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THE EFFECT OF AGGREGATING AGENTS ON OXIDATIVE METABOLISM OF RABBIT PLATELETS

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SUMMARY

The oxidation of [6-^{14}C]glucose to $^{14}CO_2$ was studied with substrate-depleted suspensions of washed rabbit platelets aggregated by ADP, polylysine or thrombin. In these platelet suspensions, oxidation and phosphorylation were tightly coupled. ADP, polylysine, and thrombin caused increased oxidation of glucose, detectable within 1 min of the addition of the stimulus and lasting for a period of 12 to 16 min.

A lack of calcium in the suspending medium prevented ADP- or thrombin-induced aggregation but did not inhibit the platelet shape change or the increased $^{14}CO_2$ production caused by these agents. Compounds that inhibited both aggregation and the platelet shape change induced by ADP caused the greatest inhibition of $^{14}CO_2$ production; these inhibitors were prostaglandin E_1, dibutyryl cyclic AMP, AMP, adenosine, or high Mg^{2+} concentration. Therefore increased oxidation of glucose was not due solely to platelet aggregation, but also appeared to be related to the shape change induced by the aggregating agents and to the release reaction induced by thrombin.

INTRODUCTION

A number of investigators have shown that both ADP-induced platelet aggregation and the release reaction require a source of metabolic energy[1–4]. There has been some uncertainty about the relative importance of glycolysis and oxidative phosphorylation in platelet metabolism[5,6], but it has been observed that inhibitors of oxidative phosphorylation alone can prevent the restoration of rabbit platelet sensitivity to aggregating stimuli that is obtained upon the addition of glucose to platelet suspensions that have become unresponsive to the stimuli because of a lack of glucose[3,4]. Several studies have shown that washed human platelets oxidize [6-^{14}C]glucose to $^{14}CO_2$ and that when the platelets are exposed to stimuli such as thrombin, ADP or latex particles, the amount of $^{14}CO_2$ produced is increased[7–9]. However, in these studies long incubation periods were used and it is difficult to relate the increase in $^{14}CO_2$ production to platelet aggregation and the release reaction.

Abbreviation: HEPES, *N*-hydroxyethylpiperazine-*N*-2-ethanesulfonic acid.

Since washed rabbit platelets deprived of glucose, that have lost their sensitivity to ADP or release-inducing stimuli, can be restored by the addition of glucose[3,4], it seemed possible that rabbit platelet suspensions could be used to examine the relationship of glucose oxidation to platelet function. The purpose of the experiments reported in this paper was to establish a method for studying the oxidation of [6-^{14}C]-glucose over a comparatively short time interval following the addition of ADP or thrombin to suspensions of washed platelets from rabbits. In these studies, we have attempted to determine whether the ADP-induced increase in $^{14}CO_2$ production is related to the change in platelet shape and/or to platelet aggregation. We have, in addition, examined thrombin-induced release of platelet constituents to determine whether increased $^{14}CO_2$ production is related to aggregation, shape change, and the release reaction or is independent of these reactions.

MATERIALS AND METHODS

Preparation of platelet suspensions

Blood was collected from rabbits as previously described[4]. The platelets were separated and washed according to the method of ARDLIE *et al.*[10], except that glucose was omitted from the washing and suspending media to facilitate depletion of endogenous substrate. In addition, 5 mM HEPES buffer (*N*-hydroxyethylpiperazine-*N*-2-ethanesulfonic acid), pH 7.35 was included in the suspending medium to prevent pH changes which were found to occur during the depletion period due to extensive lactic acid production. The suspensions were stored at room temperature in the absence of added calcium until there was no demonstrable aggregation on the addition of ADP (1 μM) and calcium (0.3 mM). This took approximately 1 h. With these preparations the addition of small quantities of glucose (167 μM) at this stage restored platelet responsiveness to ADP or other stimuli.

Platelet aggregation

Platelet aggregation was studied using a turbidimetric method wihch has been described[11].

Incubation with [6-^{14}C]glucose and the estimation of $^{14}CO_2$ released

Incubations were carried out in duplicate in wide-mouthed counting vials (24 mm outer diameter) fitted with 1 ml beakers suspended by wires from the top. Into each vial was pipetted 0.5 ml of an incubation mixture consisting of Tyrode's solution (pH 7.35) modified so that the final concentration of calcium during incubation was 0.3 mM and containing 5 mM HEPES buffer (pH 7.35), 0.35 % albumin, and [6-^{14}C]glucose (250 nmoles, 0.2 μC). One ml of platelet suspension containing approximately 10^9 platelets was added to each vial in turn at one minute intervals and after gentle mixing each vial was tightly stoppered with special rubber stoppers supplied by Kontes Glass Co. (Vineland, N. J.) to provide a closed system. Incubations were carried out with gentle agitation in a water bath maintained at 37°. 30 sec before the end of each incubation period 0.3 ml of NCS (Nuclear Chicago Solubilizer) was injected by syringe through the stopper into the suspended glass beaker. Each incubation was terminated by the injection of 0.5 ml of 2 M perchloric acid (0.5 M, final concentration) into the contents of the incubation vial. The radioactive CO_2

