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### Research Article

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Thrombin, epinephrine, and ADP resulted in platelet agglutination and contraction and increased platelet glycogenolysis (phosphorylase activity). Epinephrine appeared to be a more potent activator of phosphorylase, resulting in a 70% increase in glucose 6-phosphate levels. Thrombin and epinephrine both increased glucose uptake and lactate production. Glucose uptake decreased in the presence of ADP. Except for incubations with epinephrine, glucose 6-phosphate remained constant under conditions in which lactate flux increased 3-fold and glucose uptake increased 2-fold. Thrombin, epinephrine, and ADP decreased ATP levels in the presence or absence of glucose.

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## Studies on Human Platelet Glycolysis. Effect of Glucose, Cyanide, Insulin, Citrate, and Agglutination and Contraction on Platelet Glycolysis \*

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**Summary.** Evidence for a metabolically active plasma-free platelet system is presented. Glycogenolysis was found to be a potent pathway for lactate production. Aerobic glycolysis constituted a major fraction of glucose metabolized. Both insulin and cyanide increased lactate production in the presence of glucose. The Krebs cycle appeared to be operative for ATP synthesis when citrate was used as substrate. The first stages of gluconeogenesis were noted to be present. Glucose uptake contributed to increased lactate production.

Thrombin, epinephrine, and ADP resulted in platelet agglutination and contraction and increased platelet glycogenolysis (phosphorylase activity). Epinephrine appeared to be a more potent activator of phosphorylase, resulting in a 70% increase in glucose 6-phosphate levels. Thrombin and epinephrine both increased glucose uptake and lactate production. Glucose uptake decreased in the presence of ADP. Except for incubations with epinephrine, glucose 6-phosphate remained constant under conditions in which lactate flux increased 3-fold and glucose uptake increased 2-fold. Thrombin, epinephrine, and ADP decreased ATP levels in the presence or absence of glucose.

### Introduction

Platelets are viable cytoplasmic fragments that circulate in the bloodstream for 8 to 11 days. Their agglutination and contraction are thought to be necessary for plugging of vessel wall injury and attainment of hemostasis. Bounameaux (1) demonstrated that thrombin results in platelet agglutination and "viscous metamorphosis." Sokal (2) reported that viscous metamorphosis produced in platelet-rich plasma is accompanied by platelet contraction. Hellem (3) and Gaarder and his as-

sociates (4) demonstrated that ADP released from red blood cells causes platelet agglutination; concentrations as low as  $1 \times 10^{-7}$  mole per L were effective (5). Mitchell and Sharp discovered that epinephrine, norepinephrine, and serotonin are also effective in initiating platelet agglutination (5). It has not been established whether epinephrine also results in platelet contraction. Bettex-Galland and Lüscher (6) reported the presence of thrombosthenin, a contractile protein with ATPase activity and postulated an association with platelet contraction.

Knowledge of the control of platelet energy (ATP synthesis and expenditure) during platelet agglutination and contraction is important for a proper understanding of the mechanism of hemostasis. The purpose of this series of investigations was to study the regulation of glycolysis in human platelets during basal and functioning conditions. This required metabolically active platelets, sus-

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pended in an enzyme-free medium of known composition. This communication may be divided into two sections: 1) the biochemical evidence for the attainment of metabolically active platelets in a plasma-free system (glycolytic stimulation was initiated with glucose, insulin, citrate, and cyanide), and 2) glycolytic changes under conditions known to result in platelet agglutination and contraction (an *in vitro* system employing thrombin, ADP, and epinephrine was utilized for this purpose).

With enzymatic techniques, lactate production, glucose consumption, adenosine triphosphate, and glucose 6-phosphate levels were measured under these conditions.

### Methods

#### *Preparation of platelets*

Human platelet-rich plasma,<sup>1</sup> collected in acid-citrate-dextrose solution, was obtained within 2 to 3 hours after phlebotomy. It was kept over ice during delivery and handled in containers not made of glass: Fenwal triple pack plastic bags, cellulose nitrate centrifuge tubes, and plastic pipettes. The platelets were separated from their plasma by differential centrifugation in an International centrifuge at 5° C. The platelet-rich plasma was centrifuged at 350 *g* to remove red blood cells. The supernatant platelet suspension was decanted and centrifuged at 2,250 *g* for 25 minutes. The platelet button obtained was suspended in 30 to 40 times its volume of a modified human Ringer solution containing 4 mM KCl, 107 mM NaCl, 20 mM NaHCO<sub>3</sub>, and 2 mM Na<sub>2</sub>SO<sub>4</sub> gassed with 5% CO<sub>2</sub>-95% O<sub>2</sub> to a pH of 7.1. This suspension was accomplished by gentle aspiration and regurgitation of the Ringer solution with a plastic pipette. The suspension was resedimented for 10 minutes at 2,250 *g* and again suspended in approximately 40 times its volume of Ringer solution. This procedure was then repeated a second time. For studies with platelet agglutination and contraction, 0.1 mM EDTA was added to the Ringer solution. This eliminated to some extent the problem of platelet agglutination during the preparation of platelet suspensions. Whenever platelet agglutination occurred during the isolation and washing of platelets, the preparation was discarded. Before the 0.1 mM EDTA was introduced to the experimental design, it was first established that concentrations of EDTA in the order of 1 and 2 mmoles per L had no effect on platelet lactate production, uptake of glucose, ATP levels, or glucose 6-phosphate levels.

