Energy Metabolism in Human Platelets: Interrelationship Between Glycolysis and Oxidative Metabolism

BY J.C.G. DOERY, J. HIRSH AND I. COOPER

Glycolysis and oxidative metabolism were assessed in washed human platelets incubated in a Ca" and Mg" free Krebs-Ringer bicarbonate buffer, pH 7.4 at 37° C for 1 hour. Glycolytic rate was 45–65 per cent lower under aerobic than anaerobic conditions. Glycolysis was decreased further when albumin was present in the incubation medium and under these conditions glucose uptake, glycogen utilization and lactate production were 21.4, 15.8 and 78 μmoles/hr. per 1011 platelets, respectively. The oxidation of 6-14C glucose was 0.39 μmoles/hr. per 1011 platelets and of U-14C palmitate 0.19 μmoles/hr. per 1011 platelets, and the rate of oxidation of either substrate was increased in the absence of the other. The uncoupling agent, dinitrophenol, stimulated oxidation of glucose to a much larger extent than fatty acid. It is concluded that both glycolysis and oxidative phosphorylation are important to platelet energy metabolism, that either pathway may compensate for decreased activity of the other and that, under conditions of metabolic stress, glucose is preferred to fatty acids as a substrate for oxidative metabolism.

ALTHOUGH IT IS NOW GENERALLY ACCEPTED that platelet energy is provided by both glycolysis and oxidative phosphorylation, opinions differ on the relative importance of these two pathways in platelet energy metabolism. Thus, Waller et al.1 concluded that glycolysis was more important, although a very significant contribution from oxidative phosphorylation is suggested from their value for CO2 production. Chernyak2 and Bettex-Galland and Lüscher3 also concluded that glycolysis was more important to platelet energy production, while more recently, Karpatkin4 has emphasized the importance of oxidative metabolism.

We have investigated energy metabolism in platelets by measuring glycolysis under aerobic and anaerobic conditions and oxidation of labeled glucose and palmitic acid. The aim of the study was to determine the contribution of glycolysis and oxidative phosphorylation to platelet energy metabolism and the relative importance of glucose and fatty acids as substrates for oxidative metabolism.
**Materials and Methods**

**Isolation of Platelets**

Human platelets were prepared from venous blood collected into 2 per cent EDTA containing 0.42 per cent saline in 50 ml. cellulose nitrate centrifuge tubes; 9 parts of blood to 1 part of EDTA saline. Following collection, the blood was then further diluted with an equal volume of isotonic saline; a procedure found to improve platelet yields. Platelet-rich plasma was prepared by centrifugation of the diluted blood at 6°C at 200 X g. for 17 minutes; the supernatant was then centrifuged at 1250 X g. for 15 minutes at 6°C. The supernatant was removed and the platelet button washed once in cold EDTA-Tyrode solution (9 mM EDTA) and resuspended in a Krebs-Ringer bicarbonate buffer, pH 7.4, modified by replacing the CaCl₂ and MgSO₄ solutions with 0.9 per cent NaCl. The suspension was finally centrifuged at 6°C at 100 X g. for 12 minutes to remove any contaminating erythrocytes or leukocytes. The final suspension was free of platelet aggregates and contained approximately 1 X 10⁶ platelets per ml. Erythrocyte and leukocyte contamination was less than one cell per 20,000 platelets.

**Platelet Counting**

Platelet counts were performed in an electronic particle counter (Coulter, Model B). The diluent was 0.3 per cent potassium oxalate in 0.85 per cent sodium chloride made particle-free by passage through a 0.45 µ Metricel filter (Gelman, Mich.).

**Binding of Palmitic Acid to Albumin**

A stock albumin solution was prepared in a concentration of 45 mg./ml. of Krebs Ringer buffer. This was assayed for contaminating fatty acid and was found to contain 0.156 µEq. per milliliter. This is equivalent to 0.04 mg. palmitic acid per milliliter. A solution of palmitic acid (0.15 mg./ml.) bound to albumin was prepared fresh daily as follows: To 2 ml. of albumin solution (45 mg./ml. of Krebs-Ringer buffer) was added 0.3 mg. of palmitic acid dissolved in 0.3 ml. of acetone. The acetone was then driven off by a stream of nitrogen. The final palmitic acid concentration was calculated by subtracting the concentration of the contaminating fatty acid from the value for total fatty acid and was found to be 0.619 µEq. or 0.158 mg. per milliliter. The molar ratio of palmitic acid to albumin was 0.9. This approximates to the molar ratio of free fatty acids to albumin in human plasma which is 1.1.7