released was collected as the [^{14}C]carbamate using the NCS CO_2 trap. Diffusion was carried out at 37° for 1.5 h with shaking and at the end of this period the NCS was transferred to a standard toluene-fluor solution. Radioactivity was determined using a Phillips liquid scintillation analyzer and disint./min values were obtained by an external standard channels ratio method. Experiments with Na_2 $^{14}CO_3$ showed that these procedures gave quantitative $^{14}CO_2$ recovery. All samples were corrected for non-specific $^{14}CO_2$ production by including in each experiment duplicate vials which contained Tyrode's solution instead of platelets. The $^{14}CO_2$ produced by these samples rarely exceeded 5 % of the experimental values.

Incubations with [6-^{14}C]glucose in the presence of aggregating stimuli or inhibitors

Stimuli such as ADP, thrombin or polylysine were added to the vials by injection with syringe through the rubber stoppers and immediately thoroughly shaken. These additions were routinely made after the platelets had been incubating with [6-^{14}C]glucose for 4 min. Experiments have shown that by this time the rate of $^{14}CO_2$ production is linear.

Inhibitors were added at the beginning of the experimental period unless otherwise indicated. The appropriate solvent solution was added to the control vials.

Materials

ADP, AMP and adenosine were obtained from Sigma Chemical Corp., St. Louis, Mo. They were dissolved in glucose-free Tyrode's solution (pH 7.35) and the pH was adjusted to pH 7.35. Crude bovine thrombin was from Parke Davis and Co., Detroit, Mich., and was dissolved in distilled water to a final concentration of 500 units/ml, lyophilized and kept frozen. Poly-DL-lysine (mol. wt. 15000–20000) was obtained from Miles Laboratories, Elkhart, Indiana. Both the thrombin and polylysine were dissolved as required in glucose-free Tyrode's solution. Prostaglandin E_1 was generously supplied by the Upjohn Co., Kalamazoo, Mich., and a stock solution was prepared by dissolving 1 mg amounts in 0.1 ml 95 % ethanol and adding 0.9 ml of 0.02 % Na_2CO_3 solution. Stock solutions were stored at −20° and were diluted with glucose-free Tyrode's solution immediately before use. Dibutyryl cyclic AMP was obtained from Sigma Chemical Corp.

Antimycin A, Type III and oligomycin (15 % oligomycin A, 85 % oligomycin B) were obtained from Sigma Chemical Corp., and were dissolved and diluted in 95 % ethanol. 2,4-Dinitrophenol was dissolved in 95 % ethanol and diluted in glucose-free Tyrode's solution. HEPES buffer was from Sigma Chemical Corp. [6-^{14}C]Glucose was purchased from New England Nuclear Corp., Boston, Mass.

All concentrations given in the text are final values.

RESULTS

The oxidation of [6-^{14}C]glucose by suspensions of rabbit platelets

In preliminary experiments in which rabbit platelets were washed in the presence of 5.5 mM glucose and resuspended without added glucose, barely detectable quantities of $^{14}CO_2$ were formed when these suspensions were incubated for 10 min with [6-^{14}C]glucose (167 μM, 0.2 μC). It was found, however, that if glucose were omitted from both the washing and resuspending media and the final platelet suspen-

sions were stored at room temperature in the absence of glucose for 1 h, the formation of $^{14}CO_2$ from [6-^{14}C]glucose (167 μM, 0.2 μC) was sufficient to permit the study of citric acid cycle activity over relatively short periods of time (Fig. 1). Platelet suspensions prepared in this way were unresponsive to ADP[4], but the addition of small quantities of glucose (Fig. 1) restored responsiveness[4]. After the addition of [6-^{14}C]-glucose to these suspensions there was an initial lag phase in $^{14}CO_2$ production al-

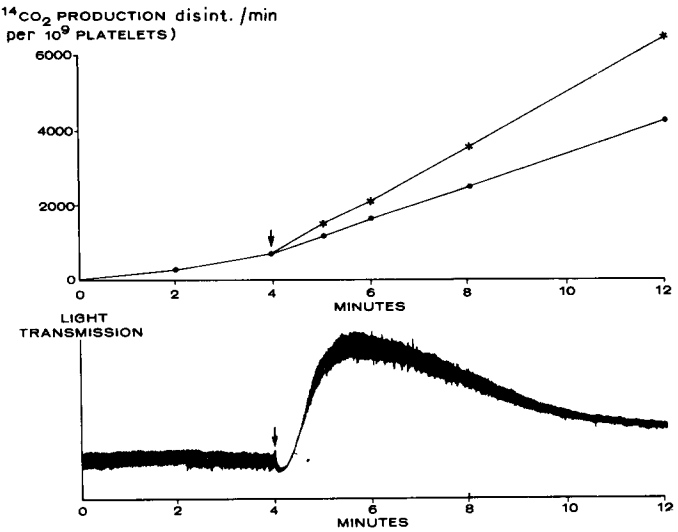

Fig. 1. Upper portion: Effect of ADP on the oxidation of [6-^{14}C]glucose by washed rabbit platelets. ●—●, tyrode; or ×—×, ADP (10 μM) added (as indicated by the arrow) 4 min after the commencement of the incubation with [6-^{14}C]glucose. Lower portion: Aggregation of the same platelet suspension following the addition of the same concentration of ADP.

