Lysolecithin Metabolism by Human Platelets

By Peter Elsbach, Penelope Pettis, and Aaron Marcus

Human platelets incubated with $^{32}$P-labeled lysolecithin convert this compound to platelet lecithin and the water-soluble product glycerylphosphorylcholine. Lecithin synthesis from lysolecithin by intact platelets has a pH optimum of 9, whereas platelet homogenates show a pH optimum of 7.5 for the same reaction. Conversion of lysolecithin to platelet lecithin takes place by direct acylation of lysolecithin with free fatty acid. Addition of thrombin or ADP to the incubation medium has no effect on lysolecithin acylation or breakdown and does not affect the degradation of $^{14}$C choline-labeled platelet lecithin. Polystyrene particles stimulate lecithin synthesis and lysolecithin breakdown to glycerylphosphorylcholine by intact platelets but have no effect when added to platelet homogenates. By converting lysolecithin to lecithin, platelets add 2% to their lecithin content in 1 hr.

The observation that platelet lipids and/or lipoproteins could serve as platelet substitutes in in vitro coagulation tests has led to extensive studies of platelet lipid composition and distribution.$^{1,2}$ More recently a series of investigations of synthesis and turnover of platelet lipids has been carried out in several laboratories.$^{3-7}$ For example, Lewis and Majerus$^6$ have demonstrated alterations in the incorporation of glycerol into various platelet phospholipids following incubation with bovine thrombin.

The rapidity with which platelets aggregate, maintain hemostasis, and contribute to coagulation suggests that these are phenomena occurring at the level of the plasma membrane. It is therefore possible that circulating lysolecithin, an agent capable of causing membrane lysis, may contribute to early platelet interactions. Indeed, Bolton and associates$^8$ have proposed that circulating lysolecithin may be involved in altering reactivity to adenosine diphosphate (ADP). Any possible effect of lysolecithin on the platelet would be modified by enzymatic activities that convert lysolecithin to membrane lecithin or to the water-soluble, nonmembrane lytic nonreutilizable catabolic product, glycerylphosphorylcholine (GPC).$^9-14$
This report concerns the identification of a number of enzymatic activities involved in the metabolism of lysolecithin by intact and homogenized platelets.

**MATERIALS AND METHODS**

**Platelet Collection**

For each experiment 425 ml of whole blood were obtained from fasting donors with normal platelet counts. Collections were made in plastic bags using either ethylenediaminetetraacetic acid (EDTA) (11 experiments) or "acidified" acid citrate dextrose (ACD) (14 experiments) as anticoagulants. The bag was centrifuged at 800 g for 20 min. (15°C). The platelet-rich plasma was transferred to polypropylene tubes and centrifuged at 1500 g for 20 min (15°C). The platelet buttons were then treated in several ways depending upon experimental requirements. For some studies the platelets were suspended in platelet-poor plasma, and in others in Tris buffer. If homogenization was anticipated the buttons were washed twice in a solution composed of 0.154 M sodium chloride, 0.154 M Tris hydrochloride buffer, pH 7.4, and 0.077 M EDTA in the proportion 90:8:2 by volume and containing 0.005 M glucose. Platelet homogenates were prepared as previously described.

When intact platelets were studied they were counted by phase microscopy just prior to incubation.

**Labeled Substrates**

Lecithin $^{32}$P and lysolecithin $^{32}$P were prepared biosynthetically as described previously. Choline-$^{14}$C was obtained commercially from Nuclear-Chicago Co. (51.8 mCi/mM).

**Incubation Procedure**

Labeled lysolecithin in chloroform:methanol (2:1, v/v) was taken to dryness in a test tube and complexed to bovine serum albumin (Armour fraction V) 20 g/100 ml in phosphate buffer (0.04 M) at pH 7.4, or in Tris HCl buffer at pH 8.5. Each incubation mixture contained $2 \times 10^9$ platelets (intact or homogenized), 0.1 ml of albumin-lysolecithin solution to yield a final albumin concentration of 4 g/100 ml in a total volume of 0.5 ml of Haslam's Tris NaCl solution. Other additions or buffers were as indicated in the text or legends. Incubations were carried out at 37°C in a shaking water bath.

