Biochemical Energetics of Simulated Platelet Plug Formation

EFFECT OF THROMBIN, ADENOSINE DIPHOSPHATE, AND EPINEPHRINE ON INTRA- AND EXTRACELLULAR ADENINE NUCLEOTIDE KINETICS

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ABSTRACT Washed human platelets were incubated in a modified Ringer's solution, pH 7.1, at 37°C for 1 hr. Intracellular basal levels for glycogen, adenosine triphosphate (ATP), adenosine diphosphate (ADP), adenosine monophosphate (AMP), and orthophosphate were 31.1, 2.52, 1.39, 0.36, and 1.2 μmoles/ml of platelets, respectively. Extracellular ATP, ADP, and AMP remained fairly constant and represented 4, 2, and 4% of total adenine nucleotide content. Total adenine nucleotide content remained unchanged during the period of control incubation. Glycogen depletion was 17.8 μmoles/ml at the end of 1 hr; lactate production was 20.7 μmoles/ml per hr. In the presence of glucose, lactate production increased 100%, and glycogen depletion was spared 13%. Approximately 55% of glucose or glycogen fuel was converted to lactate.

The agglutinating agents, thrombin, ADP, and epinephrine, resulted in increased glycogen depletion and lactate production both in the presence and absence of glucose. The effect of thrombin was greater than epinephrine. The effect of epinephrine was greater than ADP. All three agglutinating agents resulted in loss of high energy phosphates (net decline in adenine nucleotides) with release of adenine nucleotides into the extracellular environment. The effect of thrombin was greater than ADP. The effect of ADP was greater than epinephrine. In experiments with ADP addition, significant quantities of ADP were converted to AMP extracellularly. In experiments with thrombin and epinephrine appreciable quantities of extracellular orthophosphate were taken up by plateletes and could not be accounted for by changes in intracellular orthophosphate or adenine nucleotide. Sufficient ADP was released during exposure to thrombin and epinephrine to account for platelet agglutination. Changes in intracellular adenine nucleotides and orthophosphate could be correlated with the activation of regulator glycogenolytic and glycolytic enzymes.

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INTRODUCTION

Human platelets are a predominantly aerobic glycolytic tissue (1). They are a unique tissue in that their major function, platelet plug formation, is closely geared to their physical destruction. Consequently, the requirement for a return to steady-state levels of glycolytic intermediates after performance of a physiologic function does not apply. Accordingly, platelets behave biochemically with a terminal release of energy. The biochemical energetics of this platelet interaction during platelet plug formation have not yet been adequately investigated (1–5).

Adenine diphosphate (ADP), a potent agglutinator of platelets (6), is belived to be the initiator of thrombin-induced platelet agglutination and contraction (7). Thrombosthenin (8), a contractile platelet protein adenosine triphosphatase (ATPase) (similar to actomyosin of muscle), may be responsible for platelet and clot retraction. Consequently, knowledge regarding the intra- and extracellular adenine nucleotide kinetics after platelet plug formation and possible activation (1) of this ATPase is of importance. This information would provide a better understanding of the mechanism of ADP-induced platelet agglutination and thombin-induced platelet contraction (9). In an effort to provide this information, adenine nucleotide kinetics as well as associated energy requirements were investigated during platelet plug formation.

METHODS

Preparation of platelets. Human platelet-rich plasma was obtained from donor blood collected in acid citrate dextrose (ACD) solution, 1–2 hr after phlebotomy. All procedures were performed as described previously (1) at 0°C unless otherwise noted. Human Ringer's solution contained the same electrolytes as described previously except for the addition of 2 mM Na_2HPO_4. Red blood cell and white blood cell contamination was negligible (one red blood cell per 1000 platelets, one white blood cell per 1000 platelets) and platelet yield approximated 80–100% of the average theoretical yield of 1 ml of packed platelets per unit of plasma.

Incubation procedure. Platelet suspensions were incubated at 37°C under 5% CO_2–95% O_2 for varying time intervals up to 1 hr. Approximately 0.4 ml of packed platelets was suspended in Ringer's solution-0.1 mM ethylenediaminetetraacetate (EDTA) in a volume of 6 ml and incubated in tightly capped plastic tubes shaken at 25 rpm. Most experiments represented pooled platelets from three or four donors. Incubations were performed in duplicate with and without: thrombin, 1 NIH unit/ml; epinephrine hydrochloride, 0.1 mM, plus vitamin C, 1 mmole/liter; ADP, 0.1 mmole/liter, plus $CaCl_2$, 2.5 mmoles/liter. These concentrations gave maximal glycolytic effects. Macroscopic platelet agglutination was observed within 2–3 min in experimental tubes and not observed in control tubes. Incubations were terminated by centrifugation of the incubation tubes at 5°C for 10 min at 3000 *g*. For further details see reference (1).