**Incubation Procedure and Preparation of Samples for Assay**

For studies on oxidation of labeled substrates, incubations were carried out at 37°C under 5 per cent CO₂-95 per cent O₂ in Warburg flasks with latex rubber caps. The outer well of the flask contained 2 ml. of platelet suspension and either 0.2 ml. of buffer or 0.2 ml. of buffer containing added substrates or inhibitors. All flasks contained albumin in a final concentration of 4 mg./ml. Labeled substrates which were always used in association with cold carrier were added to the platelet suspensions to give a final activity of approximately 0.7 µCi per flask. A glass vial containing a 4 sq. cm square of glass filter paper (Whatman, No. GF/A) was placed in the center well. At the completion of the incubation period, metabolism was stopped by injecting 0.4 ml. of 21 per cent perchloric acid through the cap into the outer well. Evolved CO₂ was captured by addition of 0.3 ml. of hydroxide of Hyamine into the glass vial in the center well. After a further 2 hours incubation, the glass vial and contents were transferred to counting bottles containing 10 ml. of scintillating fluid (4 Gm. PPO and 40 mg. POPOP in 1 L. of toluene) and counted in a Packard Tri-Carb liquid scintillation spectrometer. Controls were run by injecting the perchloric acid at zero time.

For studies on glycolysis (glucose uptake, glycogen utilization and lactate production) 2 ml. samples of platelet suspension were incubated at 37°C, under 5 per cent CO₂-95 per cent O₂ or 5 per cent CO₂-95 per cent N₂ in stoppered glass centrifuge tubes. When
albumin was present in the incubation, a 0.2 ml. sample of albumin in buffer (45 mg./ml.) was used to coat the glass tube just prior to addition of the platelet suspension. At completion of the incubation time, the tubes were placed in melting ice and agitated for 1 minute. A platelet button was then prepared for glycogen analysis by centrifugation at 2500 x g. for 7 minutes at 0°C. The supernatant was removed and a portion added to an equal volume of cold 7 per cent perchloric acid and frozen overnight at -20°C. This was thawed the following day; lactate determinations were carried out on the supernatant. Glucose determinations were carried out on the same samples, after neutralization with anhydrous sodium bicarbonate. Glycogen was extracted from the residual platelet button and hydrolyzed to glucose as described previously.8

Assay Procedures

All assays were enzymatic methods employing nucleotide changes which were followed at 340 nm on a Gilford Model 2000 spectrophotometer. Assays were always performed in triplicate. L-Lactic acid was assayed by the method of Horn and Bruns9 utilizing lactic dehydrogenase. Glucose was assayed by the method of Pfleiderer10 utilizing hexokinase, pyruvate kinase and lactic dehydrogenase.

Materials

Glass distilled, deionized water was used throughout. Hexokinase, type III, ATP, phosphoenol pyruvate, 2-deoxyglucose, NAD, NADH were obtained from Sigma Chemical Co., St. Louis, Mo., and pyruvate kinase and lactic dehydrogenase were obtained from C. F. Boehringer & Sons; bovine albumin fraction V was obtained from Pentex. 6-14C glucose and U-14C palmitic acid were obtained from the Radiochemical Centre, Amersham, England. Acetone, spectroquality, was obtained from Matheson, Coleman & Bell, Ohio.

Recovery of Labeled CO2

It was shown that maximum recovery of labeled CO2 by Hyamine was obtained with the 2-hour incubation period used following deproteinization of the platelet suspension and that negligible amounts of radioactivity were released in the control suspensions which were deproteinized at zero time.

RESULTS

Glycolysis

The results of glucose uptake, glycogen utilization and lactate production under aerobic and anaerobic conditions in the presence and absence of albumin in the incubation medium are shown in Table 1. Under aerobic conditions in the absence of albumin, glucose uptake was 39.4 μmoles/hr. per 1011 platelets, glycogen utilization was 23.1 μmoles and lactate production 92 μmoles/hr. per 1011 platelets. Anaerobic conditions produced a significant increase in glycolysis in both the presence and absence of albumin. Thus, in the presence of albumin, glucose uptake was increased by 248 per cent (p < 0.001), glycogen utilization by 91 per cent (p < 0.01) and lactate production by 146 per cent (p < 0.01), while in the absence of albumin, the corresponding increases were 105 per cent (p < 0.01), 37 per cent (p < 0.05) and 122 per cent (p < 0.01).