TABLE I

EFFECT OF INHIBITORS OF OXIDATIVE PHOSPHORYLATION ON [6-^{14}C]GLUCOSE OXIDATION BY RABBIT PLATELETS *

Inhibitor added	*Concentration of inhibitor (μM)*	*$^{14}CO_2$ produced from [6-^{14}C]glucose in 10 min (disint./min)*	*% of control*
None	—	1806	100
Antimycin	0.03	212	12
	0.3	42	2.3
Oligomycin	0.03	488	27
	0.3	74	4.0
	3.0	49	2.7
Dinitrophenol	3.0	1967	109
	30	2507	139
	230	3553	198

* This is one of 3 similar experiments.

though within 4 min $^{14}CO_2$ formation became linear (Figs. 1–8 control values). The rate at which these platelets oxidized added glucose was determined in 30 experiments using the linear portion of the $^{14}CO_2$ production curves and the specific activity of the added [6-^{14}C]glucose. The rates ranged from 4.1 to 16.2 nmoles glucose oxidized/h per 10^9 platelets with an average of 7.2 and a median value of 6.2.

The effect of metabolic inhibitors on [6-^{14}C]glucose oxidation

The extent of [6-^{14}C]glucose oxidation by these platelet suspensions was affected by several inhibitors of oxidative phosphorylation (Table I). Antimycin, which blocks the electron transport chain, and oligomycin, which blocks a final phosphorylation step in ATP formation, almost completely inhibited [6-^{14}C]glucose oxidation. Dinitrophenol, an uncoupler of oxidative phosphorylation, stimulated $^{14}CO_2$ production. Moreover, the addition of dinitrophenol to oligomycin-inhibited platelets largely overcame the oligomycin inhibition (Fig. 2). This effect could be demonstrated both when dinitrophenol was added at the same time as the oligomycin, and when it was added 4 min after oligomycin (Fig. 2).

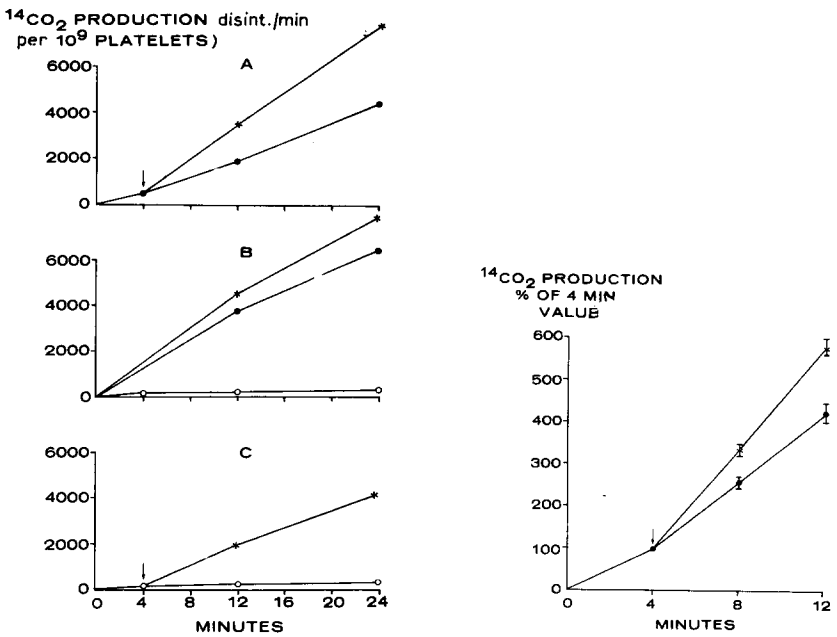

Fig. 2. Effect of inhibitors of oxidative phosphorylation on the oxidation of [6-^{14}C]glucose by washed rabbit platelets. (A) ×—×, dinitrophenol (0.2 mM) added 4 min after commencement of the incubation with [6-^{14}C]glucose; ●—●, Tyrode. (B) Effect of dinitrophenol and/or oligomycin added at the beginning of the incubation with [6-^{14}C]glucose. ○—○, oligomycin (0.1 μM); ×—×, dinitrophenol (0.2 mM); ●—●, oligomycin (0.1 μM) *plus* dinitrophenol (0.2 mM). (C) Effect of dinitrophenol on oligomycin-inhibited platelets when added 4 min after the beginning of the incubation with [6-^{14}C]glucose. ○—○, oligomycin (0.1 μM); ×—×, oligomycin (0.1 μM) added at the beginning of the incubation *plus* dinitrophenol (0.2 mM) added as indicated by arrow.