Preparation of samples. The supernatant Ringer solution was treated with 10% by volume, cold 5.9 N $HClO_4$ followed by a neutralizing mixture containing 5 N KOH, 50 mM Tris, pH 7.0. The $KClO_4$ precipitate was removed by centrifugation. Aliquots of the supernatant were assayed for ATP, ADP, AMP, lactate, and glucose. Rapid freeze techniques were employed for intracellular measurements of platelets in order to avoid enzymatic activation during extraction. The platelet pellet in the centrifuge tube was frozen with liquid nitrogen, −196°C. The plastic tube was cracked open and the frozen pellet rapidly weighed. The frozen pellet was then transferred to a precooled mortar held on dry ice, ground with sand, and thoroughly mixed with 2.4 volumes of 1 N $HClO_4$. The mortar was then transferred to an ice bucket where it was kept until the mixture thawed. The thawed slurry was then reground and the liquid transferred to a centrifuge tube and sedimented at 3020 *g* for 10 min. The supernatant was treated with neutralizing mixture and recentrifuged. Aliquots of this material were employed to measure ATP, ADP, AMP, and orthophosphate.

Assay procedures. Standards were run with all assays. These were linear with increasing concentration over the range measured. Addition of standard to platelet extract resulted in recovery of better than 90% for all assays. Tissue blanks were run whenever enzymatic reactions were initiated with tissue extract (e.g., ATP, lactate, and glucose), and these were negligible.

Glycogen. The procedure was a minor modification of that described by Hassid and Abraham (10).

Intracellular orthophosphate. Orthophosphate was measured by a method which avoided the hydrolysis of creatine-*P* and other labile phosphates during the extraction procedure. This was accomplished by extracting tissue with rapid freeze techniques in order to remain below 0°C during thawing (11, 12). The acid extract was neutralized, and 0.1 ml of the perchlorate-free extract was diluted to 2 ml with 50 mM Tris buffer, pH 8.5. The rest of the procedure was as described previously (11, 12).

Enzymatic measurements. All enzymatic measurements were performed with a Beckman-Gilford spectrophotometer (Beckman Instruments, Inc., Fullerton, Calif.) and a Honeywell recorder (Honeywell, Inc., Minneapolis, Minn.) employing nucleotide changes at 363 and 340 mμ for acetyl nicotinamide adenine dinucleotide (AcNAD) and NAD, or nicotinamide adenine dinucelotide phosphate (NADP), respectively. These are minor modifications of the methods of Lowry, Passonneau, Hasselberger, and Schulz unless otherwise noted (13). All measurements were performed in triplicate, and the expanded scale of the recorder was employed whenever necessary. Lactate, glucose, and ATP were measured as described previously (1).

ADP was measured by coupling the pyruvate kinase reaction with the lactic dehydrogenase reaction and measuring the NADH to NAD change at 340 mμ (13).

AMP was measured by coupling the myokinase reaction to the above ADP assay (13). Commercial NADH contained significant amounts of AMP. This was measured as a blank and subtracted from the sample reading.

2,3-diphosphoglycerate was measured by the Krimsky procedure (14). The procedure was sensitive to concentrations of 2,3-diphosphoglycerate of 2×10^{-7} moles/liter.

Materials. Distilled, deionized water was used at all times. All chemicals were reagent grade. Glucose-6-phos-

