Effect of Palmitate on Human Platelet Glycolysis and Aggregation[1] (35050)

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Platelet metabolism undergoes change during functional events, as is well illustrated by the effect of platelet aggregating agents on glucose utilization (1–6). Most metabolic experiments with platelets *in vitro* have involved participation of undefined plasma factors. Karpatkin (1) worked with a plasma-free platelet system in his studies on human platelet glycolysis. There, as in previous studies both with and without plasma in the system (2), glycolysis provided the major energy pathway. Factors which stimulated glycogenolysis or inhibited Krebs cycle still further increased lactate production. These included thrombin and epinephrine in modified Ringer bicarbonate buffer (1), while adenosine diphosphate (ADP) has been found to increase lactate production in bicarbonate buffer, but only in the absence of added glucose.

In the present study of human washed platelets in Krebs–Ringer phosphate buffer without added plasma, thrombin increased lactate production in the presence of added glucose, as did fluoroacetate (explored as a tricarboxylic acid cycle inhibitor). However palmitate increased lactate production to a far greater extent than did either thrombin or fluoroacetate, in the presence of added glucose.

Materials and Methods. As in previous studies (3), venous blood from human subjects, anticoagulated with ethylenediaminetetraacetic acid (EDTA), was processed to yield intact washed platelets by differential centrifugation in a siliconed system at 5°. Mammalian Krebs–Ringer phosphate calcium-free buffer, pH 7.4, was used throughout as before (4), with or without added glucose as noted in tables, and with 0.1 vol of 1% EDTA added to the washing buffer only. Erythrocyte and leukocyte contamination was quantitated and found as before. Water-lysed and sonicated platelets were prepared for some experiments by dividing platelets suspended in washing buffer into two exactly equal aliquots before final centrifuging. One platelet button was resuspended as usual in buffer for use. The second platelet button was either sonicated in buffer (9 KC per sec for 10 min in a Raytheon sonic oscillator) or resuspended in demineralized water, mixed rapidly for lysis, then immediately restored to buffer concentration exactly equal to that of the control suspension. Protein content of the two final aliquots was the same. Comparative platelet counts showed substantial but incomplete reduction in numbers of apparently intact platelets in the lysed samples.

Basic incubation mixtures consisted of 0.5 ml of washed platelet suspension (0.65 to 2.0 mg of platelet protein/ml) in buffer, plus 0.1 ml of triphosphopyridine nucleotide (NADP, 0.5 μmoles) in buffer, 0.1 ml of diphosphopyridine nucleotide (NAD, 1.2 μmole) in buffer, and, where indicated in tables, 5.0 μmoles of glucose. Further additions, where indicated, consisted of fluoroacetate (1.5 μmole in 0.05 ml of buffer), calcium or ammonium chloride (1.5 μmoles in 0.05 ml of buffer), thrombin (Upjohn, 0.45 or 0.9 units in 0.05 or 0.1 ml of buffer), palmitate (0.1 μmole/0.01 ml). Additives were adjusted to approximately pH 7.4 before addition to the buffered basic mixture. Final total volumes were made up to 0.75 ml with buffer. All flasks were run in duplicate for later lactate determinations. Lactic acid was determined as before (4) by enzymatic assay. Endogenous lactate was measured at zero incubation time in each experiment and was

[1] Supported by U.S. Public Health Service Grants HE10625 and AM12441.

deducted from total values in incubation mixtures before tabulation of data.

In mixtures identical to those used in incubation flasks, degree of aggregation was recorded in representative experiments over the first 3 to 14 min of incubation at 37°, using a Chrono-Log "aggregometer" and a Sargent SRL recorder with linear gears. The representative records, shown in Figs. 1 and 2, were consistent and easily reproducible.

Palmitate was prepared by suspending palmitic acid (Calbiochem), 100 μmoles, in 1.5 ml of 0.85% sodium chloride solution which had been brought to pH 9.0 with ammonium hydroxide. After mixing (on a Vortex Jr. Mixer) and grinding of crystals in a microagate mortar, the suspension was returned to a small glass tube, remixed, heated at approximately 60° under a hot-water tap, readjusted with ammonium hydroxide to pH 7.2, and made to 2-ml total volume with saline. This was then diluted 1:5 with buffer. The final suspension was turbid and required reheating under a hot-water tap prior to each pipetting of an aliquot, to achieve a smooth suspension. All other reagents were obtained commercially (Biochemica Boehringer and Sigma Pharmaceutical Corp.).