#### *Incubation procedure*

The packed cell volume of the platelet suspension was measured in a microhematocrit tube, and 3- to 5-ml sam-

ples of this suspension were employed for *in vitro* incubation. The packed cell volumes of these samples were 3% to 6% (1 ml packed platelets was equivalent to  $1 \times 10^{11}$  platelets). Measurements were performed in duplicate on two different dilutions and agreed within 5 to 10% of each other and the total packed cell volume. Incubations were performed at 37° C under 5% CO<sub>2</sub>-95% O<sub>2</sub> (unless otherwise noted) in tightly capped 40-ml Sorvall cellulose nitrate centrifuge tubes, in a gyratory New Brunswick shaker, model G-77, at 25 rpm. Most experiments were performed with pooled platelets from 2 to 4 U of blood. For agglutination and contraction experiments, additions consisted of the following: thrombin, 1 U per ml; ADP,  $1 \times 10^{-4}$  mole per L, plus CaCl<sub>2</sub>,  $2.5 \times 10^{-3}$  mole per L; or epinephrine,  $1 \times 10^{-4}$  mole per L, plus ascorbic acid,  $1 \times 10^{-3}$  mole per L. Ascorbic acid was required to avoid oxidation of epinephrine. All additions resulted in macroscopic platelet agglutination and contraction within 5 to 8 minutes. Paired controls were devoid of clumping. Incubations were terminated by centrifuging in the same incubation tubes at 5° C at 6,000 *g* in a Sorvall RC11 centrifuge for 10 minutes.

#### *Preparation of samples*

The supernatant Ringer solution was treated with 10% by volume 5 N perchloric acid, followed by a neutralizing mixture containing 5 N KOH, 100 mM Tris, pH 7.5, to remove KClO<sub>4</sub>. Suitably diluted samples of this neutralized solution were used to assay extracellular lactate and glucose. The platelet pellet within its centrifuge tube was treated with 3 times its volume of cold 1 N perchloric acid while immersed in an ice bucket and mixed with a glass stirring rod. The tubes were then shaken on a Vortex shaker for 1 minute and centrifuged at 6,000 *g* in the Sorvall centrifuge for 10 minutes. The supernatant obtained was treated with neutralizing mixture and again centrifuged at 6,000 *g* for 10 minutes. Undiluted samples of this neutralized tissue sample were used to assay intracellular lactic acid, ATP, and glucose 6-phosphate. It is essential that neutralizing mixture be added after removal of precipitated protein; otherwise an interfering gel is obtained.

#### *Enzymatic measurements*

All measurements were performed in microcuvettes, with a Gilford spectrophotometer and Honeywell recorder, employing nucleotide changes at 363 and 340 *mμ* for acetyl-NAD and NADP, respectively.

**Lactate.** The method employed was a minor modification of the Lowry procedure (7) and is a reproducible enzymatic method for measuring lactate provided the following precautions are observed: 1) All glassware must be free of lactate. It is suggested that glassware be immersed for several hours in 25% NaOH, rinsed in tap water, and rinsed several times in distilled water. 2) Final pH of at least 9.6 must be obtained. 3) Beef heart lactic dehydrogenase must be employed. 4) Lactate concentration should be below 0.025 mmole per L to assure adherence to Beer's law.

<sup>1</sup> Obtained from the American Red Cross and The New York Blood Center.

Base-line readings were obtained at 363  $m\mu$  in a final volume of 0.5 ml containing the following: 0.1 mM acetyl-NAD; 200 mM bicarbonate buffer, pH 9.6; and beef heart lactic dehydrogenase, 150  $\mu$ g. The reaction was initiated with 0.015 ml of tissue sample or 0.05 ml of neutralized extracellular Ringer solution. Extracellular Ringer solution was diluted 1:10 in bicarbonate buffer. Beef heart lactic dehydrogenase was stored as a 1:10 dilution of its  $(\text{NH}_4)_2\text{SO}_4$  suspension in 50 mM Tris buffer, pH 8.5, 1 mM EDTA; 0.03 ml of this dilution was used for the assay. The molar extinction coefficient for acetyl-NAD was in agreement with that obtained by Lowry,  $9.3 \times 10^4$ .

**Adenosine triphosphate.** ATP was measured by classical procedures coupling the hexokinase reaction to the glucose 6-phosphate dehydrogenase reaction, and employing the NADP-NADPH change at 340  $m\mu$ . The reagents employed in a final volume of 0.5 ml were these: 20 mM glucose, 0.46 mM NADP, 12.5 mM  $\text{MgCl}_2$ , 1  $\mu$ g of crystalline hexokinase, 2  $\mu$ g of glucose 6-phosphate dehydrogenase in 50 mM Tris buffer, 1 mM EDTA, pH 7.5. After base-line readings at 340  $m\mu$ , 0.02 ml of neutralized tissue extract was added directly to the microcuvettes. Tissue blanks in the absence of hexokinase were negligible.