Fig. 3. Effect of ADP on the oxidation of [6-^{14}C]glucose by washed rabbit platelets. Values are the means ± S.E. of 41 experiments. In each experiment $^{14}CO_2$ production at 4 min was assigned a value of 100%. ●—●, Tyrode; or ×—×, ADP (1 μM) was added at 4 min (arrow).

The effect of ADP on [6-^{14}C]glucose oxidation

The addition of ADP at a concentration which caused platelet aggregation resulted in an increase in the oxidation of glucose-6-^{14}C by these platelet suspensions (Figs. 1 and 3). In the 41 experiments in which the effect of 1 μM ADP was studied, its addition caused an average stimulation of 30 % after 4 min and 36 % after 8 min (Fig. 3). Higher concentrations of ADP caused a greater conversion of [6-^{14}C]glucose to $^{14}CO_2$. For example, in 3 experiments in which the effects of 2 concentrations of ADP were compared, 1 μM caused an average stimulation of $^{14}CO_2$ production of 22 % after 4 min with ADP and a stimulation of 44 % after 8 min. The corresponding mean values observed with 10 μM ADP were 46 % and 59 %.

The effect of ADP on [6-^{14}C]glucose oxidation appeared to be of limited duration since after approximately 12 to 16 min in the presence of ADP, $^{14}CO_2$ production returned to the original rate (Fig. 4). The increased rate of [6-^{14}C]glucose oxidation in the presence of ADP continued, however, well beyond the time when both platelet aggregation and deaggregation had occurred (Fig. 1).

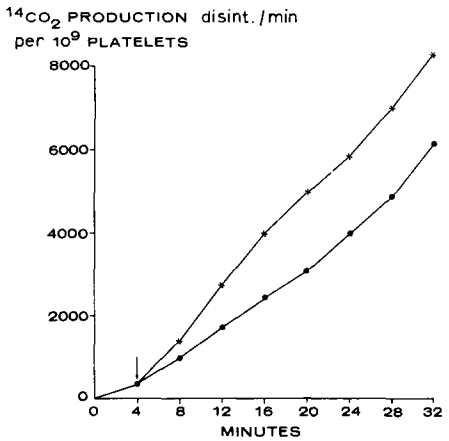

Fig. 4. Longer term effects of ADP on the oxidation of [6-^{14}C]glucose by washed rabbit platelets. Values are the average of 2 experiments. ●—●, Tyrode; ×—×, ADP (1 μM) added at 4 min (arrow).

In the absence of added calcium, the addition of ADP to a platelet suspension prepared as outlined was followed by an initial decrease in light transmission. This is generally taken to indicate that platelet shape change has occurred[6,12]. Aggregation was not observed under these conditions, but did occur in the presence of added calcium (0.3 mM). In both cases, however, a burst in [6-^{14}C]glucose oxidation was observed upon the addition of ADP (Table II, Expt. A).

At high magnesium concentrations (10 mM) and in the absence of added calcium, little shape change was observed upon the addition of 1 μM ADP[13] and no stimulation of [6-^{14}C]glucose oxidation occurred. (Table II, Expt. B). If 100 μM ADP were used under these conditions, however, platelet aggregation did take place and stimulation of [6-^{14}C]glucose oxidation was observed (Table II, Expt. C).

Prostaglandin E_1 (0.1 mM)[14], AMP (2 mM)[12,15], and dibutyryl cyclic AMP (2 mM)[16], at concentrations that completely inhibit ADP-induced aggregation and

TABLE II

EFFECT OF Mg^{2+} AND Ca^{2+} CONCENTRATIONS ON THE ADP-INDUCED STIMULATION OF [6-^{14}C]GLUCOSE OXIDATION

Expt.	*Additions to platelet suspensions (final concentration)*			*$^{14}CO_2$ production from [6-^{14}C]glucose (disint./min)* *				*Stimulation of $^{14}CO_2$ production on addition of ADP* (% of corresponding control)*	
				8 min		*12 min*			
	Mg^{2+} (mM)	*Ca^{2+} (mM)*	*ADP** (μM)*	*Control*	*+ADP*	*Control*	*+ADP*	*8 min*	*12 min*
A (2)***	1	0	1	2184	2947	3278	4958	135	151
	1	0.3	1	2458	3505	3930	5238	142	133
B (3)	1	0	1	1576	2076	2461	3053	131	124
	10	0	1	1412	1360	2415	2385	96	99
C (2)	1	0	100	1014	1247	1718	2783	123	162
	10	0	100	997	1278	1833	2328	128	127

* Mean values.
** Not added to controls.
*** The value in parentheses indicates the number of experiments which have been averaged.

shape change, decreased the stimulation of $^{14}CO_2$ production from [6-^{14}C]glucose-observed on the addition of ADP alone (Table III). Even at lower inhibitor concentrations, some reduction of ADP-induced stimulation of $^{14}CO_2$ production was apparent, although platelet shape change was evident. Adenosine, which generally is not a good inhibitor of the platelet shape change[12,15], did inhibit the ADP-induced shape change at the concentration (1.0 mM) and under the conditions used in these experiments. This concentration of adenosine inhibited the ADP-induced stimulation of $^{14}CO_2$ production (Table III). It should be noted that, with the exception of AMP, the inhibitors themselves reduced $^{14}CO_2$ production by platelets that were not exposed to ADP.

