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**Research Article**

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# Biosynthesis of Cytidine Diphosphate Diglyceride by Human Platelets

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ABSTRACT Homogenates of human platelets contain an enzyme which catalyzes the formation of cytidine diphosphate diglyceride from cytidine triphosphate and phosphatidic acid. The enzymatic activity could not be dissociated from platelet particles and the greatest specific activity was found in the membrane fraction. The $K_m$ for cytidine triphosphate was 0.16 mmole/liter and the apparent $K_m$ for phosphatidic acid was 6.2 mmoles/liter. The pH optimum was 7.0 and the most effective buffers were triethanolamine-HCl and Tris-HCl. The reaction was dependent on the presence of divalent cations, magnesium being the most effective of those investigated. Monovalent cations did not alter the reaction rate. Evidence is presented that the cytidine diphosphate diglyceride produced can serve as a precursor for the synthesis of phosphatidylinositol. No difference was found in the enzymatic activity in platelets from normal subjects and from patients with diseases known to interfere with platelet thromboplastic function.

## INTRODUCTION

Previous studies have shown that human platelets incorporate radioactive phosphate into phospholipids both *in vivo* and *in vitro* (1–5). In the *in vitro* studies, phosphatidic acid and phosphatidylinositol were labeled more rapidly than other phosphatides, suggesting that these compounds were synthesized rapidly and that they might be responsible for some important metabolic function. For example, the incorporation of radioactive phosphate into phosphatidic acid and phosphatidylinositol is stimulated in certain cell types performing active transport or secretion, and it has been postulated that these phosphatides are linked intimately to this process (6–9).

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Phosphatidic acid is known to be a precursor of cytidine diphosphate diglyceride (CDP-diglyceride), an intermediate in the synthesis of phosphatidyl glycerol and phosphatidylinositol in mammalian tissues as well as a number of phosphatides in bacteria (10–15). However, little is known regarding the metabolic role of phosphatidic acid in platelets, and the present study was undertaken to determine whether or not this compound is an intermediate in platelet phospholipid synthesis. This report describes experiments in which it was found that platelets form CDP-diglyceride from phosphatidic acid. The characteristics of the reaction were determined and the enzymatic activity was studied in platelets from patients with diseases in which platelet function may be impaired.

## METHODS

*Materials.* Disodium adenosine triphosphate ($Na_2ATP$), disodium cytidine triphosphate ($Na_2CTP$), cytidine-5′-monophosphomorpholidate, and *Crotalus adamanteus* venom were products of the Sigma Chemical Co., St. Louis, Mo. Myoinositol was obtained from Pfanstiehl Labs., Inc., Waukegan, Ill. Tetralithium cytidine-5′-triphosphate-$^3H$ (1 Ci/mmole) and myoinositol-$^{14}C$ (160 Ci/mole) were purchased from Schwartz Bio Research Inc., Orangeburg, N. Y. and Nuclear-Chicago Corporation, Des Plaines, Ill. Mallinckrodt silicic acid, 100–200 mesh, and aluminum oxide (Merck & Co., Inc., Rahway, N. J.) suitable for chromatographic adsorption were used for column chromatography. The silica gels with and without $CaSO_4$ binder used for thin-layer chromatography were Camag products (Camag, Inc., Milwaukee, Wis.). All solvents were reagent grade. Calcein was obtained from Fisher Scientific Company, Pittsburgh, Pa.

*Platelet separation.* Human platelets were prepared from volumes of 25–100 ml of whole blood by the method of Aster and Jandl (16), and from volumes of 500 ml by the method of Cohen and Gardner using additional acid citrate dextrose solution (17). Specimens containing more than 20 leukocytes or 100 erythrocytes/mm$^3$ were recentrifuged in 50 ml plastic tubes at room temperature for 3 min at 1350 rpm in an International PR-2 centrifuge. The final platelet-rich plasma contained 200,000–500,000 platelets/mm$^3$ as determined by the method of Brecher and Cronkite (18). All

subsequent procedures were carried out at 4°C using plastic or siliconized glass materials. The platelets were sedimented by centrifuging at 2000 *g* for 20 min. They were resuspended and washed twice with 0.15 M NaCl containing $10^{-3}$ M disodium ethylenediaminetetraacetate ($Na_2EDTA$) and $10^{-2}$ M 2-mercaptoethanol, buffered with 0.05 M Tris-maleate pH 6.5 (subsequently referred to as buffered 0.15 M NaCl). The platelet button was resuspended to an estimated concentration of $1.5 \times 10^9$ platelets/ml in 0.25 M sucrose containing $10^{-3}$ M $Na_2EDTA$ and $10^{-2}$ M 2-mercaptoethanol adjusted to a final pH of 7.0 with NaOH (subsequently referred to as buffered 0.25 M sucrose). The suspension was routinely frozen (solid $CO_2$) and thawed six times, and used as the enzyme source.

*Preparation of platelet subcellular fractions.* Washed platelet pellets were suspended in 0.44 M sucrose containing $10^{-3}$ M $Na_2EDTA$ and $10^{-2}$ M 2-mercaptoethanol buffered with 0.02 M Tris-HCl pH 7.0, and were homogenized according to the technique outlined by Marcus, Zucker-Franklin, Safier, and Ullman (19). Granule and membrane fractions were prepared on continuous 30–60% sucrose gradients. Platelet homogenates were also fractionated by sequential centrifugation at 1000 *g* for 20 min, 12,000 *g* for 12 min, and 100,000 *g* for 60 min. All pellets were washed once and then suspended in buffered 0.25 M sucrose. All platelet fractions were assayed immediately or within 24 hr if stored at —20°C.