**Glucose.** Glucose was measured with the use of hexokinase and ATP coupled to the glucose 6-phosphate dehydrogenase reaction. The reagents employed were in a final volume of 0.5 ml: 10 mM ATP, 12.5 mM  $\text{MgCl}_2$ , 0.46 mM NADP, 1  $\mu$ g of hexokinase, and 2  $\mu$ g of glucose 6-phosphate dehydrogenase in 50 mM Tris buffer, 1 mM EDTA, pH 7.5. The neutralized extracellular incubating Ringer solution was diluted 1:10 in 50 mM Tris buffer, 1 mM EDTA, pH 7.5, and 0.05 ml employed for the initiation of the reaction, after the attainment of base-line levels.

**Glucose 6-phosphate.** The reaction mixture in 0.5 ml final volume contained the following: 0.46 mM NADP in 50 mM Tris buffer, pH 7.5, 1 mM EDTA, and 0.15 ml neutralized tissue extract. The reaction was started by the addition of 2  $\mu$ g of glucose 6-phosphate dehydrogenase in 0.02 ml. For these measurements it was necessary to use the expanded scale on the Honeywell recorder, employing a 2-fold amplification.

**Calculations.** Measurements were expressed per milliliter of packed platelets. In calculation of intracellular values, the platelet mass contribution to the perchloric acid volume was assumed to be 80% of its packed cell volume (8). In calculation of intracellular lactate, the contribution of entrapped lactate in the extracellular space of the platelet button was considered negligible.

**Materials.** All chemicals employed were reagent grade. Glucose 6-phosphate dehydrogenase type V,<sup>2</sup> crystalline hexokinase,<sup>2</sup> ATP,<sup>2</sup> ADP,<sup>2</sup> NADP,<sup>2</sup> acetyl-NAD,<sup>2</sup> beef heart lactic dehydrogenase,<sup>3</sup> and glucagon-free insulin<sup>4</sup> were obtained commercially. Purified human thrombin

was a gift.<sup>5</sup> Epinephrine hydrochloride<sup>6</sup> was obtained commercially (intravenous injection) and was freshly diluted in 10 mM ascorbic acid.

## Results

Basal levels for ATP, intracellular lactate, and glucose 6-phosphate at 0° C before incubation were  $2.7 \pm 0.11$ ,  $1.95 \pm 0.06$ , and  $0.089 \pm 0.004$   $\mu$ moles per ml packed platelets, respectively, and may be seen from the ordinate of Figure 1. In the absence of glucose, lactate production proceeded at a linear rate of  $15.6 \pm 0.95$   $\mu$ moles per ml per hour for at least 60 minutes, and in over one-half the experiments for 90 minutes. Thus, platelets with respect to their glycolytic end-product, lactate, remain metabolically active for at least 60 to 90 minutes. It should be noted that glucose is not required and that glycogenolysis is an active metabolic pathway. Although ATP, intracellular lactate, and glucose 6-phosphate measurements remained fairly similar in different platelet pools, total lactate production varied considerably from 7 to 22  $\mu$ moles per ml per hour. This may be a reflection of glycogen stores. Intracellular lactate rose slightly, reaching its peak level of 3.1  $\mu$ moles per ml at 90 minutes, and then declined gradually for the next 90 minutes. Glucose 6-phosphate levels remained constant over an 180-minute interval. ATP, however, declined to 38% of its initial level after 2 hours.

Platelets were responsive to increasing temperatures: 17°, 27°, and 37° C. For total lactate production  $Q_{10}$  (17 to 27°) was 2.0 and  $Q_{10}$  (27 to 37°) was 2.7.

In the presence of 5 mM glucose, lactate production increased 2-fold to  $34.8 \pm 1.6$   $\mu$ moles per ml per hour (range 21 to 45) and remained constant for 90 minutes (Figure 2 and Table I). Glucose 6-phosphate levels remained constant and ATP levels declined as in the absence of glucose. Intracellular lactate levels rose to twice their basal levels. Glucose uptake was 21  $\mu$ moles per ml per hour. In the presence of 5 mM NaCN, an inhibitor of cytochrome oxidase, glucose uptake increased 2-fold, to  $42.3 \pm 6.9$  (5 experiments); lactate production increased 1.9-fold, to  $64.0 \pm 15.6$  (4 experiments).

In the presence of glucose and insulin, 0.4 U per

<sup>2</sup> Sigma Chemical Co., St. Louis, Mo.

<sup>3</sup> Worthington Biochemical Corp., Freehold, N. J.

<sup>4</sup> Eli Lilly, Indianapolis, Ind.

<sup>5</sup> From Dr. Kent Miller.

<sup>6</sup> Parke Davis, Detroit, Mich.