The effect of thrombin on [6-^{14}C]glucose oxidation

The addition of low concentrations of thrombin to rabbit platelet suspensions led to a stimulation of [6-^{14}C]glucose oxidation (Figs. 5 and 6). In 9 experiments in which the thrombin concentration was 0.006 unit/ml, a mean stimulation of 45 % was observed after 4 min in the presence of thrombin and a 46 % stimulation was noted after 8 min.

Adenosine, prostaglandin E_1 and dibutyryl cyclic AMP at concentrations which completely inhibited thrombin-induced platelet aggregation, decreased the stimulation of [6-^{14}C]glucose oxidation which occurred on the addition of thrombin alone (Table IV). At the concentrations of inhibitors used, however, some stimulation of glucose oxidation was always observed and under these conditions, platelet shape change also occurred.

In the presence of 0.1 mM EDTA the addition of thrombin (0.006 unit/ml) caused platelet aggregation but with 1 mM EDTA, only platelet shape change was observed. Similar increases in [6-^{14}C]glucose oxidation, however, were observed under both conditions (Fig. 7).

TABLE III

EFFECT OF INHIBITORS OF PLATELET AGGREGATION ON THE ADP-INDUCED STIMULATION OF [6-^{14}C]GLUCOSE OXIDATION

Expt.	*Additions to platelet suspension (final concentration)*	*Aggregation on addition of ADP*	*Shape change*** *on addition of ADP*	$^{14}CO_2$ *production from [6-^{14}C]glucose (disint./min)*		*Stimulation of $^{14}CO_2$ production on addition of ADP (% of control)**	
				8 min	*12 min*	*8 min*	*12 min*
A (2)**	Tyrode			914	1412		
	ADP, 1 μM	+	+	1175	2120	129	150
	Adenosine, 0.01 mM			811	1358		
	Adenosine, 0.01 mM *plus* ADP, 1 μM	−	+	967	1521	119	112
	Adenosine, 1.0 mM			870	1327		
	Adenosine, 1.0 mM *plus* ADP, 1 μM	−	−	856	1531	99	116
B (4)	Tyrode			1394	2439		
	ADP, 1 μM	+	+	1959	3307	141	136
	Prostaglandin E_1, 0.05 μM			1322	2127		
	Prostaglandin E_1, 0.05 μM *plus* ADP, 1 μM	−	+	1709	2527	129	119
	Prostaglandin E_1, 100 μM			1279	2226		
	Prostaglandin E_1, 100 μM *plus* ADP, 1 μM	−	−	1388	2359	108	106
C (2)	Tyrode			2591	4058		
	ADP, 1 μM	+	+	3352	5786	129	142
	AMP, 2 mM			2674	4182		
	AMP, 2 mM *plus* ADP, 1 μM	−	−	2694	4631	101	111
D (1)	Tyrode			1314	2205		
	ADP, 1 μM	+	+	1762	2866	134	130
	Dibutyryl cyclic AMP, 2 mM			1213	1895		
	Dibutyryl cyclic AMP, *plus* ADP, 1 μM	−	−	1278	2064	105	109

* Disint./min obtained with controls containing all additions except ADP were assigned a value of 100%.

** The number of experiments in which similar results were obtained is indicated in parentheses.

*** + indicates a decrease in light transmission following the addition of ADP. − indicates no change.

TABLE IV

EFFECT OF INHIBITORS OF PLATELET AGGREGATION ON THE THROMBIN-INDUCED STIMULATION OF [6-^{14}C]GLUCOSE OXIDATION

Expt.	*Additions to platelet suspension (final concentration)*	*Aggregation on addition of thrombin*	*Shape change on addition of thrombin*	$^{14}CO_2$ *production from [6-*^{14}*C]glucose (disint./min)*		*Stimulation of* $^{14}CO_2$ *production on addition of thrombin (% of control)* *	
				8 min	*12 min*	*8 min*	*12 min*
A (2)**	Tyrode			1900	3403		
	Thrombin, 0.026 unit	+	+	2464	4803	130	141
	Adenosine, 0.1 mM			1746	2808		
	Adenosine, 0.1 mM *plus* thrombin 0.026 unit	−	+	2092	3767	120	134
	Adenosine, 1.0 mM			1746	2808		
	Adenosine, 1.0 mM *plus* thrombin 0.026 unit	−	+	1957	3355	111	122
B (2)	Tyrode			1374	2291		
	Thrombin, 0.05 unit	+	+	1706	3132	125	137
	Prostaglandin E_1, 1 μM			1146	1845		
	Prostaglandin E_1, 1 μM *plus* thrombin 0.05 unit	−	+	1253	2101	110	114
	Prostaglandin E_1, 10 μM			1277	1826		
	Prostaglandin E_1, 10 μM *plus* thrombin 0.05 unit	−	+	1329	2148	104	118
C (1)	Tyrode			2666	4384		
	Thrombin 0.01 unit	+	+	3560	6230	133	142
	Dibutyryl cyclic AMP 2 mM			1424	3268		
	Dibutyryl cyclic AMP 2 mM *plus* thrombin 0.01 unit	−	+	1685	4140	118	126

* Disint./min obtained with controls containing all additions except thrombin were assigned a value of 100%.