The addition of albumin to the incubation medium had no significant effect on glycolysis measured under anaerobic conditions (glucose uptake p < 0.1, glycogen utilization p < 0.05 and lactate production p < 0.1), but a 46 per cent decrease in glucose uptake (p < 0.05), a 32 per cent decrease in
Table 1.—Effect of Albumin and Oxygen on Glycolysis

<table>
<thead>
<tr>
<th>Experiment No.</th>
<th>Glucose Uptake</th>
<th>Glycogen Utilization</th>
<th>Lactate production</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No Albumin</td>
<td>Albumin</td>
<td>No Albumin</td>
</tr>
<tr>
<td></td>
<td>Anaerobic</td>
<td>Aerobic</td>
<td>Anaerobic</td>
</tr>
<tr>
<td>1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>90.0</td>
<td>37.0</td>
<td>74.0</td>
</tr>
<tr>
<td>3</td>
<td>55.1</td>
<td>30.8</td>
<td>52.7</td>
</tr>
<tr>
<td>4</td>
<td>65.5</td>
<td>49.1</td>
<td>63.1</td>
</tr>
<tr>
<td>5</td>
<td>98.0</td>
<td>35.5</td>
<td>94.8</td>
</tr>
<tr>
<td>6</td>
<td>86.3</td>
<td>36.4</td>
<td>84.0</td>
</tr>
<tr>
<td>6</td>
<td>90.0</td>
<td>47.6</td>
<td>77.6</td>
</tr>
<tr>
<td>Mean ± SE</td>
<td>80.8 ± 6.8</td>
<td>39.4 ± 3.0</td>
<td>74.4 ± 6.1</td>
</tr>
</tbody>
</table>

Data is expressed as μmoles per hour per 10¹¹ platelets. Initial glucose concentration was 5 mM, albumin 4 mg./ml. and NaCN 5 mM.
ENERGY METABOLISM IN HUMAN PLATELETS

Fig. 1.—Rate of evolution of labeled CO₂ from (left) 6_14C glucose (5 mM) and (right) U_14C palmitate (56 μM) in presence of glucose (5 mM).

glycogen utilization (p < 0.001) and 15 per cent decrease in lactate production (p < 0.05) were observed under aerobic conditions. The effect of albumin on glycolysis was determined because it was necessary to use albumin as carrier in the experiments using fatty acids described below. Because it is difficult to totally eliminate oxygen from the incubation medium, the completeness of the anaerobic conditions was checked by measuring lactate production in the presence of added cyanide. There was a further slight increase in lactate production (Table 1, experiments 5 and 6) but this was not sufficient to influence the conclusions drawn from the experiments.

Glucose Oxidation

After approximately 20 minutes the rate of labeled CO₂ production from 6_14C glucose was linear for at least a further 40 minutes (Fig. 1). The rate of oxidation of 6_14C glucose measured between 30 and 60 minutes was 0.385 μmoles/hour/10¹¹ platelets (Table 2). The addition of unlabeled palmitic acid (56 μM) to the incubation medium produced a decrease of 30 per cent (p < 0.001) in rate of glucose oxidation.

Palmitate Oxidation

When platelets were incubated with U_14C palmitate (56 μM) there was an initial delay of approximately 25 minutes before the rate of production of labeled CO₂ was linear (Fig. 1). In the presence of unlabeled glucose (5 mM) the rate of oxidation of 14C palmitate measured between 30 and 60 minutes was 0.191 μmoles/hour per 10¹¹ platelets (Table 3). When glycolysis was inhibited by omitting glucose and adding 2-deoxyglucose (7 mM) to the incubation medium there was a 31 per cent increase (p < 0.001) in the rate of palmitate oxidation. The addition of cyanide (5 mM) completely inhibited the oxidation of 14C palmitate.
Table 2.—Oxidation of 6-C\textsuperscript{14}G glucose and the Effect of Palmitate