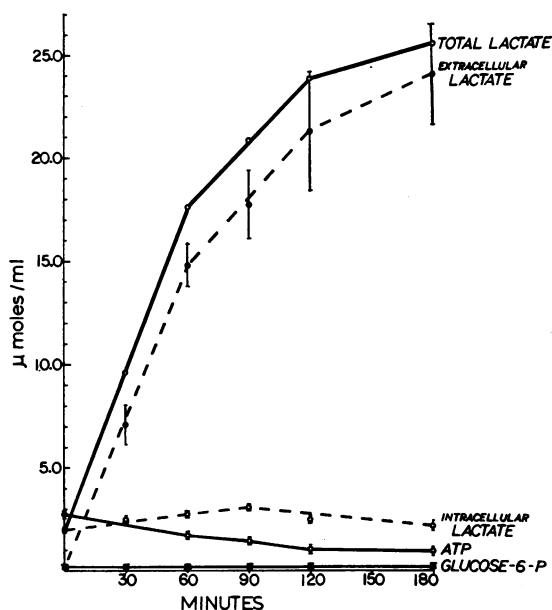


FIG. 1. LACTATE (INTRACELLULAR AND EXTRACELLULAR), ATP, AND GLUCOSE 6-PHOSPHATE LEVELS OF PLATELETS INCUBATED IN THE ABSENCE OF GLUCOSE. Each lactate point represents 6 to 14 experiments, except the 60-minute point, which represents 51 experiments. Each ATP point represents 6 to 21 experiments, except the zero time, which represents 26 experiments. Each glucose 6-phosphate point represents 6 to 30 experiments. Standard error of the mean is given.

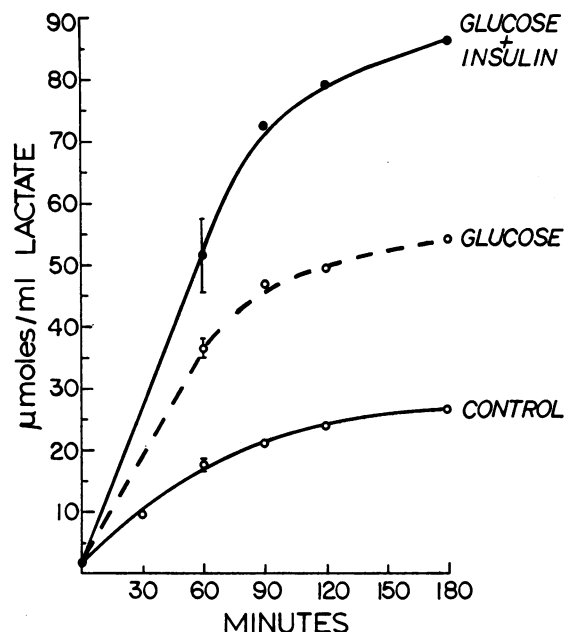


FIG. 2. TOTAL LACTATE LEVELS OF PLATELETS INCUBATED IN THE ABSENCE OF GLUCOSE, PRESENCE OF 5 mM GLUCOSE, AND PRESENCE OF GLUCOSE PLUS 0.4 U PER ML INSULIN. Standard error of the mean is given for 60-minute points, which represent 51, 40, and 5 experiments for control, glucose, and glucose plus insulin, respectively. Other points from glucose and glucose plus insulin experiments represent the average of 2 to 4 experiments.

ml (Figure 2), lactate production increased 3-fold over basal rates and remained constant for 60 to 90 minutes. Again, ATP and glucose 6-phosphate remained the same as for glucose alone (Table I),

and intracellular lactate rose to 4  $\mu$ moles per ml at 1 hour. Insulin was effective at 0.004 to 0.4 U per ml in the presence of glucose. In the absence of glucose, insulin had no effect on increased lac-

TABLE I  
Comparison of lactate production and ATP and glucose 6-phosphate levels after 1-hour incubation\*

	Basal 0° C	Control	Glucose	Insulin + glucose	Citrate	Citrate + glucose
ATP, $\mu$ moles/ml	2.70 $\pm$ 0.11 (18)	1.74 $\pm$ 0.12 (17)	1.75 $\pm$ 0.10 (15)	1.72 $\pm$ 0.12 (3)	2.19 $\pm$ 0.05 (3)	2.19 $\pm$ 0.06 (4)
Glucose 6-phosphate, $\mu$ mole/ml	0.089 $\pm$ 0.004 (30)	0.091 $\pm$ 0.005 (23)	0.094 $\pm$ 0.005 (18)	0.078 $\pm$ 0.005 (6)	0.092 $\pm$ 0.007 (9)	0.092 $\pm$ 0.007 (8)
Extracellular lactate, $\mu$ moles/ml/hr	0	14.8 $\pm$ 0.95 (51)	33.1 $\pm$ 1.6 (40)	47.4 $\pm$ 6.1 (5)	20.7 (2)	43.4 $\pm$ 3.8 (4)
Intracellular lactate, $\mu$ moles/ml/hr	1.95 $\pm$ 0.06 (19)	2.77 $\pm$ 0.11 (17)	3.61 $\pm$ 0.23 (15)	4.0 $\pm$ 0.28 (7)	2.6 (2)	4.38 $\pm$ 0.43 (4)
Lactate production, $\mu$ moles/ml/hr	0	15.6	34.8	49.5	21.4	45.8

\* Basal values at 0° C are compared with values obtained after changes in incubation medium at 37° C: absence of glucose, 5 mM glucose, glucose plus 0.4 U per ml insulin, 40 mM citrate, and citrate plus glucose. Standard error of the mean is given. Number of experiments is denoted in parentheses.

tate production (control  $16.5 \pm 0.8$  vs. insulin  $16.8 \pm 1.3$   $\mu$ moles per ml per hour, 5 experiments).