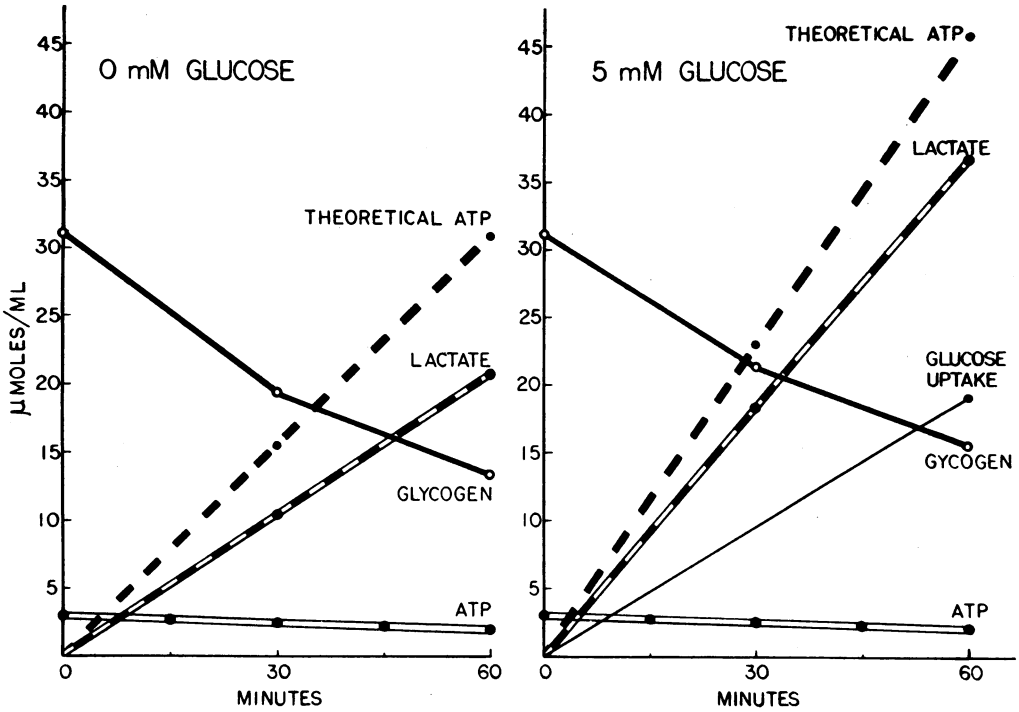

FIGURE 1 Theoretical adenosine triphosphate (ATP) generation from the Embden-Meyerhof pathway, in the absence and presence of glucose, 5 mmoles/liter. Glycogen, lactate, and ATP levels are expressed as μmoles/ml of platelets. Glucose uptake at 1 hr is expressed as μmoles/ml of platelets per hr. All data have SEM of less than 10%. Glycogen time points represent 18 experiments; lactate time points represent 50 experiments. ATP time points represent 20 experiments. Glucose time points represent 35 experiments. Lactate, ATP, and glucose experiments represent duplicate incubations assayed in triplicate. Glycogen experiments represent duplicate incubations.

phate dehydrogenase, type X, EDTA, NADP, acetyl NAD, ATP, ADP, and AMP were obtained from the Sigma Chemical Co., St. Louis, Mo. 2,3-diphosphoglycerate, phosphoglycerate mutase, enolase, pyruvate kinase, hexokinase, myokinase, phosphoenolpyruvate, and NADH were obtained from the Boehringer Mannheim Corp., New York. Beef heart lactic dehydrogenase was obtained from the Worthington Biochemical Corp., Freehold, N. J. Purified human thrombin was a gift of Dr. Kent Miller.

Calculations. Measurements were determined per gram wet weight for ATP, ADP, AMP, orthophosphate (Pi), and per milliliter of packed platelets for glycogen, glucose, and lactate measurements. Gram wet weight and milliliter of packed platelets were found to be interchangeable (15). All values are consequently 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 (1, 15). Per cent glycogen conversion to lactate was determined from the formula: GlycLac × 100/2 Glyc, where GlycLac = μmoles/ml per hr of lactate production in the absence of glucose, and Glyc = μmoles/ml of glycogen depletion at the end of 1 hr. Glycogen is expressed in terms of glucose units hydrolyzed. The assumption was made that insignificant lactate production was coming from sources other than glycogen, that the Embden-Meyerhof pathway was the only significant lactate production pathway, and that the 2,3-diphosphoglycerate pathway (16, 17) was inoperative (see below). Per cent glucose conversion to lactate was obtained from the following formula: (GlucLac − GlycLac × 0.87) × 100/2 Gluc, where GlucLac = lactate production, μmoles/ml per hr in the presence of glucose, 5 mmoles/liter; Gluc = μmoles/ml per hr of glucose uptake; GlycGluc = μmoles/ml per hr of glycogen depletion in the presence of glucose at the end of 1 hr; and 0.87 represents a constant value obtained empirically of the per cent GlycGluc/Glyc. Values for average hourly changes of ATP, ADP, AMP, and Pi were calculated in the following manner: levels at 5, 15, 30, and 60 min were totaled and averaged in the absence of thrombin and compared to the similar average in the presence of thrombin. The difference was taken as the change. In this manner a more composite "integration" of the changes was obtained over the 1 hr period. Values for theoretical ATP generation via the Embden-Meyerhof pathway were calculated from the assumption that 1 glucose-glycogen

TABLE I

Comparison of Glycolytic Changes after Platelet Agglutination

	Glycogen depletion		Glucose uptake	Lactate production		% glycogen → lactate	% glucose → lactate
5 *mmoles/liter* of glucose	0	+	+	0	+	0	+
Control, *μmoles/ml per hr*	17.8	15.6	19.0	20.7	36.6	58%	49%
Increments or decrements							
ADP	4.4	5.4	−4.0	3.3	0.8	54%	63%
Thrombin	8.6	9.7	15.6	8.0	18.7	54%	48%
Epinephrine	8.2	7.7	9.3	8.1	16.4	53%	58%

ADP, adenosine diphosphate.

moiety → 2 lactates → 3 ATPs; and 1 extracellular glucose → 2 lactates → ATPs.