<table>
<thead>
<tr>
<th>Experiment No.</th>
<th>Glucose Oxidized</th>
<th>Percentage Decrease with Palmitate</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>Palmitate</td>
</tr>
<tr>
<td>1</td>
<td>0.438</td>
<td>0.324</td>
</tr>
<tr>
<td>2</td>
<td>0.396</td>
<td>0.217</td>
</tr>
<tr>
<td>3</td>
<td>0.422</td>
<td>0.331</td>
</tr>
<tr>
<td>4</td>
<td>0.473</td>
<td>0.336</td>
</tr>
<tr>
<td>5</td>
<td>0.298</td>
<td>0.211</td>
</tr>
<tr>
<td>6</td>
<td>0.282</td>
<td>0.190</td>
</tr>
<tr>
<td>Mean ± SE</td>
<td>0.385±0.032</td>
<td>0.268±0.028</td>
</tr>
</tbody>
</table>

Rates of oxidation are expressed as \(\mu\)moles of glucose oxidized per hour per 10\textsuperscript{11} platelets. These values represent minimum rates of glucose oxidation and have not been adjusted to compensate for rate of glycogen entry into glycolysis. (See Discussion). Final concentration of glucose was 5 mM, palmitate 56 \(\mu\)M and albumin 4 mg./ml.

Table 3.—Oxidation of U-\textsuperscript{14}C Palmitic Acid and the Effect of Glucose

<table>
<thead>
<tr>
<th>Experiment No.</th>
<th>Palmitate ((\mu)M)</th>
<th>Palmitate oxidation</th>
<th>Percentage Increase in Absence of Glucose</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Glucose</td>
<td>Deoxyglucose</td>
</tr>
<tr>
<td>1</td>
<td>28</td>
<td>0.155</td>
<td>0.184</td>
</tr>
<tr>
<td>2</td>
<td>28</td>
<td>0.200</td>
<td>0.265</td>
</tr>
<tr>
<td>3</td>
<td>28</td>
<td>0.167</td>
<td>0.211</td>
</tr>
<tr>
<td>4</td>
<td>56</td>
<td>0.211</td>
<td>0.277</td>
</tr>
<tr>
<td>5</td>
<td>56</td>
<td>0.219</td>
<td>0.283</td>
</tr>
<tr>
<td>6</td>
<td>56</td>
<td>0.194</td>
<td>0.277</td>
</tr>
<tr>
<td>Mean ± SE</td>
<td>0.191±0.010</td>
<td>0.250±0.017</td>
<td></td>
</tr>
</tbody>
</table>

Rates of oxidation are expressed as \(\mu\)moles of palmitate oxidized per hour per 10\textsuperscript{11} platelets. Glucose concentration was 5 mM and deoxyglucose, 7 mM.

Effect of 2,4-Dinitrophenol on Rates of Glucose and Palmitate Oxidation

The effect of increasing concentrations of the uncoupling agent 2,4-dinitrophenol (DNP) on oxidation of labeled glucose and palmitate is shown in Table 4. DNP at a concentration of 0.05 mM produced a marked increase (approximately 300 per cent) in glucose oxidation and a smaller increase (approximately 25 per cent) in palmitate oxidation. At a concentration of 0.5 mM, DNP markedly inhibited oxidation of either substrate.

DISCUSSION

Discussion
Table 4.—Effect of 2,4-Dinitrophenol on Rate of Oxidation of Labeled Substrates

<table>
<thead>
<tr>
<th>Exp. No.</th>
<th>Added Substrates</th>
<th>Labeled</th>
<th>Unlabeled</th>
<th>Concentration of DNP (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>0</td>
<td>0.005</td>
<td>0.05</td>
</tr>
<tr>
<td>1</td>
<td>6-C¹⁴-glucose</td>
<td>0.374 (100)</td>
<td>2.214 (392)</td>
<td>0.052 (14)</td>
</tr>
<tr>
<td>2</td>
<td>6-C¹⁴-glucose</td>
<td>0.334 (100)</td>
<td>0.368 (110)</td>
<td>1.489 (446)</td>
</tr>
<tr>
<td>3</td>
<td>6-C¹⁴-glucose</td>
<td>0.358 (100)</td>
<td>0.374 (104)</td>
<td>1.378 (385)</td>
</tr>
<tr>
<td>4</td>
<td>U-¹⁴C-palmitate</td>
<td>0.580 (100)</td>
<td></td>
<td>0.001 (0.2)</td>
</tr>
<tr>
<td>5</td>
<td>U-¹⁴C-palmitate</td>
<td>0.232 (100)</td>
<td>0.235 (101)</td>
<td>0.295 (127)</td>
</tr>
<tr>
<td>6</td>
<td>U-¹⁴C-palmitate</td>
<td>0.286 (100)</td>
<td>0.269 (101)</td>
<td>0.286 (108)</td>
</tr>
</tbody>
</table>