The addition of citrate increased lactate production and prolonged the duration of linearity from 60 to 90 minutes to 120 to 180 minutes (Figure 3). Table II depicts the effect of increasing citrate concentration on lactate production and protection of ATP levels. Citrate plus glucose increased lactate production 3-fold, intracellular lactate rose to 4.4  $\mu$ moles per ml at 1 hour, and glucose 6-phosphate remained constant (Table I). Figure 4 depicts the kinetics of the effect of citrate on ATP decline. Citrate had a potent deterring action on ATP decline, whereas glucose alone had no effect. This would suggest that citrate penetrates the platelet membrane and is utilized by Krebs cycle enzymes to maintain ATP

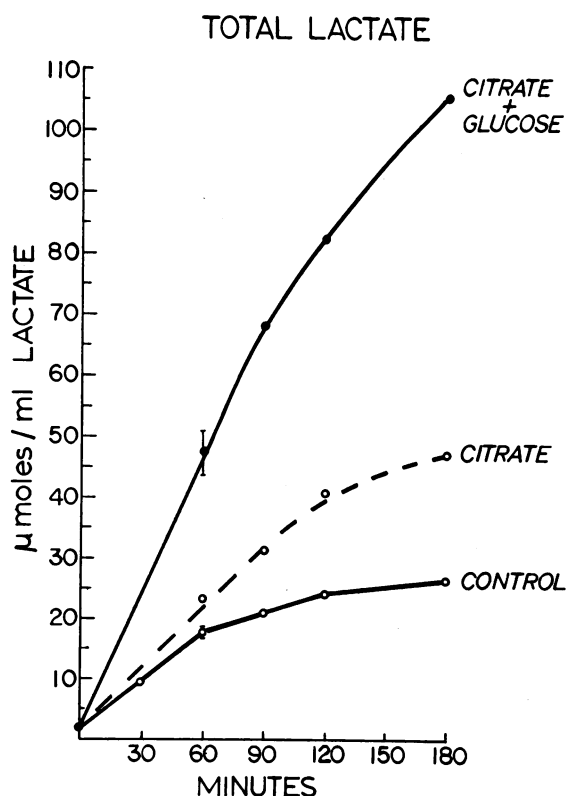


FIG. 3. TOTAL LACTATE LEVELS OF PLATELETS INCUBATED IN THE ABSENCE OF GLUCOSE, PRESENCE OF 40 mM CITRATE, AND PRESENCE OF 40 mM CITRATE PLUS 5 mM GLUCOSE. Standard error of the mean is given for 60-minute points for control and citrate plus glucose experiments, which represent 51 and 4 experiments, respectively. All other citrate points represent the average of 2 experiments.

TABLE II

*Effect of citrate concentration on ATP levels and lactate production after 1 hour of incubation\**

Citrate, mM:		0	5	10	20	40	60	80
Experiment 1	ATP	%	%	%	%	%	%	%
	Lactate	45	90	100	90	90		
Experiment 2	ATP	40		60		67	53	53
	Lactate	100		100	125	164	109	109

\* Percent of initial ATP levels and per cent of 1-hour control lactate production is given.

levels and increase lactate production. To substantiate this hypothesis the following experiment was employed. In the presence of 40 mM citrate, platelets were incubated with and without 2 mM, 2,4-dinitrophenol under anaerobic conditions. Both conditions were employed in order to assure cessation of ATP synthesis. (Because of technical difficulties, anaerobiosis was not always attainable.) Inhibition of the ATP-synthesizing enzymes linked to the Krebs cycle prevented the effect of citrate on increased lactate production and ATP levels (Table III). As first reported by Luganova, Seits, and Tedorovich (9), 2,4-dinitrophenol did not decrease ATP levels in platelets.

Experiments were performed to study the effect of platelet agglutination and contraction on glycolysis.