RESULTS

The basal level, 0°C, for platelet glycogen was 31.1 ± 1.0 μmole/ml [1] of packed platelets (17 experiments). This value is similar to that described in primate skeletal muscle (18). In the absence of glucose, glycogen depletion reached 17.8 μmoles/ml per hr (Fig. 1). Lactate production was linear, 20.7 ± 1.3 μmoles/ml per hr (35 experiments). In the presence of 5 mM glucose, lactate production increased to 36.6 ± 3.0 μmoles/ml per hr (24 experiments), and glycogen depletion was spared approximately 13% ($P < 0.02$). Glucose uptake was 19.0 ± 3.5 μmoles/ml per hr (24 experiments). Intracellular glucose was not detectable. From these data (see calculations) it is apparent that approximately 55% of glucose or glycogen is converted to lactate (Table I).

Theoretical ATP generated by the Embden-Meyerhof pathway (glycolytic) was 31 and 48 μmoles/ml, respectively in the absence and presence of glucose at the end of 1 hr. Nevertheless, intracellular ATP levels could not be maintained (Fig. 1); ATP declined from 2.52 to 2.11 μmoles/ml at the end of 1 hr or 0.41 μmoles. Thus compartmentation of ATP storage and utilization is suggested.

Thrombin, ADP, and epinephrine all resulted in increased glycogen depletion and lactate production both in the presence and absence of glucose (Table I). It is again apparent that approximately 55% of glucose or glycogen fuel is converted to lactate.

[1] All ± values refer to standard error of the mean (SEM).

Adenine nucleotide and orthophosphate changes. Basal levels for ATP, ADP, AMP were 2.52 ± 0.11, 1.39 ± 0.06, and 0.36 ± 0.03 μmole/ml of packed platelets for 18, 14, and 17 determinations, respectively. These values are similar to that reported in skeletal muscle and brain tissue (13, 19). If the assumption is made that the adenylate kinase reaction $2\,ADP \rightleftharpoons ATP + AMP$ regulates the ratios of these adenine nucleotides as it does in other tissues, one can calculate the apparent equilibrium constant for this reaction. If one averages the equilibrium constant (Keq) values obtained at 0, 5, 15, 30, and 60 min one obtains a value of 0.57 ± 0.05 which is very similar to that reported by Lowry and associates (13) in adult rat brain tissue.

During control incubation, at the end of 1 hr, intracellular ATP decline averaged 0.19 μmole/ml or 8%, ADP increase averaged 0.08 μmole/ml or 5%, and AMP increase averaged 0.02 μmole/ml or 6%. Extracellular ATP, ADP, and AMP remained fairly constant for the hour of incubation and represented approximately 7, 6, and 31%, respectively, of average total ATP, ADP, and AMP, 4, 2, and 4% of total adenine nucleotide content. Glucose, 5 mmoles/liter had no effect on changes in adenine nucleotides. Thus under control conditions, total adenine nucleotides did not change appreciably and averaged 4.64 μmoles/ml for the varying time intervals. The apparent Keq for adenylate kinase did not change appreciably.

The basal, 0°C, value for Pi was 1.2 ± 0.10 μmole/ml (29 experiments). The average 1-hr control value for Pi was 1.94 μmoles/ml. Addition of 5 mM glucose had a sparing effect on Pi levels, lowering the average 1-hr control value to 1.72 μmoles/ml (Table II). It is of interest to note that

TABLE II

Comparison of Adenine Nucleotide and Orthophosphate Changes during Platelet Agglutination

	ATP		ADP		AMP		Net change	Orthophosphate		Ortho-phosphate Net change
	IC	EC	IC	EC	IC	EC		IC	EC	
Control*	2.33	0.16	1.47	0.10	0.38	0.18		1.94		
(G)								1.72		
Thrombin‡	−1.48	0.80	−1.14	1.03	−0.08	0.24	−0.63	1.57	−3.2	−1.6
(G)								1.14	−7.5	−6.4
Epinephrine	−0.86	0.16	−0.40	0.38	0.23	0.11	−0.34	0.98	−1.6	−0.6
(G)	−0.78	0.16	−0.25	0.38	0.17	0.11	−0.21	0.42	−3.1	−2.7
ADP	−0.79	0.58	−0.39	1.10	0.06	0.76	−0.40	0.56		
(G)								0.45		

ATP, adenosine triphosphate; ADP, adenosine diphosphate; AMP, adenosine monophosphate; IC, intracellular; Ec, extracellular; (G), glucose.

* Control data represent composite average values during hr of incubation.