In some experiments bovine thrombin (5 units/ml) or ADP (0.25 μmoles/ml) was added to the incubation mixture in order to evaluate the effects of these agents on the biochemical reactions under investigation.

**Lipid Extraction and Thin-Layer Chromatography (TLC)**

These techniques have been described in previous publications. All results are expressed as nmoles of lecithin or GPC formed per $10^{10}$ platelets per hour and are normalized for a lysolecithin concentration of 0.2 mM. The water soluble product of the reactions was identified as GPC by standard techniques. Protein determinations were carried out by the method of Lowry et al.

**RESULTS**

All experiments were carried out with lysolecithin complexed to bovine serum albumin. The lysolecithin concentrations used in these experiments were within the range encountered in plasma where lysolecithin also occurs in association with albumin.

Figure 1 shows the effect of pH on conversion of $^{32}$P lysolecithin to lecithin and to GPC by intact and homogenized platelets. The incubations were usually
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Fig. 1.—Effect of pH on lecithin and GPC formation from lysolecithin by intact or homogenized platelets. Incubation was carried out for 1 hr as described in Materials and Methods. The lysolecithin concentrations in different experiments ranged from 0.04 to 0.2 mM. Acetate buffer was used in the pH range 5.0 to 6.0, phosphate buffer in the range of 6.0 to 7.5, and Tris HCl in the range 7.5 to 9.0, all in a final concentration of 0.04 M. Adjustments of pH were made by dropwise addition of 1 N NaOH or HCl. To incubation mixtures that contained homogenate, ATP, CoA, and MgCl₂ were added to give a final concentration of 10, 0.2, and 10 mM, respectively. Triangles indicate incubation mixtures not supplemented with ATP, CoA, and MgCl₂. The symbols and the vertical bars indicate the mean and SEM.

carried out in a buffered salt solution containing bovine serum albumin (4%). However, in several experiments the platelets were incubated in plasma with similar results. The findings of both procedures have been combined in Fig. 1. The pH dependence of lecithin synthesis from lysolecithin was different for intact and homogenized platelets (Fig. 1, lower panels). Lecithin synthesis by intact platelets increased with increasing pH and had not reached a plateau at pH 9.0. By contrast, lecithin synthesis by homogenized platelets showed a pH optimum of 7.5. Addition of adenosine triphosphate (ATP) and coenzyme A (CoA) stimulated conversion of lysolecithin to lecithin by homogenates but had a slightly inhibitory effect on intact platelets (not shown).

Degradation of lysolecithin to GPC by both intact and homogenized platelets increased as the pH was raised. No attempt was made to study the metabolism of lysolecithin at pH higher than 9.0 since nonenzymatic breakdown occurs under such conditions.

It was found that thin-layer chromatography of extracts of ³²P lysolecithin in Tris HCl buffer at pH 9.0 showed a higher $R_f$ value for the lysocompound than at lower pH. We therefore considered the possibility of a pH related chromatographic artifact as an explanation for the apparent greater conversion of lysolecithin to lecithin at alkaline pH.

Lecithin formation by intact platelets was therefore also examined in barbital buffer, which does not produce the $R_f$ changes at pH 9.0 seen in Tris HCl.
buffer. Although less radioactive lecithin was formed in barbital than in Tris buffer at all pH's examined, the amount of radioactive material with an $R_f$ of lecithin was still high at pH 9.0. Further, neutralization of the pH of the reaction mixture in Tris buffer at pH 9.0 just prior to extraction, which, in the absence of platelets restored the usual $R_f$ of lysolecithin, did not reduce the radioactivity in the lecithin area on the chromatogram when platelets were present. It should also be pointed out that formation of radioactive lecithin by intact platelets at pH 9.0 increased with time, whereas the $R_f$ change of $^{32}$P lysolecithin at pH 9.0 in Tris buffer alone is instantaneous. These observations render it less likely that the increased conversion of lysolecithin to lecithin at alkaline pH by intact but not by homogenized platelets can simply be ascribed to an artifact. Nevertheless, unless otherwise specified, the experiments reported herein were carried out at physiological pH.

Figure 2 depicts the effect of time on formation of lecithin and GPC from lysolecithin. Since activities varied from experiment to experiment, results are presented as a percentage of the 60-min value. It is apparent that formation of both products leveled off with time but had not reached a plateau at 90 min.

