The Influence of Buffers on Human Platelet Aggregation and Energy Metabolism[1] (35286)

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The metabolism of human platelets in various artificial media is known to vary. Rossi (1) has explored the effect of various buffers, including hyperphysiologic concentrations of phosphates, on the metabolism of rat platelets. Further understanding of the effect of physiologic buffers on the energy metabolism of human platelets is important not only from the point of view of survival of stored platelets but in achieving further understanding of platelet mechanisms in functional events where energy is required.

The present study showed striking comparative differences in human platelet metabolism and aggregative function when washed platelets were incubated in physiologic Krebs–Ringer-bicarbonate (KRC) and Krebs–Ringer–phosphate (KRP) buffers, respectively. Lactate production was significantly greater in KRC buffer than in KRP buffer. The reverse was true of $^{14}CO_2$ production from pyruvate-2-^{14}C, significantly more $^{14}CO_2$ being evolved in KRP than in KRC buffer. In the presence of added adenosine diphosphate (ADP) or of palmitate, degree of aggregation of platelets in KRC buffer exceeded that in KRP buffer.

Earlier studies (2) demonstrated suppression of lactate production by human blood platelets in KRC buffer when exogenous adenosine triphosphate (ATP) was added. The present study showed this effect to be due in part to contribution of phosphate to the system. An additional and as yet undefined effect was operative also. In contrast, augmentation of $^{14}CO_2$ production from pyruvate-2-^{14}C when exogenous ATP was added to washed human platelets was fully attributable in the absence of added glucose to the addition of phosphate moiety.

Other earlier studies (3) showed striking enhancement of lactate production in phosphate buffer mixture when palmitate was added to human washed platelets. The present study showed that this effect was at least in considerable part phosphate-dependent and due to competition with pyruvate in furnishing acetyl CoA to the tricarboxylic acid (TCA) cycle.

Materials and Methods. Venous blood from human subjects, anticoagulated with ethylenediaminetetraacetic acid (EDTA) was processed as in earlier studies (2) to yield intact washed platelets by differential centrifugation in a siliconed system at 5°. Washing buffers as described earlier (4) conformed to the final buffer of the incubation mixture. EDTA was omitted from the final suspending buffer, as before. Erythrocyte and leukocyte contamination was quantitated and found as before (2).

Basic incubation mixtures were in Krebs–Ringer–phosphate (KRP) or Krebs–Ringer–bicarbonate (KRC) buffer (calcium-free, pH 7.4), as specified in Tables I and II. Each contained, in final 0.82-ml volume, 1.2 μmoles of added diphosphopyridine nucleotide (NAD), 0.5 μmole of triphosphopyridine nucleotide (NADP), 0.013 μmole of pyruvate-2-^{14}C, and 2.0 μmoles of either unlabeled glucose or unlabeled pyruvate. Further additions noted in Tables I and II consisted of final concentrations of 3×10^{-3} M adenosine triphosphate (ATP), 3×10^{-5} M adenosine diphosphate (ADP), thrombin (0.56 U/ml), or palmitate (0.61 μmole/ml). Palmitate was prepared for use as described in detail earlier (3). All flasks were run in duplicate, incubating in a Dubnoff metabolic shaker water bath at 30° for 1 hr before the reaction in each flask was stopped by addition of acid (4).

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$^{14}CO_2$ production was determined as before (4), and lactic acid assays were done as before (3) by enzymatic method. As in (3), degree of platelet aggregation was recorded in representative experiments at 37°, using a Chrono-Log "aggregometer" and a Sargent SRL recorder with linear gears.

Results. Table I shows that lactate production by human platelets was significantly greater in basic incubation mixtures in physiologic KRC buffer than in KRP buffer, whether with glucose or pyruvate substrate. As in earlier studies (2), addition of ATP led to marked decrease in lactate production by platelets from either substrate and in either buffer, and production in KRC buffer then no longer exceeded that in KRP buffer. Addition of ADP was without significant effect on lactate production. Addition of thrombin was without effect on lactate production by platelets incubated in KRC buffer but significantly increased lactate production in KRP buffer over the level noted without added thrombin. Addition of palmitate resulted as in previous studies (3) in markedly increased lactate production by platelets incubated in KRP buffer but did not significantly increase lactate production in KRC buffer.