‡ Values below Control represent increments or decrements of experimental composite average values when compared to composite average control values obtained from the same incubation. Control and thrombin composite averages were obtained from 5-, 15-, 30-, and 60-min time points. (These "averages" did not differ significantly from 30–60-min averages). Epinephrine and ADP composite averages were obtained from 30–60-min time points. Each time point employed in the calculation of these composite averages was obtained from a minimum of four incubations performed in duplicate and assayed in triplicate. SEM was less than 10%. SD was less than 20%.

extracellular Pi declined appreciably resulting in an average Pi decline of 4.6–3.8 μmoles/ml (not shown in table), in the absence and presence of 5 mM glucose, respectively.

Thrombin agglutination and contraction. The kinetics of thrombin-mediated platelet agglutination and contraction were studied at several time intervals. Upon the addition of thrombin, biochemical changes were fairly complete by the first 5 min and then appeared to level off (Fig. 2). Thus for the 1 hr period, intracellular ATP decline averaged 64% or 1.48 μmoles/ml, ADP decline averaged 78% or 1.14 μmoles/ml, and AMP decline averaged 20% or 0.077 μmole/ml (Table II). Extracellular ATP increased 6-fold during the 1 hr period or an average increment of 0.8 μmole/ml; ADP increased 11-fold or an average increment of 1.03 μmoles/ml; and AMP increased 2.3-fold or an average increment of 0.24 μmole/ml. Again, glucose had no effect on changes in adenine nucleotides. The *K*eq for adenylate kinase did not change appreciably and averaged 0.58 ± 0.05. From these data it is suggested that extracellular ATP was converted to ADP + AMP + X. Thus high energy phosphates have been converted to lower energy states with a net loss of 0.63 μmole/ml of adenine nucleotide.

In order to investigate the contribution of Pi to X, changes in true orthophosphate were examined. Net total ATP and ADP loss was 0.79 μmole/ml and may be compared to the intracellular Pi gain of 1.1–1.6 μmoles/ml. Average intracellular Pi declined appreciably, 3.2–7.5 μmoles/ml in the absence and presence of glucose, respectively. Net total Pi change resulted in a loss of 1.6–6.4 μmoles/ml of Pi in the absence and presence of glucose, respectively.

Epinephrine agglutination. Measurements of adenine nucleotide changes during epinephrine-induced platelet agglutination were obtained at 30 and 60 min. Average changes are tabulated in Table II for comparison with thrombin. Contrary to thrombin experiments, addition of 5 mM glucose did have a sparing effect on adenine nucleotide depletion. The adenine nucleotide changes were less than those with thrombin-induced platelet agglutination and contraction for both intra- and extracellular AMP. However, intracellular AMP increased 60% in the absence of glucose and 45% in the presence of glucose compared to a 20%