*Preparation of erythrocyte membranes.* Erythrocytes were separated from heparinized whole blood using siliconized glass-bead columns (20). Cells were sedimented at 375 *g* for 7 min and washed twice with buffered 0.15 M saline. No leukocyte or platelet contamination was detected by phase microscopy. The cells suspended in buffered 0.25 M sucrose were frozen and thawed three times and the membranes collected by centrifugation at 37,000 *g* for 45 min. The pellet was resuspended and washed twice with buffered 0.25 M sucrose. Alternately, membranes were prepared by lysis and washing with hypotonic phosphate buffer (21). The washed pellets were suspended in buffered 0.25 M sucrose. The protein concentration was adjusted to 4 mg/ml, and the suspensions were frozen at —20°C until assayed.

*Separation of leukocytes.* Lymphocytes and polymorphonuclear (PMN) leukocytes were separated by methods minimizing platelet contamination in an effort to assess their contribution to the enzyme activity of the frozen and thawed platelet preparations (22, 23). To prepare lymphocytes, heparinized whole blood was layered on Ficoll gradients and subjected to isopycnic centrifugation (22). The lymphocytes in the supernate were then sedimented by rate-zonal centrifugation in a discontinuous sucrose gradient. The pellet contained 2–5 erythrocytes and 2–4 platelets/100 lymphocytes. No other type of leukocytes were detected on stain smears. PMN leukocytes were separated from heparinized whole blood by isopycnic centrifugation in discontinuous gradients of colloidal silica-polyvinylpyrrolidone (23). The PMN leukocyte band was washed six times with buffered 0.15 M saline. The final pellet contained 2–30 erythrocytes and 2–4 platelets/100 leukocytes. No mononuclear cells were detected. The leukocyte preparations were adjusted to $5 \times 10^7$/ml, and the suspension was frozen and thawed six times. Specimens were frozen at —20°C until assayed.

*Chemical analysis.* Lipid phosphorus, esters, and cytidine were determined by established techniques (24–26). Nitrogen was measured by perchloric acid digestion of the lipid (27) followed by nesslerization (28). Lipid choline was quantitated by hydrolysis with barium hydroxide (29), and precipitation according to the Reineckate method (30). Calcium was measured by titration with $Na_2EDTA$ in the presence of the indicator calcein (31). Lipids were dissolved in cyclohexane and the degree of oxidation determined by ultraviolet absorption at 235 and 255 mμ (32). The quality of aqueous emulsions of lipid was estimated by the degree of light absorption at 420 and 540 mμ using 2–4 mM lipid-buffer emulsions (33). Protein was determined by the method of Lowry, Rosebrough, Farr, and Randall (34).

*Phosphatidylcholine.* Phosphatidylcholine was isolated from hen egg yolks using sequential aluminum oxide and silicic acid column chromatography (35). Thin-layer chromatography showed one spot (36), and the phosphorus: choline molar ratio was 1 : 1.047.

*Phosphatidic acid.* Phosphatidic acid was prepared from phosphatidylcholine by the method of Davidson and Long using Savoy cabbage as the source of phospholipase D (37). The reaction was terminated by adding 0.1 volume of cold 1 N HCl. The mixture was filtered through a bed of celite with suction. The ether phase was separated, washed twice with 0.01 M $Na_2EDTA$, and taken to dryness under reduced pressure in an atmosphere of nitrogen. The lipid was resuspended in chloroform and purified by silicic acid column chromatography (38). The phosphatidic acid eluted with methanol : chloroform (6 : 94 v/v) was taken to dryness as before, and the sodium salt prepared (39). The Tris salt was prepared similarly but it was insoluble in small volumes of ether, so it was suspended in ether and washed with the same organic solvents used to reprecipitate the sodium salt. The phosphatidate salts were dried under reduced pressure and stored at —20°C under nitrogen in a desiccator. These compounds migrated as a single spot on thin-layer chromatography (40). The phosphorus : ester : nitrogen molar ratio of the sodium salt was 1.0 : 0.97 : 0.05. The calcium content of the lipid was less than 0.01 mole/mole of lipid (41). The molar absorption in cyclohexane was 0.893 at 235 mμ and 0.277 at 255 mμ, indicating limited oxidation during the preparatory procedures.

*Lysophosphatidic acid.* Lysophosphatidic acid was prepared by converting phosphatidylcholine to lysophosphatidylcholine with *Crotalus adamanteus* venom (42) followed by treatment with Savoy cabbage phospholipase D in the absence of ether. The reaction was stopped with 0.1 volume of 1 N HCl and the lipids extracted with chloroform : methanol (2 : 1 v/v). The extract was dried as before and the sodium salt prepared. The salt was dissolved in chloroform and purified by silicic acid column chromatography (43). The lipid was reprecipitated as the sodium salt and stored at —20°C. The phosphorus : ester ratio was 1.0 : 0.99. Thin-layer chromatography showed a trace of phosphatidic acid.