Table II demonstrates an effect of test conditions on production of $^{14}CO_2$ from pyruvate-2-^{14}C which is the reverse of that shown in Table I on lactate production. Production of labeled CO_2 was significantly greater in basic incubation mixtures in KRP buffer than in KRC buffer. When ATP was added, the difference attributable to buffer was absent. Production of $^{14}CO_2$ from pyruvate-2-^{14}C was markedly increased by ATP in both buffers when glucose was added and in KRC buffer but not KRP buffer when only pyruvate was added.[2] Addition of ADP or thrombin did not significantly affect $^{14}CO_2$ production from pyruvate-2-^{14}C by platelets incubated in either buffer. Addition of palmitate very significantly diminished $^{14}CO_2$ production from pyruvate-2-^{14}C when platelets were incubated in KRP buffer. This did not occur to a significant degree in KRC buffer.

Figure 1A and B compare degree of aggregation occurring at 37° on addition of ADP to platelet mixtures which were identical other than for the physiologic buffer used. Each had a flat base line recorded (not shown) for 11 min prior to addition of ADP. Degree of aggregation was consistently greater in KRC than in KRP mixtures. Palmitate produced a similar effect when added to washed platelet mixtures in KRC (Fig. 2A) and KRP (Fig. 2B) buffer. Degree of aggregation in KRC buffer again consistently exceeded that in KRP buffer. Such an effect was not striking when thrombin was added to KRC and KRP buffer mixtures after an 11-min base line incubation of platelets at 37°. As shown in Fig. 3A and B, a precipitous degree of aggregation of platelets occurred in both buffer mixtures on addition of thrombin.

Discussion. The differences observed here in lactate production and in evolution of $^{14}CO_2$ from pyruvate-2^{14}C by human platelets incubated in different physiologic buffers emphasize the importance of selection of buffer in studying energy pathways of these cytoplasmic fragments *in vitro*. The presence of inorganic phosphate markedly decreased lactate production and increased use of the TCA cycle (as reflected in increased production of CO_2 from pyruvate). Furthermore the previously observed effect of exogenous ATP on these parameters (2) was seen to be due in large part to addition of phosphate moiety to the system. The observed effect of added ATP was not entirely explicable on this basis, however. Thus presumably an additional effect beyond addition of phosphate moiety was operable also. Possible explanations have been discussed previously (2).

Thrombin did not alter the metabolic parameters studied during 1 hr of incubation of platelets at 30° in bicarbonate buffer. However, it eliminated the usual depressing effect of phosphate buffer on lactate production when added prior to incubation of plate-

[2] As noted briefly in Table II footnote, the apparent excess of $^{14}CO_2$ produced when unlabeled pyruvate was added over that recorded when unlabeled glucose was added simply reflects dilution of the ^{14}C label by unlabeled pyruvate derived from the glucose. This unlabeled pyruvate could not be taken into account in subsequent calculations, in contrast to the situation where a known quantity of unlabeled pyruvate was added.

TABLE I. Effect of Mammalian Krebs–Ringer–Bicarbonate (KRC) and –Phosphate (KRP) Buffers and of Various Additions on Lactate Produced (μmoles/g of platelet protein in 1 hr at 30°) in the Presence of Added (2 μmoles/assay) Glucose or Pyruvate.