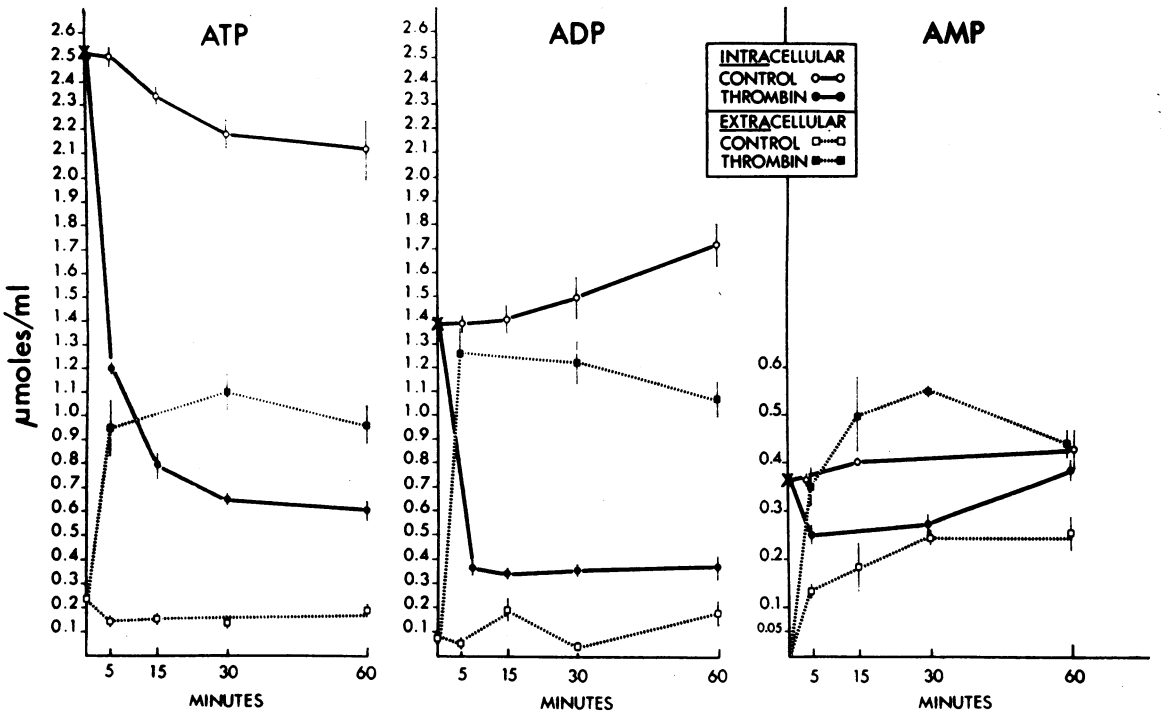

FIGURE 2 Kinetics of intra- and extracellular ATP, adenosine diphosphate (ADP), and adenosine monophosphate (AMP) after thrombin-induced platelet agglutination. Values are expressed as μmoles/ml of platelets. Time points represent incubations from 6–10 experiments, performed in duplicate and assayed in triplicate. SEM is given.

decline in the presence of thrombin. Intracellular ATP and ADP declined 37 and 27% in the absence of glucose and 34 and 17%, respectively, in the presence of glucose. Extracellular adenine nucleotide changes were unaffected by glucose. Thus extracellular ATP, ADP, and AMP increased 2-, 5-, and 1.6-fold, respectively. Again it is suggested that extracellular ATP has been converted to ADP + AMP + X, and that considerable intracellular AMP has been formed from ATP and (or) ADP. The loss of high energy phosphate (nucleotide deficit of 0.21–0.34 μmole/ml) is considerably less than that observed with thrombin. In the absence of glucose there is a net decline of ATP and ADP of 0.72 μmole/ml which is within the range of intracellular Pi gain of 0.98 μmole/ml. In the presence of glucose there is a net loss of ATP and ADP of 0.49 μmole/ml which is again within the range of intracellular Pi gain, 0.42 μmole/ml. Again, average extracellular Pi declined 1.6 and 3.1 μmoles/ml in the absence and presence of glucose. Net change in total Pi resulted in a loss of 0.6 and 2.7 μmoles/ml, respectively, for the absence and presence of glucose.

ADP agglutination. Measurements of ADP-mediated platelet agglutination were obtained at 30 and 60 min and compared to thrombin and epinephrine (Table II). Addition of 5 mM glucose had no sparing effect on adenine nucleotide loss. Intracellular ATP and ADP declined 34 and 27%, respectively. AMP increased 16%. Since ADP was added to the media, extracellular adenine nucleotide changes should be interpreted with caution. Initial extracellular ADP concentration when expressed per milliliter of platelets was 1.74 μmoles/ml. The final average extracellular ADP concentration was 1.10 μmoles/ml. Accordingly, 0.64 μmole/ml of ADP was utilized during ADP-mediated platelet agglutination. The relatively high extracellular AMP of 0.76 μmole/ml is consistent with the extracellular ADP being converted to AMP. If one considers the net addition of 1.74 μmoles/ml of ADP to the system, then a net adenine nucleotide loss of 0.40 μmole/ml is obtained.

Search for 2,3-diphosphoglycerate shunt. An enzymatic technique for the measurement of 2,3-diphosphoglycerate was employed which was sen-

sitive to concentrations of 2×10^{-7} moles/liter and not inhibited by tissue extract. Measurements of platelet extracts revealed concentrations of 0.024 ± 0.001 μmole/ml or 1/200 the concentration found in red blood cells (eight experiments, 32 measurements). These values were not affected by 3-fold changes in glycolytic flux (e.g., control vs. glucose or control vs. glucose plus thrombin after an hour of incubation at 37°C). Since the content of red blood cell 2,3-diphosphoglycerate is so high, e.g., 3.5–4.4 μmoles/ml of red blood cells (20), red blood cell contamination in the order of 1 red blood cell per 1000 platelets could account for platelet values of 0.02 μmole/ml. When considerable precaution [2] was taken to avoid red blood cell contamination, 2,3-diphosphoglycerate was not measureable, e.g., the tissue concentration was less than 4.5×10^{-6} moles/liter (three experiments, nine measurements).

DISCUSSION

The human platelet is a predominantly aerobic glycolytic tissue (1). Its high aerobic glycolytic rate is 13 times that of human red blood cells (20) and 4.7 times that of primate skeletal muscle (18). Approximately 55% of glucose or glycogen was converted to lactate. There was no appreciable change in per cent of conversion after platelet agglutination and contraction induced by ADP, thrombin, and epinephrine. The theoretical contribution of the Embden-Meyerhof pathway towards the generation of ATP was 31 and 48 μmoles/ml per hr in the absence and presence of glucose, respectively. It should be recognized that these calculations are based on the assumption that glycolytic flux through the 2,3-diphosphoglycerate shunt is negligible. A significant accumulation of 2,3-diphosphoglycerate was not observed, as is the case with red blood cell (20). Indeed the minute concentration found may be attributed to red blood cell contamination. This suggests that the utilization of such a shunt pathway as an "energy clutch" (20) is negligible or absent. Accordingly, this assumption made for the calculation of Embden-Meyerhof ATP generation is justified.

