Relationship of Glucose Oxidation to Aggregation of Human Platelets

By Asif A. Chaudhry, Arthur L. Sagone, Jr., Earl N. Metz, and Stanley P. Balcerzak

The effect of aggregating agents on the hexose monophosphate shunt (HMPS) and the relationship of $^{14}$CO$_2$ production to platelet aggregation were studied in normal volunteers. Platelets collected in ACD were suspended in modified Ringer's bicarbonate buffer without washing and were studied before and after the addition of collagen, adenosine diphosphate (ADP), epinephrine, or thrombin. HMPS and Krebs cycle activities were estimated by the yields of $^{14}$CO$_2$ from glucose-1-$^{14}$C (C$_1$) and glucose-6-$^{14}$C (C$_6$). $^{14}$CO$_2$ production from each substrate was measured continuously during experiments using paired, vibrating reed electrometers and incubation flasks. Both flasks contained aliquots of the same platelet suspension. Baseline $^{14}$CO$_2$ production averaged $22 \pm 4.5$ $\mu$moles/hr/10$^9$ platelets from C$_6$ as compared to $33 \pm 6$ $\mu$moles/hr/10$^9$ platelets from C$_1$. Each aggregating agent gave a prompt and striking increase in $^{14}$CO$_2$ production from C$_1$. In contrast, the increase in $^{14}$CO$_2$ production from C$_6$ was not detectable for 10 min and then production increased slowly. Inhibition of $^{14}$CO$_2$ production (25 mM) did not interfere with platelet aggregation. Stimulation of $^{14}$CO$_2$ production from C$_1$ by aggregating agents was unaffected by malonate. These data indicate that platelet aggregation coincides with stimulation of the HMPS, but the increase in Krebs cycle activity occurs later and is not essential for platelet aggregation.

A SOURCE OF METABOLIC ENERGY is essential for most platelet functions, including aggregation. Kinlough et al. have shown that the processes of platelet aggregation, disaggregation, and maintenance of discoid shape depend on metabolic energy that can be supplied by glucose. Doery et al. and Karpatkin have shown that both glycolysis and oxidative phosphorylation are important to platelet energy metabolism, but their relative importance is controversial. Murer et al. studied the effects of metabolic inhibitors of glycolysis and oxidative phosphorylation and concluded that the continuous

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energy production necessary for the various platelet functions can be main-
tained either by glycolysis or through oxidative phosphorylation.

A number of aggregating agents are known to stimulate the Embden-
Meyerhof pathway and the Krebs cycle, but stimulation of platelet hexose
monophosphate shunt (HMPS) in response to aggregating agents has not been
reported. Steiner et al. have reported that platelet aggregation is associated
with, and possibly mediated by, a burst of activity in the Krebs cycle. Mustard
et al. have presented evidence showing that inhibition of ADP-induced aggre-
gation also inhibits this increased Krebs cycle activity, and they have suggested
that stimulation of the Krebs cycle is essential for aggregation and is not its
consequence.

The present study was undertaken with three aims: (1) to study the suit-
ability of an ionization chamber method for continuous measurement of
glucose-14C oxidation by platelets, (2) to study the effects of different
aggregating agents on the HMPS of platelets, and (3) to study the relationship
of increased glucose oxidation and platelet aggregation.

MATERIALS AND METHODS

Subjects
Normal human volunteers who had not taken any medication for at least 1 wk were
studied.

Preparation of Platelets
Siliconized glassware and plastic syringes were used throughout. Blood and platelet
suspensions were kept at room temperature until incubation with radioactive glucose.
Venous blood drawn into one-sixth volume of ACD9 was centrifuged at 200 g for 15 min
to obtain platelet-rich plasma (PRP). Contaminating red and white cells were removed by
centrifugation of PRP at 120 g for 10 min. The resulting ratio of platelets to red and white
cells was always greater than 100 to 1. Platelets were then concentrated by centrifugation
of PRP at 1000 g for 20 min. Within 2 hr of collection of blood, 1.7 X 109 unwashed,
aggregate-free platelets were resuspended in 0.2 ml of autologous plasma and 2.8 ml of
modified Ringer’s solution (30 mM NaHCO3, 4 mM KCl, 107 mM NaCl, and 2 mM
Na2PO4) containing a total of 9 μ mole of glucose, 5 μCi of 14C-glucose (specific activity
3.0 mCi/mM), 300 μg of streptomycin, and 300 U of penicillin G at pH 7.45.

Experimental Procedure
The platelet suspension was incubated at 37°C in a 25 ml flask with three airtight ports
(Fig. 1). Ninety-five per cent O2 and 5% CO2 entered through one port at a rate of
65 ml/min and exited through a second port connected to a Cary-Tolbert ionization
chamber. The third opening was closed at the beginning of each experiment by a rubber
stopper through which various reagents were added to the incubation mixture by means
of a syringe and spinal needle. The platelet suspension was mixed constantly by a
magnetic stirrer.