Platelets plus:	No additive (17–20)[a]	ATP (3×10^{-3} *M*) (5–7)	ADP (3×10^{-5} *M*) (3–5)	Thrombin (0.56 U/ml) (4–7)	Palmitate (0.625 μmole/ml) (3)
In KRC buffer:					
With glucose	647	*142*	608	573	855
SD	±137	±52	±88	±37	±157
p[b]		<.001	>0.5	<0.2	>0.1
With pyruvate	323	*114*	398	413	402
SD	±79	±34	±35	±60	±102
p[b]		<.001	>0.1	<.025	>0.1
In KRP buffer:					
With glucose	*416*	*159*	518	*568*	*1245*
SD	±99	±38	±91	±34	±393
p[c]	<.001[b]	<.001	>0.1	<.001	<.001
With pyruvate	*250*	*130*	327	*370*	*542*
SD	±62	±20	±54	±59	±190
p[c]	<.01[b]	<.001	<.025	<.005	<.001

[a] Number of experiments.
[b] Compared to corresponding KRC buffer mixture with no additives.
[c] Compared to corresponding KRP buffer mixture with no additive, except as noted.[b]

TABLE II. Effect of Mammalian Krebs–Ringer–Bicarbonate (KRC) and –Phosphate (KRP) Buffers and of Various Additives on $^{14}CO_2$ Produced (μmoles/g of platelet protein in 1 hr at 30°) from Pyruvate-2-^{14}C (13.5 mμmoles/assay) in the Presence of Added Excess (2 μmoles/assay) of Glucose or Pyruvate.

Platelets plus:	No additive (17–18)[a]	ATP (3×10^{-3} *M*) (5–6)	ADP (3×10^{-5} *M*) (3–5)	Thrombin (0.56 U/ml) (4–6)	Palmitate (0.625 μmole/ml) (3)
In KRC buffer:					
With glucose[b]	0.094	*1.851*	0.111	0.166	0.073
SD	±.067	±.469	±.019	±.045	±.038
p[c]		<.001	>0.5	<.025	>0.5
With pyruvate	11.01	*26.27*	12.86	16.63	7.27
SD	±4.56	±7.01	±0.81	±5.27	±4.86
p[c]		<.001	>0.5	<.025	>0.2
In KRP buffer:					
With glucose[b]	*0.470*	*1.405*	0.321	0.264	*0.121*
SD	±.173	±.429	±.159	±.056	±.039
p[d]	<.001[c]	<.001	>0.1	<.025	<.005
With pyruvate	*28.01*	24.02	27.52	23.93	*11.08*
SD	±8.69	±5.91	±1.09	±7.80	±5.15
p[d]	<.001[c]	>0.2	>0.5	>0.4	<.005

[a] Number of experiments.
[b] With marked dilution of ^{14}C label resulting from the unlabeled pyruvate formed from the excess of unlabeled glucose added. This did not enter into subsequent calculations.
[c] Compared to corresponding KRC buffer mixture with no additive.
[d] Compared to corresponding KRP buffer mixture with no additive, except as noted.[c]

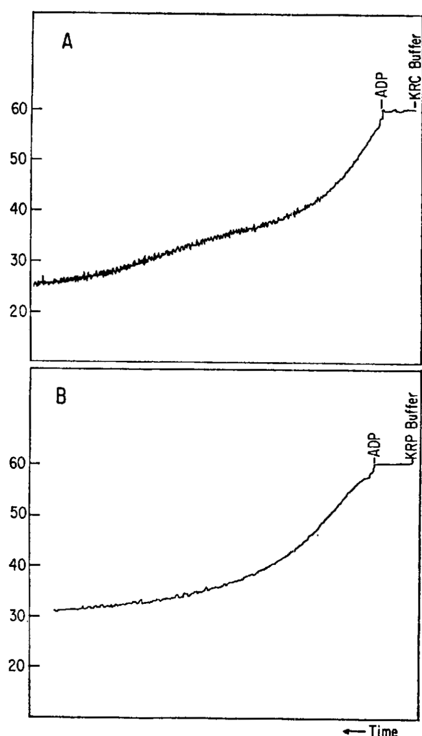

FIG. 1. Aggregation of human platelets by ADP at 37°, in KRC buffer (A) and KRP buffer (B), respectively. Total incubation span shown is 6 min, 56 sec, recorded from right to left.

lets in phosphate buffer.