** The number of experiments in which similar results were obtained is indicated in parentheses.

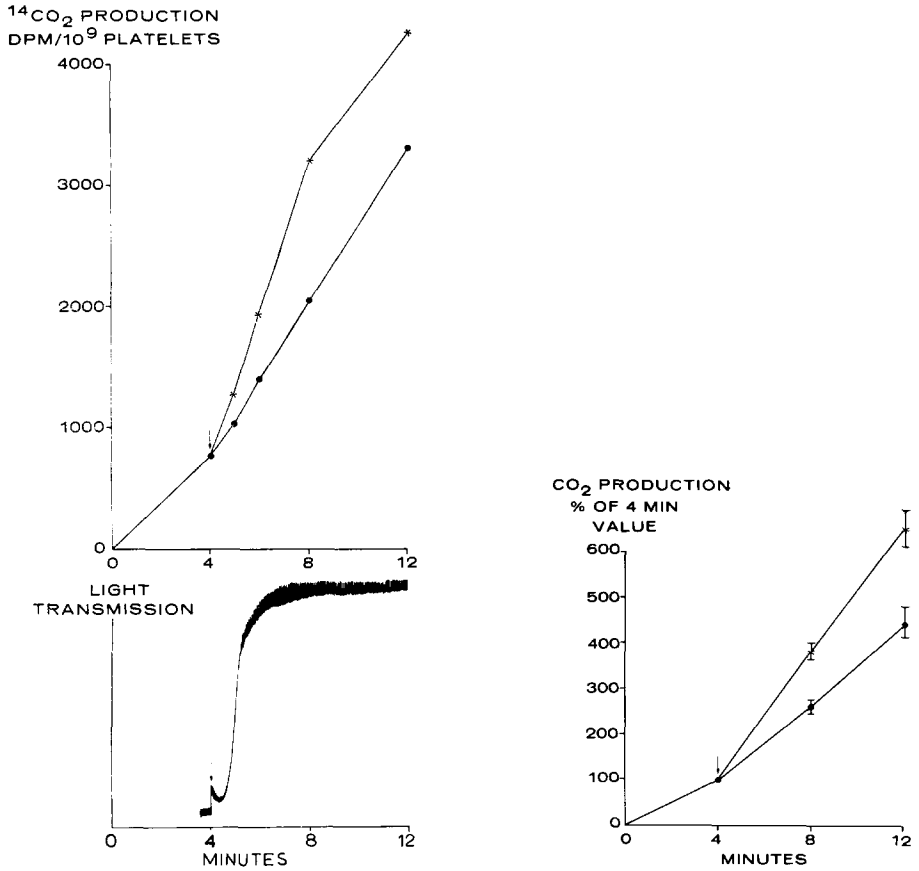

Fig. 5. Upper portion: Short term effect of thrombin on [6-^{14}C]glucose oxidation by washed rabbit platelets. ●—●, Tyrode; or ×—×, thrombin (0.033 unit/ml) added at 4 min (arrow). Lower portion: Aggregation of the same platelet suspension following the addition of the same concentration of thrombin.

Fig. 6. Effect of thrombin on [6-^{14}C]glucose oxidation by washed rabbit platelets. Values are the means ± S.E. of 9 experiments. In each experiment $^{14}CO_2$ production at 4 min was assigned a value of 100%. ●—●, Tyrode; or ×—×, thrombin (0.006 unit/ml) added at 4 min (arrow).

The effect of polylysine on [6-^{14}C]glucose oxidation

Polylysine (0.06 mg/ml) caused rapid and extensive platelet aggregation and also stimulated [6-^{14}C]glucose oxidation (Fig. 8). In 8 experiments in which this concentration of polylysine was added, an average stimulation of 20 % was observed after 4 min in the presence of polylysine and an average stimulation of 40 % was observed after 8 min.