Radioactive Measurement
14CO2 from 14C-glucose was quantitated using modifications of the method of Davidson
et al. previously described in our laboratory. Krebs cycle and HMPS activities were
estimated by the yields of 14CO2 produced from platelets metabolizing glucose-6-14C (C6)
or glucose-1-14C(C1). 14CO2 production from each substrate was measured continuously
using paired, vibrating reed electrometers and incubation flasks. Both flasks contained
aliquots of the same platelet suspensions.
Fig. 1. Diagram of apparatus for continuously measuring $^{14}\text{CO}_2$ from platelet metabolism of $^{14}\text{C}$ glucose.

After each experiment, the best fitting curve was drawn through the recording tracing, and the $^{14}\text{CO}_2$/hr/$10^9$ platelets was calculated according to the following equation:

$$14\text{CO}_2 \text{ production (mmoles/hr/10}^9\text{ platelets)} = (MV) \times (k \times \frac{1}{A}) \times \frac{1}{10^9 \text{ platelets}}$$

Where: MV, millivolt reading; k, chamber constant ($5.73 \mu\text{Ci/MV/hr}$ ($10^{-3}$)); and A, specific activity of glucose.

The rate of $^{14}\text{CO}_2$ production was calculated for points at 5-min intervals along the curve. These points were replotted and connected by a continuous curve.

Platelet Aggregation

In each metabolic experiment, platelet aggregation was confirmed by light microscopy 5 min after the addition of aggregating agents. Platelet aggregation of duplicate platelet suspensions was also studied at $37^\circ\text{C}$ by a turbidimetric method. Aggregation was studied in fresh platelet suspensions, platelet suspensions incubated for 5 hr under the condition of the metabolic experiments, and in the presence of $25 \text{mM}$ sodium malonate.

Platelet Glucose Consumption

Glucose consumption was measured by a glucose-oxidase method in duplicate experiments. Results were expressed as mmoles glucose consumed/hr/$10^9$ platelets. All cells were counted with a Coulter Counter, Model B.

Preparation of Collagen

Collagen was obtained from Sigma (St. Louis, Mo.). Two grams were suspended in 100 ml of Ringer's buffer and blended in a Waring Blendor for 5 min. The suspension was centrifuged at 800 g for 15 min, and the resulting supernatant was diluted to a concentration that gave maximum aggregation.

Materials

Commercial chemicals were reagent grade. ADP and sodium malonate were obtained from Sigma (St. Louis, Mo.), thrombin and epinephrine from Parke-Davis (Detroit, Mich.), Glucostat from Worthington Biochemical (Freehold, N. J.) and $^{14}\text{C}$-glucose from Amersham/Searle (Arlington Heights, Ill.).
RESULTS

Platelet Aggregation

Aggregation of duplicate platelet suspensions studied by the turbidimetric method usually gave typical biphasic curves in response to ADP and epinephrine and was similar to results reported by other investigators (Fig. 2). Occasionally, maximum platelet aggregation occurred in a monophasic fashion. Platelet suspensions incubated for 5 hr under the conditions used in the metabolic experiments gave aggregation curves similar to fresh platelet suspensions (Fig. 3). This was true regardless of the aggregating agent used. Preincubation of these platelet suspensions with 25 mM sodium malonate gave similar aggregation curves in response to ADP, epinephrine, thrombin, and collagen. Duplicate platelet suspensions from five different donors gave quantitatively similar aggregation curves on incubation with sodium malonate or equimolar sodium chloride.

Fig. 2. Aggregation of platelets immediately after preparation. Epinephrine was added at 2 sec.

Fig. 3. Aggregation of platelet after 5-hr incubation under conditions described for metabolic experiments. Epinephrine was added at 2.75 sec.
Fig. 4. Pattern of $^{14}$CO$_2$ production by normal human platelets from glucose-1-$^{14}$C(C$_1$) and glucose-6-$^{14}$C(C$_6$). Number of experiments performed is shown in parenthesis. These curves represent continuous measurements of $^{14}$CO$_2$ production.

$^{14}$C-Glucose Oxidation

Figure 4 shows curves of the continuous $^{14}$CO$_2$ production by normal human platelets from $^{14}$C-glucose. $^{14}$CO$_2$ production from C$_1$ was detectable within 10 min following the addition of labeled substrate. $^{14}$CO$_2$ production followed a hyperbolic curve during the first hour, then increased linearly, and reached equilibrium in about 5 hr. In contrast, $^{14}$CO$_2$ production from C$_6$ was not detectable during the first hour, then increased linearly, and also reached equilibrium in about 5 hr. In 22 experiments, the rate of $^{14}$CO$_2$ production after 5 hr of incubation was $33 \pm 6$ mmoles/hr/10$^9$ platelets from C$_1$ and $22 \pm 4.5$ mmoles/hr/10$^9$ platelets from C$_6$. pH remained constant at 7.4 during these and all other experiments. Bacterial cultures were negative at the conclusion of each experiment.