[2] Platelet-rich plasma was first centrifuged at 320 *g* for 30 min. The supernatant plasma was then recentrifuged at 320 *g* for 10 min. This supernatant plasma was again recentrifuged at 385 *g* for 7.5 min. The supernatant material was then spun at 2000 *g* for 30 min (see Methods).

Haslam (5) reported enzymatic measurements of ATP and ADP after exposure to thrombin and concluded that ADP release into the media could not be accounted for by the intracellular ATP loss. In this respect, our observations are in agreement. Decrease in intracellular ATP levels after exposure to thrombin has also been reported by Born (2), Bettex-Galland and Luscher (3), Zucker and Borrelli (4), and Karpatkin (1). However, quantitative enzymatic measurements have not been available on intra-and extracellular adenine nucleotide kinetics after exposure to thrombin, epinephrine, and ADP.

Our data reveal that a washed platelet system is adequate for measurements of adenine nucleotides since during 1 hr of control incubation total adenine nucleotides were conserved. Furthermore, the apparent *K*eq for adenylate kinase was unaltered during control conditions and exposure to thrombin.

Thrombin, epinephrine, and ADP were in some respects similar in their effects on platelet glycogenolysis and glycolysis. Thrombin had the most pronounced effect on glycogenolysis, lactate production, and glucose uptake, whereas epinephrine and ADP had a more moderate effect. All three agents decreased intracellular ATP and ADP and increased extracellular ATP, ADP, and AMP. These measurements suggest disruption of the internal platelet environment after agglutination and contraction with the release of high energy phosphate as well as conversion of high energy phosphate to lower energy states. Epinephrine resulted in increased intracellular AMP levels and in this respect behaved differently from thrombin or ADP. It is apparent that enzymes are available for the extracellular conversion of ATP and ADP to lower energy states and Pi. This fact is particularly evident after the addition of ADP to the media when a considerable per cent of this agent is converted to AMP. Of interest are the observations of Salzman, Chambers, and Neri (21), and of Spaet and Lejnieks (22) which indicate the presence of enzymes in platelet-poor plasma which are capable of converting ADP to AMP and to adenosine. The latter authors have also demonstrated the presence of platelet enzymes capable of converting extracellular ADP to ATP and AMP. The contribution of conversion to inosine mono-

phosphate (IMP) and adenosine was not investigated in our studies. However this contribution may be implied from the net loss in adenine nucleotide affected by the agents tested. It is apparent that both epinephrine and thrombin result in the extracellular release of sufficient ADP over the 1 hr period to give increments of 1 and 0.4 mM ADP, respectively, per milliliter of platelets (total values of 1.13 and 0.48 mM ADP). When corrected for physiological concentrations of platelets these values become 2.8 and 1.2×10^{-6} moles/liter, respectively. These concentrations are 10–30 times that required for ADP induced platelet agglutination (6). Since AMP and adenosine have been shown to be competitive inhibitors (23) of ADP agglutination, their concentrations must also be considered in considering the ADP effect on agglutination.

Changes in adenine nucleotides have been shown to be intimately related to the regulation of glycolysis in other tissues by their allosteric effects on regulator enzymes. This has been referred to as the adenylate control hypothesis (24). If similar assumptions are made for these enzymes in human platelets, then reasonable correlations can be noted between the observed and predicted glycogenolytic and glycolytic rates. There are three regulator enzymes which have been universally shown to be extremely sensitive to changes in adenine nucleotides as well as certain glycolytic intermediates. (*a*) Phosphorylase *b* exists as an inactive dimer in skeletal muscle, as well as human platelets (25–27), requiring AMP for activation and Pi as one of its substrates. Under appropriate conditions the enzyme from both tissues can undergo conversion into an active form (phosphorylase *a*) (25–27). A change from the inactive *b* to the active *a* has been noted in skeletal muscle after muscle contraction (25) or exposure to epinephrine but has not been noted in human platelets (26, 27) after agglutination and contraction induced by thrombin or epinephrine. However, phosphorylase from both tissues in inhibited by ATP and ADP (26–28). Accordingly, a decrease in intracellular ATP and ADP with an associated increase in Pi (noted with all three agents) would result in increased phosphorylase activity and glycogenolysis as was the case. (*b*) Hexokinase requires ATP for one of its substrates and is inhibited by ADP and glucose-6-*P* (29, 30). The inhibition by glucose-6-*P* may be overcome by the presence of Pi (31, 32). Glucose-6-*P* does not change appreciably with all three agents[3] (1). Although a decrease in ATP to intracellular water levels of 1.5–2.8 mmoles/liter was noted, this may have been counterbalanced by the increase in Pi and decrease in inhibitory ADP level. Furthermore, since the K_m for ATP for most hexokinases is in the range of 0.3–1.5 mmoles/liter (32–34) this would predict a minimum of $\frac{1}{2} V_{max}$ for these substrate concentrations, providing other effectors were not operating. (*c*) Phosphofructokinase is an enzyme which again requires ATP for one of its substrates but which has a low ATP K_m, 0.1 mmole/liter (35) and is extremely sensitive to inhibition by relatively high concentrations of ATP, e.g., that present in the cell (36). This enzyme would thus increase in activity with decreasing ATP. Reversal of this ATP inhibition is extremely sensitive to Pi and AMP (35).