DISCUSSION

The suspensions of washed rabbit platelets used in the present study to investigate oxidative metabolism afford several advantages over preparations previously used for this purpose[7,8,17]. In the substrate-depleted platelets used in our experiments

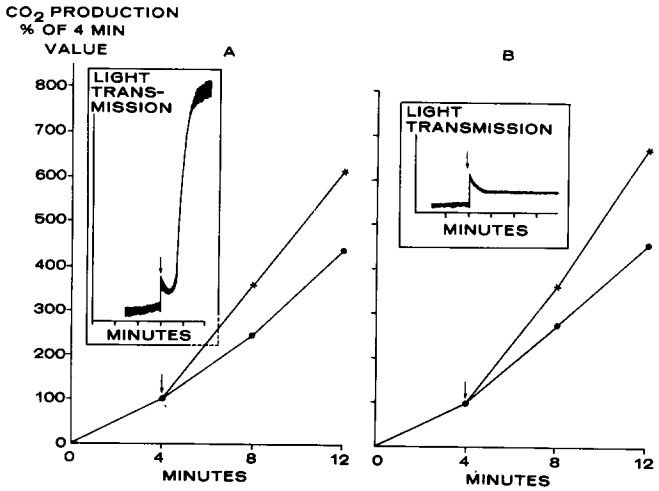

Fig. 7. Effect of EDTA on thrombin-induced stimulation of [6-^{14}C]glucose oxidation. (A) EDTA (0.1 mM) at a concentration at which aggregation occurred (see inset). ●—●, Tyrode; or ×—×, thrombin (0.006 unit/ml) added after 4 min (arrow). (B) EDTA (1 mM) at a concentration which prevented aggregation but not shape change (see inset). ●—●, Tyrode; or ×—×, thrombin (0.006 unit/ml) added after 4 min (arrow).

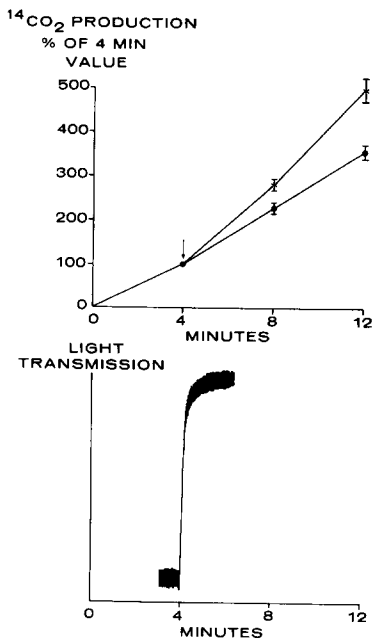

Fig. 8. Upper portion: Effect of polylysine on [6-^{14}C]glucose oxidation by washed rabbit platelets. Values are the mean ± S.E. of 8 experiments. In each experiment $^{14}CO_2$ production at 4 min was assigned a value of 100%. ●—●, Tyrode; or ×—×, polylysine (0.06 mg/ml) was added at 4 min (arrow). Lower portion: Platelet aggregation following the addition of polylysine (0.06 mg/ml) under identical conditions.

sufficient $^{14}CO_2$ is evolved from [6-^{14}C]glucose to make it possible to detect changes in the activity of the citric acid cycle over relatively short time intervals. In addition, a linear rate of $^{14}CO_2$ production is established by 4 min. In contrast, in studies with washed human platelets reported by other investigators[7,8,17], incubations of at least 30 min with [6-^{14}C]glucose were frequently required before $^{14}CO_2$ evolution was both readily detectable and linear. The rate of oxidation of added glucose observed for rabbit platelets, however, is in the same range as that reported for human platelets[7,17]. Platelets prepared by the method used in the present study are probably largely depleted of endogenous substrate since the glycogen and ATP levels are low[4] and the platelets do not respond to aggregating stimuli in the absence of added substrate[3,4]. Any contributions made by glycogenolysis or fatty acid oxidation to the 2-carbon pool feeding the citric acid cycle, therefore, may be considered as minimal. Hence any effects which the stimuli or inhibitors under study might have on these pathways would not seriously influence the observed effects on the citric acid cycle itself. Varying dilutions of radioactive precursor pools by endogenous substrate normally pose a serious problem in interpretation when specific metabolic pathways are studied using whole cells. Despite the extended period of time required for substrate depletion, these platelet suspensions retain the ability to aggregate on the addition of low concentrations of stimuli such as ADP and thrombin, if glucose is also added. Hence the interrelationship between aggregation and oxidative metabolism can be assessed more readily than has been possible previously.

HASLAM[5] has pointed out that before a valid assessment can be made of the role of oxidative metabolism in supporting platelet function, some idea of the efficiency of oxidative phosphorylation in platelets is required. At present there are conflicting views as to the actual tightness of the coupling of respiration to phosphorylation in platelets. Using a crude mitochondria-rich fraction from human platelets, CHERNYAK[18] concluded that these processes were actually uncoupled. ROCK AND NEMERSON[19] have also provided evidence for uncoupling in washed human platelet suspensions. On the other hand, DOERY and associates[17], using washed human platelets, have reported and reviewed several pieces of evidence for at least partial coupling of oxidative phosphorylation. These include their finding that dinitrophenol, an uncoupler of oxidative phosphorylation, produces a stimulation of glucose oxidation. In the present study, similar results were obtained on the addition of dinitrophenol to rabbit platelet preparations. In addition, it was found that dinitrophenol stimulates the oxidation of glucose in oligomycin-inhibited platelets. In isolated mitochondrial suspensions from other tissues, oligomycin inhibits and dinitrophenol stimulates respiration and substrate utilization but only under conditions of tight coupling[20]. By analogy it is possible to conclude that respiration and phosphorylation are tightly coupled in the platelet preparation used in the present study. Furthermore, in isolated mitochondria the rate of oxidative phosphorylation is related to the concentration of available ADP which in turn controls citric acid cycle activity[21] but again these interdependencies operate only in the presence of tight coupling. Because of the evidence for coupling obtained in the present studies it is therefore possible with these suspensions to use observed effects on citric acid cycle activity as evidence for effects on the oxidative generation of ATP by these platelets. This would not be valid in uncoupled preparations.