*Cytidine diphosphate diglyceride.* CDP-diglyceride was prepared as described by Agranoff and Suomi (26), except final separation was initiated by drying the pyridine mixture under reduced pressure in an atmosphere of nitrogen, resuspending the lipid in chloroform, and washing once with cold $10^{-3}$ N HCl. After drying over $Na_2SO_4$, the extract was purified by silicic acid column chromatography (13). The material eluted with methanol : chloroform (1 : 1 v/v) was taken to dryness as before, dissolved in ether, emulsified in water, and the ether removed under reduced pressure. The pH was raised to 7.5 with 1 N $NH_4OH$ and the water removed under reduced pressure. The precipitate was washed twice with ether and stored at —20°C. This compound had a phosphorus : ester : cytidine molar ratio of 2.0 : 1.85 : 0.93 and migrated as one spot on thin-layer chromatography in two systems (26, 36).

*Preparation of lipid emulsions.* Sodium phosphatide was dissolved in ether and the appropriate buffer added followed by removal of the ether in a vigorous stream of nitrogen. The Tris phosphatidate was added directly to the buffer followed by shaking. The lipid emulsions were cloudy, and light absorption combined with centrifugation was used to estimate the character of the emulsions (33). A typical emulsion of 3.87 mM sodium phosphatidate in 0.25 M Tris-HCl buffer, pH 7.8 gave OD readings of 0.53 and 0.99 at 540 and 420 mμ respectively. After centrifugation at 10,000 *g* for 30 min, the supernate contained 2.88 mM lipid phosphorus and the OD readings were 0.055 and 0.136.

*Enzyme assay.* The procedure employed was similar to that by Carter and Kennedy (40). An assay mixture of 0.5 ml was incubated in a Dubnoff shaker at 37°C in 50 ml glass-stoppered conical centrifuge tubes. The buffer containing a phosphatidate salt, $Na_3ATP$ adjusted to pH 7 with NaOH, and a mixture of $Li_4CTP$-$^3H$ and $Na_2CTP$ adjusted to pH 7.0 with NaOH (final SA 1 Ci/mole) were combined at 4°C. The platelet suspension in buffered 0.25 M sucrose was added and the tubes mixed. The tubes were placed in the incubator at 37°C for 1 min and $MgCl_2$ added with mixing. At the end of the incubation, 5 ml of 0.1 N HCl in methanol was added followed by 10 ml of chloroform. The organic layer was washed three times with 20 ml of 2 M KCl and a chloroform aliquot dried in a scintillator vial. 15 ml of toluene: methoxyethanol (2:1 v/v) containing 2,4 bis[2-(5-*tert*-butylbenzoxazolyl)]thiophene 4 g/l (w/v) was added and the radioactivity determined in a Packard Tri-Carb liquid scintillator counter. An aliquot of the CTP-$^3H$ or inositol-$^{14}C$ mixture was counted and the value used to convert the dpm in the assay vials to a molar value.

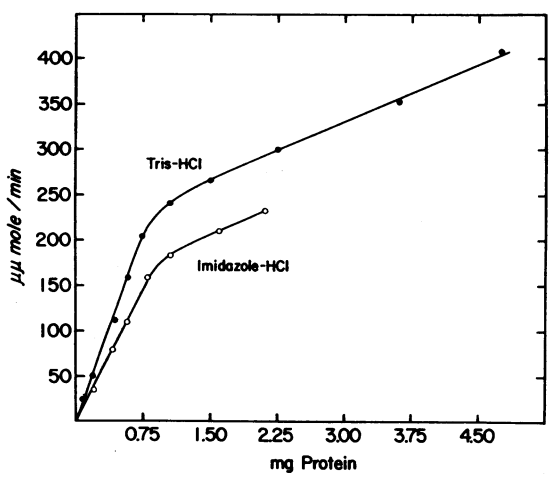


FIGURE 1 The production of cytidine diphosphate (CDP)-diglyceride at varying protein concentrations. The reaction mixture contained protein derived from platelets disrupted by repeated freezing and thawing in the amount indicated, 0.1 M sucrose, 0.4 mM NaEDTA, 4 mM 2-mercaptoethanol, 4 mM Na phosphatidate, 6 mM NaATP, 1 mM CTP-$^3H$ (SA 1 Ci/mole), 0.04 M $MgCl_2$, and 0.1 M Tris-HCl or imidazole-HCl buffer in a total volume of 0.5 ml at a final pH of 7.0. The reaction mixture was incubated at 37°C for 45 min.

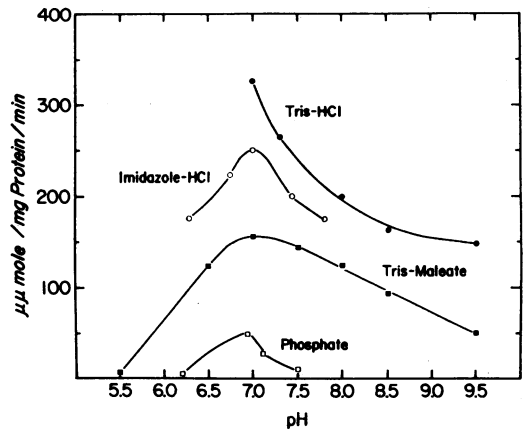


FIGURE 2 Effects of pH and buffer salts on CDP-diglyceride production. The conditions are identical with those described in Fig. 1 except the reaction mixtures contained 0.6 mg of protein. The buffer salt and pH were varied as indicated, but the final buffer salt concentration was always 0.1 mole/liter.

## RESULTS

*Preliminary experiments.* When isolated platelets suspended in buffered 0.25 M sucrose were incubated in the presence of Na phosphatidate emulsified in Tris-HCl buffer pH 7.5, CTP-$^3H$, and $MgCl_2$, radioactivity was found in the chloroform extract indicating the synthesis of a lipid product containing cytidine. Platelets disrupted by repeated freezing and thawing were six times more effective in catalyzing this reaction.