Thus epinehrine would have been very effective in increasing total phosphorylase activity as well as phosphofructokinase activity since AMP increased considerably. Thrombin would have been very effective in increasing total phosphorylase activity and perhaps hexokinase activity since Pi increased considerably. ADP resulted in small adenylate changes compared with thrombin but did not appreciably increase AMP levels as did epinephrine. Consequently one might predict from these data only moderate glycogenolytic and glycolytic changes with ADP, as was the case. The 13% sparing effect noted with glucose on the rate of glycogenolysis during control conditions might be attributed to the decrease in intracellular Pi of 11%.

Thrombin, an agent which results in severe distortion and disorganization of the platelet internal structure, had the most pronounced effects glycolytically.[4] ADP which has a minimal disorganiza-

[3] In a previous report (1) glucose-6-*P* levels did not change with ADP or thrombin but did increase with epinephrine. These measurements were made with extraction procedures at 4°C. When this was performed with liquid nitrogen frozen pellets −196°C, glucose-6-*P* levels did not change with either ADP, thrombin, or epinephrine.

[4] With respect to thrombin, it is conceivable that proteolytic degradation or activation of platelet surface enzymes may also be operative in the regulation of adenine nucleotide kinetics. In this regard, preliminary observa-

tional effect (37, 38) on platelet structure had a mild glycolytic and glycogenolytic effect. Epinephrine, which macroscopically and electron microscopically appears to have an effect similar to ADP,[5] did however result in more moderate glycolytic and glycogenolytic changes. This might be attributed to the rise in AMP noted in contrast to the decline with thrombin which could have resulted in a stronger activation of phosphorylase and phosphofructokinase.

It is intriguing to note similarities between platelets and skeletal muscle. Both tissues have considerable stores of glycogen and high energy phosphate. Both have high glycolytic rates and undergo active contraction during the performance of their physiologic function. The contractile protein of platelets, thrombosthenin (8), is a calcium-magnesium dependent ATPase and has properties similar to that of actomyosin of skeletal muscle. Although it has not been proven that thrombosthenin is required for platelet contraction the obvious similarities and association with skeletal muscle actomyosin becomes apparent (8). In this respect, an ATPase (39) has been localized by specific histochemical techniques (39) as well as kinetic measurements (40, 41) to the outer platelet membrane. Furthermore, thrombosthenin has also been localized to the outer platelet membrane by specific antibody techniques (41, 42). Our studies indicate that active "ATPases" as well as "ADPases" are sufficiently close to the extracellular environment to result in considerable flux from one high energy state to another.

It is conceivable that thrombosthenin may be the ATPase which is activated (1), resulting in the release of energy for the maintenance of ionic gradients as well as the performance of platelet function. It is also plausible that platelets require energy to maintain their discoid shape. This could all be reflected by a continuous utilization and recycling of ATP to provide energy for the maintenance of the platelet integrity as well as performance of platelet function. Our data on theoretical ATP generated reveal a considerable quantity of ATP generated with no increment in ATP stores. This must reflect a high rate of ATP utilization. A clue may be obtained from the observation of considerable extracellular Pi disappearance during incubation. This Pi could have been utilized for the synthesis of ATP from ADP via the Embden-Meyerhof pathway or the Krebs cycle.

It is also conceivable that platelet membrane phospholipid may be turning over at a rapid rate in order to repair continual breaks and tears in the membrane. Thus phospholipid synthesis (43) could account for unexplained ATP utilization and extracellular decrement of Pi. It is recognized that membrane injury may be more prevalent when washed platelets are employed; however, we have no evidence for this.

Agents which result in tears to the platelet membrane can also sufficiently disrupt the internal environment so as to result in release of stored energy. The consequent metabolic depletion and physical disintegration of the platelet, most obvious with thrombin, epinephrine, and ADP, may take place at a more subtle level in vivo secondary to other unknown agents or stimuli.

tions (unpublished data) have revealed that thrombin inhibits the production of extracellular AMP from extracellular ADP by 60% during a 1 hr incubation period at 37°C.

[5] White, J. G. Personal communication.

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