Although inorganic phosphate was observed to have a depressing effect on lactate production and an augmenting effect on TCA cycle activity, inorganic phosphate appeared to be important in the reverse effect produced on these pathways by added palmitate. Possible explanations of palmitate effect on platelet glycolysis have been discussed previously (3). The present study suggested that competition with pyruvate in furnishing acetyl CoA to the TCA cycle may be of major importance in the observed effect, more pyruvate then being available for conversion to lactate.

The increased degree of aggregation of platelets by ADP or palmitate when added to a bicarbonate buffer incubation mixture (as contrasted to a phosphate buffer mixture) was unexplained but consistent. The difference was not striking when thrombin was used as the aggregating agent. ADP did not alter the metabolic parameters measured in the present studies. However aggregation is known to produce metabolic alterations in platelets (5–7), and marked alteration of the parameters measured did occur when palmitate was added. It would seem probable that selection of buffer for platelet aggregation studies is a matter of some importance.

Summary. Washed human platelets suspended in Krebs–Ringer–bicarbonate and Krebs–Ringer–phosphate buffers, respectively, showed significant differences in lactate production, in $^{14}CO_2$ production from pyruvate-2-^{14}C, and in degree of aggregation by ADP. Degree of aggregation of platelets by ADP or palmitate was greater in bicarbonate than in

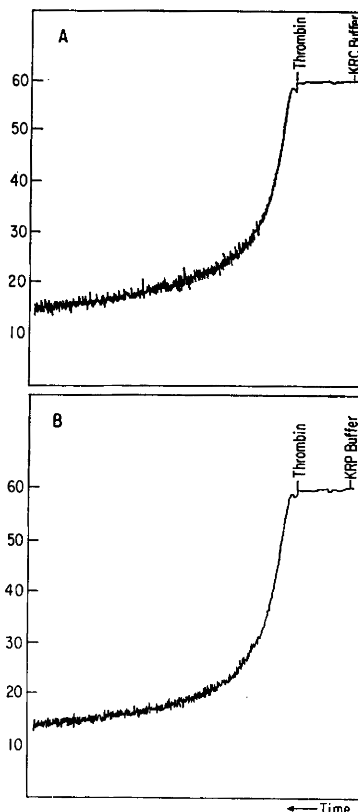

FIG. 2. Aggregation of human platelets (same platelet preparation as for Fig. 1 incubation mixtures) by thrombin at 37°, in KRC buffer (A) and KRP buffer (B), respectively. Total incubation span shown is 6 min, 3 sec, recorded from right to left.

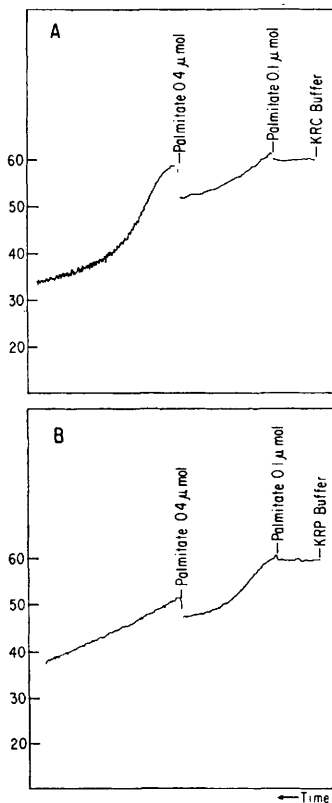

FIG. 3. Aggregation of human platelets (same platelet preparation as for Fig. 1 and 2 incubation mixtures) by palmitate at 37°, in KRC buffer (A) and KRP buffer (B), respectively. Total incubation span shown is 5 min, 53 sec, recorded from right to left.

phosphate buffer, as was production of lactate by platelets from added glucose or pyruvate. The reverse was true of $^{14}CO_2$ production.

The effect of exogenous ATP previously reported (2) was shown to be in large part but not completely due to addition of phosphate moiety.

The effect of added palmitate previously reported (3) was shown to require inorganic phosphate for significance and to be due chiefly to competition with pyruvate in furnishing acetyl CoA to the tricarboxylic acid cycle.

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