*Thrombin experiments.* In the presence of thrombin, lactate production increased 8.0  $\mu$ moles per ml per hour or 37% (Table IV). This in-

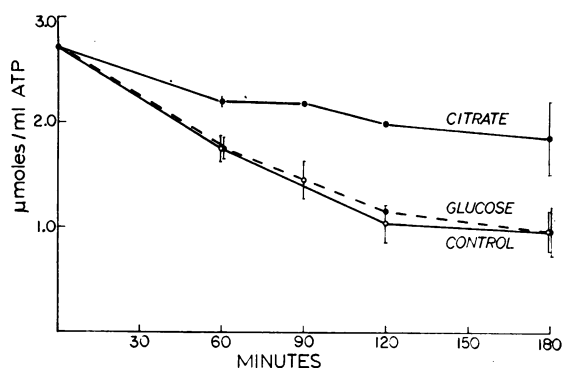


FIG. 4. ATP LEVELS FROM A 3-HOUR PERIOD OF INCUBATION IN THE ABSENCE OF GLUCOSE, PRESENCE OF 5 mM GLUCOSE, AND PRESENCE OF 40 mM CITRATE. The citrate curve combines experiments with citrate alone and citrate plus glucose (both were identical with respect to ATP decline) and represents 3 to 7 experiments. The glucose curve represents 3 to 15 experiments. Standard error of the mean is given.

TABLE III  
Reversal of citrate effect with 2 mM 2,4-dinitrophenol  
and anaerobiosis\*

Experiment	Glucose		Glucose + citrate		Glucose + citrate + NAD + An		Glucose + NAD + An	
	$\mu\text{moles/ml}$	(%)	$\mu\text{moles/ml}$	(%)	$\mu\text{moles/ml}$	(%)	$\mu\text{moles/ml}$	(%)
1	31.4	(40)	57.3	(67)	30.0	(40)		
2	37.9	(65)	55.1	(83)	37.9	(65)		
3	45.3	(65)					55.1	(65)

\* Lactate production is after 1 hour of incubation. Per cent ATP of initial level is given in parentheses. Anaerobiosis (An) was obtained with 95% argon and 5% CO<sub>2</sub>.

crease in glycolysis may be predominantly attributed to glycogenolysis, since glucose was absent from the media. ATP declined to 32% of its 60-minute control level, and glucose 6-phosphate levels remained constant. In the presence of 5 mM glucose plus thrombin, lactate production increased 51% from 37.0 to 55.7 or 18.7  $\mu\text{moles per ml per hour}$ . This was accompanied by an 82% increase in glucose uptake. ATP declined similarly to 37% of its 60-minute control level, and glucose 6-phosphate remained constant.

*Adenosine diphosphate experiments.* In the presence of ADP and CaCl<sub>2</sub>, lactate production increased 15% or 3.3  $\mu\text{moles per ml per hour}$ . This increase in lactate production was noted in 10 of 11 experiments ( $p < 0.01$ ). ATP declined to 75% of its 60-minute control value, and glucose 6-phosphate remained constant. In the pres-

ence of glucose, a significant increase in lactate production could not be demonstrated in a total of 8 experiments. Glucose uptake by the platelets declined 25%. This decline in uptake was noted in 6 of 8 experiments ( $p < 0.05$ ). ATP declined to 73% of its 60-minute control value, and glucose 6-phosphate remained constant.

*Epinephrine experiments.* In the presence of epinephrine and ascorbic acid, lactate production increased 37% or 8.1  $\mu\text{moles per ml per hour}$ . ATP declined to 68% of its 60-minute control value, and glucose 6-phosphate remained constant. Ascorbic acid alone did not result in platelet agglutination or affect ATP levels or lactate production. In the presence of 5 mM glucose plus epinephrine, lactate production increased 43%, from 38.2 to 54.6 or 16.4  $\mu\text{moles per ml per hour}$ . This was accompanied by a 52% increase in glucose uptake. ATP declined to 61% of its 60-minute control value, and glucose 6-phosphate rose to 70% above its control value ( $p < 0.05$ ).

### Discussion

Previous investigations of glycolytic enzymes, basal glycolytic rates, and nucleotide changes in human platelets have been published (9-13). Various methods were employed to measure ATP and lactate production, and different levels were obtained. Those for ATP were 1.61 (9), 2.5 (11), 3.8 (12), and 5.2 (13)  $\mu\text{moles per ml}$ . Lac-