Percentage of the control values is denoted in parentheses. The concentration of palmitate was 56 μM and glucose, 5 mM.
and resuspension in artificial media. Thus, Waller found that repeated washing in artificial media caused a gradual decline in glucose uptake, oxygen uptake and ATP levels; Scott found increased glycogen breakdown when platelets were resuspended in saline and Wu found that similar conditions enhanced aerobic glycolysis. In addition, Nishizawa et al. found that the amount of incorporated into membrane phospholipid was increased during preparation unless special precautions were taken to prevent platelet surface interactions. This increase in phospholipid synthesis would be expected to stimulate energy production and is presumably a response to membrane damage. It is likely, therefore, that our results for energy metabolism and indeed those of other workers represent values above basal levels. Nevertheless, the relationships between aerobic and anaerobic metabolism found are probably relevant to metabolism in the undamaged platelet.

Albumin was found to decrease glycolysis under aerobic conditions. Albumin has been shown to bind very firmly to platelets and to be singularly effective in reducing the interaction of platelets with foreign surfaces. Thus it may be that albumin is reducing glycolysis by reducing surface stimulation. Alternatively the decreased glycolysis could be due to utilization of fatty acids in the commercial albumin preparation.

Our result of 0.39 μmoles/hour per 10^11 platelets for 6-14C glucose oxidation was calculated over the 30–60 minute incubation period, during which time the rate appeared to be linear. A similar figure for the rate of 6-14C glucose oxidation over the same period could be calculated from data published by Warshaw et al. However, in their study the rate of oxidation continued to show a considerable increase for at least 4 hours and their calculated mean value was 0.6 μmoles/hour per 10^11 platelets. Oxidation of 14C palmitate has been reported by Rosenzweig and Wais who did not calculate rates of oxidation and by Donabedian and Nemerson who obtained a rate of 0.47 μmoles/hr. per 10^11 platelets, a result which is approximately double our figure for rate of palmitate oxidation.

From the data on glycolysis under anaerobic conditions in the presence of albumin, total ATP was estimated to be between 128 and 192 μmoles/10^11 platelets per hour. There was a decrease of 60 per cent in lactate production when measured under aerobic conditions (Table 1), suggesting that 60 per cent of total ATP is derived from oxidative phosphorylation. These results are consistent with those reported by Karpatkin who found that lactate production under aerobic conditions was doubled when cyanide was added.

In any consideration of the relative contributions of aerobic and anaerobic metabolism to energy production it is necessary to ascertain the efficiency of mitochondrial oxidation. This is particularly important in view of the recent suggestion that washing and resuspending platelets in artificial media produces uncoupling. Several aspects of our results strongly suggest at least partial coupling of oxidative phosphorylation in the platelet suspensions used. Thus, glycolysis was doubled under anaerobic conditions, palmitate oxidation was increased when glycolysis was inhibited and the uncoupling agent DNP produced a fourfold increase in glucose oxidation. Others using washed platelet suspensions have reported a Pasteur effect, a Crabtree effect and that
incorporation of orthophosphate into platelet phosphatides is dependent on mitochondrial activity. Findings which support the view that significant coupling of oxidative phosphorylation may occur in washed platelet suspensions.

Although platelets can oxidize fatty acids in addition to glucose, it is uncertain which of these two substrates is more readily utilized by the platelet. Either substrate was spared in the presence of the other, suggesting that both are normally used. When the relative rates of oxidation was measured in the presence of DNP, it was found that while glucose increased approximately 300 per cent, palmitate was increased by only 25 per cent. This would appear to suggest that at least under the conditions of the stress imposed by uncoupling, glucose is the preferred substrate for oxidative metabolism in platelets.

Our results and those reported by others indicate that both glycolysis and oxidative phosphorylation are important to platelet energy production. Detwiler has recently shown that the enzyme phosphofructokinase is a primary site of regulation for platelet glycolysis. The activity of this enzyme is regulated by the levels of both ADP and ATP, and it is likely that, as in other cells, the interrelationship between glycolysis and oxidative phosphorylation in platelets is mediated by the ADP/ATP ratio. Both pathways appear to be necessary for normal platelet function. Thus, although energy derived from either pathway can support certain platelet reactions, the synthesis of platelet phosphatides and uptake of amino acids requires oxidative metabolism.

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REFERENCES


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