Platelet Aggregation and Glucose Oxidation

The effect of epinephrine on $^{14}$CO$_2$ production from $^{14}$C-glucose is shown in Fig. 5. After addition of $10^{-4}$ M of epinephrine, platelet aggregation occurred coincident with a prompt and striking increase in $^{14}$CO$_2$ production from C$_1$. In contrast, $^{14}$CO$_2$ production from C$_6$ increased slowly after a lag period of 10 min.

The effect of collagen on $^{14}$CO$_2$ production from $^{14}$C-glucose is shown in Fig. 6. The prompt increase in $^{14}$CO$_2$ production from C$_1$ after addition of collagen was similar to the epinephrine effect but was of lesser magnitude. The slow increase in $^{14}$CO$_2$ production from C$_6$ beginning 10 min after addition of collagen was also similar to the epinephrine effect.

The effect of ADP ($10^{-4}$ M) and thrombin (0.25 U) on platelet metabolism of $^{14}$C-glucose was similar to that of collagen. Each of these aggregating agents was studied using platelets of four different normal donors.

All aggregating agents studied stimulated oxidation of glucose-2-$^{14}$C in a pattern similar to C$_1$.

Effect of Malonate on Glucose Oxidation and Platelet Aggregations

Sodium malonate, at a final concentration of 25 mM, was added simultaneously to aliquots of the same platelet suspension containing either C$_1$ or C$_6$. In control experiments, equimolar concentrations of sodium chloride were used. As shown in Fig. 7, malonate effectively inhibited $^{14}$CO$_2$ production from C$_6$.\[\text{\textcopyright 1974, American Society of Hematology. All rights reserved.}\]
Fig. 5. Effect of epinephrine on $^{14}$CO$_2$ production from metabolism of $^{14}$C-glucose by normal human platelets. Typical example of four experiments using platelets from different donors is shown. Glucose-1-$^{14}$C (C1) and glucose-6-$^{14}$C (C6) metabolism was studied simultaneously on identical aliquots of the same platelet suspension. $^{14}$CO$_2$ production during entire experiment is depicted in insert. Dotted and dashed rectangle encloses that portion of the entire experiment that has been enlarged and comprises major portion of the figure.

Fig. 6. Effect of collagen on $^{14}$CO$_2$ production from metabolism of $^{14}$C-glucose by normal human platelets. Typical example of the four experiments using platelets from different donors is shown. Conditions are similar to those shown in Fig. 5.

Fig. 7. Effect of sodium malonate on $^{14}$CO$_2$ production from metabolism of $^{14}$C-glucose by normal human platelets. Typical example of three experiments using platelets from different donors is shown. Sodium malonate was added after 5 hr of incubation, and epinephrine was added after 6 hr. Macroaggregation was seen at time indicated.
After the addition of epinephrine at this point, a prompt and striking increase occurred in $^{14}$CO$_2$ production from C$_1$ but not from C$_6$. In each experiment, platelet aggregation occurred with or without the addition of malonate.

Three similar experiments were performed with platelets from different donors in which effects of ADP, collagen, and thrombin were studied after the addition of malonate. These aggregating agents gave similar responses to that of epinephrine in regard to metabolism and aggregation.

**Platelet Glucose Consumption**

Glucose consumption by platelets before and after exposure to aggregating agents is shown in Fig. 8. Before aggregation, platelet glucose consumption was constant at a rate of 0.2 µmoles/hr/10$^9$ platelets for 7 hr. After the addition of aggregating agents, approximately an eightfold increase in the rate of glucose consumption occurred during the first 15 min. After 2 hr, the rate of glucose consumption dropped toward that seen prior to aggregation. ADP (10$^{-4}$ M) and thrombin (0.25 U) gave results similar to collagen and epinephrine.

**DISCUSSION**

The method described herein has certain advantages over the standard method of $^{14}$CO$_2$ trapping in Warburg flasks. (1) The results of the experiments can be monitored continuously so that the addition of metabolic inhibitors and stimulants may be made at appropriate times. (2) The $^{14}$CO$_2$ trapping method gives only one value for each flask. If the time course of glucose oxidation is to be ascertained, multiple flasks are required. The present method needs only one flask for such a record. (3) With the present technique platelets are accessible at all times for sampling. (4) Each experiment may serve as its own control.