*Identification of product.* Platelets disrupted by repeated freezing and thawing (0.5 mg of protein) were incubated for 60 min at 37°C in a reaction mixture identical with that described in Fig. 1 except the CTP-$^3H$ was 0.2 mmole/liter (SA 80 Ci/mole) and the final pH was 7.5. The reaction was terminated as described in the Methods, and 1 mmole of chemically synthesized CDP-diglyceride was added to the chloroform extract. The chloroform was reduced in volume under a stream of nitrogen and aliquots used for thin-layer chromatography. Thin-layer plates coated with silica gel (slurried in 1 mM $Na_2CO_3$) were developed with chloroform: methanol: acetic acid: water (25:15:4:2 v/v). Plates coated with silica gel containing $CaSO_4$ binder were developed with isobutyl ketone: acetic acid: water (40:30:7 v/v). Lipids were detected by iodine vapor. The portions of the silica gel containing the lipids were separated and the lipids eluted as described by Skipski, Peterson, and Barclay (36). The radioactivity was determined as outlined in Methods. Chemically synthesized CDP-diglyceride had an $R_f$ of 0.48 and 0.32 respectively in the two systems. 96 and 98% of the radioactivity eluted from the thin-layer plates was present in the CDP-diglyceride areas.

TABLE I

*Effect of Omission of Reagents from the Complete Reaction Mixture*

| Omissions from reaction mixture | CDP-diglyceride formed |
|---|---|
| | *μμmoles/min* |
| None | 165 |
| NaATP | 164 |
| NaEDTA | 99 |
| 2-Mercaptoethanol | 145 |
| Phosphatidic acid | 0.2 |
| $MgCl_2$ | 0.8 |
| Enzyme | 0.15 |

The complete reaction mixture was identical with that detailed in Fig. 1 except 0.1 M Tris-HCl buffer was used and the reaction mixtures contained 0.5 mg of protein derived from platelets disrupted by repeated freezing and thawing except where indicated.

*Time course.* The formation of CDP-diglyceride increased in a linear fashion for 120 min in Tris-HCl and imidazole-HCl buffers at pH 7.0.

*Enzyme concentration.* The enzyme activity was proportional to protein concentration up to 0.75 mg/0.5 ml assay volume in either Tris-HCl or imidazole-HCl. At higher protein concentrations, CDP-diglyceride production continued to increase at a slower rate (Fig. 1).

*pH optimum and effect of buffer salts.* The reaction occurred at optimal rates in 0.1 M Tris-HCl (Fig. 2) or 0.1 M triethanolamine-HCl buffer at pH 7.0. These two salts buffered the reaction mixture poorly below pH 7.0, and although smaller amounts of CDP-diglyceride were formed, the results were of uncertain significance and are not illustrated. Increasing the concentration of Tris-HCl to 0.2 and 0.4 mole/liter caused an 18% and 25% decrease in the reaction rate. In imidazole-HCl, the optimal activity occurred at pH 7.0, but the rate was only 80% that achieved in Tris-HCl (Fig. 2). 0.1 M maleate and 0.1 M phosphate were inhibitory to the reaction when used as buffers alone and with Tris. The pH optima in Tris-maleate (Fig. 2) and Tris-phosphate buffers (not shown) were similar to the pH optimum of imidazole-HCl. Phosphate buffer was inhibitory at a concentration as low as 0.04 mole/liter which resulted in a reaction rate only 24% of that found with Tris-HCl.

*Substrates and cofactors.* Table I shows the effect of omission of some of the reagents from the complete reaction mixture. 4 mM 2-mercaptoethanol had no effect alone, but in the presence of $Na_2EDTA$ is increased activity by about 13%. 6 mM ATP did not change the activity of the enzyme but was routinely used. No significant CDP-diglyceride production occurred unless a divalent cation was added. Magnesium as the chloride or sulfate salt was the most effective divalent cation investigated. The optimal concentration was 0.04 mole/liter. There were 7 and 29% losses in activity at Mg concentrations of 0.08 and 0.16 mole/liter respectively. Addition of $MgCl_2$ to the lipid emulsions before adding the enzyme caused precipitation of the lipid and a 90% decrease in product formation. $MnCl_2$ could replace $MgCl_2$. The optimal concentration of $MnCl_2$ was 0.08 mole/liter, but this was only 31% as effective as 0.04 M $MgCl_2$. Calcium, zinc, and copper salts did not activate the enzyme. In the absence of $Na_2EDTA$, 5 mM $CaCl_2$ caused a 40% fall in the reaction rate measured in the presence of 0.04 M $MgCl_2$.

$Na_2EDTA$ increased the reaction rate (Table I). The optimal concentration of $Na_2EDTA$ was 1 mmole/liter. Concentrations of 5 mmoles/liter or higher caused a complete inhibition of CDP-diglyceride production.