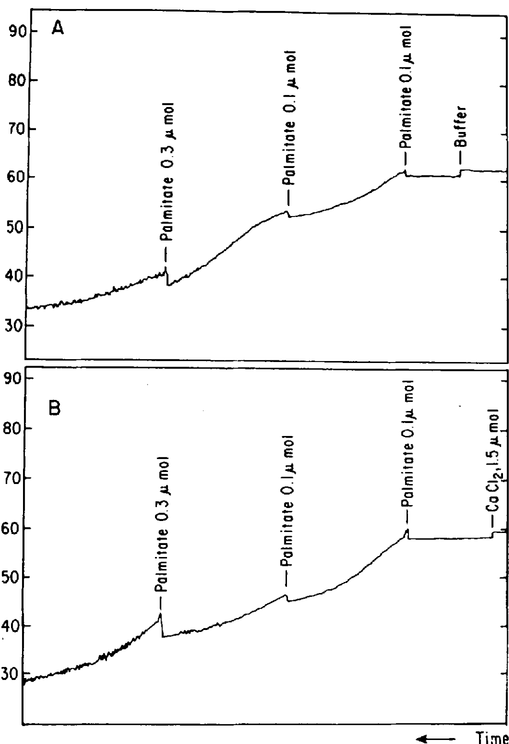

FIG. 1. Effect of palmitate on aggregation of human platelets in Krebs-Ringer phosphate calcium-free buffer at 37°, without (A); and with (B) preaddition of $CaCl_2$ to the mixture. Total incubation span shown is 9 min, 12 sec, recorded from right to left.

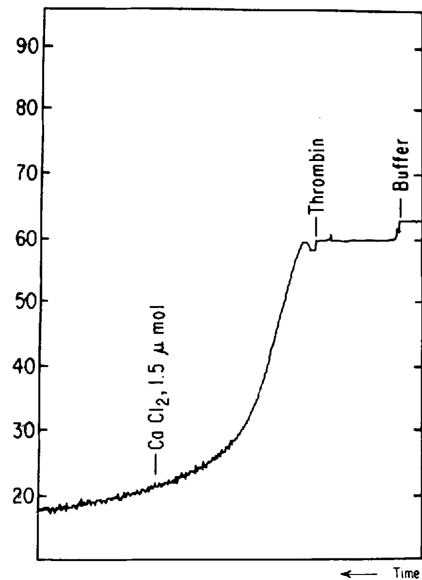

FIG. 2. Aggregation of human platelets (same platelet preparation as for Fig. 1 incubation mixtures) by thrombin at 37°, with later addition of $CaCl_2$ as indicated. Total incubation span shown is 5 min, 30 sec, recorded from right to left.

Results. Table I shows a mild but highly significant increase ($t = 5.87$ and 5.16, respectively; $p < 0.001$) in lactate production on addition of either thrombin or fluoroacetate to human washed platelets in a balanced phosphate buffer mixture containing added glucose. Addition of palmitate resulted in a strikingly greater increase in lactate production than with either thrombin or fluoroacetate. Ammonium ion (present in the palmitate preparation) was without significant effect on lactate production. Calcium chloride slightly increased lactate production but, when added with palmitate, greatly diminished the degree of increased production seen with palmitate alone.

TABLE I. Influence of Various Additives on Micromoles of Lactate Produced per Gram of Platelet Protein by Human Washed Platelets in Mammalian Krebs-Ringer Calcium-Free Phosphate Buffer with Added Glucose at pH 7.4 in 1 hr at 30°.

Addition to basic mixture[a]				
No addition	Fluoroacetate	Thrombin	Ammonium chloride	Calcium chloride
302	384	378	286	
254	327	461	288	
207	267	389		229
250	388	348		348
284	411	334		311
274	338	358		
220	379			
252	432			
271	314			
312	357			

Palmitate added (μmoles/assay)									
None	0.1	0.2	0.3	0.4	0.5	1.0	2.0	3.0	4.0
284	555	803	891	921					
250		830	900	870					
252	710				1019				
271	843				915				
312	742				854				
274	570[b]				792[c]				
302					808	808			
254					831	848			
207						406	548	593	602
220							935		

[a] See Methods.

[b,c] When calcium chloride and palmitate both were added to the mixture, lactate production declined to 317[b] and 378[c] μmoles/g of platelet protein.

Figure 1A illustrates degree of aggregation occurring in identical platelet mixtures at 37° on addition of palmitate and of thrombin. The increasing degree of aggregation with increasing amounts of added palmitate is evident. Aggregation on addition of palmitate was essentially unchanged by preaddition of calcium chloride which by itself had no demonstrable aggregating effect on washed platelets (Fig. 1B). Thrombin added alone produced precipitous change as expected, as shown in Fig. 2, and this was unchanged by later addition of calcium chloride. No aggregation could be recorded on addition of fluoroacetate or ammonium chloride. Prior incubation with fluoroacetate had no effect on subsequent palmitate aggregation. There was no parallelism between degree of aggregation and alteration of lactate production.