The effect of lysolecithin concentration on lecithin and GPC formation by intact platelets is shown in Fig. 3. The lysolecithin concentrations found in plasma range from 0.2 to 0.5 mM. Below this range lecithin and GPC formation increased linearly with increasing lysolecithin concentrations, but at concentrations above 0.2 mM, usually encountered in plasma, neither lecithin synthesis nor GPC formation showed a further increment.

Figure 4 demonstrates that conversion of lysolecithin to lecithin and GPC increased linearly with increasing numbers of platelets up to approximately $2 \times 10^9$ per incubation mixture.

Table 1 shows the mean values of lecithin synthesis and GPC formation by intact platelets from 30 donors.

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**Fig. 2.** Effect of time on lecithin and GPC formation by intact platelets. The reaction was carried out at pH 7.4 as described in Materials and Methods and in the legend of Fig. 1. Results are given as percentage of the 60-min value.

**Fig. 3.** Effect of lysolecithin concentration on formation of lecithin and GPC. Incubation was carried out for 1 hr at pH 7.4, as described in Materials and Methods.
The unusual finding of lecithin formation by intact cells at a pH as high as 9.0 prompted an examination of the possibility that the conversion of lysolecithin to lecithin might be due to a reaction other than direct acylation (Lands' pathway). In liver, erythrocytes, and leukocytes, the direct conversion of lysolecithin to lecithin can also take place by transfer of a fatty acid moiety of one lysolecithin molecule to another. It has been demonstrated that this reaction in leukocyte homogenates has an acid pH optimum. The two pathways leading from lysolecithin to lecithin can be distinguished by use of a mixture of lysolecithin labeled with 14C-1-palmitate and 32P. Acylation of lysolecithin, according to Lands, requires free fatty acid. Thus, in the presence of unlabeled free fatty acid the ratio of 14C and 32P in the newly formed lecithin should be the same as in the lysolecithin substrate. If the fatty acid of the lecithin formed is derived from lysolecithin, however, the 14C/32P should be twice that of the lysolecithin. In Table 2 it can be seen that the 14C/32P of lecithin and of lysolecithin remains closely similar both at pH 7.0 and at pH 9.0, signifying that unlabeled fatty acid occupies the free-OH group of lysolecithin. This finding strongly suggests that the mechanism of lecithin synthesis is the one described by Lands.

**Effect of Thrombin and ADP**

Figure 5 shows the effect of thrombin on the conversion of medium lysolecithin to lecithin and on the degradation of platelet lecithin previously labeled with 14C choline. The results shown are the average values of two closely similar experiments. It is apparent that thrombin exerts no detectable effect on acylation of lysolecithin nor on degradation of 14C choline-labeled platelet lecithin.

During the 1-hr incubation period no turnover of 14C choline-labeled lecithin was observed. However, it was noted that platelet homogenates demonstrated distinct phospholipase A and lysolecithinase activity as evidenced by the
Table 2.—Incorporation of Radioactivity From Lysolecithin Labeled With Palmitate-1-14C and 32P Into Lecithin by Intact and Homogenized Platelets

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<th>14C/32P in Lecithin</th>
<th>32P in Lysolecithin</th>
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<td>(pH 7.0)</td>
<td>(pH 9.0)</td>
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<tr>
<td>Intact platelets</td>
<td>1.3</td>
<td>1.1</td>
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<tr>
<td>Homogenized platelets</td>
<td>1.2</td>
<td>1.6†</td>
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† The determination of this ratio is less accurate because of low incorporation of labeled lysolecithin by homogenate at this pH.

accumulation of both lysolecithin and GPC during incubation with 32P-labeled lecithin (unpublished observations). Degradation of lysolecithin to GPC was also unaffected by addition of thrombin. Similar results were obtained in several experiments in which the effect of added ADP (0.25 mM) on these parameters was examined.