No significant amount of CDP-diglyceride was produced in the absence of added phosphatidic acid (Table I). The concentration of phosphatidic acid producing a half-maximal velocity under the conditions shown was 1.4 mmoles/liter (Fig. 3). Determination of the $K_m$ by a Lineweaver-Burk plot of the reciprocal of velocity against the reciprocal of substrate concentration gave an approximate value of 6.2 mmoles/liter. Differences in these values have been noted in other reactions involving lipid emulsions (44), although no clear explanation of this discrepancy has been presented. Emulsions of Tris or sodium phosphatidate served equally well as a substrate. When lysophosphatidate was used as the lipid substrate no radioactivity appeared in the chloroform extract, indicating that the reaction

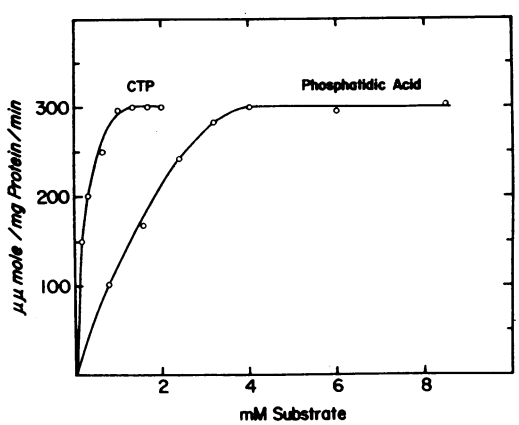


FIGURE 3 The production of CDP-diglyceride at varying concentrations of CTP and Na phosphatidate. The conditions are identical with those detailed in Fig. 1 except 0.1 M Tris-HCl buffer was used, and the reaction mixtures contained 0.6 mg of protein. In one experiment, 1 mM CTP-$^3$H was used and Na phosphatidate concentration was varied as indicated. In the other experiment, 4 mM Na phosphatidate was used, and the CTP-$^3$H concentration was varied as indicated.

TABLE II

*Effect of Temperature on the Rate of CDP-Diglyceride Formation*

| Temperature of incubation | CDP-diglyceride produced |
|---|---|
| | *μμmoles/min* |
| 4°C | 8.3 |
| 25°C | 54 |
| 37°C | 160 |
| 55°C | 210 |
| 65°C | 64 |

The conditions of the experiment were identical with those detailed in Fig. 1 except 0.1 M Tris-HCl buffer, pH 7.0, was used and the reaction mixtures contained 0.6 mg of protein at the temperature indicated.

rates described are related only to the substrate phosphatidic acid. The $K_m$ for CTP was 0.16 mmole/liter (Fig. 3).

*Temperature effects.* The production of CDP-diglyceride in Tris-HCl buffer pH 7.0 was investigated at different temperatures and the rate of reaction compared with that obtained at 37°C (Table II). Enzyme which was boiled 5 min and assayed at 37°C gave values only 1–1.5% of those found with the same enzyme assayed before boiling. Platelets disrupted by repeated freezing and thawing and stored at −20°C lost no activity over 30 days. At 4°C there was a 55% loss of activity in 7 days.

*Miscellaneous observations.* 0.5 mM pyrophosphate caused a 64% decrease in the reaction rate. 0.016 mM CDP-diglyceride decreased enzyme activity by 25% and 0.116 mmole/liter caused a 60% decrease.

0.04 M KCl, 0.04 M $NH_4Cl$, or 0.1 M NaCl did not change the reaction rate in either Tris-HCl or phosphate buffer. 1 mM KF had no effect on the reaction rate but 4 mM or higher concentrations were inhibitory.

When platelet homogenates were precipitated with acetone or methanol at −20°C and resuspended in buffered 0.25 M sucrose, no enzyme activity was present. Attempts to solubilize the enzyme with organic solvents, detergents, and urea were unsuccessful and resulted in enzyme inactivation.

Detergents depressed the reaction rate. CDP-diglyceride production was 28, 0, 23, and 51% as high as control values, respectively, in the presence of 3.2 mM sodium cholate, 1 mg/ml Cutscum (isooctylphenoxy polyoxyethylene ethanol), 0.5 mg/ml Triton X-100 (octylphenoxy polyethoxyethanol), and 0.1 mg/ml Tween 80 (polyoxyethylene sorbitan monooleate).

*Enzyme activity in platelet subcellular fractions.* Platelets were homogenized and the subcellular components separated as described in Method. Platelet material which sedimented between 1000 and 12,000 *g* had a specific SA 2.8 times greater than material sedimented at 1000 *g*. The component sedimented between 12,000 and 100,000 *g* had a SA 3.9 times greater than the 1000 *g* sediment. The supernate from the 100,000 *g* centrifugation was inactive and did not contain inhibitors when mixed with the other fractions. When platelets were sonicated for time intervals less than 10 sec, more enzyme activity and protein remained in the supernate after centrifugation at 2000 *g* compared with the other methods of platelet disruption. Sonication did not solubilize the enzyme and the use of high energy levels or longer time intervals caused loss of enzyme activity.

When platelet "membranes" and "granules" were separated on sucrose density gradients (19), the membranes contained four times the activity of the granules.

*Enzyme activity in other blood cells.* Lymphocytes or PMN leukocytes, disrupted by repeated freezing and thawing, contained an enzyme which catalyzed the conversion of CTP-$^3$H into a chloroform-soluble product. The reaction rate for lymphocytes was 203 μμmoles/mg of protein per min or 1005 μμmoles/$10^8$ cells per min. Values for PMN leukocytes were 120 μμmoles/mg of protein per min or 600 μμmoles/$10^8$ cells per min. Erythrocyte membranes did not catalyze the conversion of CTP-$^3$H into a chloroform-soluble product. These results indicate that 1% or less of the activity which was demonstrated in the platelet preparations could be attributed to contamination by other cell types.

