomyomatosis of the gall bladder and underwent cholecystectomy; M.T. suffered from idiopathic choledochus dilatation and underwent cholecystectomy together with choledochojejunostomy. In all patients, liver function tests (SGOT, SGPT, and serum alkaline phosphatase) were within normal range.

DISCUSSION

Previous studies have shown that platelets exhibit an active lipid metabolism. They are capable of de novo fatty acid synthesis,^{9-11,13} chain elongation of preformed fatty acids,^{9,11} and de novo phospholipid synthesis.^{12,16} With regard to the enzymic apparatus of de novo PA synthesis, the presence of GP-acylating activity in platelet extracts was briefly mentioned, but no data were given.^{11,12} The present investigation has clearly demonstrated that human platelets possess the enzyme activity responsible for the conversion of GP to PA. Studies on the subcellular distribution of this enzyme activity in human platelets have revealed that the fraction sedimenting between 12,000 g and 105,000 g has the highest specific activity. This is in agreement with the previous report that the rat liver enzyme is located in the micro-

Table 3. Level of GP-Acylating Activity in Human Platelets as Compared with That in Human Liver

Subject	Specific Activity*		
	Platelet	Liver	Platelet/Liver
T.M.	0.364	0.839	0.434
K.A.	0.380	0.847	0.449
M.T.	0.308	0.797	0.386

*nmole of GP incorporated per minute per milligram of protein; Fr. 3 of the sonicated platelets or the microsomal fraction of the liver was used for enzyme assay.

somal fraction.³¹ Some properties of the enzyme from human platelets have been shown to be similar to those of the enzyme from other mammalian tissues. The apparent Michaelis constant of the platelet enzyme for GP (0.48 mM) approximated that of the rat liver enzyme (0.5 mM)³² and of the rat brain enzyme (0.4 mM).³³ Higher concentrations of acyl-CoA thioesters inhibited the platelet enzyme, as is the case with the enzymes from rat liver and brain.^{32,33} The platelet enzyme exhibited a broad pH optimum as reported also with the rat liver enzyme.³² The lipid product formed by particulate preparations from human platelets was mainly PA, but a small amount of LPA was also found together with it. Particulate preparations from other mammalian tissues were likewise shown to yield PA as principal product,^{4,32,33} while it was reported that LPA was formed as main product by rat liver microsomes at pH 6.5.³⁴

The level of GP-acylating activity in human platelets has been found to be about 40% that in human liver, as compared on the basis of the specific activity of the microsomal fraction. This suggests that the glycerophosphate pathway makes an essential contribution to the de novo synthesis of phospholipids in human platelets. The relationship between GP-acylating activity and the function of platelets is not understood. In an attempt to gain an insight into this problem, the level of the enzyme activity in platelets from patients with diseases known to affect the function and phospholipid content of platelets is under investigation.

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