Effect of Addition of Polystyrene Particles on Metabolism of Lysolecithin

Earlier studies in our laboratory showed that acylation of medium lysolecithin by human and rabbit polymorphonuclear leukocytes is markedly stimulated during phagocytosis of polystyrene latex particles. Platelets are also capable of ingestion of polystyrene particles in vitro. Figure 6 depicts a representative experiment. Both lecithin synthesis from lysolecithin and breakdown of lysolecithin to GPC were stimulated by polystyrene particles. The extent of stimulation is comparable for the two reactions. It must be emphasized that in several experiments less or no stimulation of either lecithin or GPC formation was observed. This variability was not related to the methods of platelet collection or processing, as it occurred irrespective of

Fig. 5.—Effect of thrombin on acylation of lysolecithin and on degradation of lecithin. Lecithin synthesis (determined as described in Materials and Methods.) Degradation of platelet lecithin previously labeled with choline-14C was examined in the absence and presence of thrombin (2.5 U/0.5 ml of each incubation mixture). Total radioactivity in lecithin of platelets in each incubation mixture has been expressed as a percentage of the value at zero time.
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Fig. 6.—Effect of addition of polystyrene particles on lecithin and GPC formation. For experimental conditions, see Materials and Methods and the legend of Fig. 1. Polystyrene particles (1.1 μ diameter) were purchased from Dow Chemical Co. and added as a suspension in saline (0.1 ml representing 3.6 × 10⁹ particles).

the procedure employed. Addition of polystyrene particles had no effect on metabolism of lysolecithin by homogenized platelets over a pH range from 5.0 to 9.0.

DISCUSSION

The results demonstrate that human blood platelets are capable of converting lysolecithin to lecithin and to GPC. Thus, platelets have in common with erythrocytes and leukocytes the ability to metabolize the membrane-lytic agent lysolecithin to either an important membrane constituent or to a non-lytic, water-soluble product. However, there were several differences between the platelet activities and those of erythrocytes and leukocytes. Notable was the marked increase in both lecithin-forming activity and lysolecithinase activity with increasing pH. To our knowledge this phenomenon has not been reported before. It was particularly surprising that the alkaline pH optimum for lecithin synthesis was clearly evident in experiments with intact but not with homogenized platelets.

The Lands pathway has been demonstrated in numerous tissues, and in all instances where a pH optimum was established this was at approximately physiological pH. Since GPC formation by intact platelets also increased with an increase in pH, we considered the possibility that the reaction: 2 lysolecithin → lecithin + GPC might operate under the conditions of these experiments. This reaction has been shown to have an acid pH optimum in rabbit leukocytes.¹²,¹³ The available evidence indicates, however, that lecithin synthesis by intact platelets at pH 9.0 is by acyl transfer, according to Lands. This conclusion is based mainly on experiments with doubly labeled lysolecithin (Table 2) and on the observations in several tissues that GPC does not serve as an acyl acceptor.

Lecithin synthesis by platelets, when expressed per mg protein, is roughly comparable to that by human peripheral blood leukocytes,²⁴ but less than that by rabbit leukocytes.¹⁴ Conversion of lysolecithin to lecithin in 1 hr adds an average of 2% to platelet lecithin. Acylation of circulating lysolecithin can therefore provide a very appreciable source of new platelet lecithin.

The functional significance of this apparently important pathway of syn-
thesis of the platelet's major membrane phospholipid\textsuperscript{2} is not clear. The profound morphological changes in platelets that are brought about in vitro by addition of thrombin or ADP had no detectable effect on acylation of lysol-
lecithin, nor on breakdown of platelet lecithin previously labeled during incubation with choline-\textsuperscript{14}C (Fig. 5).

On the other hand, as previously shown for granulocytes, phagocytosis of polystyrene particles can cause marked stimulation of lecithin formation from lysollecithin by human platelets, at both physiological pH and at pH 9.0. However, unlike leukocytes, the extent of stimulation was variable and was not apparent until after a lag period of 15 min following addition of particles to the platelet suspension. Further, while phagocytizing leukocytes exhibit marked stimulation of lecithin synthesis but no increased breakdown of lysollecithin to GPC, addition of polystyrene particles to platelets results in stimulation of \textit{both} lecithin synthesis and GPC formation (Fig. 6). The relation of enhanced metabolism of lysollecithin to particle ingestion by platelets needs further exploration.

Whatever the role of lysollecithin metabolism may be in platelet physiology, the studies reported indicate that the platelet possesses both degradative and synthetic pathways to convert potentially harmful lysollecithin, whether endog-

\textbf{Acknowledgment}

The skillful assistance of Harris Ullman and Lenore Saifer is gratefully acknowledged.

\textbf{References}

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