*Enzyme activity in the platelets from normal subjects and patients.* Platelets from 10 normal subjects disrupted by repeated freezing and thawing (protein concentration 2.2–3.0 mg/ml) catalyzed the production of 261–377 μμmoles of CDP diglyceride/mg of protein per min. The values for eight uremic patients (BUN > 100 mg/100 ml) ranged from 255 to 388 μμmoles/mg of protein per min. Platelets from two patients with polycythemia vera, two patients with Marfan's syndrome, and one patient each with chronic myelogenous leukemia, Glanzmann's thrombasthenia, and homocystinuria gave enzyme activities in the normal range.

*Influence of CDP-diglyceride production on the incorporation of inositol into platelet lipids.* CDP-diglyceride may be converted to phosphatidylinositol by an enzyme from mammalian tissues, and in order to determine whether this occurred in the platelet system, an experiment was performed to compare the rate of incorporation of inositol-$^{14}$C and CTP-$^3$H into chloroform-soluble products. Platelets disrupted by repeated freezing and thawing (0.5 mg of protein) were incubated with 4 mM Na phosphatidate, 2 mM inositol-$^{14}$C (SA 28 mCi/mole), 1 mM CTP-$^3$H (SA 1 Ci/mole), 0.04 M $MgCl_2$, 0.4 mM NaEDTA, 4 mM 2-mercaptoethanol, 0.1 M sucrose, and 0.1 M Tris-HCl buffer in a total volume of 0.5 ml at a final pH of 7.5. The reaction mixtures

were incubated for varying time intervals up to 120 min and then extracted and counted as described in Methods. The incorporation of cytidine was 20–40% greater than the incorporation of inositol for the first 60 min. Thereafter, inositol incorporation exceeded the cytidine incorporation by 8–10%. Reaction mixtures from which either CTP or phosphatidic acid was omitted converted no inositol-$^{14}C$ to a chloroform-soluble product. These results indicate that the synthesis of phosphoinositols in this system was dependent on CDP-diglyceride production.

## DISCUSSION

These studies have demonstrated that human platelets have the enzymatic apparatus necessary to convert phosphatidic acid to CDP-diglyceride, a well-established intermediate in the biosynthesis of phosphatidylinositol and phosphatidyl glycerol in mammalian tissues (10–13). CDP-diglyceride is also an intermediate in the synthesis of other phospholipids in microorganisms and possibly could fulfill that role in platelets as well, but to data there is no evidence for this in mammalian tissues (14, 15). The characteristics of the enzyme (CTP: diacyl glycerophosphate–cytidyl transferase) prepared from platelets were similar to those from other mammalian sources (40, 45). The reaction proceeded optimally if Mg was present in the reaction mixture, although Mn could substitute partially, as has been reported for liver (40). In embryonic chick brain CDP-diglyceride formation occurred with Mn, and Mg in the presence of Mn inhibited the enzyme (45). The inhibition of the platelet enzyme by maleate or Tris-maleate buffers is of interest in view of the use of this latter buffer system in studies of the brain enzyme (45). The activity of the platelet enzyme per milligram of protein was 25–33% that described for liver microsomes and 3–4 times that found in embryonic chick brain mitochondria. The activity was a small fraction (0.2%) of that found with *Micrococcus cerificans* (46). Further, it differed from the bacterial and yeast systems in not being activated by univalent cations (46, 47), and it was inactivated by precipitation with organic solvents at low temperature and by the detergent Cutscum (46).

The phosphatidic acid used in these experiments was derived from egg phosphatidylcholine and therefore does not necessarily represent the "natural" substrate for the enzyme. The fatty acids of egg phosphatidylcholine are highly unsaturated (48, 49) but contain only 2–5% arachidonic acid. Marcus, Ullman, and Safier (50) have recently reported on the fatty acid composition of platelet phospholipids and approximately 42% of the molecular weight of phosphatidylinositol was composed of arachidonic acid. Phosphatidic acid with arachidonic acid esterified in the beta position may be the preferred substrate for the enzyme.

These studies extend those previously reported in which it was found that $^{32}PO_4$ is incorporated rapidly into phosphatidic acid and phosphoinositide in platelets in vitro (1). The present results demonstrate that platelets can convert phosphatidic acid to phosphoinositide with CDP-diglyceride as an intermediate, and thus support the possibility that the incorporation of $^{32}P$ into phosphoinositide in the previous studies represented synthesis of new phosphoinositide at least in part. Details of the conversion of CDP-diglyceride to phosphatidylinositol will be presented in a forthcoming report.[1]

These studies do not bear on the origin of phospholipids in platelets, since the initial substrate is phosphatidic acid. Majerus, Smith, and Clamon (51) have demonstrated that platelets have a complete fatty acid synthesizing system, and more recently Lewis, Kilburn, and Majerus (52) have reported that platelets can synthesize phospholipids *de novo* from labeled α-glycerophosphate. Platelets are known to exchange phospholipids with plasma as well, and the relative contribution of these two sources remains to be evaluated.

The role of the present enzyme system in the platelet is not clear. Phospholipids of platelets are essential reactants in blood coagulation, but must also subserve important functions in the maintenance of the cell. Abnormalities of lipid synthesis might influence any of these functions. In the small group of patients with diseases known to influence platelet function in blood coagulation (53–55), the enzyme was present in the platelets in normal quantities. Further work will be necessary to determine the significance of this reaction in the normal or diseased platelet.