TABLE IV  
Effect of thrombin, ADP, and epinephrine on glycolysis\*

	Lactate production		Glucose uptake	Adenosine triphosphate		Glucose 6-phosphate	
	Control	Glucose		Control	Glucose	Control	Glucose
	$\mu\text{moles/ml/hr}$	$\mu\text{moles/ml/hr}$	$\mu\text{moles/ml/hr}$	$\mu\text{moles/ml}$	$\mu\text{moles/ml}$	$\mu\text{moles/ml}$	$\mu\text{moles/ml}$
Control	21.5 $\pm$ 1.3 (8)	37.0 $\pm$ 4.5 (7)	19.0 $\pm$ 6.0 (7)	2.00 $\pm$ 0.24 (8)	1.89 $\pm$ 0.17 (8)	0.070 $\pm$ 0.007 (5)	0.080 $\pm$ 0.010 (5)
Thrombin	29.5 $\pm$ 3.2 (8)	55.7 $\pm$ 6.4 (7)	34.6 $\pm$ 5.6 (7)	0.64 $\pm$ 0.08 (5)	0.69 $\pm$ 0.10 (5)	0.065 $\pm$ 0.011 (3)	0.071 $\pm$ 0.003 (5)
Control	21.6 $\pm$ 1.3 (11)	34.6 $\pm$ 2.5 (8)	16.1 $\pm$ 1.4 (7)	2.11 $\pm$ 0.57 (11)	2.10 $\pm$ 0.16 (6)	0.069 $\pm$ 0.014 (5)	0.080 $\pm$ 0.013 (6)
ADP	24.9 $\pm$ 1.4 (11)	35.4 $\pm$ 3.1 (8)	12.1 $\pm$ 2.1 (8)	1.59 $\pm$ 0.15 (11)	1.54 $\pm$ 0.12 (6)	0.076 $\pm$ 0.009 (6)	0.069 $\pm$ 0.006 (6)
Control	22.1 $\pm$ 0.36 (15)	38.2 $\pm$ 3.0 (9)	17.8 $\pm$ 2.9 (9)	1.77 $\pm$ 0.17 (9)	1.82 $\pm$ 0.16 (7)	0.068 $\pm$ 0.010 (5)	0.077 $\pm$ 0.004 (5)
Epinephrine	30.2 $\pm$ 2.6 (15)	54.6 $\pm$ 5.0 (9)	27.1 $\pm$ 4.5 (9)	1.21 $\pm$ 0.12 (9)	1.12 $\pm$ 0.10 (7)	0.095 $\pm$ 0.017 (5)	0.131 $\pm$ 0.025 (5)

\* Platelets were incubated for 1 hour at 37° C at 25 rpm in the presence and absence of 5 mM glucose. Additions consisted of thrombin, 1 U per ml; ADP,  $1 \times 10^{-4}$  mole per L, plus CaCl<sub>2</sub>,  $2.5 \times 10^{-3}$  mole per L; epinephrine,  $1 \times 10^{-4}$  mole per L, plus ascorbic acid,  $1 \times 10^{-3}$  mole per L. ATP and glucose 6-phosphate levels are given at 1 hour. Standard error of the mean is given along with number of experiments in parentheses.

tate production in the absence of glucose was 27 (9) and in the presence of glucose was 70 (9) and 76.4 (10)  $\mu$ moles per ml per hour at 37° C. The ATP levels of the present investigation correspond to the middle range of these values. Lactate production is approximately one-half. Waller, Löhr, Grignani, and Gross (10) reported the presence of glycolytic, Krebs cycle, and hexose-monophosphate shunt enzymes in human platelets. Platelets converted 50% of glucose uptake to lactate (glycogenolysis was not measured). Both Waller and associates (10) and Lukanova and associates (9) indicated that the principal source of ATP in the platelet is the glycolytic pathway. Chernyak and Totskaya (14) also reported that the oxidative pathway is not so important as glycolysis in the generation of high energy phosphate. Bettex-Galland and Lüscher (11) first reported that thrombin increases lactate production and glucose consumption. Born (12), Bettex-Galland and Lüscher (11), and Zucker and Borrelli (13) have described ATP decline in the presence of thrombin. Little information is available on the regulation of glycolysis in a plasma-free platelet system in which glycolysis has been activated by known stimulators or platelets have been subjected to agglutination and contraction.

The results of this study confirm the importance of aerobic glycolysis in platelets but also emphasize the significance of glycogenolysis and Krebs cycle enzymes. Thus glycogen in freshly collected platelets is sufficient to maintain lactate production for at least 60 to 90 minutes. This is not to imply that the decrease in lactate production is due to decline of metabolic activity. Control mechanisms or changes in intracellular pH may be regulating the rate of lactate production (extracellular pH did not change).

In the rat (15) and anaerobic turtle (16) heart the rate of maximal glycogenolysis is dependent on the initial glycogen level. Reeves (16), employing elegant isotopic studies, demonstrated that maximal glycogenolysis proceeds at a constant rate with increasing work loads and is independent of glucose uptake. Under anoxic conditions, control dog hearts demonstrated similar 1-hour glycogen disappearance rates as did hearts from animals previously transfused with glucose and insulin (17). The assumption is made that platelet glycogenolysis similarly proceeds at a con-

stant rate in the presence and absence of glucose. The contribution of glucose conversion to lactate can then be calculated by deducting lactate production in the absence of glucose and assuming that one glucose may be converted to two lactates. The value obtained was 46%, similar to that reported in skeletal muscle of the rhesus monkey (18). This suggests that under basal conditions 54% of glucose is metabolized by some pathway other than the Embden-Meyerhof. Waller and associates (10) reported that 20% of metabolized glucose is recovered as CO<sub>2</sub> and water. This leaves 34% of glucose utilization unaccounted for. Conversion to glycogen, amino acids, or lipids might explain this.