The present study confirms that both the Krebs cycle and HMPS are active in intact platelets in the absence of aggregating agents. The time course of the production of $^{14}$CO$_2$ from C$_1$ and C$_6$ glucose differ significantly. $^{14}$CO$_2$ from C$_1$ is detectable within 10 min of incubation, while $^{14}$CO$_2$ from C$_6$ is not detectable during the first hour. Since $^{14}$CO$_2$ is produced from C$_1$ both as a result of the oxidative portion of the HMPS and the Krebs cycle, the $^{14}$CO$_2$
detected from C₁ during the first hour of incubation represents a direct measure of the HMPS since no ¹⁴CO₂ could be detected from C₆ during this period. From the second to fifth hour of incubation, there is an equal and linear increase in ¹⁴CO₂ from both C₁ and C₆ until steady state conditions are reached at 5 hr. These results indicate that increased ¹⁴CO₂ detected from both substrates during this period is the result primarily of the Krebs cycle activity, while the ¹⁴CO₂ produced by the HMPS from C₁ is almost constant during this period. It is of interest that the rate of HMPS activity predicted by the ¹⁴CO₂ yield at 1 hr from ¹⁴C₁ of 2.17% is very similar to the value of 2.19% using the steady state of 33 for C₁ and 22 for C₄ in the Wood equation four.*¹⁵

\[
\frac{G₁ \text{ CO₂} - G₆ \text{ CO₂}}{1 - G₆ \text{ CO₂}} = \frac{3 \text{ PC}}{1 + 2 \text{ PC}}
\]

The reason for the delay in detection of ¹⁴CO₂ from C₆ is uncertain. This delay could occur if CO₂ diffused less readily from mitochondria than from other areas of the cell. Such a differential in diffusion seems unlikely for at least two reasons. (1) A similar delay in reaching constant ¹⁴CO₂ production from labeled Krebs cycle substrates occurs when methods that would not be influenced by differentials in CO₂ diffusion are used to study platelets.¹⁶,¹⁷ (2) The present method can detect ¹⁴CO₂ from labeled Krebs cycle substrates within minutes when certain tissues other than platelets are examined.¹⁸,¹⁹ The slower appearance of ¹⁴CO₂ from C₆ may well represent greater dilution of labeled substrate with unlabeled intermediates. Presumably, the 5-hr period required for ¹⁴CO₂ from either C₁ or C₆ to reach equilibrium represents the time required for all intermediates in the glycolytic pathway and Krebs cycle to be uniformly labeled.

Our results of C₁ and C₂ oxidation differ from those reported previously in that substantial stimulation of ¹⁴CO₂ production from the HMPS was found in response to standard aggregating agents. Addition of aggregating agent resulted in prompt increase in ¹⁴CO₂ from C₁ and C₂, while no increase in ¹⁴CO₂ from C₆ occurred indicating that the increased ¹⁴CO₂ during this period was produced primarily by the HMPS. The increase in ¹⁴CO₂ from C₆ did not occur until 10 min after the addition of aggregating agent, indicating that increased Krebs cycle activity does not occur until after aggregation is complete. The coincident occurrence of platelet aggregation and stimulation of HMPS activity, as well as delayed stimulation of the Krebs cycle, differs from previous studies. The reason for these differences is not certain, but the technique of preparing platelet suspensions differs appreciably from previous studies. Because Rock and Nemerson²⁰ have shown that uncoupling of oxidative phosphorylation may occur in washed platelets, this study employed platelets that were not washed. The use of unwashed platelets also allows quantitative measurement of platelet aggregation of aliquots of the same platelet suspensions used for study of glucose oxidation. Platelets were collected in ACD at a pH 6.5 and kept at room temperature prior to glucose studies because such conditions appear to be optimal for platelet integrity as judged by survival studies. Two other possible reasons for the difference in results...
between this and earlier studies relate to the method of measurement of \(^{14}\)CO\(_2\) production and to the fact that in our experiments aggregating agents were not added until a stable baseline had been established by 5 hr of incubation with continuous monitoring.

The delayed response of the Krebs cycle to aggregating agents suggests that stimulation of that pathway is not a prerequisite for aggregation. In fact, the results of experiments with sodium malonate indicate that aggregation can occur when his pathway is inhibited. In the presence of malonate, which inhibits succinate dehydrogenase,\(^{21}\) Krebs cycle activity decreased markedly, but platelet aggregation was unaffected. These results differ from those reported by Mustard et al.\(^{8}\) who studied washed rabbit platelets and from those of Steiner et al.\(^{7}\) who studied unwashed human platelets. Although the metabolic requirements for aggregation in vivo cannot be determined with certainty from studies in vitro, the present investigation demonstrates that platelets may aggregate in response to physiologic stimuli without an increase in Krebs cycle activity. Whether the simultaneous increase in activity of the HMPS we have demonstrated is a requirement for aggregation remains to be determined by further study.

REFERENCES


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