## ACKNOWLEDGMENTS

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## REFERENCES

1. Firkin, B. G., and W. J. Williams. 1961. The incorporation of radioactive phosphorus into the phospholipids of human leukemic leukocytes and platelets. *J. Clin. Invest.* **40**: 423.
2. Westerman, M. P., and W. N. Jensen. 1962. The *in vitro* incorporation of radiophosphorus into the phosphatides of normal human platelets. *Blood.* **20**: 796.
3. Grossman, C. M., R. Kohn, and R. Koch. 1963. Possible errors in the use of $P^{32}$ orthophosphate for the estimation of platelet life span. *Blood.* **22**: 9.

[1] Lucas, C. T., F. L. Call II, and W. J. Williams. Biosynthesis of phosphatidylinositol in human platelets. In preparation.

4. Grossman, C. M., and R. Kohn. 1965. Enzymatic characteristics of *in vitro* incorporation of $P^{32}$ orthophosphate into human platelet phosphatide. *Thromb. Diath. Haemorrhag.* **13**: 126.
5. Grossman, C. M., and F. Bartoš. 1968. Succinate dependence of *in vitro* incorporation of $^{32}P$-orthophosphate into human platelet phosphatide. *Arch. Biochem. Biophys.* **128**: 231.
6. Hokin, L. E., and M. R. Hokin. 1958. Phosphoinositides and protein secretion in pancreas slices. *J. Biol. Chem.* **233**: 805.
7. Hokin, M. R., L. E. Hokin, M. Saffran, A. V. Schally, and B. U. Zimmermann. 1958. Phospholipids and the secretion of adrenocorticotropin and of corticosteroids. *J. Biol. Chem.* **233**: 811.
8. Hokin, M. R., B. G. Benfey, and L. E. Hokin. 1958. Phospholipids and adrenaline secretion in guinea pig adrenal medulla. *J. Biol. Chem.* **233**: 814.
9. Hokin, M. R., and L. E. Hokin. 1967. The formation and continuous turnover of a fraction of phosphatidic acid on stimulation of NaCl secretion by acetylcholine in the salt gland. *J. Gen. Physiol.* **50**: 793.
10. Agranoff, B. W., R. M. Bradley, and R. O. Brady. 1958. The enzymatic synthesis of inositol phosphatide. *J. Biol. Chem.* **233**: 1077.
11. Paulus, H., and E. P. Kennedy. 1960. The enzymatic synthesis of inositol monophosphatide. *J. Biol. Chem.* **235**: 1303.
12. Kiyasu, J. Y., R. A. Pieringer, H. Paulus, and E. P. Kennedy. 1963. The biosynthesis of phosphatidylglycerol. *J. Biol. Chem.* **238**: 2293.
13. Prottey. C., and J. N. Hawthorne. 1967. The biosynthesis of phosphatidic acid and phosphatidylinositol in mammalian pancreas. *Biochem. J.* **105**: 379.
14. Kanfer, J., and E. P. Kennedy. 1964. Metabolism and function of bacterial lipids. II. Biosynthesis of phospholipids in *Escherichia coli*. *J. Biol. Chem.* **23**: 1720.
15. Stanacev, N. Z., Y. Chang, and E P. Kennedy. 1967. Biosynthesis of cardiolipin in *Escherichia coli*. *J. Biol. Chem.* **242**: 3018.
16. Aster, R. H., and J. H. Jandl. 1964. Platelet sequestration in man. I. Methods. *J. Clin. Invest.* **43**: 843.
17. Cohen, P., and F. H. Gardner. 1966. Platelet preservation. IV. Preservation of human platelet concentrates by controlled slow freezing in a glycerol medium. *N. Engl. J. Med.* **274**: 1400.
18. Brecher, G., and E. P. Cronkite. 1950. Morphology and enumeration of human blood platelets. *J. Appl. Physiol.* **3**: 365.
19. Marcus, A. J., D. Zucker-Franklin, L. B. Safier, and H. L. Ullman. 1966. Studies on human platelet granules and membranes. *J. Clin. Invest.* **45**: 14.
20. Rabinowitz, Y. 1964. Separation of lymphocytes, polymorphonuclear leukocytes and monocytes on glass columns, including tissue culture observations. *Blood.* **23**: 811.
21. Dodge, J. T., C. Mitchell, and D. J. Hanahan. 1963. The preparation and chemical characteristics of hemoglobin-free ghosts of human erythrocytes. *Arch. Biochem.* **100**: 119.
22. Perper, R. J., T. W. Zee, and M. M. Michelson. 1968. Purification of lymphocytes and platelets by gradient centrifugation. *J. Lab. Clin. Med.* **72**: 842.
23. Pertoft, H., O. Bäck, and K. Lindahl-Kiessling. 1968. Separation of various blood cells in colloidal silica-polyvinylpyrrolidone gradients. *Exp. Cell Res.* **50**: 355.
24. Fiske, C. H., and Y. SubbaRow. 1925. The colorimetric determination of phosphorus. *J. Biol. Chem.* **66**: 375.
25. Antonis, A. 1960. The colorimetric determination of ester groups in lipid extracts. *J. Lipid Res.* **1**: 485.
26. Agranoff, B. W., and W. D. Suomi. 1963. Cytidine diphosphate-DL-dipalmitin. *Biochem. Prep.* **10**: 47.
27. Long, C., and D. A. Staples. 1961. Chromatographic separation of brain lipids; cerebroside and sulphatide. *Biochem. J.* **78**: 179.
28. LePage, G. A. 1959. Methods for the analysis of phosphorylated intermediates. *In* Monometric Techniques. W. W. Umbreit, R. H. Burris, and J. F. Stauffer, editors. Burgess Pub. Co., Minneapolis. 274.
29. Entenman, C., A. Taurog, and I. L. Chaikoff. 1944. The determination of choline in phospholipids. *J. Biol. Chem.* **155**: 13.
30. Glick, D. 1944. Concerning the Reineckate method for the determination of choline. *J. Biol. Chem.* **156**: 643.
31. Diehl, H., and J. L. Ellingboe. 1956. Indicator for titration of calcium in presence of magnesium using disodium dihydrogen ethylenediamine tetraacetate. *Anal. Chem.* **28**: 882.
32. Lea, C. H. 1956. Some observations on the preparation and properties of phosphatidylethanolamine. *In* Biochemical Problems of Lipids. G. Popjak and E. LeBreton, editors. Butterworth & Co. (Publishers), London. 81.
33. Mushett, C. W., D. P. J. Goldsmith, and K. L. Kelly. 1954. Studies on lipide anticoagulants I. assays *in vitro*. *J. Biol. Chem.* **211**: 163.
34. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with the folin phenol *J. Biol. Chem.* **193**: 265.
35. Rhodes, D. N., and C. H. Lea. 1957. Phospholipids. IV. On the composition of hen's egg phospholipids. *Biochem. J.* **65**: 526.
36. Skipski, V. P., R. F. Peterson, and M. Barclay. 1964. Quantitative analysis of phospholipids by thin-layer chromatography. *Biochem. J.* **90**: 374.
37. Davidson, F. M., and C. Long. 1958. The structure of the naturally occurring phosphoglycerides. IV. Action of cabbage-leaf phospholipase D on ovolecithin and related substances. *Biochem. J.* **69**: 458.
38. Hübscher, G., and B. Clark. 1960. Metabolism of phospholipids. II. Isolation and properties of phosphatidic acid from mammalian liver. *Biochim. Biophys. Acta.* **41**: 45.
39. Kates, M. 1955. Hydrolysis of lecithin by plant plastid enzymes. *Can. J. Biochem. Physiol.* **33**: 575.
40. Carter, J. R., and E P. Kennedy. 1966. Enzymatic synthesis of cytidine diphosphate diglyceride. *J. Lipid Res.* **7**: 678.
41. Abramson, M. B., R. Katzman, C. E. Wilson, and H. P. Gregor. 1964. Ionic properties of aqueous dispersions of phosphatidic acid. *J. Biol. Chem.* **239**: 4066.
42. Robertson, A. F., and W. E. M. Lands. 1962. Positional specificities in phospholipid synthesis. *Biochemistry.* **1**: 804.
43. Pieringer, R. A., and L. E. Hokin. 1962. Biosynthesis of phosphatidic acid from lysophosphatidic acid and palmityl coenzyme A. *J. Biol. Chem.* **237**: 659.
44. Barden, R. E., and W. W. Cleland. 1969. 1-Acylglycerol 3-phosphate acyltransferase from rat liver. *J. Biol. Chem.* **244**: 3677.
45. Petzold, G. L., and B. W. Agranoff. 1967. The biosynthesis of cytidine diphosphate diglyceride by embryonic chick brain. *J. Biol. Chem.* **242**: 1187.