Thrombin- and epinephrine-induced platelet agglutination and contraction are associated with an increase in glycogen breakdown (phosphorylase activity), lactate production, and glucose uptake. It is recognized that increased glucose transport may be related to disintegration of the platelet membrane secondary to agglutination and contraction. In the presence of glucose, a further increase of lactate production was noted and can be attributed to the increase in glucose uptake. If glycogenolysis can be assumed to be independent of glucose penetration, this becomes more evident. Thus for thrombin, the theoretical lactate increase for an increase in glucose uptake of 1.8-fold should be 46% of 3.6-fold, or 1.7-fold. The actual value obtained was 1.7-fold. Similarly, the same applies for epinephrine agglutination and contraction, where glucose uptake increased 1.5-fold, the theoretical increase in lactate production being 1.4-fold. The actual increase obtained was 1.5-fold. Thus both epinephrine and thrombin can further increase glycolysis (hexokinase activity) by increasing glucose uptake.

It has been reported that a plasma factor and calcium are required for ADP agglutination of pig platelets (19). This factor does not appear to be required for human platelets although its presence increases their sensitivity to ADP (20). ADP-induced platelet agglutination resulted in a minimal but definite increase in lactate production. In the presence of glucose this effect could not be demonstrated. Surprisingly, glucose uptake decreased by 25%. Perhaps the ADP-mediated agglutination in some way impairs the sites for glucose entry.



Enhanced glucose uptake by the platelet contributed to increased lactate production. Addition of glucose to the media increased lactate production 2-fold. Cyanide increased glucose uptake 2-fold and was associated with a parallel increase in lactate production, 1.8-fold. Since free glucose was not found intracellularly, glucose disappearance from the media is a reflection of glucose utilization. This appears to be the first report of the effect of insulin on human platelets. Since insulin was not operative in the absence of glucose, the effect of insulin was probably on glucose transport. The effects of cyanide, insulin, thrombin, and epinephrine would indicate that hexokinase is not working at maximal capacity and that transport of glucose contributes to the increase of lactate production.

Skeletal muscle contraction results in increased glucose transport (21–23) and activation of the glycolytic chain (21, 22, 24, 25). The latter can be shown to be a result of the apparent activation of phosphorylase, hexokinase, and phosphofructokinase. Danforth, Helmreich, and Cori (25) have demonstrated an increase in the conversion of phosphorylase *b* to its active phosphorylase *a* form after muscle contraction. Platelet glucose 6-phosphate levels, except for incubations with epinephrine, remained constant under conditions in which lactate flux increased 3-fold and glucose uptake 2-fold. This would indicate that phosphorylase and hexokinase, key enzymes involved in the production of glucose 6-phosphate, remain closely geared to phosphofructokinase and glucose 6-phosphate dehydrogenase, the regulator enzymes controlling its removal. The assumption is made that glucose 6-phosphate is in equilibrium with fructose 6-phosphate (24, 26). Similar findings have been reported in skeletal muscle (24) and red blood cells (27). With epinephrine plus glucose, glucose 6-phosphate rose 70% above its control level. A similar finding has been reported in frog skeletal muscle (24), in which it was postulated that epinephrine activated the phosphorylase *b* to *a* conversion. With platelet phosphorylase, epinephrine appears to be a more potent activator than ADP or thrombin. As with skeletal muscle, key enzymes removing glucose 6-phosphate become rate limiting in the case of epinephrine (24).

Synthesis of ATP is considerably more efficient

via the Krebs cycle than the Embden–Meyerhof pathway. This could explain the significant protection of ATP levels by a pathway contributing no more than 20% of glucose utilization. Aster and Jandl (28) reported that collection of blood in acid–citrate–dextrose solution containing increased citrate, 22 mM, enhanced platelet recovery and viability after platelet transfusion. This was explained on the basis of lowered pH (6.5), which led to reduction of platelet clumping. Perhaps maintenance of ATP levels can also explain their results. These data also suggest that enzymes capable of converting citrate to lactate are present in platelets. It appears that citrate is able to penetrate mitochondria, contribute to ATP production, and be converted to lactate via the gluconeogenic enzymes (possibly phosphoenolpyruvate carboxykinase). Thus platelets are capable of at least the first stages of gluconeogenesis.

The observation of ATP decline during incubation has also been made by Zucker and Borrelli (13). This phenomenon may result from decreased ATP synthesis or increased ATP utilization. Glucose alone or glucose plus insulin were incapable of preventing decline of ATP levels. Furthermore, lactate production did not fall during a significant span of the ATP decline. This would suggest that ATP synthesis did not decrease, but utilization increased. ATP declined further with all 3 agents: thrombin > epinephrine > ADP. These agents were capable of increasing ATP expenditure above that of control platelets. Platelet agglutination and contraction were associated with this ATP decline. It is conceivable that activation of thrombosthenin, the contractile protein ATPase, is required for platelet agglutination and contraction.

Platelet agglutination and contraction whether mediated by ADP, epinephrine, or thrombin result in increased glycolytic flux and expenditure of ATP. This universality of response was noted despite the variety of agents employed to induce agglutination and contraction. It would be interesting to speculate that this activation of the glycolytic chain and associated expenditure of ATP are a result of the cellular reorganization inherent in platelet agglutination and contraction, rather than specific agents activating specific enzymes.

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