In Table II, base line lactate production by human washed platelets in buffer with no added glucose is seen to approach that achieved in the experiments recorded in Table I where glucose was added to the buffer. However, in the absence of added glucose, the very high levels of lactate previously produced on addition of palmitate to the basic mixture were not seen, although lactate production was increased slightly by addition of palmitate. As before, the increase was somewhat suppressed by preaddition of calcium chloride.

Table III reveals persistence of lactate production in water-lysed platelet mixtures, with some increased production on addition of palmitate. Water-lysed platelets retained

TABLE II. Effect of Omission of Added Glucose on Lactate Production by Human Washed Platelets in the Presence of Various Additives.[a]

Platelets in buffer with	Expt.:	Lactate (μmoles/g of platelet protein) 1	2	3
No addition		210	208	243
Thrombin (0.9 U)		228	285	307
Fluoroacetate		242	306	293
Calcium chloride ($CaCl_2$)		214	246	255
Palmitate (0.1 μmole)		254	289	371
+ $CaCl_2$		195	253	285
Palmitate (0.5 μmole)		284	400	436
+ $CaCl_2$		204	247	314

[a] See Methods. Each vertical column of data represents a separate experiment.

ability to produce lactic acid to at least the same extent that platelets which appeared intact on phase microscopy remained in the lysed suspension. In two experiments, *e.g.*, reductions in platelet count following hypoosmosis were 65 and 51%, respectively, while reductions in lactate production were 36 and 51% respectively. Sonication in contrast, eliminated significant lactate production.

Discussion. Platelet aggregating agents have marked effects on glucose utilization by platelets (1–6). Karpatkin (1) has speculated that activation of the glycolytic chain and associated expenditure of ATP under the influence of agglutinating and contracting agents may be the result of cellular reorganization inherent in platelet agglutination and contraction rather than activation of specific enzymes. Troup *et al.* (7) reviewed platelet lipids and their essentiality to function. Okuma *et al.* (8) have reemphasized this recently in their investigation of lipid peroxides in platelets. The present study has shown a degree of increase in lactate production in the presence of palmitate which exceeded by far that seen with thrombin. The effect required the presence of added glucose. Karpatkin (1) has shown the importance of enhanced glucose uptake by the platelet in increased lactate production. It is possible that the striking increase in lactate production in the presence of palmitate simply reflects alteration of normal glucose uptake secondary to damage of the cell membrane. On the other hand, deliberate very brief hypoosmotic exposure of platelets did not result in increased lactate production nor in abolition of palmitate effect. Rather the remaining palmitate effect was very roughly coincident with the number of grossly intact platelets remaining in the preparation. Another possibility could be that palmitate competes with glucose-derived pyruvate in furnishing acetyl-CoA to the tricarboxylic acid cycle, thereby shunting more pyruvate to lactate. Still another interesting possible explanation of the striking increase in lactate production with added palmitate, however, may be along lines of Karpatkin's speculations (1), in that the palmitate may present moieties to the cell which are important in attempts at cellular reorganization. Rosenzweig and Ways (9) have emphasized the ability to platelets to incorporate and oxidize long-

TABLE III. Effect of Water-Lysis and of Sonication of Platelets on Micromoles of Lactate Produced per Gram of Platelet Protein in Mammalian Krebs–Ringer Calcium-Free Phosphate Buffer with Added Glucose 1 hr at 30°.

Expt.:	4		5		6	
Platelets[a]	Int.	W-L	Int.	W-L	Int.	Son.
No addition	252	162	271	132	312	6
Fluoroacetate	432	213	314	166	357	20
Palmitate (0.1 μmole)	710	277	843	347	742	24
(0.5 μmole)	1019	315	915	360	854	20

[a] See Methods; Int. = intact platelets; W-L = water-lysed platelets; Son. = sonicated platelets; each pair of vertical columns of data represents a separate experiment.

chain fatty acids, supplied either as salt in buffer or bound to albumin. Whatever the mechanisms may be, it is clear that addition of palmitate under the conditions of the present *in vitro* experiments did result in marked augmentation of activity of the principal energy pathway in human platelets, namely glycolysis.

Summary. Washed human platelets in balanced phosphate buffer mixture showed markedly increased lactate production in the presence of added palmitate and glucose. The degree of augmentation far exceeded that seen with added thrombin, although the aggregative effect of thrombin far exceeded that of palmitate in the amounts used. The palmitate effect was striking only when glucose also was added to the mixture.

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Received May 25, 1970. P.S.E.B.M., 1970, Vol. 135.