46. McCaman, R. E., and W. R. Finnerty. 1968. Biosynthesis of cytidine diphosphate-diglyceride by a particulate fraction from *Micrococcus cerificans*. *J. Biol. Chem.* **243**: 5074.
47. Hutchison, H. T., and J. E. Cronan, Jr. 1968. The synthesis of cytidine diphosphate diglyceride by cell-free extracts of yeast. *Biochim. Biophys. Acta.* **164**: 606.
48. Tattrie, N. H., and R. Cyr. 1963. Fatty acid compositions of naturally occuring lysolecithins and lecithins. *Biochim. Biophys. Acta.* **70**: 693.
49. Kai, M., T. Joshita, and M. Saga. 1963. Fatty acid composition of lecithin from beef brain and egg yolk. *J. Biochem.* **54**: 403.
50. Marcus, A. J., H. L. Ullman, and L. B. Safier. 1969. Lipid composition of subcellular particles of human blood platelets. *J. Lipid Res.* **10**: 108.
51. Majerus, P. W., M. B. Smith, and G. H. Clamon. 1969. Lipid metabolism in human platelets. I. Evidence for a complete fatty acid synthesizing system. *J. Clin. Invest.* **48**: 156.
52. Lewis, N., and P. W. Majerus. Lipid metabolism in human platelets. II. *De novo* phospholipid synthesis and the effect of thrombin on the pattern of synthesis. *J. Clin. Invest.* **48**: 2114.
53. Lewis, J. H., M. B. Zucker, and J. H. Ferguson. 1956. Bleeding tendency in uremia. *Blood.* **11**: 1073.
54. McClure, P. D., G. I. C. Ingram, R. S. Stacey, U. H. Glass, and M. O. Matchett. 1966. Platelet function tests in thrombocythaemia and thrombocytosis. *Brit. J. Haematol.* **12**: 478.
55. Caen, J. P., P. A. Castaldi, J. C. Leclerc, S. Inceman, M. H. Larrieu, M. Probst, and J. Bernard. 1966. Congenital bleeding disorders with long bleeding time and normal platelet count. I. Glanzmann's thrombasthenia. *Amer. J. Med.* **41**: 4.