ADP and thrombin, in concentrations which cause platelet aggregation, were

found to stimulate $^{14}CO_2$ production from [6-^{14}C]glucose. On the basis of the preceding discussion this can be taken as evidence that these aggregating stimuli increase both the activity of the citric acid cycle and the rate of oxidative phosphorylation in these platelet preparations. Several reports of the stimulation of the citric acid cycle upon the addition of aggregating stimuli have been made. WARSHAW and co-workers[7] have demonstrated increased $^{14}CO_2$ formation from [6-^{14}C]glucose using relatively high concentrations of thrombin (0.4 units/ml) with washed human platelets. Since little effect could be detected in their studies until after 30 min in the presence of thrombin, it was impossible to correlate these changes with aggregation. STEINER and associates[8,22] have also reported increased $^{14}CO_2$ formation from [6-^{14}C]glucose upon the addition of ADP, thrombin, collagen or epinephrine. These workers used unwashed human platelets suspended in plasma and their studies were also carried out at time intervals of 30 min or more. In neither study was evidence for tight coupling of oxidation and phosphorylation given.

The stimulation of glucose oxidation observed upon the addition of ADP to platelets could reflect increased requirements for ATP to support adherence, the platelet shape change which takes place prior to aggregation, or some subsequent event such as the regaining of the discoid shape during deaggregation. Results from the present studies indicate that the ADP-induced increase in glucose oxidation is independent of platelet aggregation or deaggregation. However, events related to the initial shape change may be involved since the greatest inhibition of increased $^{14}CO_2$ production was caused by compounds which inhibit the shape change (0.1 mM prostaglandin E_1, 2 mM AMP, 2 mM dibutyryl cyclic AMP, 10 mM magnesium).

Although polylysine does not induce platelet aggregation by a mechanism involving ADP, and added glucose is not required for polylysine-induced aggregation[23], it was found to cause increased $^{14}CO_2$ production from [6-^{14}C]glucose. In the case of polylysine, it seems likely that the observed increase in glucose oxidation is a consequence of the platelet changes caused by the polylysine, such as the change in shape[23]. It may be that it is only when the platelets are in an altered shape that they require increased glucose oxidation.

In addition to confirming the findings of others that thrombin stimulates the oxidation of glucose by platelets[7,8], the present studies demonstrate that this stimulation is not dependent on aggregation but is also noted under conditions where platelet shape change occurs but aggregation does not. In the presence of 1 mM EDTA and the concentrations of thrombin used in this study, the release of platelet nucleotides and serotonin is inhibited[24], so it is unlikely that the release reaction was the stimulus for increased glucose oxidation in this experiment. It seems probable that the increased glucose oxidation was related to the change in shape of the platelets.

Metabolic energy is known to be required for the release reaction[1,3]. When inhibitors of the release reaction (prostaglandin E_1[14], adenosine[25], or dibutyryl cyclic AMP[25]) were used, in concentrations which inhibit the release reaction but not the shape change, the thrombin-induced increase in $^{14}CO_2$ production from [6-^{14}C]glucose was inhibited to some extent. It seems likely that stimuli such as thrombin increase glucose oxidation by causing both the release reaction and a change in platelet shape. Prostaglandin E_1 is believed to stimulate adenyl cyclase activity and hence increase the concentration of cyclic AMP in platelets[26–28], while adenosine is believed to act by inhibiting the phosphodiesterase[29] in platelets. The present data are in keeping

with the concept that increasing the platelet cyclic AMP levels decreases the response of platelets to stimuli. This may be related to an effect of cyclic AMP on metabolism, since it has been observed with disrupted platelets that cyclic AMP inhibits the citric acid cycle[30]. Our observations are compatible with those of WOLFE AND SHULMAN[31] who found that increasing platelet cyclic AMP levels reduced the thrombin-induced increase in lactate production.

Although an increase in glucose oxidation could be detected 1 min after the addition of thrombin in the present investigations, the increased rate of glucose oxidation was maintained for at least 8 min. These findings are at variance with those of MÜRER[2] who reported a burst in oxygen consumption upon the addition of thrombin or latex particles to washed human platelets which lasted little more than 1 min.

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